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ACTIVATION AND DYSREGULATION OF INNATE IMMUNITY IN HIV-1 AND HIV-2 INFECTIONS

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بنی آدم اعضای یکدیگرند
که در آفرینش ز یک گوهرند
چو عضوی بدرد آورد روزگار
دگر عضوها را نماند قرار
توکز محنت دیگران بی غمی
نشاید که نامت نهند آدمی

- سعدی شیرازی

Human beings are members of a whole
In creation of one essence and soul,
If a member is afflicted with pain
Other members uneasy will remain,
If you have no sympathy for human pain
The name of human you cannot retain

-Saadi Shirazi

To my Grandparents

ABSTRACT

Toll-like receptors (TLRs) are pattern recognition receptors, expressed by antigen presenting cells (APCs) that recognize conserved molecular patterns of diverse microorganisms. TLR triggering activates APCs, leading to expression of innate effector molecules and signals that initiate adaptive immune responses. Studies have revealed that the use of TLR agonists might offer novel approaches for the development of therapeutic and prophylactic measures. Two types of human immunodeficiency virus (HIV) can cause acquired immune deficiency syndrome (AIDS): HIV-1, which is found worldwide and HIV-2, which is mostly detected in West Africa and known to be less transmissible and less pathogenic. During HIV infection, the constant battle between the virus and the immune system because of rapid viral turnover results in chronic immune activation that is thought to exhaust several compartments of the immune system. The interactions between HIV and the innate immune system remain, however, relatively unexplored.

Studies in this thesis show that the replication of both HIV-1 and HIV-2 strains can be suppressed *in vitro* by the TLR9 agonist, CpG oligodeoxynucleotide (ODN). Additionally, conjugation of ODNs with a phosphorothioate backbone to cholera toxin B subunit enhanced the anti-HIV activity. These results indicate that the use of TLR agonists might have implications for the development of new HIV intervention strategies. Furthermore, studies on the impact of HIV on TLR stimuli responsiveness reveal that both advanced HIV-1 and HIV-2 infections were associated with defective IFN-α responses after *in vitro* TLR9 stimulation. In addition, defective IL-12 expression after TLR7/8 stimulation was observed in HIV-1-infected individuals. Moreover, levels of microbial translocation, measured as concentrations of lipopolysaccharide (LPS) in plasma, were elevated in both HIV-1- and HIV-2-infected individuals with AIDS. The plasma LPS levels correlated with CD4+ T cell count and viral load, in addition to TLR responsiveness. These results suggest that alterations in innate immune responses and microbial translocation are associated with the pathogenesis of both HIV-1 and HIV-2 infections. Studies on the immunological consequences of treatment interruption- (TI) associated viremia in HIV-1-infected individuals, showed that circulating dendritic cells were reduced and that TLR stimuli responsiveness was dysregulated. Moreover, analyses of immune activation markers showed that the frequency of HLA-DR+ T cells and spontaneously released IL-12 increased during TI, whereas microbial translocation remained unaffected. Hence, innate immunity and T cell markers of immune activation are affected during HIV-1 infection even after short-term viremia, but prolonged viremia appears to be required for the detection of microbial translocation.

This thesis adds knowledge on the potential use of TLR agonists for the development of novel approaches to prevent HIV infection, but also emphasizes the need for better understanding of the role of TLR responsiveness during the pathogenesis of HIV-1 and HIV-2 infections.

POPULÄRVETENSKAPLIG SAMMANFATTNING

I den här avhandlingen har medfött immunförsvar studerats i förhållande till humant immunbrist virus (hiv) infektion.

Vårt immunförsvar är uppdelat i två olika system; det medfödda immunsystemet, som aktiveras inom få minuter efter att kroppen blivit invaderad av främmande mikroorganismer; samt det förvärvade immunsystemet som börjar verkar efter några dagar till veckor och även bildar ett minnessvar för framtida skydd. Toll-liknande receptorer (TLR) som uttrycks av celler i vårt medfödda immunförsvar, t ex dendritiska celler, känner igen olika molekyler som är gemensamma för mikroorganismer. Det medfödda immunförsvaret aktivera bland annat genom att TLR stimuleras. Vid stimulering av TLR, aktiveras cellen som den uttrycks av, och cellen skickar i sin tur ut signaler för att aktivera det förvärvade immunsystemet.

Hiv är ett virus som efter många års infektion kan orsaka förvärvat immunbristsyndrom (aids). Det finns två typer av hiv; hiv-1 som är spritt över hela världen samt hiv-2 som inte är ett lika aggressivt virus och som främst finns i Väst Afrika. Aids är ett tillstånd då immunförsvaret har blivit så försvagat att det inte längre kan skydda oss mot vanligt förekommande infektioner. Anledningen till varför aids uppstår beror till viss del på att hiv infekterar celler som ingår i vårt immunförsvar. En annan bidragande ordsak till aids utveckling är att hiv infektionen leder till en kronisk aktivering av immunförsvaret som till slut utmattas och vars kvalitet försämras. Än idag finns det inget vaccin tillgängligt för att förhindra en hiv infektion och det finns heller inget sätt att bota en hiv infektion. Däremot finns bromsmediciner som kan hålla tillbaka viruset och hindra det från att föröka sig allt för snabbt i kroppen. Det har visat sig att det medfödda immunförsvaret kan ha inverkan på sjukdomsförloppet hos hiv infekterade. Dock är kunskapen relativt ny och området relativt outforskat.

I den här avhandlingen visar vi att infektion av både hiv-1 och hiv-2 kan nedregleras i cellodlingar genom att stimulera en TLR, TLR9, med CpG-DNA molekyl. Kolerabakterien utsöndrar en molekyl, CTB, som har visat sig aktivera immunförsvaret. Vi visar även att nedreglering av hiv infektion kan förbättras om CpG kopplas till CTB. Anti-hiv effekten av CpG-CTB kan antingen bero på att CpG-CTB binder till hiv och förhindrar viruset från att infektera celler, men det kan även bero på att CpG-CTB förstärker aktiveringen av immunförsvaret som framkallar signaler som hindrar hiv från att föröka sig. Dessa resultat antyder att TLR stimulering skulle kunna utnyttjas för att utveckla sätt att förhindra en hiv infektion.

Vi har vidare studerat TLR funktionen hos redan hiv infekterade individer. Vi visar här att signaler som skickas ut av celler efter TLR7/8 och TLR9 stimulering var försämrade hos hiv-1 infekterade individer och att hiv-2 infekterade individer med framskriden sjukdom också har nedreglerade signalsvar efter TLR9 stimulering. Vi har även kunnat visa att läckage av mikroorganismer till blodsystemet p.g.a. skadade tarmväggar var förhöjda hos individer med aids (dvs individer med försvagat immunförsvar), och detta var kopplat till nivåerna av virus i blodet. Detta indikerar att läckage av mikroorganismer kan påverka sjukdomsförloppet hos hiv infekterade individer.

Hos bromsmedicin-behandlade hiv-1 patienter som genomgick behandlingsuppehåll, kunde vi visa att antalet dendritiska celler sjönk i blodet som en konsekvens av ökade virusnivåer i blodet. Vi såg även att förhöjda virusnivåer i blodet under behandlingsuppehåll resulterade i att celler antingen visade försämrad eller extrem TLR signalering beroende på typ av stimulering. Vi kunde även visa att immunaktiveringen hos vissa av immunförsvarets celler var uppreglerade, men att nivån av läckage av mikroorganismer från tarmarna till blodsystemet var oförändrad under behandlingsuppehållet. Detta tyder på att högre nivåer av cirkulerande hiv partiklar i blodet vid kortare behandlingsuppehåll bidrar till försämrad TLR funktion och därmed risk för sämre sjukdomsprognos.

Sammantaget tyder resultaten på att TLR stimulering kan bidrar till anti-hiv aktiviteter och därför är det möjligt att detta skulle kunna utnyttjas vid utveckling av metoder för att blockera hiv infektion. Däremot, om en hiv infektion redan har etablerats så kan viruset påverka TLR funktioner negativt och istället bidra till ett snabbare sjukdomsförlopp hos hiv infekterade

با قطع داروی بیمارانی که از داروهای کند کننده استفاده میکردند، توانستیم نشان دهیم که مقدار سلول های دندریتیک خون بعلت بالا رفتن سطح ویروس در خون کاهش یافتند. ما همچنین مشاهده کردیم که بالا رفتن سطح ویروس در خون در دوره قطع دارو سبب بدتر شدن سلولها و یا تشدید TLR بخاطر تحریک پیاپی گشت. ما همچنین توانستیم نشان دهیم که فعال شدن سیستم ایمنی نزد بعضی سلول های دفاعی تقویت شده اما سطح نشت میکرو ارگانیسمها از روده ها به سیستم خونی در زمان قطع دارو بلا تغییر بوده است. این بدان معنی است که سطح بالای ذرات HIV که در خون در گردش هستند در دوران کوتاه قطع دارو باعث بدتر شدن عملکرد TLR و در نتیجه خود بیماری میشود.

بطور کلی نتیجه بدست آمده نشان میدهد که تحریک TLR میتواند باعث فعالیت های ضد HIV شده که میتواند امکان استفاده از آن به ایجاد روشهایی برای جلوگیری از ابتلا به HIV شود.

بالعکس، اگر آلودگی HIV پیشرفته باشد، این ویروس میتواند روی TLRها اثر منفی گذاشته که در آن صورت سبب سریع تر شدن دوره بیماری بعلت تضعیف سیستم ایمنی در مقابل میکروارگانیسم های دیگر میگردد.

مقدمه فارسی/ PERSIAN SUMMARY

در این پایان نامه ایمنی مادرزادی در ارتباط با آلودگی به (HIV) مورد بررسی قرار گرفته است.

ایمنی به دو دسته تقسیم میشود. نخست ایمنی مادرزادی است که ظرف چند دقیقه پس از اینکه بدن بوسیله میکرو ارگانیسم های خارجی مورد تهاجم قرار گرفته است، فعال میشود و دیگری ایمنی اکتسابی است که ظرف چند روز یا چند هفته شروع به اثر کردن میکند و همچنین یک پاسخ حافظه ای برای پیشگیری های آینده ایجاد میکند.

گیرنده های تل مانند (TLR) موجود در سطح یا داخل سلول ها در سیستم ایمنی مادرزادی مانند سلول های دندریتیک مولکولهای مشابه در میکرو ارگانیسم ها را شناسایی میکنند. ایمنی مادرزادی زمانیکه این گیرنده ها تحریک شوند فعال میشود. زمان تحریک گیرنده ، سلولی که بوسیله آن شناسایی میشود فعال شده و به نوبه خود منجر به فعال کردن سیستم ایمنی اکتسابی میشود. ویروس HIV ویروسی است که پس از چند سال آلودگی باعث ایدز (سندرم نقض ایمنی اکتسابی) میشود. دو گونه HIV وجود دارد: HIV-1 که در تمام دنیا گسترش پیدا کرده است و HIV-2 که چندان تهاجمی نیست و در درجه نخست در آفریقای غربی وجود دارد.

ایدز مرحله ایی است که ایمنی بدن به اندازه ایی ضعیف شده است که دیگر نمیتواند بدن را در مقابل میکروبهای بیماری زا حفاظت کند. دلیل بوجود آمدن ایدز تا اندازه ایی به خاطر این است که HIV سلولهای مربوط به ایمنی بدن را آلوده میکند. ایدز همچنین میتواند بخاطر اینکه آلودگی HIV مرتبا" سیستم ایمنی بدن را فعال میکند که به نوبه خود باعث خستگی و پایین آمدن کیفیت ان میشود، بوجود آید.

امروزه هنوز هیچ واکسنی جهت پیشگیری یا درمان HIV وجود ندارد. در مقابل، داروهایی هستند که میتوانند تکثیر ویروسها را متوقف نموده و از افزایش سریع آنها در بدن جلوگیری کنند.

سیستم ایمنی مادرزادی نشان داده شده است که بر دوره بیماری اثر دارد، هرچند اطلاعات ما در این مورد نسبتا" تازه است و در این زمینه پژوهش چندانی انجام نگرفته است.

در این پایان نامه نشان داده شده است که تکثیر هر دو HIV-1 و HIV-2 میتواند از طریق فعال کردن TLR9 توسط مولکولهای CPG در کشت سلولی کاهش داده شوند. همچنین کاهش آلودگی HIV میتواند بهتر انجام شود اگر ترکیب CPG و CTB (CTB-CPG) (بخشی از توکسین میکروب و یا بدون آثار سمی توکسین) مورد استفاده قرار گیرد. تاثیر ضد HIV در CPG-CTB میتواند بسته به این باشد که CPG-CTB در HIV میماند و از آلودگی سلول ها بوسیله این ویروس جلوگیری میکند، اما این نیز میتواند بسته به این باشد که CPG-CTB سیستم ایمنی را تقویت کرده که علائمی را بوجود می آورد که از افزایش تکثیر HIV جلوگیری میکند.

ما همچنین کارکرد TLR را در بیماران آلوده به HIV مورد بررسی قرار داده ایم. ما نشان دادیم که تحریک سلولهای ایمنی توسط TLR 7/8 و TLR 9 در بیماران مبتلا به HIV-1 به مقدار قابل ملاحظه ای کاهش نشان میدهد. همچنین بیماران مبتلا به HIV-2 و دارای بیماری پیشرفته به میزان کمتری قادر به ایجاد پاسخ ایمنی بعد از تحریک TLR9 میباشند .

ما همچنین توانسته ایم نشان دهیم که نشت میکرو ارگانیسم ها در سیستم خونی بعلت آسیب دیدگی دیواره روده ها نزد بیماران مبتلا به ایدز بیشتر بوده و این پدیده در ارتباط مستقیم با میزان ویروس در خون مبتلایان بوده است.

این بدان معنی است که نشت میکرو ارگانیسم ها میتواند روی دوره مریضی در بیماران آلوده به HIV تاثیر گذار باشد.

LIST OF PUBLICATIONS

I. **Nowroozalizadeh S,** Jansson M, Adamsson J, Lindblad M, Fenyö EM, Holmgren J, and Harandi AM. *Suppression of HIV replication in vitro by CpG and CpG conjugated to the non toxic B subunit of cholera toxin*. Current HIV Research, 6:230-8, 2008

II. **Nowroozalizadeh S,** Månsson F, da Silva Z, Repits J, Dabo B, Pereira C, Biague A, Albert J, Nielsen J, Aaby P, Fenyö EM, Norrgren H, Holmgren B, Jansson M. *Studies on toll-like receptor stimuli responsiveness in HIV-1 and HIV-2 infections*. Cytokine, 46:325-31, 2009

III. **Nowroozalizadeh S,** Månsson F, da Silva Z, Repits J, Dabo B, Pereira C, Biague A, Albert J, Nielsen J, Aaby P, Fenyö EM, Norrgren H, Holmgren B, Jansson M. *Microbial translocation correlates with the severity of both HIV-1 and HIV-2 infections.* Journal of Infectious Diseases, 201:1150-4, 2010

IV. **Nowroozalizadeh S,** Gudmundsdotter L, Hejdeman B, Andersson L, Sandström E, Gaines H, Wahren B, Jansson M. *Viremia during short-term HIV-1 treatment interruption is associated with dendritic cell decline, altered TLR-stimuli responsiveness and elevated immune activation.* Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cells
cART	Combinational antiretroviral therapy
CA	Capsid protein
CCR	CC chemokine receptor
CpG	Cystidine-phosphate-guanosine
CT	Cholera Toxin
CTA	Cholera toxin A subunit
CTB	Cholera toxin B subunit
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
CXCR	CXC chemokine receptor
DC	Dendritic cell
EC	Elite controller
ER	Endoplasmic reticulum
GALT	Gut-associated lymphoid tissue
GM	Monosialoganglioside
GM-CSF	Granulocyte macrophage colony stimulating factor
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
LPS	Lipopolysaccharide
LTNP	Long-term-non-progressors
mDC	Myeloid dendritic cell
mRNA	Messenger RNA
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
nAb	Neutralizing antibodies
NF-κB	Nuclear factor-kappa B
NK	Natural killer cell
NKT	Natural killer T cell
ODN	Oligodeoxynucleotide
PAMP	Pathogen associated molecular pattern
pDC	Plasmacytoid dendritic cell
PBMC	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
PMN	Polymorphnuclear leukocytes
PO	Phosphodiester
PRR	Pattern recognition receptors
PS	Phosphorothioate
RANTES	Regulated on activation normal T cell expressed and secreted
RT	Reverse transcriptase
SIV	Simian immunodeficiency virus
SU	Surface glycoproteins
TCR	T cell receptor
T_H cells	T helper cells
TI	Treatment interruption
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TM	Transmembrane glycoproteins
T_{reg} cells	Regulatory T cells
WBS	Whole blood stimulation

1 AIM

The overall objectives of this thesis were to examine if anti-human immunodeficiency virus (HIV) responses can be triggered by innate immune stimulation and how HIV-1 and HIV-2 infections influences the responsiveness of immune cells to toll-like receptor (TLR) agonists.

Specifically:

- To examine the effects of CpG and cholera toxin B subunit (CTB) conjugated to CpG (CTB-CpG) on *in vitro* HIV replication in peripheral blood mononuclear cells (PBMCs) (**Paper I**)

- To analyze innate stimuli responsiveness by assessing the expression of innate cytokines in whole blood after TLR-agonist stimulation and to relate this to the type of HIV infection, HIV-1 or HIV-2, the degree of immunocompetence, and viral load (**Paper II**)

- To evaluate the levels of microbial translocation in HIV-2-infected individuals, as compared with HIV-1-infected individuals and to relate these to viral load, CD4+ T cell count and innate stimuli responsiveness (**Paper III**)

- To study how innate immunity, including responses to TLR stimuli and frequencies of circulating dendritic cell (DC) populations, and chronic immune activation in HIV-1-infected patients are affected by short-term viremia, as a result of treatment interruption (**Paper IV**)

2 THE IMMUNE SYSTEM

The immune system is our defence against invasion of foreign agents, such as microorganisms. Immune responses are activated by the recognition of foreign agents and the elicitation of various molecules from different cells within the immune system. This activation leads to a cascade of events where the different cells communicate and collaborate with each other to eliminate the foreign agents from the body.

Although the aim of the immune system is to defend us, it can, in some situations, cause more harm than aid and consequently lead to different diseases. One example is when immune responses are made against environmental agents, such as food, and initiate allergic and hypersensitive reactions. Other types of unwanted immune responses occur when the immune system reacts to antigens from the cells and tissues of one's own body, termed autoimmunity, or to transplanted organs, termed graft rejection. In addition, some microbial pathogens have found ways to take advantage of the immune system for survival. There are pathogens that target the immune cells for infection and can use the activation of the immune system to recruit more cells for reproduction. Other pathogens can use different molecules on the surface of immune cells to be transported to the site where more target cells can be found for infections. Pathogens have also found strategies to evade the immune system to survive within their host.

The immune system is divided into two parts, the innate and the adaptive immune system. The innate immune system represents the primary defence mechanism against invading pathogens, acting within hours after infection, whereas the adaptive immune system acts as a second line of defence after the initial pathogen encounter. The adaptive immune response is established days after the infection and develops a memory response so that, during a second encounter, it can act faster. In addition, these two systems interact, i.e., cells or components of the innate immune system influence the adaptive immune system and vice versa by cell-to-cell contact or cytokine signals for positive or negative feedback [26, 165, 182].

2.1 INNATE IMMUNITY

The skin acts as the first line of protection against invading pathogens with the help of epithelial cells, which form a barrier that is difficult to penetrate. The mucous membrane barriers in the respiratory, gastrointestinal, and genital tracts are also physiological barriers where pathogens are trapped and processed by the cells within the epithelial layer of the mucosa. The chemical barriers that inhibit the growth of microbes include the low pH in sweat, gastric and female genital secretions, and fatty acids in sweat [26]. Another component of the innate immune system, complement, carries out its function after activation by pathogens or antibodies, where several serum proteins react via enzymatic cascades, leading to lysis of the pathogen. Because of its interaction with antigens and antibodies, complement also plays a role in the adaptive immune system. The innate

immune system also includes the inflammation response, during which various cells are recruited to the site of infection (or injury) by the release of chemokines, such as CXCL8, CCL3 and 4 (also known as macrophage inflammatory protein (MIP)-1α and MIP-1β) and CCL5 (also known as regulated on activation normal T cell expressed and secreted, RANTES), resulting in the destruction of pathogens by different anti-microbial approaches. Different cells also express a variety of molecules with anti-microbial qualities that are considered as part of the innate immune response. One group of these molecules is the anti-microbial peptides (AMP), such as cathelicidin LL-37 and the α- and β-defensins. AMPs are cationic peptides that can bind to the negatively charged membranes of microbes, disrupting the membrane and resulting in lysis of the microbe [67, 124]. Natural killer cells (NK cells) recognize virus-infected cells and tumor cells based on down-regulation of major histocompatibility complex (MHC) class I and lyse the cells by releasing different cytotoxic molecules. Natural killer T cells (NKT cells) express markers of both NK cells and T cells and can interact with the antigen-presenting molecule, CD1d on antigen presenting cells (APCs), and recognize glycolipids. Studies have shown that mice lacking either NKT cells or CD1d are more susceptible to infections by various bacteria and viruses [248]. NKT cells also have cytotoxic qualities, similar to NK cells. The γδ T cells are a subset of T cells having a γ chain and δ chain in their T cell receptor (TCR). The γδ T cells are found mostly at epithelial surfaces where they can recognize conserved non-peptide antigens induced by stressed cells in an MHC-independent manner [37]. The neutrophil-, basophil-, and eosinophil-granulates are polymorphnuclear leukocytes (PMNs) that can, together with mast cells, release histamines along with other specific functions that eventually destroy the pathogen. In addition to killing of extracellular pathogens, PMNs, especially neutrophils, can also kill microbes by phagocytosis. Phagocytosis is an action during which cells engulf the pathogen and destroy it. Other cells that also are capable of phagocytosis of pathogens are the monocytes and macrophages. Another important feature of the monocytes and macrophages is their ability to act as APCs by presenting antigen to T cells, thus linking the innate and adaptive immune system [26, 165, 182]. Dendritic cells (DCs) are professional APCs that can activate naïve T cells and regulate T cell responses [250]. By capturing antigen, DCs mature and become activated by producing different cytokines and expressing co-stimulatory molecules, such as CD80 and CD86. DCs derive from two different progenitor cells (myeloid or lymphoid), which differentiate into myeloid DCs (mDCs) expressing CD11 and plasmacytoid DCs (pDCs) lacking myeloid markers including CD11. The myeloid derived DCs are present in various tissues and compartments, and can be discriminated on their expression of different surface markers [227]. The skin contains Langerhans cells (LC) in the epidermis and interstitial DCs (intDCs) in the dermis and different mucosal tissues and organs also contain mDCs. In addition, mDCs together with pDCs circulate in the peripheral blood and secondary lymphoid tissue.

The interaction of cells with pathogens also results in the production and release of cytokines with anti-microbial activity. Interferons (IFN), which are more or less produced

by all cells after virus encounter, are cytokines with antiviral activity. At the time of infection, cells secreting IFN as well as the affected neighbouring cells will enter into an antiviral-state, in addition to activating other immune cells such as the NK cells, resulting in inhibition of the infection [52, 53, 182]. There are three types of IFNs in humans based on their location on the chromosome and which receptor they signal through. Type I IFNs, mainly IFN-α (produced at high concentrations by pDCs [91, 162]) and IFN-β, in addition to type III IFN, including IFN-λ, are one of the innate immune system's most important cytokines, as they are produced and act rapidly after viral infection [40, 52, 53]. Type II IFN, IFN-γ, is produced only by the immune cells, such as T cells and NK cells, and has a more central role during the adaptive immune responses. Interleukin (IL)-12, another cytokine that plays an important role in innate immune responses, is secreted mainly by mDCs upon stimulation. IL-12 is an immunoregulatory cytokine that induces T helper 1 (T_H1) responses [267]. Other essential cytokines produced by cells within the innate immunity are IL-1, IL-6, IL-10, granulocyte macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor alpha (TNF-α) [182]. Although the innate immune system is relatively non-specific, it is able to recognize different pathogen-associated molecular patterns (PAMP), from microbes, or damage-associated molecular patterns (DAMP), released from damaged cells, through a set of transmembrane pattern recognition receptors (PRRs) [239]. PRRs include the scavenger receptors, carbohydrate receptors, mannose receptors, RIG-I like receptors, NOD-like receptors and Toll-like receptors (TLRs) [165, 182].

2.1.1 Toll-like receptors

To date, ten TLRs have been identified in humans, with each TLR recognizing a distinct PAMP/ ligand (Fig. 1, Table 1) [127, 149, 219, 227, 239].

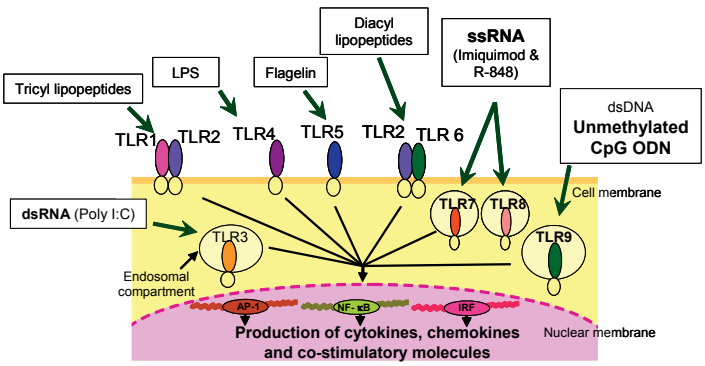

Figure 1. Toll-like receptors and their ligands (agonists).

TLR2 forms a heterodimer with either TLR1 or TLR6, resulting in the recognition of different types of lipopeptides and carbohydrates on bacterial cell wall. TLR4 recognizes the lipopolysaccharide (LPS) in the Gram-negative bacterial cell wall, and TLR5 recognizes flagellin on bacteria. Unlike the TLR1, 2, 4, 5, and 6, which are located on the cell surface, TLR3, 7, 8, and 9 are localized in endosomal compartments where they can recognize

different nucleic acid derivates associated with viral infections. TLR3 recognizes double-stranded RNA, TLR7 and TLR8 recognizes single-stranded RNA, and TLR9 recognizes unmethylated cystidine-phosphate-guanosine (CpG) DNA [45, 148]. TLRs are expressed on/in various cell types, including the APCs (Table 1) [127, 149, 219, 227, 239]. The most common response upon TLR activation is the expression of different cytokines and up-regulation of co-stimulatory molecules such as CD80 and CD86 on DCs, triggered by binding of the PAMP to the TLR (Fig. 1) [250]. The cytokines produced can, in turn, activate the adaptive immune system. Triggering of TLRs initiates signalling pathways leading to activation of the transcription factors including nuclear factor (NF)-κB, activator protein 1 (AP-1) and interferon regulatory factors (IRFs), resulting in cytokines production. All TLRs, except for TLR3, induce their signalling through the adaptor protein myeloid differentiation factor 88 (MyD88), which in turn will recruit IL-1 receptor associated kinase (IRAK) and activate TNF receptor associated factor (TRAF). TLR3, and sometimes TLR4, instead, uses toll/IL-1 receptor domain containing adapter inducing interferon-β (TRIF) for signalling [137, 149, 219].

Table 1. TLR expression* on immune cells and their ligands/ agonist for stimulation [127, 149, 219, 227, 239]

TLR	Location of TLR	PAMPS/ Agonists	Cell type
TLR1	Plasma membrane (Cell surface)	Triacyl lipopeptides (in combination with TLR2)	DCs, B cells, neutrophils, eosinophils, mast cells, monocytes/macrophages,
TLR2	Plasma membrane (Cell surface)	Lipopeptides, Peptidogycans, lipoarabinomannan	mDC, pDC PMNs, mast cells, monocytes/macrophages, B cells, NK cells
TLR3	Endosomes	dsRNA, poly I:C	mDC, mo-DC, NK cells
TLR4	Plasma membrane (Cell surface)	LPS	mDC, mo-DC, PMNs, monocytes/macrophages, B cells,
TLR5	Plasma membrane (Cell surface)	Flagellin	mDC, mo-DC, neutrophils, monocytes/macrophages, NK cells, T cells
TLR6	Plasma membrane (Cell surface)	Diacyl lipopeptides (in combination with TLR2)	mDC, mo-DC, B cells, neutrophils, eosinophils, mast cells, monocytes/macrophages,
TLR7	Endosomes	ssRNA, Imiquimod, R-848	DC, neutrophils, eosinophils, B cells,
TLR8	Endosomes	ssRNA, R-848	mDC, mo-DC, neutrophils, monocytes/macrophages, NK cells, T cells
TLR9	Endosomes	Unmethylated CpG ODN, dsDNA	pDC, B cells, NK cells neutrophils, eosinophils, monocytes/macrophages,
TLR10	Plasma membrane (Cell surface)	Unknown	mDC, pDC, B cells, neutrophils, eosinophils, monocytes/macrophages,

*TLRs expressed either as mRNA or protein.
LPS: lipopolysaccharide, PMN; polymorphnuclear leukocytes, DC; dendritic cells, mDC; myeloid DC, pDC; plasmacytoid DC, Mo-DC; monocyte derived DC, NK cells; natural killer cells

2.1.1.1 TLR7/8

TLR7 and TLR8 are expressed by DCs (TLR7 mainly by pDCs and TLR8 by mDCs), monocytes, B cells, NK cells, and T cells and are localized intracellularly [25]. TLR7 is activated by the synthetic imidazoquinoline compounds imiquimod and resiquimod (R-848) [113], while TLR8 is stimulated only by R-848 [131]. In addition to the imidazoquinoline compounds, single-stranded RNA (ssRNA), including HIV-derived ssRNA, has been shown to activate cells through TLR7 in mice and TLR8 in humans [70, 112]. Stimulation through TLR7/8 results in activation of cells and, depending on which cell type is activated, production of various cytokines. The most important antiviral cytokines expressed after TLR7/8 stimulation are type I and type III interferons [264], and IL-12, although other cytokines, such as TNF-α and IL-1, can also be produced.

2.1.1.2 TLR9

In humans, TLR9 is mainly expressed in B cells and pDCs, although studies have shown that other immune cells, glioma cells, as well as some epithelial cells may also express TLR9 [16, 80, 178]. Similar to TLR7/8, TLR9 is located within the endosomal compartments of the cells [25]. Microbial DNA and oligodeoxynucleotides (ODN) containing CpG motifs were shown to be ligands for TLR9. There are three classes of CpG-ODN in humans (Table 2): CpG A induces high levels of IFN-α by stimulating pDCs, CpG B is a strong stimulator of B cell Ig production and also induces the maturation of pDCs, and CpG C, exhibiting a combination of CpG A and B properties, is capable of stimulating both pDCs and B cells [147, 258]. In addition to the production of type I IFNs, stimulation with CpG ODN induces the production of IL-6, IL-12, and TNF-α. Because of its immunostimulatory properties, CpG ODNs have been used for the development of immunotherapy and vaccine adjuvants [108, 114]. Mucosal administration of CpG has been shown to increase both innate immune responses, such as the CC chemokines, as well as antigen-specific immune responses.

Table 2. Sequences of different unmethylated CpG ODN motifs.

CpG ODN	Sequens	Cell types	Function
CpG A	GGG GGA CGA TCG TCG GGG GG	pDC	Induces high levels of IFN-α
CPG B	TCG TCG TTT TGT CGT TTT GTC GTT	B cells	Strong stimulators of B cells
CpG C	TCG TCG TCG TTC GAA CGA CGT TGA T	pDCs and B cells	Mixture of A and B

2.2 ADAPTIVE IMMUNITY

The adaptive immune system is activated by an encounter with pathogens, either presented by APCs or soluble antigens. When the antigen is first presented to the adaptive immune system, a series of reactions, including different numbers of cells and molecules, is set off to eventually develop a specific response against the pathogen. The second time the host has been invaded by the same pathogen, the adaptive immune system will react rapidly and

more effectively because a specific memory response has developed against that specific antigen after the first encounter. The adaptive immune responses include cellular immune responses, involving T cells and humoral immune responses, involving B cells [26, 165, 182].

2.2.1 Cellular immune responses

Adaptive cellular responses are activated when APCs present antigens to T cells in the lymph nodes. Antigens are presented by MHC molecules, referred to as human leukocyte antigen (HLA) in humans, to T cell receptors (TCRs) on T cells. The MHC class I molecules, HLA-A, HLA-B, HLA-C and HLA-E, present short endogenous peptides, which can be derived from viruses, to cytotoxic T cells (CTLs, CD8+ T cells). The MHC class II molecules, HLA-DP, HLA-DR and HLA-DQ, present longer peptides, derived from the extracellular environment, to helper T cells (T_H cells, CD4+ T cells). In addition to the MHC-TCR interaction, additional signals are needed between a T cell and an APC, such as binding of CD80/CD86 on APCs to CD28/ cytotoxic T lymphocyte antigen-4 (CTLA-4) on T cells, as well cytokine/ chemokine signalling from APCs to T cells (Fig. 2). When T_H cells have been activated, they start secreting effector molecules, such as cytokines, to activate surrounding lymphocytes, including the cytotoxic activity of CTLs, phagocytosis of macrophages and antibody secretion by B cells. T_H cells are divided into T_H1, T_H2 and T_H17 cells. T_H1 cells produce the IL-2, IFN-γ, GM-CSF and TNF-α cytokines and mediate protection against intracellular pathogens by the activation of CTLs, induction of isotype switching (see below) and activation of microbicidal mechanisms in macrophages. T_H2 cells secrete the IL-4, IL-5, IL-10 and IL-13 cytokines to induce protection against extracellular pathogens, such as parasitic worm infections, by mediating isotype-switching into IgE production (see below). T_H17 cells, which mainly produce the cytokines IL-17 and IL-22, are involved in protection against infections at mucosal sites but also play a role in autoimmune diseases [76, 146, 245]. Another group of CD4+ T cells are called regulatory T cells (T_{reg}) that act by suppressing the activation of DCs and T cells, by the production of IL-10 and transforming growth factor (TGF)-β. The function of CTLs is to kill pathogen infected cells, by inducing apoptosis in infected/damaged cells through the secretion of cytotoxic effector molecules, to inhibit the replication and spreading to other cells and tissues [26, 165, 182].

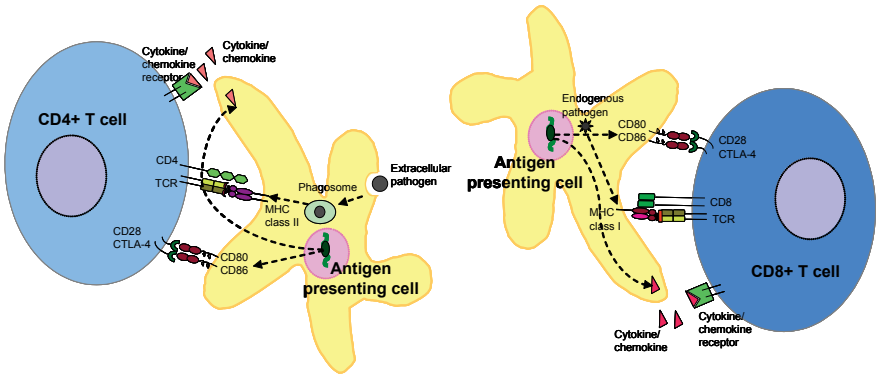

Figure 2. Schematic view of APC interactions with T cells.

2.2.2 Humoral immune responses

Adaptive humoral immune responses are initiated when B cells have been activated either by binding of the B cell receptor (BCR) to an antigen or by T_H cells. Upon activation, B cells differentiate into plasma cells and start secreting BCRs, also called antibodies or immunoglobulins (Ig), which bind to antigens and eliminate pathogens. The effector functions of antibodies are as follows: neutralization, where an antibody binds to an antigen and inhibits it from attaching to the target cells; opsonisation, where an antibody coats an antigen/ pathogen and enhances phagocytosis; and coating of antigens to activate the complement system. Infected cells expressing antigens bound to antibodies can also be eliminated by antibody-dependent, cell-mediated cytotoxicity (ADCC), which is triggered by the binding of the Fc region of an antibody to Fc receptors (FcRs) on NK cells and activating the cytotoxic mechanisms of the NK cell. There are five isotypes of Ig: IgM, IgA, IgG, IgE and IgD. B cells initially express IgM, but after stimulation and differentiation, the cells undergo isotype-switching, leading to expression and secretion of new isotypes from the plasma cells. The different isotypes are present in diverse compartments of the body and vary in their functions. IgM is the primary antibody response to pathogens encountered for the first time and is found at higher concentration during the early phases of an infection. In addition to IgM, IgD is also found on the membranes of inactivated B cells and helps in the differentiation of B cells. IgG is found at the highest concentration in the peripheral blood and its main tasks are opsonisation of antigens and activation of complement. In addition, IgG can also cross the placenta to the fetus and help protect the newborn baby against infections. IgA is mainly found at mucosal surfaces and in body secretions, including genital secretions, tears, saliva and breast milk. IgE acts together with mast cells and is involved in the defence against parasitic infections and interact with allergens [26, 165, 182].

3 HIV

In 1980, physicians in the United States noted that young homosexual men were suffering from opportunistic infections along with immune suppression [3, 4, 104, 105, 123]. Shortly thereafter, reports described these symptoms in other groups, such as intravenous drug users and patients with hemophilia, leading to the decision that these symptoms would be called acquired immune deficiency syndrome (AIDS) [8, 172]. In the search for the infectious agent responsible for AIDS [1] a new retrovirus was isolated from the lymph node of an AIDS patient by French scientists in 1983 [24]. In 1984, an American research group also published their studies on a new retrovirus in AIDS patients [99, 200, 220, 228]. Both the French and the American groups also showed that the newly discovered retrovirus infected CD4-expressing T-cells [144, 200], suggesting its involvement in immune suppression and AIDS. It was later acknowledged that the viruses identified by the different groups were in fact the same [204, 253], and in 1986, a new name was suggested by the International Committee on the Taxonomy of Viruses: human immunodeficiency virus (HIV) [56]. In 1986, another human retrovirus, related to but distinct from the previously discovered AIDS virus, was isolated from West African individuals [55, 134] and later named HIV-2. HIV-1 is present worldwide and responsible for the HIV pandemic [7, 174, 238], whereas HIV-2 is mostly found in West Africa, Portugal, and countries and regions formerly colonized by the Portuguese [206, 238]. By the end of 2008, about 33.4 million people were living with HIV, with the highest prevalence in sub-Saharan Africa [7]. Since its discovery more than 25 million people have died due to HIV, and the pandemic spread of the virus is continually growing. The first case of AIDS in Sweden was reported in 1982 [5] and by the end of December 2009 a total of 8935 HIV-infected individuals have been identified in Sweden [6].

3.1 ORIGIN OF HIV

Shortly after the discovery of HIV, non-human primate retroviruses were identified and named simian immunodeficiency viruses (SIVs), followed by the suggestion that HIV could have arisen from SIV through cross-species transmission [256]. More than 40 different primate species, all originating from African, have been shown to be infected with SIV. HIV-1-related SIVs have been identified in chimpanzees, *Pan troglodytes troglodytes* (SIVcpz*Ptt*) [100], and western gorillas, *Gorilla gorilla* (SIVgor) [255], while HIV-2 has been related to SIVsm from the sooty mangabey monkey, *Cercocebus atys* (Fig. 3, Table 3) [115]. HIV-1 consists of three different genetic groups, M (major), O (outlier) and N (non-M/non-O), indicating that HIV-1 was introduced into the human host on three occasions. HIV-1 group M, which clusters with SIVcpz*Ptt* from the chimpanzee population in Cameroon, is responsible for the current pandemic [238, 256]. HIV-1 group M is further classified into nine different subtypes (A-D, F-H, J and K). In addition to the subtypes, circulating recombinant forms (CRFs) have also been identified due to recombination between different subtypes. Since SIVsm display genetic similarities to HIV-2 (Fig. 3) and the sooty mangabey is a resident of West Africa, HIV-2 is believed to have originated from

the same area. HIV-2 was transferred into humans on several occasions resulting in groups A-H, which are as diverse as the HIV-1 groups M, N and O. However, to date, only groups A and B are accountable for the HIV-2 epidemic, with group A circulating in western West Africa and group B in central and eastern West Africa [238, 256]. Guinea-Bissau, a country in West Africa, has had the highest prevalence of HIV-2 with a peak during the late 1990s (Table 3) [60, 155, 166, 212].

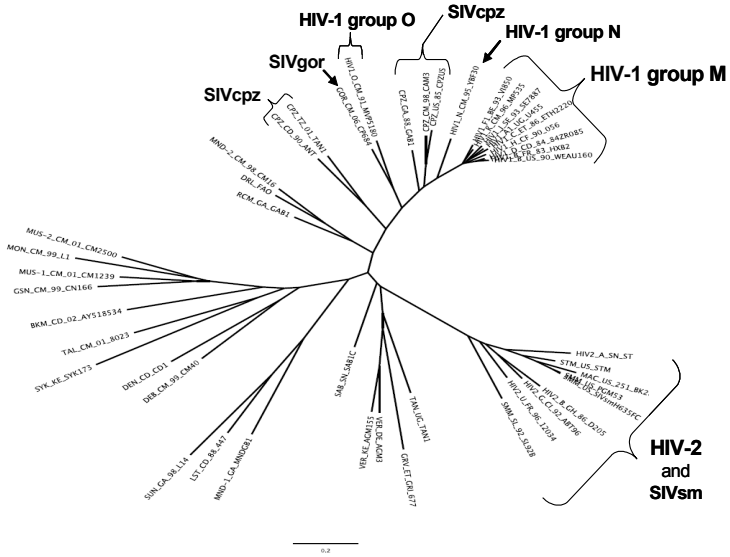

Figure 3. Phylogenetic tree showing the relationship between HIV and SIV strains, based on the *pol* gene. Kindly provided by Helena Skar.

3.2 HIV STRUCTURE

HIV-1 and HIV-2 belong to the *Retroviridae* family and the *Lentivirinae* genus, which are enveloped plus-stranded RNA viruses. The virus particles are spherical with a diameter of approximately 100nm and contain two identical single-stranded RNAs that are surrounded by structural proteins that form the nucleocapsid. The lipid envelope enclosing the particle is derived from the host cell membrane and contains viral envelope glycoproteins in addition to host cell proteins. Like all retroviruses, HIV-1 and HIV-2 replicate through a DNA intermediate, during which time the viral DNA is integrated into the host genome [94, 183].

3.2.1 HIV genome and proteins

The HIV genome (Fig. 4), like other retroviruses, consists of a promoter gene called the long terminal repeat (LTR). The LTR is situated in the 5' and 3' region of the virus genome and is involved in the integration of the viral DNA into the host genome. Other HIV genes that are also common among other retroviruses are *env*, *gag*, and *pol* [158, 183].

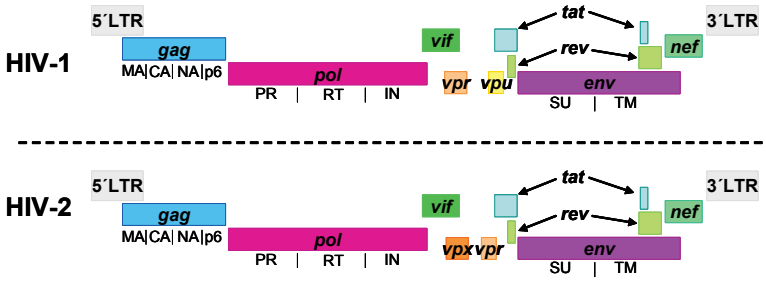

Figure 4. Genomic organization of HIV-1 and HIV-2. Modified from [158].

The *env* gene encodes the precursor envelope glycoproteins gp160 in HIV-1 and gp140 in HIV-2 (Fig. 5). The glycoproteins are cleaved into gp120 (HIV-1)/ gp125 (HIV-2) and gp41 (HIV-1)/ gp36 (HIV-2). gp120/gp125 are the surface glycoproteins (SU) that mediates virus binding to the host cell surface receptors. gp41/gp36 are the transmembrane glycoproteins (TM) and contain two α-helical regions and a fusion peptide [103, 206]. *Gag* encodes four proteins: matrix, capsid, nucleocapsid, and p6 [96, 158]. The matrix protein (MA; p17) is involved in assembly of the HIV particle, viral entry, and infection of non-dividing cells [111]. The capsid protein (CA; p24 for HIV-1 and p26 for HIV-2) encloses the viral genome and enzymes. The nucleocapsid protein (NC; p7) and p6 protect the viral RNA during assembly and virus budding. The *pol* gene encodes the following viral enzymes: protease (PR), reverse transcriptase (RT), and integrase (IN). PR cleaves the Gag and Gag-Pol precursor proteins into their sub-proteins. The RT enzyme has three enzymatic functions: (i) RNA-dependent DNA polymerase, (ii) RNase H domain and (iii) DNA-dependent DNA polymerase activity. The function of IN is to integrate the synthesised double-stranded proviral DNA into the host genome [96, 158, 183]. In addition to the *gag, pol* and *env*, HIV contains genes encoding two regulatory proteins, Tat and Rev, and four accessory proteins, Nef, Vpr, Vif, Vpx (in HIV-2), and Vpu (in HIV-1). The Tat protein plays an important role in the *trans*-activation of HIV transcription, and the Rev protein is required for stabilisation and transport of viral messenger RNA (mRNA) from the nucleus to the cytoplasm [96, 158, 229, 237]. The Nef protein has several functions of which the most important are the downregulation of CD4 receptor and MHC-I molecules, leading to immune evasion of the infected cell. Nef from HIV-2, but not HIV-1, has also been shown to down-regulate TCR-CD3, which, in turn, suppresses T cell activation [222]. The functions of HIV-1 Vpr include delay or cell cycle arrest and apoptosis. In addition, the Vpr of HIV-1 mediates the transport of the HIV pre-integration complex (PIC) into the host cell nucleus. In HIV-2, Vpr induces cell cycle arrest, while Vpx, which is expressed only in HIV-2, is responsible for the import of PIC into the nucleus [93]. The Vif protein interacts with and neutralizes the intracellular anti-HIV protein apolipoprotein B mRNA-editing enzyme catalytic polypeptide 3G (APOBEC3G), that otherwise may cause hypermutations. The Vpu protein (expressed only in HIV-1) is involved in release of the virus particles and degradation of CD4 in the

endoplasmic reticulum (ER). The HIV-2 counterparts of Vpu are represented by two different domains within the Env protein involved in virus release (Fig. 5) [9, 39].

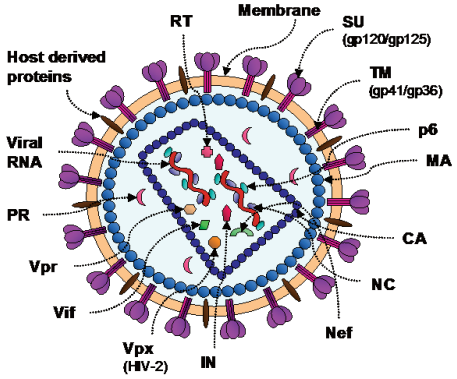

Figure 5. Structure of HIV.

3.3 HIV REPLICATION

3.3.1 Receptors and cell tropism

Early observations of CD4+ T cell depletion during AIDS suggested CD4 to be the likely receptor for HIV [62, 144, 145, 200]. It is now well established that CD4 is the cell surface receptor utilized for entry of both HIV-1 and HIV-2, explaining why the T_H lymphocytes (CD4+ T cells) are target cells for HIV. In addition, cell types such as macrophages, monocytes, microglia, and different subsets of DCs can also be infected by HIV. Subsequent observations demonstrated that additional receptors were required for HIV target cell entry. The chemokine receptors CXCR4 and CCR5 were identified as the major coreceptors [14, 87], and viruses utilizing these receptors were classified as X4 or R5 viruses, respectively [28]. Soon after the discovery of CCR5 as a coreceptor, individuals possessing a 32-base-pair deletion in the CCR5 gene were reported to exhibit lower susceptibility to HIV-1 R5 infection [160, 216]. CXCR4 and CCR5 are also used by HIV-2, although HIV-2 is more promiscuous with regard to coreceptor usage, and reports have indicated that HIV-2 can enter cells via a CD4-independent pathway [206]. DCs are susceptible to HIV infection, however, virus replication in DCs appear to be relatively ineffective. It has also been reported that mDCs and pDCs are less susceptible to HIV-2 [78]. In addition, DCs can mediate *trans*-infection, in which HIV binds to DC expressed C-type lectins such as dendritic cell-specific ICAM-3 non-integrine receptor (DC-SIGN). During *trans*-infections, HIV may use the DCs in order to be transported from the mucosal tissues to the lymph nodes where it can spread more efficiently to T cells [102, 199].

3.3.2 Entry

Attachment of SU (gp120 in HIV-1 and gp125 in HIV-2) to the CD4 receptor is a high affinity interaction for HIV-1 and a low affinity interaction for HIV-2 [54, 158, 206]. Once SU has attached to the CD4 antigen, the envelope glycoprotein complex undergoes

structural changes, allowing it to interact with the coreceptor, e.g., the chemokine receptor. Coreceptor binding results in additional conformational changes in SU, allowing the TM (gp41 in HIV-1 and gp36 in HIV-2) to unfold, insert its hydrophobic N-terminal fusion peptide into the cell membrane, and facilitate fusion of the virus and target cell membranes.

3.3.3 Reverse transcription and integration

Once inside the cytoplasm, the capsid breaks open and the viral RT enzyme makes a cDNA copy of the viral RNA genome via its reverse transcriptase activity. This process of reverse transcription is extremely error-prone, since the RT activity lacks a proof-reading mechanism, and it is during this step that mutations may occur. Additionally, during cDNA synthesis, RT can "jump" from one viral RNA strand to the other, leading to recombined DNA if the RNA strands are not identical [242]. As a consequence, many HIV variants, so called quasi-species, may develop in an individual. As cDNA is formed, RT degrades the RNA strand via its RNase H activity. A complementary DNA strand is then synthesized by the DNA polymerase activity of RT. The cDNA, as a component of PIC, is transported into the host cell nucleus. IN then cleaves the cellular DNA and integrates the ends of the viral DNA into the host DNA. At this point, the integrated viral DNA is now referred to as proviral DNA. Following integration, the proviral DNA may remain latent [94, 183].

3.3.4 Transcription, translation and assembly

Upon host cell activation, the integrated viral DNA can be transcribed into viral mRNA by the host cell transcription machinery. The earliest transcribed mRNAs are doubly spliced and encode for regulatory proteins. Tat up-regulates the production of viral RNA. Singly or unspliced viral mRNA is then transported into the cytoplasm after binding Rev. Viral mRNA is translated in the cytoplasm of the cell, yielding viral structural proteins. CA assembles Gag-Pol precursor proteins and, with the aid of the MA domain of Gag assembly of the virus proteins takes place at the plasma membrane of the cell. The Env glycoproteins, TM and SU, are inserted into the host cell membrane and the virion is released by budding. During or after release of the virus particle, PR cleaves the Gag and Gag-Pol precursor proteins into their active sub-proteins, making the virus infectious [94, 183].

3.3.5 HIV disease course

The pathogenesis of an HIV infection depends on several factors including the virus, e.g., the level of virulence and the quantity of viruses in the infected individual, and the host, e.g., age, genetic differences, immune responses and environmental factors. Co-infection with other pathogens can also affect the rate and severity of the disease progression [158].

The process of disease progression takes approximately 10 years without treatment in HIV-1-infected individuals (Fig. 6), although there are reports of those individuals who can

control the virus and delay disease progression, so-called long-term-non-progressors (LTNPs) or elite controllers (EC). But there are also individuals who progress to AIDS within two to three years after infection, termed rapid progressors [193]. Although HIV-1 and HIV-2 are biologically similar, HIV-2 is less transmissible, and HIV-2 infected individuals take a longer period of time to develop AIDS (Table 3) [17, 126, 135, 168, 201]. Why HIV-2 is less pathogenic still remains largely unknown.

3.3.6 HIV transmission routes

The main route of HIV infection is through sexual contact, with heterosexual activity being the major cause [2, 158]. Sexual transmission of HIV occurs when HIV-infected body fluids, such as semen, vaginal secretions or blood, containing either free virus particles or virus-infected cells, come in contact with the genital, oral, or rectal mucosa. As HIV is present in the blood, transmission of the virus can also occur by this route, via blood transfusions, or blood products, intravenous drug use, or accidents in health care or laboratory work environments. Mother-to-child transmission (MTCT) is an additional route of HIV transmission. This type of transmission usually takes place during either late pregnancy or at the time of delivery. Caesarean section and treatment with antiretroviral drugs during pregnancy can, however, decrease MTCT HIV transmission. Children can also be infected by their mothers through the breast milk, although breast milk has been shown to also contain antiviral factors [158].

3.3.7 Acute phase

At the site of infection in the mucosa, it is thought that, beside infection of CD4+ T cells directly, HIV can be taken up by DCs and presented to CD4+ T cells, which are subsequently infected [102, 116]. The virus-engaged DCs or infected CD4+ T cells then migrate to the lymph nodes, where further HIV infection of CD4+ T cells occurs. Primary HIV infection (acute phase) is followed by a burst of viremia, which in some cases is accompanied by flu-like symptoms. The number of CD4+ T cell targets, mainly those in the gut-associated lymphoid tissue (GALT), decreases rapidly [106, 132, 198]. However, after the increase in innate and HIV-specific adaptive immune responses, the peripheral CD4+ T cells are recovered, and the viremia is reduced to a level called the set point, which is predictive of disease progression [177]. The infection then enters its asymptomatic chronic phase.

3.3.8 Chronic phase

During the chronic phase the immune system of HIV-1-infected individuals is constantly active believed to be due to high viral turnover (10^9 virus particles/ day [117]) resulting in general immune activation. During the chronic phase, HIV-specific immunity, including CTL and neutralizing antibodies (nAb), may partially control the virus. However, due to integration of HIV provirus into the cellular DNA and high antigenic variation resulting in constantly emerging escape variants, the infection can persist. The chronic phase is also

accompanied by a gradual loss of CD4+ T cells, dysregulated B-cell responses and defect responsiveness of several innate and adaptive immune functions [65, 74, 158].

3.3.9 AIDS

AIDS occurs when the number of CD4+ T cells drops below 200 cells per µl. At this point, opportunistic infections, due to microbes such as *Pneumocystis*, *Candida, Toxoplasma*, and *Mycobacterium*, as well as different herpes infections and rare cancers like Kaposi's sarcoma, are observed. Thus, upon the depletion or inefficiency of important immune cells, the virus and infectious microbes take over, and the patient eventually dies if left untreated.

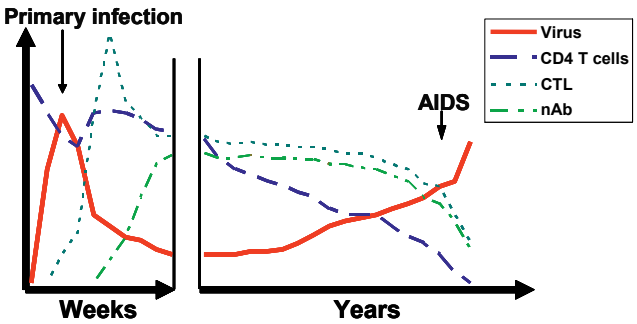

Figure 6. Course of an HIV infection.

3.4 HIV AND IMMUNE DEFENSE

The host immune responses against HIV, as for other microbes, are mediated via collaboration between innate and adaptive immune responses, including humoral and cellular immunity.

3.4.1 Innate immunity in HIV

During recent years, the role of the innate immune system in HIV infection has been acknowledged, and several mechanisms have been shown to elicit anti-HIV activity. These can be categorized into three groups: cellular, extracellular, and intracellular.

Cells comprising innate anti-HIV immunity include NK cells, NKT cells, γδ T cells and APCs, such as DCs and monocytes. NK cells target and lyse cells that exhibit down-regulated MHC-I molecules, including virus infected cells, and especially the MHC-I alleles HLA-C and HLA-E can inhibit NK cell lysis [165]. The Nef protein of HIV down-regulates HLA-A and HLA-B, but not HLA-C and HLA-E, thereby partially evading the NK cell lysis [57]. Both NK and NKT cells can also suppress viral replication as result of their anti-HIV properties, such as the production of β-chemokines and other cytokines [251]. Similar to NK cells, the γδ T cells possesses cytotoxic activities and, furthermore, can also produce several anti-HIV cytokines and chemokines [37].

The extracellular innate anti-HIV components include proteins involved in the complement system and a variety of cytokines such as the type I interferons, as well as the antimicrobial peptides. Chemokines can also be considered as part of innate anti-HIV immunity, especially those that are natural ligands of the HIV coreceptors. Thus, binding of their respective receptor will block the attachment of HIV to the cell and inhibit infection. MIP-1α, MIP-1β and RANTES are the β-chemokines being the natural ligands of CCR5 [14], while the α-chemokine stromal cell derived factor 1 (SDF-1) is the natural ligand of CXCR4 [33]. In addition to the production of β-chemokines, CD8+ T cells have non-cytotoxic anti-HIV effects through molecules referred to as CD8 antiviral factor (CAF); the characteristics of these molecules, however, remain elusive [232].

Apolipoprotein B mRNA-editing enzyme catalytic polypeptide 3G (APOBEC3G) is an intracellular protein that is packaged into virions and transferred to subsequent cells where it causes deamination and hypermutations of the retroviral DNA making the virus non-infectious [158]. The Vif protein of HIV can neutralize APOBEC3G such that it can no longer be incorporated into virus particles. However, it has been reported that the Vif protein of HIV-2 is less active against APOBEC3G than Vif of HIV-1 [208]. Another intracellular protein, tripartite motif protein 5α (TRIM-5α), has been shown to inhibit HIV infection after virus cell entry by interacting with the capsid protein [158]. The TRIM-5α of rhesus monkey cells can block HIV replication, although human TRIM-5α has little effect on HIV-1 and HIV-2 replication [266]. Lentivirus restriction factor 2 (Lv2) is a similar restriction factor shown to mainly inhibit the replication of HIV-2 by interacting with the envelope and capsid proteins, although it is also capable of blocking HIV-1 [167].

3.4.1.1 Toll-like receptors and HIV

TLR stimulation mediates different anti-HIV activities, such as production of cytokines and expression of co-stimulatory molecules that induce adaptive immune responses. TLR7/8-triggered anti-HIV activity is mediated through the interaction of DCs with CD8+ T cells and NK cells and the production of different cytokines and chemokines [225]. Schlaepfer *et al* demonstrated the inhibiting activity of the TLR9 ligand CpG ODNs on *in vitro* HIV-1 replication [224]. In addition, several groups have reported enhanced HIV-specific immune responses when CpG ODN was used as an adjuvant combined or conjugated to the antigen [61, 77, 203]. However, in parallel, reports have revealed activation of HIV-1 replication in latently infected cells via TLR stimulation [20, 83, 221, 223]. Since stimulation of TLRs leads to activation of the NF-κB transcription factor, HIV LTR may, in turn, be stimulated in latently infected cells, implying that innate immune stimulation may lead to immune activation during different co-infections [20, 83, 221, 223]. During the viremic phase of HIV-1 infection, TLR2-4 and TLR6-8 mRNA expression has been shown to be augmented, and high levels of virus have been correlated with increased TLR responsiveness as measured by expression of pro-inflammatory cytokines [157]. However, in another study it was revealed that the secretion of IFN-α was defective while the pro-inflammatory responses were preserved, suggesting a differential impact of the

virus on the TLR triggered cytokine expression [214]. Furthermore, TLR stimulation has been shown to activate the memory and effector CD4+ and CD8+ T cells [98]. As a consequence the CD4+ T cells enter into cell cycle and apoptosis, which could contribute to increased pathogenesis during HIV infection [98]. Polymorphisms in the TLRs have been shown to affect the course of HIV-1 infection. Two single-nucleotide polymorphisms (SNPs) in the *TLR9* gene [36] and one in the *TLR7* gene were linked to faster disease progression [189], and an SNP in the *TLR8* gene was associated with slower HIV-1 progression, which could be due to its reduced ability to activate NF-κB [190]. To date, the function of TLR and HIV infection is mostly limited to studies of HIV-1 infection. However, one study suggests that HIV-2 envelope glycoproteins can suppress T cell activation via TLR4 triggering of monocytes [49].

3.4.2 Adaptive humoral responses

Antibodies against HIV can be detected 1-2 weeks after initial infection [158]. The antibody responses are directed against several viral proteins, including the Env and Gag proteins. Some of the antibodies are nAbs, since they can bind to the virus and inhibit the infection. The nAbs are directed against the Env proteins of HIV, including CD4 or coreceptor binding sites on SU and the membrane proximal external region of TM. However, HIV has adopted several mechanisms that allow it to escape from nAbs, such as masking of the receptor binding sites, glycosylation of the SU, and the high mutation rate leading to antigenic variation [122]. HIV-2 exhibits a less glycosylated V3 domain compared with HIV-1 [231]. This may enable better access of nAbs to HIV-2 and, in this way, more efficiently control viral replication. Furthermore, the frequency of autologous nAbs is believed to be higher, and the escape variants are believed to be fewer, in HIV-2-infected compared to HIV-1-infected individuals [30, 231]. It has also been reported that nAbs in HIV-2-infected individuals have a broader response compared to those of HIV-1-infected individuals, although, the nAb responses among HIV-1-infected persons were of a higher magnitude (Table 3) [211]. Both neutralizing and non-neutralizing antibodies can activate ADCC mechanisms in which different effector cells trigger events that lead to the lysis of the infected cell. The ADCC activity present in HIV-2-infected individuals appears to be broader and of higher frequency compared to that of HIV-1–infected individuals [161].

3.4.3 Adaptive cellular responses

Both HIV-specific CTL and T_H cell responses are important for viral control, and, among HIV-1-infected individuals, i.e. more polyfunctional HIV-specific responses have been noted in LTNPs and ECs [32]. Both HIV-1-specific and HIV-2 specific T cell responses target several epitopes in different virus proteins, although the Gag proteins appear to elicit the more potent responses [156]. The memory T cells (antigen-experienced cells) are subdivided into different types depending on their phenotype (surface markers); central memory (T_{CM}), mainly in the secondary lymphoid tissue; and effector memory (T_{EM}), circulating in the periphery [109]. Depending on the stage of HIV disease, different cellular

phenotypes can be found [109, 155]. Studies have also been performed to assess the function of HIV-specific responses depending on their release of various cytokines and chemokines. In HIV-1-infected individuals, polyfunctional CD4+ and CD8+ T cell responses have been associated with more benign disease progression, and HIV-2-specific T cells have been shown to be more polyfunctional than HIV-1-specific T cells (Table 3) [79]. Furthermore, it was recently reported that the HIV-2-specific CD8+ T cells were at an early stage of differentiation, which in turn can contribute to the longer viral control in HIV-2 infected individuals [154].

3.5 HIV AND IMMUNOPATHOGENESIS

Systemic immune activation during HIV infection has in several studies been shown to be an independent marker of disease progression [51, 68, 85, 88, 97, 164, 202, 215]. Constant activation of the immune system eventually leads to exhaustion of the immune system, which can be displayed by either a reduction in the number of peripheral leukocytes or functional abnormalities [34]. The systemic immune activation caused by HIV-2 has been reported to be reduced compared to HIV-1 and takes place during later disease stages, when viral loads are detectable in plasma [153, 179, 259].

3.5.1 Innate cellular defects during HIV infection

Both mDCs and pDCs can be infected by HIV-1, but they appear to be less susceptible to HIV-2 (Table 3) [78]. The number of circulating mDCs and pDCs is reduced and their responses are impaired during the course of HIV-1 infections [15, 140, 171, 235]. Similarly, the number of circulating pDCs has been shown to be reduced in HIV-2-infected individuals and could be associated with CD4+ T cell decline, as well as T cell activation [48]. In addition, the pDCs of viremic HIV-2-infected individuals also displayed reduced IFN-α production after TLR9 stimulation [48]. HIV-1-infected individuals demonstrate reduced numbers and diminished function of NK cells, while the NK cells of asymptomatic HIV-2-infected individuals display normal functions (Table 3) [188].

3.5.2 Adaptive cellular defects during HIV infection

The main characteristic of HIV infection is a decrease in CD4+ T cell numbers measured during the primary infection. In addition, functions of both CD4+ and CD8+ T cells have been shown to be dysregulated, including lower polyfunctional activity and decreased proliferation [34]. Furthermore, the expression of several activation markers on T cells of HIV-infected individuals have been shown to differ from HIV-negative individuals, such as upregulation of the activation markers HLA-DR and CD38 [34, 51]. However, in HIV-2-infected individuals with undetectable plasma viral load, the expression of HLA-DR or CD38 on CD4+ or CD8+ T cells does not differ compared to HIV-negative individuals [153]. Similar to upregulation of activation markers, CD4+ T cells in HIV-1-infected individuals have been shown to express higher levels of the inhibitory molecules CTLA-4 and programmed death-1 (PD-1), whereas HIV-1-specific CD8+ T cells only show increased expression of PD-1 [136]. The numbers of T_{reg} are similarly increased in both

HIV-1 and HIV-2-infected individuals [95] where they contribute to decreased HIV-specific T cell responses [230]. Reduced numbers of T_H17 cells have been detected in the GALT of HIV-1-infected individuals, which, in turn, has been suggested to lead to damage of the epithelial barrier of GALT due to decreased mucosal immune responses [41, 143]. Apart from T cells, B cells have also been reported to display signs of dysregulation, such as hyperactivation and differentiation [181].

3.5.3 Soluble markers of immune activation during HIV infection

Besides dysregulation of cells within the immune system, immune activation during HIV infection can be detected by the measurement of different soluble markers in the serum/ plasma of HIV-infected individuals. Beta-2 microglobulin (β_2M) is a component of MHC class I molecules and has been found to be increased in HIV-infected individuals as an indication of activated lymphocytes. HIV-2-infected individuals display lower levels of β_2M compared to HIV-1-infected individuals [153, 179]. Neopterin is another soluble marker of immune activation produced by activated macrophages, and its concentration is higher in both HIV-1- and HIV-2-infected individuals compared with HIV-negative individuals [85, 88, 97, 125, 197, 202, 215]. Hyperactivation of B cells can be measured as elevated levels of immunoglobulins, known as hypergammaglobulinemia, which was one of the first markers of activation to be reported in HIV-1-infected individuals [181]. Soluble high mobility group box protein 1 (HMGB1), which can be released by either damaged cells or activated monocytes/ macrophages, is an inducer of pro-inflammatory cytokines and is elevated in the plasma of HIV-1-infected individuals [187]. Furthermore, high levels of spontaneously released pro-inflammatory cytokines and chemokines in plasma/ serum have been reported during HIV infection, where they contribute to activation and recruitment of target cells for HIV.

3.5.4 Microbial translocation

It has been suggested that chronic immune activation in HIV-infected individuals may result from microbial translocation into the peripheral blood after disruption of the mucosal barrier in the gut [42, 129]. Damage to the epithelial layers of the mucosa can be induced by reduced mucosal immune responses [41, 143] or by direct interactions with the virus [184]. The leakage of microbial products into the peripheral blood, in addition to HIV itself, can consecutively activate the innate immune system by triggering different pattern recognition receptors (PRRs), such as the TLRs, leading to the production of pro-inflammatory cytokines [180], and, as previously described, the innate immune system will, in turn, activate the adaptive immune system. However, microbial translocation could also be a consequence of chronic activation during HIV infection, where phagocytosis by immune cells becomes dysregulated and unable to destroy circulating pathogens [21, 138, 234]. Activated monocytes/ macrophages secrete soluble CD14 (sCD14), which binds LPS. HIV-1-infected individuals have increased levels of sCD14 compared to HIV-negative individuals, with the highest levels in those with more progressed disease [42, 185]. Levels of sCD14 have been correlated to LPS levels in the plasma of HIV-1-infected

individuals [42]. Furthermore, LPS binding protein (LBP) was increased in HIV-1-infected individuals with progressed disease [42] and continued to increase over time [205].

3.6 TREATMENT AND DEVELOPMENT OF HIV INTERVENTIONS

There are currently no vaccines available for the prevention of HIV infection; however, antiretroviral treatment may reduce the viral load of infected individuals, prolonging the asymptomatic phase of an infection. Because of the high mutation rate of HIV, it is essential that antiretroviral drugs are administered to HIV-infected individuals in combinations, consisting of different classes or drugs. Effective treatment with combinations of antiretrovirals is often referred to as highly active antiretroviral therapy (HAART) or combination antiretroviral therapy cART. The accessible classes of antiretroviral drugs include reverse transcriptase inhibitors (RTIs), protease inhibitors (PIs), fusion inhibitors (FIs) [158] and integrase inhibitors [241]. The RTIs are further divided into two classes: the nucleoside/nucleotide RT inhibitors (NRTIs), which are nucleoside analogues that inhibit RT activity via incorporation into viral DNA, and the non-nucleoside RTIs (NNRTIs), which inhibit RT function by binding to the enzyme directly. HIV-2 has been described as less sensitive to NNRTIs compared to HIV-1 [207, 260] and to exhibit natural polymorphisms that are resistant to the action of some of the PIs [59, 63]. The consequence of extended cART can be associated with severe side effects and drug resistance [110]. Therefore, structured treatment interruption (TI) has been introduced with the aim to reduce the drug-related severities, to identify whether so called drug holidays are beneficial and to allow drug resistant viruses to switch to wild type strains again [27]. Furthermore, TI has also been advocated as a strategy to re-boost the HIV-1-specific immune responses and to lower the treatment costs [27, 150]. It has, however, been reported that structured TI or poor adherence to cART results in a rebound of viremia and CD4+ T cell decline [27, 64, 81, 101, 195]. Efforts have also been made aiming to develop a therapeutic vaccines that can boost the immune response in HIV-infected individuals in order to control or eliminate the virus in the infected host [73, 252]. In addition to efforts to identify effective treatments, there have been attempts to produce a preventive vaccine that can inhibit HIV infection. However, so far, limited success has been made in this area, with two Phase III clinical trials, Vaxgene and STEP that have failed, and the most recent trial, the RV144 Thai HIV vaccine trial, showing marginal 31% efficacy [141]. Given that HIV is a virus that is mainly transmitted during sexual contact, development of a microbicide that can be administered in the vaginal or rectal mucosa has been proposed. Desired characteristics of a potential microbicide include potent antiviral activity that does not induce excessive inflammation to avoid summoning target cells for HIV infection [43].

Table 3. Comparison of HIV-1 and HIV-2 infections.

	HIV-1	HIV-2	References
Origin	SIV of chimpanzee	SIV of sooty mangabey	[100, 115]
Distribution	Pandemic	Endemic in West Africa	[238, 256]
Transmission	Reduced in HIV-2 compared to HIV-1-infected		[135]
CD4 decline	Faster in HIV-1-infected than in HIV-2-infected		[126]
Progression to AIDS	± 10 years	Acts as LTNP	[168, 193, 201]
Plasma viral load	High, detectable most of time	Approx. ten times lower than HIV-1, below detection level	[17]
Coreceptor usage	Narrow, mainly CXCR4 and CCR5	Broad, reports of CD4-independence	[206]
Immune activation	Reduced in HIV-2-infected compared to HIV-1-infected		[179]
Innate responses	Reduced function of NK cells; relative effective infection of mDCs and pDCs,	Preserved function of NK cells; ineffective infection of mDCs and pDCs,	[48, 78, 188]
Humoral responses	Low number of autologous nAbs; Higher magnitude nAb responses	Little escape and frequent autologous nAbs; Broader nAb responses	[161, 211, 231]
Cellular responses	More polyfunctional virus specific T cells in HIV-2-infected than in HIV-1-infected, HIV-2-specific CD8+ T cells were at an early stage of differentiation		[79, 154]

4 MATERIALS AND METHODS

Paper I was performed in collaboration with colleagues at the University of Gothenburg. Here, the anti-HIV activity of CpG, conjugated to the non-toxic subunit B of the cholera toxin (CT), was examined. CT contains two subunits, the toxic subunit A (CTA) and the non-toxic subunit B (CTB). CTB exists as a pentamer and is responsible for binding of the toxin to the ganglioside GM1 receptor, which is present in the lipid rafts of several cell types, including epithelial cells [217] (Fig. 7). The CTB molecule is tightly packaged by hydrogen bonds and salt bridges, making it stable. After binding to GM1, CT, including CTA and CTB, is endocytosed by the cell and transported to the ER through a retrograde pathway via the Golgi. Once in the ER, CTA is separated from CTB. GM1 is also expressed on APCs, which can take up the CT/ CTB and present it to cells of the immune system. CT has been shown to be a potent immunogen and a powerful mucosal adjuvant [217]. Because of its non-toxic characteristics, CTB has been widely utilized without exerting any side effects. For example, CTB has been used in the oral cholera vaccine Dukoral®. CTB was also shown to enhance antigen-specific immune responses when linked to antigen [119, 120, 217]. It was also recently demonstrated that, when conjugated to CpG ODN, CTB enhances the immunostimulatory effects of CpG ODN, in both mouse and human immune cells [10].

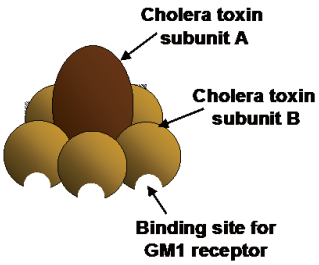

Figure 7. The cholera toxin with the toxic subunit A and the non-toxic subunit B.

In **Paper I**, PBMCs from healthy donors and CCR5- or CXCR4-expressing U87.CD4 cells were infected with defined HIV-1 and HIV-2 strains *in vitro* and simultaneously treated with different prototypes of CpG ODN (A, B, and C) and CTB-CpG (CpG A, B and C conjugated to CTB). The HIV strains used in this study have been previously described [13, 31, 128]. Viral replication was monitored in the culture medium by analysis of the p24 antigen content, using enzyme linked immuno-sorbent assay (ELISA) or functional RT activity, as determined by the synthesis of cDNA from RNA templates by RT present in virions that were released into the culture medium (CAVIDI HS-kit Lenti RT). Cells were also stimulated with CpG ODN or CTB-CpG in the absence of HIV infection to analyze their effects on cell viability using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay. Furthermore, experiments on the mechanisms behind CTB-CpG-induced anti-HIV activity included analysis of HIV receptor expression detected by flow cytometry and the production of chemokines by ELISA.

Paper II and **Paper III** were performed in collaboration with colleagues at the National Laboratory for Public Health and The Bandim Health Project in Bissau, Guinea-Bissau,

Lund University and Swedish Institute for Infectious Disease Control. Blood samples from HIV-1 and/or HIV-2-infected and HIV-negative controls were obtained from two study populations, a prospectively-followed cohort of police officers and a population-based cohort from the Bandim Project [118, 186]. HIV and human T cell leukemia virus type I (HTLV-I) status, as well as CD4+ T cell count in the infected and controls groups were obtained by ELISA and flow cytometry, respectively. Plasma viral loads of both HIV-1- and HIV-2-infected individuals were determined by analysis of RT enzymatic activity present in the plasma sample, based on the synthesis of cDNA strands from RNA templates (CAVIDI ExaVir® Load kit). Table 4 shows an overview of the study population, including their immunological and virological statuses.

Table 4. HIV-status, gender, age, CD4+ T cells and viral load distribution of the study subjects.[1]

HIV status	Gender		Immunological and virological parameters		
	Male N (Age median: iqr[2])	Female N (Age median: iqr[2])	CD4+T cells/µl (n)[a]	Viral Load log10 RNA/ml (n)[a]	Spearman rank Correlation[b]
HIV-1+2	5 (49: 48-53)	11 (54: 31-62)	360: 76-408 (16)[c]	3.95: 3-478 (14)[g]	*P<0.05*
HIV-1	34 (40: 33-46)	32 (32: 28-38)	301: 186-425 (66)[d,e]	3.95:3.25-4.95 (53)[h]	*p<0.001*
HIV-2	32 (48: 43-60)	52 (55: 45-61)	376: 259-597 (84)[f]	3: 3-3.2 (70)	*p<0.001*
Neg	31 (48: 34-54)	37 (42: 31-55)	605: 410-749 (69)	-	-

[1] Study subjects included two subpopulations within Guinea-Bissau, where one was a population-based study group followed within the framework of the Bandim Health Project [118] and the other a professional cohort of police officers [186].
[2] Numbers (median age in years: age interquartil range)
[a] Median: interquartil range
[b] Correlation between CD4+ T cell count and viral load.
[c, e & f] Lower CD4+ T cell counts as compared to Neg (*p<0.001*),
[d] Lower CD4+ T cell counts as compared to HIV-2 infected (*p<0.005*).
[g & h] Higher viral load as compared to HIV-2 infected (*p<0.05* and *p<0.001* respectively)

Whole blood stimulation (WBS) assays were established to analyze innate immune stimuli responsiveness by analysis of cytokine expression after TLR stimulation (Fig 8a). Whole blood was stimulated with different TLR agonists, including R-848 and CpG ODN, which are known to trigger TLR7/8 and 9, respectively. Supernatants were harvested 20 hrs after stimulation and cryopreserved. The cytokine content of the whole blood culture supernatants was subsequently measured using multiplex Luminex analysis, which is based on antibody-conjugated beads with up to 100 spectral properties detected by a laser that allows up to 100 different analytes to be examined in one sample. WBS assays have been reported in studies examining cytokine responses as an alternative method for assays based on purified PBMCs [69, 82, 84, 142]. The benefit of WBS is the lack of prior cell manipulation and, accordingly, a retained peripheral cell repertoire. In addition, WBS allows for the usage of fresh blood cells, which is essential when working with innate immune responses. Furthermore, the whole blood assay is robust and less labor intensive than conventional purified cell population assays, and thus, it is useful for laboratories in which financial resources and logistics are limited. Upon establishment of

the WBS assay for monitoring innate immune responsiveness, we noted that unmethylated CpG ODN A gave rise to the highest levels of IFN-α, whereas the best expression of IL-12 was detected after stimulation with R-848 (Fig 8b-c). Therefore, R-848-induced IL-12 expression and CpG ODN A-induced IFN-α expression were selected as readouts for the analysis of TLR7/8 and TLR9 responsiveness, respectively, in **Paper II** and **Paper III**.

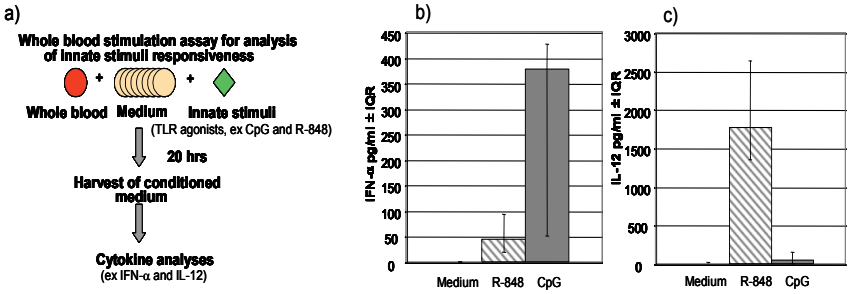

Figure 8. Schematic view of whole blood stimulation for analysis of innate stimuli responsiveness. Expression of b) IFN-α and c) IL-12 in whole blood of HIV uninfected controls (n= 50 vs 69) after 20hrs stimulation with CpG ODN A and R-848.

In addition, in **Paper III**, diluted and heat inactivated plasma was used to measure the gram-negative bacterial endotoxin LPS levels. Concentration of LPS was determined by the use of the limulus amebocyte lysate assay (LAL), which is based on the activation of an enzymatic reaction by the presence of endotoxin. The LPS plasma levels were calculated in relation to an *E. coli* endotoxin standard provided with the assay after background subtraction.

Paper IV made use of blood samples collected during a therapeutic vaccination study including TI conducted at Venhälsan, Department of Infectious Diseases, Karolinska University Hospital. Here, sequential samples from HIV-1-infected patients were obtained during cART and the course of vaccination and TI. Patients were subjected to three cycles of short-term TI followed by a longer TI (Fig. 9).

Innate stimuli responsiveness and markers of immune activation were studied in samples obtained from the participants before and during the study course, including cART periods and TI. A similar method to the one used in **Papers II** and **IV** was used for the analysis of innate stimuli responsiveness; except that PBMCs were stimulated using a panel of innate stimuli reagents, including TLR agonists (R-848 and unmethylated CpG ODN 2216). Expression of innate cytokines was determined using a multiplex Luminex assay. In collaboration with colleagues at Swedish Institute for Infectious Disease Control, the frequencies of CD4+ T cells, mDCs and pDCs in whole blood were determined by flow cytometry. Immune activation was assessed by the analysis of LPS levels in the plasma and frequencies of CD38+ and HLA-DR+ T cells. In this study, plasma viral load was

determined by a method based on quantitative PCR technology (Amplicor HIV-1 monitor test).

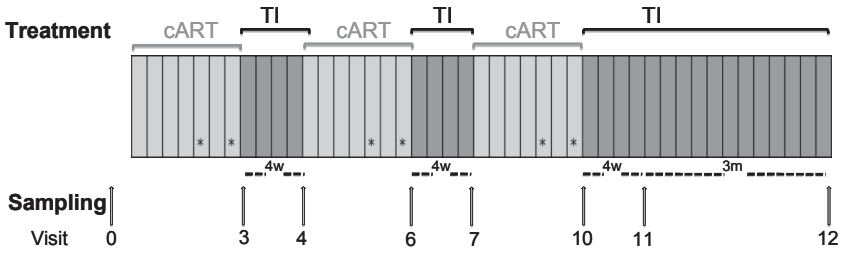

Figure 9. Schedule of cART and TI cycles during the study period. Samples were taken before the start of the study (visit 0), at the end of each cART cycle of seven weeks (visits 3, 6, and 10), at the end of each short-term TI cycle of four weeks (visits 4, 7 and 11) and after four months of extended TI (visit 12).

For more details, see the materials and methods sections of the respective papers.

4.1 ETHICAL CONSIDERATIONS

In the studies where patient blood samples were used, **Papers II** through **IV**, they were obtained in agreement with an ethical approval. Whole blood and plasma from HIV-1- or HIV-2-infected and uninfected controls in **Papers II** and **III** were obtained in agreement with the ethical approval of local authorities in Guinea-Bissau and ethical committee decisions Dnr KI 00-083 and LU 845:03. The study performed in **Paper IV** was approved by Karolinska Institutet regional ethics committee and the Swedish Medical Products Agency with ethical decisions Dnr 2005/1475-31/2 (supplementary approval 2006/1202-32 and 2008/836-31). Informed consent was obtained from all participants.

5 RESULTS AND DISCUSSION

The results of the papers included in this thesis will be presented and briefly discussed here in five different sections. First, I will discuss an approach on how to use TLR agonists, in an *in vitro* system, to suppress HIV infection (**Paper I**). In the second and third section (**Papers II** and **IV**) the attention will be on the impact that HIV-1 and HIV-2 have on the activation and dysregulation of the innate immune system, focusing mainly on TLR7/8 and TLR9 functionality. The fourth section will be about the immune activation and microbial translocation and how this relates to TLR responsiveness during HIV infection (**Papers III** and **IV**). In the fifth section, the role of gender in relation to TLR responsiveness and immune activation during HIV infection will be discussed (**Papers II** and **III**, in addition to data not presented in the papers but included in the thesis for further support and discussion).

5.1 THE SUPPRESSIVE CAPACITY OF CpG ODN AND CTB-CpG CONJUGATES ON *IN VITRO* REPLICATION OF HIV (PAPER I)

The TLR9 agonist CpG ODN may mediate innate immune protection against pathogens in animal models [108, 147]. This induced CpG protection was linked to rapid and potent production of T_H1 cytokines and the CC chemokines RANTES, MIP-1α, and MIP-1β, which are known as natural ligands of CCR5 in the female genital tract and the regional lymph nodes [107, 243]. Furthermore, CpG ODN has been shown to reduce HIV-1 replication in human lymphoid cells [224]. In **Paper I**, we demonstrated that replication of both CCR5 (R5) and CXCR4 (X4), using HIV-1 isolates, could be suppressed by different human CpG ODN prototypes. Notably, the inhibitory activity of CpG motifs was also observed in HIV-2 infections (Fig. 10).

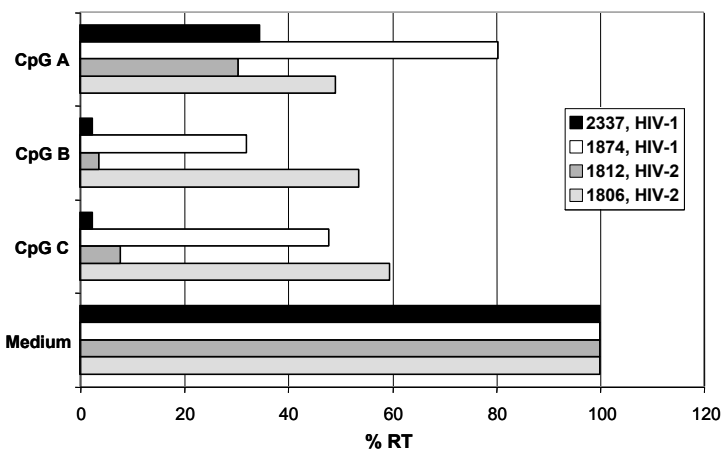

Figure 10. Effect of different human CpG prototypes on HIV-1 and HIV-2 replication in human PBMCs. Viral replication of HIV-1 and HIV-2, assessed as % RT of control, in infected PBMC cultures stimulated with different CpGs.

Enhanced anti-HIV-1 effects of CpGs conjugated to CTB

It has been reported that the conjugation of CpG ODN to CTB (CTB-CpG) enhanced the immunostimulatory effects of CpG ODNs, causing stronger innate immune responses in both mouse and human immune cells [10]. Here, we could detect that the three different CpG ODN prototypes conjugated to CTB displayed enhanced inhibition of both HIV-1 R5 and X4 viruses *in vitro* without affecting cell viability. CTB-CpG increased the production of MIP-1α and MIP-1β in PBMC cultures, which could contribute to increased anti-HIV-1 activity against the R5 isolate. However, this could not explain the enhanced anti-HIV-1 activity against the X4 isolate, nor the inhibition of HIV-1 replication in U87.CD4 indicator cell cultures, where MIP-1α and MIP-1β were not detected. Furthermore, the expression of IFN-α was not enhanced when CpG ODNs were conjugated to CTB, which cannot explain the increased HIV inhibitory activity (data not shown). The expression of HIV receptors on the target cell surface was also unaffected by CpG or CTB-CpG stimulation. Multimerization [262] and endosomal retention [265] of CpG ODN have been shown to play an important role in TLR9 activation. TLR9 has been shown to be located in the ER prior to CpG exposure [152], and CTB traffics intracellularly via a retrograde vesicular pathway to the ER [217]. It is thus possible that conjugation of CpG to CTB could lead to a more effective interaction of CpG with TLR9, by the efficient uptake via the CTB receptor GM1, multimerization of CpG, endosomal retention, and targeting to the ER. CTB-CpG may further activate several immunomodulatory genes that, in turn, can directly or through bystander mechanisms interfere with HIV replication.

The impact of the CpG motif and ODN backbone on the anti-HIV activity of CpG and CTB-CpG conjugates

The results obtained in **Paper I** also show that the presence of the CpG motif in ODN was not required for the observed HIV-suppressive effects of CTB conjugates because the oligo control (a CpG B control lacking the CpG motif) conjugated to CTB, but not oligo control alone, also suppressed replication of both R5 and X4 viruses (Fig. 3, Paper I). Subsequently, we also noted that the oligo control-CTB also enhanced the production of MIP-1α and MIP-1β. It has been reported that an oligo control combined with GM-CSF triggered monocytes to express increased levels of MIP-1α and MIP-1β, similar to CpG B ODN [257]. The anti-HIV activity of CTB conjugated to the oligo control observed in **Paper I** could also be attributed to the ability of CTB to bind the GM1 receptor, which can lead to more efficient transfer of oligo to the endosomal compartments, where TLR9 is likely to be encountered. Supporting this theory, it has been observed that the pentameric structure of CTB is important for the binding to GM1 receptor on cells and enhancing the immunogenicity of CTB conjugated HIV-peptides [35]. Alternatively, TLR9 may not be involved in the observed anti-HIV effects of CTB-CpG. Non-CpG-containing ODNs were reported to display certain immunostimulatory activities that were shown to be dependent on the presence of the phosphorothioate (PS) backbone in ODNs [210, 261]. The PS backbone makes the CpG motif nuclease-resistant, whereas CpG motifs with

a phosphodiester (PO) backbone can be degraded [147]. In **Paper I**, the anti-HIV effects of both CpG and CTB-CpG against R5 and X4 virus replication were reduced when CpG with a PO backbone was used, indicating that the presence of the PS backbone is important for anti-HIV-1 activity (Fig. 3, Paper I). Previous studies suggest that ODNs can act directly on the HIV envelope by binding to the V3 loop on gp120 [11, 173, 224, 263]. The direct interaction between ODNs with a PS backbone and HIV virions can, therefore, contribute to the anti-HIV effects of ODNs. As demonstrated in **Paper I** and by others, stimulation with CpG ODN can suppress HIV infections *in vitro* [224], but it may also induce the replication of HIV in latently infected cells [221]. Accordingly, further studies are needed to understand the precise mode of anti-HIV activity of CTB-conjugated ODNs and their potential use in HIV interventions.

5.2 TLR7/8 AND TLR9 STIMULI RESPONSIVENESS DURING HIV-INFECTION (PAPERS II AND IV)

In recent years, the effect that HIV may have on the innate immune function mediated by TLR stimulation has been examined to try to better understand the role of TLRs during the course of HIV infection. In **Papers II** and **IV**, we have focused on studying the *in vitro* functions of TLR7/8 and TLR9, given that they recognize microbial nucleosides and their derivates to trigger cells to produce effector cytokines within the innate immune system.

5.2.1 HIV-1- and HIV-2-infected untreated individuals (Paper II)

The pathogenicity of an HIV-2 infection is reduced, i.e., the progression toward AIDS development is slower, as compared to an HIV-1 infection. The majority of HIV-2 studies examine the adaptive immune responses showing, for instance, broader neutralizing antibodies, higher numbers of polyfunctional T cells, and an early stage of differentiation of HIV-2-specific T cells [60, 79, 154, 155, 166, 212]. The effects that HIV-2 infection has on the innate immune responses are, however, less well known. The innate immune responsiveness in HIV-1-, HIV-2- and dual HIV-1/2-infected individuals, compared to uninfected individuals, was investigated using a WBS assay, in which different TLRs were stimulated with their respective agonists. The study population in **Paper II** comprised individuals living in Guinea-Bissau, a country which has had the highest prevalence of HIV-2, with a peak during the late 1990s [60, 155, 166, 212] but has also experienced an HIV-1 epidemic during the past years.

In line with other studies [12, 17], we observed that HIV-2-infected individuals had higher CD4+ T cell counts and lower viral load than HIV-1-infected individuals. We also noted that, when matched for CD4+ T cell counts, HIV-2-infected individuals had approximately 1 log lower plasma viral loads (Fig. 11). These data suggest that, although the plasma viral load is low, CD4+ T cells continue to decline during the course of an HIV-2 infection [236].

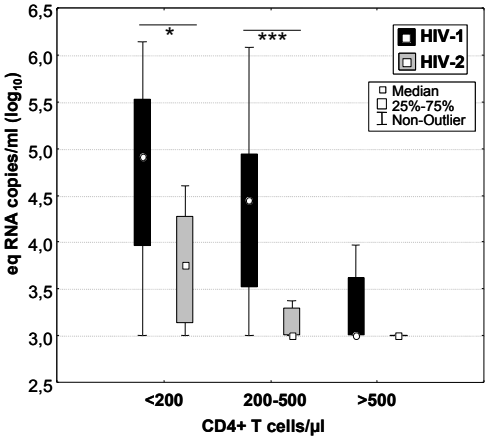

Figure 11. Viral load in relation to CD4+ T cell counts of HIV-1 or HIV-2 infected. Plasma viral load (eq RNA copies/ml) of HIV-1 or HIV-2 infected in relation to intervals of CD4+ T cell counts, < 200 (n= 14 vs 8), 200-500 (n= 28 vs 38), and >500 (n= 11 vs 23). * $p<0.05$, *** $p<0.001$ according to Mann-Whitney U-test.

Defective TLR7/8 stimuli responsiveness of HIV-1-infected measured as reduced in vitro release of IL-12

HIV-1-positive individuals demonstrated lower levels of IL-12 in whole blood cultures after stimulation of TLR7/8 with R-848 compared to HIV-negative individuals, but this was not noted in HIV-2- or dual-infected individuals (Fig. 4, Paper II). In fact, multivariate statistical analysis showed that the HIV-1-infected individuals displayed reduced TLR7/8 responsiveness compared to those infected with HIV-2. Higher levels of NK cell activity have been reported in HIV-2-infected individuals with CD4+ T cell counts >500 compared to those with HIV-1-infection [188]. Because IL-12 can regulate NK cell lysis activity [246], one could speculate that the dysregulated TLR7/8 responsiveness observed in the HIV-1-infected individuals may account for the decreased NK cell function. Furthermore, antigen-specific CD8+ T cells have been reported to maintain an early differentiation phenotype in HIV-2-infected individuals [154]. It would, therefore, be of interest to study the capacity to produce TLR7/8-triggered IL-12 in relation to NK cell and CD8+ T cell activity in both HIV-1- and HIV-2-infected individuals. Neither CD4+ T cell counts nor viral loads in HIV-1- or HIV-2-infected subjects significantly correlated with TLR7/8-triggered IL-12 responses. mRNA levels of TLR7 and TLR8 in PBMCs are upregulated during HIV-1 infection [157]. However, despite higher expression of TLR7/8, pDCs of HIV-1-infected patients have impaired functions after TLR7 stimulation [214]. Our findings of defective IL-12 production in HIV-1-infected, but not in HIV-2-infected individuals, after TLR7/8 stimulation needs further evaluation, including studies of longitudinally-followed cohorts, to be able to conclude if the impairment in TLR7/8 responsiveness observed in HIV-1-infected individuals is linked to the pathogenesis of this infection.

Defective TLR9 responsiveness measured as release of IFN-α in HIV-infected individuals is related to number of CD4+ T cells

IFN-α concentrations in whole blood after *in vitro* TLR9 stimulation with CpG ODN were reduced in HIV-1-infected individuals compared to uninfected individuals (Fig. 2, Paper II), confirming previous findings [130, 133]. Similar findings were observed in dual HIV-1/2-infected individuals. In addition, IFN-α production after CpG stimulation was correlated with CD4+ T cell numbers in both HIV-1- and HIV-2-infected individuals (Fig. 12a). In HIV-2-infected individuals, with CD4+ T cells below 200 cells/μl, TLR9 responsiveness was defective compared to uninfected individuals. The influence of CD4+ T cell counts on herpes simplex virus (HSV)-triggered IFN-α and the numbers of pDC, the main producers of CpG A-triggered IFN-α, was previously described for HIV-1 infections [86, 89, 133]; the present study shows that this is also valid for CpG-triggered IFN-α in HIV-2 infections. It has been suggested that pDC are less susceptible to HIV-2 than HIV-1 in *in vitro* infections [78]. However, in support of our data, it was recently shown that pDCs in HIV-2-infected individuals had lower IFN-α production after TLR9 stimulation, and this reduction was mainly related to a decrease in the frequency of circulating CD4+ T cells [48]. An inverse correlation between IFN-α production after CpG stimulation and viral load has been reported in HIV-1 infection [130]. HIV-2-infected individuals with viral loads greater than 10,000 copies eq./ml had lower CpG-triggered IFN-α levels compared to those with viral loads less than 1,000 copies eq./ml (Fig. 12b). Despite the lower levels of plasma viral loads in HIV-2-infected, as compared to HIV-1-infected individuals, the reduced CD4+ T cells counts linked to immunodeficiency were predictive of the responsiveness to TLR9 stimuli in both HIV-1 and HIV-2 infections, as determined by multivariate statistical analysis.

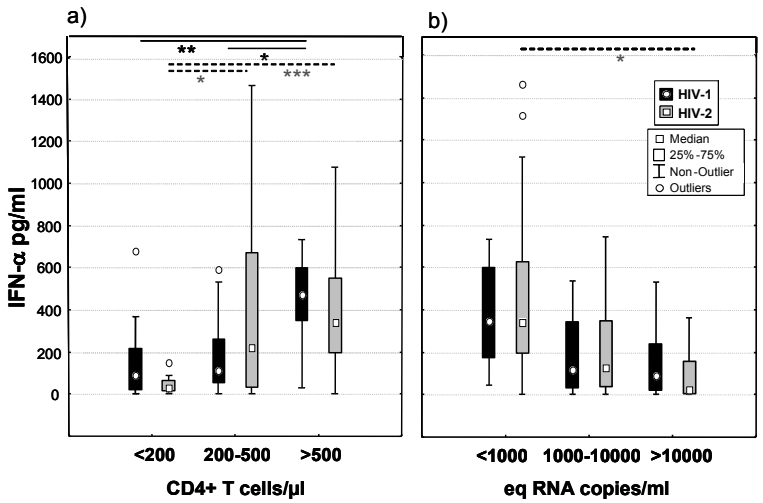

Figure 12. TLR9 stimuli responsiveness in relation to CD4+ T cell counts and viral loads of HIV-1 or HIV-2 infected individuals. CpG triggered IFN-α expression in whole blood of HIV-1 or HIV-2 infected in relation to intervals of a) CD4+ T cell counts, < 200 (n= 17 vs 8), 200-500 (n= 28 vs 34), and >500 (n= 7 vs 23) and b) plasma viral load (eq RNA copies/ml) < 1000 (n= 5 vs 29), 1000-10000 (n= 17 vs 16), and >10000 (n= 21 vs 29) * P<0.05, ** P<0.01, *** P<0.001 according to Mann-Whitney U-test, HIV-1 solid lines and HIV-2 dotted lines.

Furthermore, Sachdeva et al. showed impairment of pDCs and production of IFN-α following CpG stimulation in discordant HIV-1-infected individuals (individuals showing continuous low viral loads and low CD4+ T cell counts) [214]. However, concordant HIV-1-infected individuals (continuous low viral loads and normal CD4+ T cell counts) showed higher levels of both pDCs and IFN-α production [214]. Several studies have shown that the numbers of pDCs decrease during primary HIV-1 infection [140, 192, 213]. It would, therefore, be interesting to examine the impact that primary HIV-2 infection may have on the frequency of pDCs and the impact of primary HIV-1 and HIV-2 infection on TLR9 responsiveness, in order to study if there are any potential differences between HIV-1 and HIV-2 during the early events of these two infections.

5.2.2 HIV-1-infected individuals undergoing treatment interruptions (Paper IV)

In this study, HIV-1-infected individuals living in Sweden were subjected to repeated TI cycles during cART, as part of a therapeutic vaccine study, and sequential peripheral blood samples were taken and analyzed (Fig 9). The results obtained in **Paper IV** were independent of vaccination, and for this reason, the following discussion will not focus on vaccination in detail.

Similar to previous studies on TI in HIV-1-infected individuals [27, 64, 81, 101, 195], we also noted rebound of viremia and decrease in the total number of circulating CD4+ T cells during the periods of TI (Fig. 2, Paper IV).

Hyperactived TLR7/8 responsiveness as a result of TI

TLR7/8 stimulation of PBMC showed that the levels of IL-12 production significantly increased after the first short-term TI and remained elevated throughout the study period. The peak of IL-12 secretion after TLR7/8 stimulation occurred during the second TI and decreased to a significantly lower level after four months of extended TI (Fig. 4a, Paper IV). The production of IL-12 after TLR7/8 stimulation did not correlate with CD4+ T cell counts or to plasma viral loads. Because stimulation of TLR7/8 also results in the production of IFN-α [264], we analyzed the secretion of IFN-α after stimulating PBMCs with a TLR7/8 ligand. We observed that the expression pattern of TLR7/8-triggered IFN-α was similar to that of TLR7/8-triggered IL-12, except that peak secretion was measured during the third TI (Fig. 4b, Paper IV). In contrast to the results obtained with TLR7/8-triggered IL-12, we detected a correlation between TI-associated viremia and increased secretion of IFN-α after TLR7/8 stimulation. In summary, our results imply that TI results in elevated cytokine levels after TLR7/8 stimulation. These results are supported by a study showing upregulated TLR7 and TLR8 mRNA expression during HIV-1 viremia [157]. However, because we detected a correlation between viral load and TLR7/8-triggered IFN-α, but not IL-12, one could suggest that the virus, in addition to affecting the mRNA levels of TLRs, also affects the TLR signalling pathway. In other words, depending on which TLR signalling pathway is required for the production of the different cytokines, the cytokine profile pattern will be different and its relationship to viral load will

also differ. Furthermore, our findings also suggest that the prolonged viremia during the four months of extended TI results in exhaustion of cytokine secretion in response to TLR7/8 stimuli, implying a state of anergy due to prior hyperactivation.

Dysregulated TLR9 responsiveness as a result of TI

Levels of TLR9-triggered IL-12 were comparable to that of TLR7/8-triggered IL-12, with an increase after the first TI and during cART with a peak after the second TI (Fig. 13a). Similarly to the TLR7/8-triggered IFN-α, elevated expression of IL-12 after TLR9 stimulation correlated to increased viral load during cycles of TI. In contrast, the capacity to produce IFN-α after TLR9 stimulation decreased after the first TI, but recovered when cART was resumed, and moderate fluctuations in the IFN-α secretion were noted during the remaining TI cycles, with the exception of a drop during the extended TI (Fig 13b). We also noted that increased viral load and a decrease in CD4+ T cells correlated with reduced IFN-α expression after TLR9 stimulation.

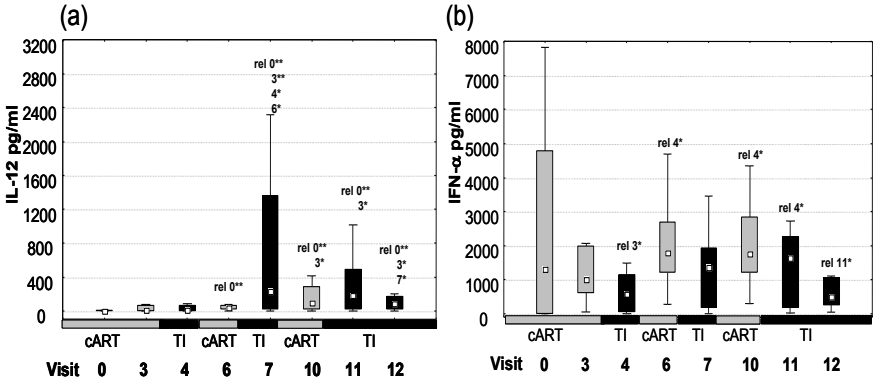

Figure 13. Fluctuation of TLR9 stimuli responsiveness during cycles of TI. TLR stimuli responsiveness assessed as net-secretion of a) TLR9 (CpG) triggered IL-12 b) TLR9 (CpG) triggered IFN-α in cultures of PBMC obtained during cART at visits 0, 3, 6 and 10 (gray boxes) and during TI at visits 4, 7, 11 and 12 (black boxes). Significant differences are indicated in relation (rel) to the prior visit, *p<0.05, **p<0.01.

The triggering of TLRs induces a cascade of events within cells, including the activation of different IRF for the production of interferons and pro-inflammatory cytokines [58, 121, 196]. HIV can upregulate or downregulate the expression of certain IRFs thereby affecting immune responses [71, 170, 191]. In summary, our results suggest that the capacity of PBMC to secrete IL-12 and IFN-α, depending on type of TLR stimuli, is dysregulated as a result of viral load fluctuations during cycles of TI in HIV infection.

5.3 EFFECTS OF TREATMENT INTERRUPTIONS ON CIRCULATING DENDRITIC CELLS AND THEIR FUNCTION IN TREATED HIV-1-INFECTED INDIVIDUALS (PAPER IV)

The numbers of circulating mDCs and pDCs are reduced during the course of HIV-1 infection [15, 171, 235]. This observation could possibly explain the defective innate responsiveness observed in **Papers II** and **IV**, because mDCs and pDCs are thought to be

the main producers of IL-12 and IFN-α after TLR7/8 and TLR9 stimulation, respectively [162]. Nevertheless, dysregulated TLR responsiveness can also be a direct consequence of the virus on cells since it was recently suggested that the impaired function of pDCs could be specifically linked to their ability to produce IFN-α, but not pro-inflammatory cytokines [214]. For this reason, we examined the frequency of DC subpopulations during the course of short-term TI and their relationship to TLR responsiveness.

Reduction of circulating dendritic cells during TI

Reduced numbers of mDCs and pDCs, as a consequence of TI, correlated with an elevated viral load and declining CD4+ T cell counts. The number of mDCs and pDCs in the peripheral blood declined during cART following the first TI at visit 6 (Fig. 3a-b, Paper IV). After a phase of delay, where the frequency of mDCs and pDCs remained unchanged, the numbers significantly decreased again during the following cART cycle, from visit 7 to visit 10 and subsequently dropped again one month after the final TI at visit 11. In addition, pDC frequencies remained reduced during the extended TI. These results suggest that the initial viral rebound did not result in an immediate loss of DC subpopulations in peripheral blood but may have activated the subsequent dissemination or cytotoxic effects of these cell populations. During the following TIs, however, the impact of viremia on blood DC levels was faster. Our findings are consistent with earlier studies that describe the decline of circulating mDCs and pDCs at the onset of viremia during primary HIV-1 infection [140, 192, 213]. Accumulation of DCs in lymphoid tissue during acute infection has been reported [163]. Thus, dissemination of peripheral DC subsets to lymph nodes could contribute to the perturbation of peripheral DC subsets during acute viremia. However, it has also been shown that DCs can become infected by HIV-1 [72, 175, 233] suggesting that the loss of DCs may be due to cytopathic effects, either mediated by the virus itself or by CD8+ cytotoxic T lymphocytes. Taken together, these results reveal longitudinal fluctuations in the frequency of DC subsets linked to viremia during short term TI, which, in turn, may contribute to faster disease progression.

Frequency and function of pDCs impact net secretion of TLR-induced IFN-α during TI-associated viremia

Levels of IL-12 and IFN-α secretion in PBMC cultures after TLR stimulation were analyzed in relation to the number of peripheral blood mDC and pDC. Correlations were detected between the number of pDCs to TLR9-triggered IFN-α and TLR7/8-triggered IFN-α (Table III, Paper IV). IFN-α secretion was analyzed on a per pDC basis, to explore if the reduced net secretion of IFN-α after TLR stimulation was linked exclusively to the depletion of pDCs or to perturbed function of these cells. The reduced levels of TLR9-induced IFN-α correlated with declining pDC numbers, as well as fluctuating secretion of TLR9-triggered IFN-α per pDC along with cycles of TI (Fig. 5a, Paper IV). These results suggest that the expression of TLR9-induced IFN-α is affected by a loss of pDCs and by a functional defect of pDCs, which is in agreement with previous studies [92, 214, 244]. Reduced secretion of IFN-α per pDC after TLR9 stimulation also correlated with

increased viral load. In contrast, both net production of IFN-α and IFN-α secretion per pDC after TLR7/8 stimulation were upregulated during cycles of short-term TI (Fig. 5b, Paper IV) and correlated with increased viral load. Hence, these results imply that the ability of pDCs to secret IFN-α after TLR7/8 or TLR9 stimulation is altered as a consequence of TI-associated viremia and that net secretion of IFN-α in TLR-stimulated PBMC cultures depends on both pDC frequency and differential dysregulation of TLR signalling pathways within pDCs [91, 226]. It has been reported that *in vivo* production of IFN-α by pDC during primary HIV-1 infection is hyperactivated (reviewed in [50]), implying that this activation may dysregulate the *ex vivo* TLR stimuli responsiveness of pDCs.

5.4 IMMUNE ACTIVATION AND MICROBIAL TRANSLOCATION IN RELATION TO DYSFUNCTIONAL TLR RESPONSES DURING VIREMIC HIV-INFECTION (PAPERS II, III AND IV)

Immune activation has been described in both HIV-1- and HIV-2-infected individuals in the context of disease progression [66, 75]. Furthermore, because microbial products can activate cells through various TLRs, it has recently been suggested that during HIV-1-infection, microbial translocation into the peripheral blood after disruption of the mucosal barrier in the gut can initiate a cascade of immune activation events [42, 129]. In **Papers II, III** and **IV**, we have examined the extent of microbial translocation during HIV-2 infection compared to HIV-1 infection and the impact of short-term viremia on chronic immune activation during HIV-1-infection. The level of immune activation was further related to CD4+ T cell count, viral load and innate immunity.

5.4.1 Microbial translocation in HIV-1- and HIV-2-infected untreated individuals (Papers II and III)

LPS was measured in the plasma of individuals resident in Guinea-Bissau, infected with HIV-1 or HIV-2, and control subjects who were HIV negative to compare the levels of microbial translocation. Notably, we could, for the first time, as found in HIV-1-infected individuals, show that HIV-2-infected individuals with AIDS also had significantly higher plasma LPS levels compared to HIV-negative individuals (Fig. 1A, Paper III). However, no significant difference was observed between HIV-1- and HIV-2-infected individuals within the same disease stage, classified as either chronic or AIDS. Elevated plasma LPS levels correlate with high plasma viral loads for both HIV-1- and HIV-2-infected individuals (Fig. 14a). Supporting our data, Jiang et al. have shown that microbial translocation, measured by the presence of bacterial 16S ribosomal DNA in plasma, correlated to the plasma viral load of untreated HIV-1-infected individuals [129]. In addition, a recent study reported on a correlation between viremia and immune activation in HIV-2-infected individuals [153]. However, despite lower plasma viral loads in HIV-2-infected individuals compared to HIV-1-infected individuals within the same CD4+ T cell range (Fig. 11), elevated levels of plasma LPS along with decreased the CD4+ T cell

counts were noted in both HIV-1- and HIV-2-infected individuals (Fig. 14b). Similar to our results, the study of Cassol et al. from South Africa detected higher levels of plasma LPS in HIV-1-infected individuals with CD4+ T cell counts below 200 cells/µl, where individuals with opportunistic infections showed the highest concentration of plasma LPS [46]. Contrary, a longitudinal study done in Uganda showed that plasma LPS levels did not change during HIV-1 disease progression [205]. Additional studies are needed to evaluate changes in the levels of microbial translocation during the course of HIV infection. The study by Trøseid et al., but not that of Redd et al., showed that plasma LPS levels of individuals with African or Oriental origin was higher [205, 247], indicating that studies should also take into account the comparisons between different study populations descending from diverse geographical areas, to exclude the effects of other intestinal infections typical for those areas.

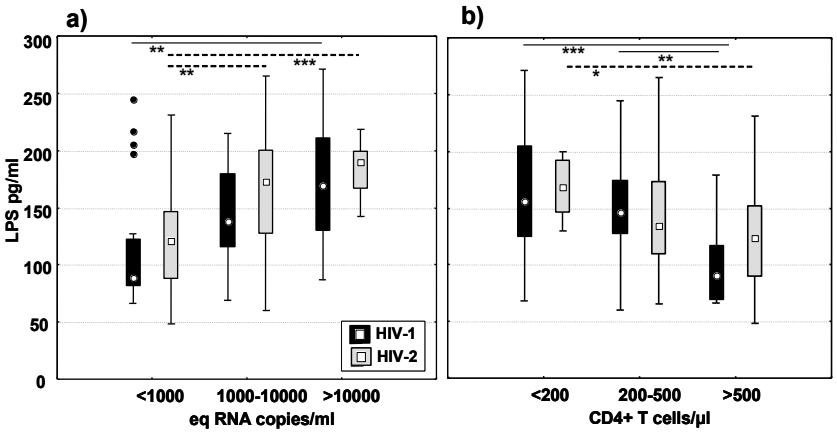

Figure 14. Levels of plasma LPS in relation to a) plasma viral load and b) CD4 + T cell counts in HIV-1 and HIV-2 infected, where box plots denote median, 25-75% interquartil range, non-outlier range and outliers, and *P< .05, **P< .01, ***P< .001, solid lines = HIV-1 and dotted lines = HIV-2.

Furthermore, we also demonstrated that high levels of plasma LPS correlated to defective innate stimuli responsiveness, i.e., reduced expression of IL-12 and IFN-α after TLR7/8 and TLR9 stimulation in HIV-infected individuals (Fig. 1D-E, Paper III) and reduced T cell mitogen responsiveness, i.e. reduced MIP-1β secretion (data not shown). A study of chronically immune-activated patients diagnosed with intestinal parasites demonstrated that TLR9 expression and function was defective in these individuals [19]. The findings of Ayash-Rashkovsky et al. [19] and our study suggest that TLR stimuli responsiveness may be linked to immune activation caused by different infectious agents, including HIV, which affect the intestinal tract. Based on the study in **Paper III**, we suggest that microbial translocation may contribute to the loss of CD4+ T cells, increase viral load and lead to defective immune stimuli responsiveness during both HIV-1 and HIV-2 infections. However, it is difficult to determine the cause and the consequence of microbial translocation in HIV infection in cross-sectional studies. Thus, longitudinal studies where the time points of infection are known are desirable to understand the relationship

between microbial translocation and HIV disease progression. Additionally, in **Paper II**, we noted an inverse correlation between CD4+ T cell counts and spontaneously released IL-12 in unstimulated whole blood cultures. In line with this observation, it was reported that IL-12, measured in whole blood cells by intracellular staining, was only detected in samples from HIV-1-infected individuals and not in uninfected controls [218]. These results imply that such responses can be triggered by the general immune activation associated with advanced HIV disease.

5.4.2 T cell Immune activation, but not microbial translocation, is a consequence of viremia during short term TI in HIV-1-infected individuals (Paper IV)

The impact of TI on systemic immune activation was analyzed by monitoring changes in the numbers of HLA-DR+ T cells and microbial translocation during the different TI cycles. The number of HLA-DR+ T cells increased gradually during the study period, with significant increases during the first and second TI cycles (Fig. 15a). Furthermore, increased T cell activation, detected as expression of both HLA-DR and CD38, was noted in both CD4+ and CD8+ T cell populations. We also noted that elevated numbers of HLA-DR-expressing T cells strongly correlated with increased viral loads and reduced numbers of CD4+ T cells (Table II, Paper IV). Our findings are supported by earlier reports that demonstrated an increase in CD8+ HLA-DR+ CD38+ T cells as a result of TI [47, 194, 240]. In contrast, microbial translocation, as analyzed by plasma LPS levels, was not affected by viral rebound during cycles of short-term and four-month extended TI (Fig 15b). HIV-1-infected patients with detectable viral load during cART have elevated plasma LPS levels compared to individuals with undetectable plasma viral load [22]. A delayed increase of plasma LPS levels after extended TI has been described; however, the time span of TI required for elevation of plasma LPS was not specified in this study [194]. Nevertheless, similar to our study, no increase in LPS was noted after a short-term TI that was less than two months long [22]. Hyperactivation of innate immune cells during viremia associated with primary HIV-1 infection results in broad and elevated innate cytokine release, including both type I IFNs and IL-12 [23, 44, 209]. Along with TI-associated viremia, we also detected increased spontaneous release of IL-12 and IFN-α in unstimulated PBMC cultures (Fig. 4e-f, Paper IV). In addition, elevated levels of spontaneously released IL-12 were associated with increased frequencies of HLA-DR+ T cells, but not with plasma LPS levels (Fig 6c-d, Paper IV). These findings imply that viremia associated with short term TI evokes elevation of T cell activation and spontaneous cytokine release independent of microbial translocation. Furthermore, viremia associated with primary HIV-1 infection does not result in increased plasma LPS levels [42], suggesting that prolonged viremia is needed for detection of elevated LPS levels in peripheral blood.

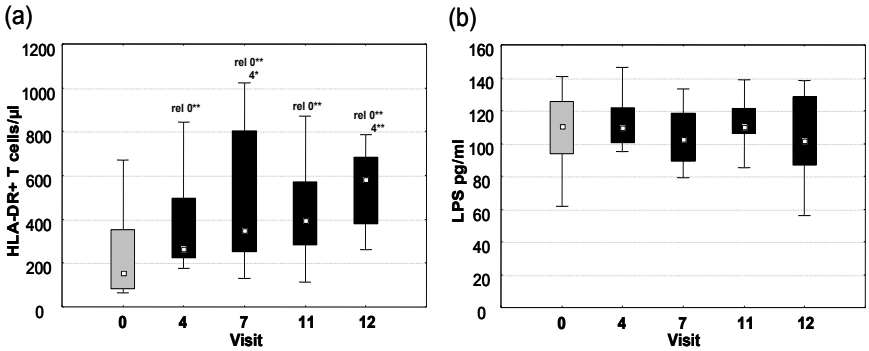

Figure 15. Markers of immune activation assessed at baseline and during cycles of TI. Levels of a) HLA-DR+ T cells and b) plasma LPS as markers of immune activation obtained at baseline, visit 0 (gray boxes) and during TI at visits 4, 7, 11 and 12 (black boxes). Significant differences are indicated in relation (rel) to the prior visit, *p<0.05, **p<0.01.

5.5 THE INFLUENCE OF GENDER ON TLR RESPONSIVENESS AND IMMUNE ACTIVATION (PAPERS II AND III)

Gender differences have been reported in expression of innate cytokines [90, 169]; for example, concentrations of IL-12 in cultures of LPS-stimulated monocytes from men have been elevated compared to women, suggesting that men display a higher state of innate immune excitation [38]. In regard to this, we decided to study TLR stimuli responsiveness in relation to gender. In **Paper II**, multivariate statistical analysis showed that TLR7/8 stimulation with R-848 and TLR9 stimulation with CpG ODN resulted in increased production of both IL-12 and IFN-α, respectively, in men compared to women. However, whether or not the increased innate immune responses present in men contribute to a better control of HIV or opportunistic infections requires further longitudinal investigations. In other papers, investigators have shown that whole blood, PBMCs and pDCs from HIV-negative women produced higher levels of IFN-α when stimulated with TLR7 ligand compared to HIV-negative men [29, 139, 176]. In unpublished data, we could show that, similar to what has been found for HIV-uninfected individuals, when whole blood was stimulated with TLR7/8 ligands, the production of IFN-α was higher in HIV-1-infected women than in HIV-1-infected men ($p<0.01$, Fig. 16). Meier et al. have suggested that the sex differences in the TLR7 activation of pDCs may be due to higher immune activation in women than in men [176]. To support this, they also showed that CD8+ T cells of HIV-1-infected women displayed increased levels of activation markers compared to CD8+ T cells of men. In agreement with the data of Meier et al., the multivariate analysis performed in **Paper III** revealed that the plasma LPS levels in HIV-infected women tended to be higher than the plasma LPS levels in HIV-infected men (Table 1, Paper III), suggesting higher immune activation in HIV-infected women than men. Our results suggest that TLR stimulation responses differ between women and men, but the gender differences also depend on what type of responsiveness that is measured

after TLR7/8 and TLR9 stimulation. In regard to our results, gender should, therefore, be one of the variables to consider when studying the TLR stimuli responsiveness in relation to immune activation and disease progression in HIV-infected individuals.

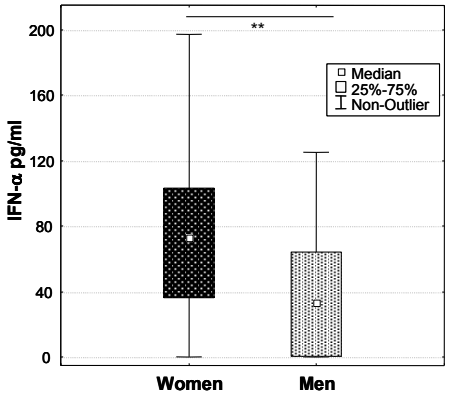

Figure 16. Higher levels of TLR7/8 triggered IFN-α in HIV-1-infected women (n=32) than HIV-1-infected men (n=34). **p<0.01

6 CONCLUDING REMARKS

Despite increasing knowledge about HIV and its interaction with the immune system, 27 years after the discovery of HIV, there exists no vaccine to prevent the infection or any treatment to eradicate established infections. Studies have indicated that innate immunity is important for activation of anti-HIV immune responses and protection against disease progression in HIV-1-infected individuals [151, 159]. In contrast, HIV may trigger immunopathogenic processes and dysregulate innate immune responses, yet knowledge regarding HIV interference with innate immunity remains relatively unexplored, especially in HIV-2 infections. HIV-2, being a less pathogenic and less transmissible virus than HIV-1, might serve as a model of controlled HIV infection; thus, better understanding of the causes behind milder HIV-2 infection could help in the development of future vaccines and therapies against HIV-1.

In the present thesis, the potential HIV suppressive effects mediated by innate immune stimuli have been examined in an *in vitro* model. Furthermore, innate immune responses and their relationship to immune activation during untreated HIV-1 and HIV-2 infections have been evaluated. In addition, this thesis includes studies on the consequences that TI may have on immune activation and innate immune responses in treated HIV-1-infected individuals.

In **Paper I**, we demonstrated that CpG ODN might suppress the replication of both HIV-1 and HIV-2 strains *in vitro*. Furthermore, conjugation of ODNs with a PS backbone to non-toxic CTB enhanced anti-HIV-1 activity. These results suggest that applications using ODNs or ODN derivates could be explored as a tool to prevent HIV infection. However, as demonstrated by us and others, ODN stimuli may also trigger secretion of cytokines and chemokines with inflammatory properties [210, 261]. Accordingly, because chronic activation of the immune system may contribute to the pathogenesis of HIV [18] and that inflammatory responses induced by vaccines or microbicides have been reported to increase HIV susceptibility [249, 254], caution should be exercised when using immunotherapies or vaccines capable of inducing inflammatory responses. Efforts should, therefore, be focused on developing new anti-HIV applications capable of suppressing HIV replication in the absence of an excessive inflammatory response. As demonstrated in **Paper I** and by others, stimulation with CpG ODN can suppress HIV infections *in vitro* [224], but it may also induce the replication of HIV in latently-infected cells [221] increasing the disease progression in HIV-infected individuals.

In **Paper II**, where innate immune functions were monitored by the analysis of TLR stimuli responsiveness during ongoing infections, it was revealed that when CD4+ T cell counts have declined during advanced disease, IFN-α secretion after TLR9 stimulation was reduced in whole blood of both HIV-1- and HIV-2–infected individuals. HIV-1-

infected individuals, but not HIV-2-infected, also displayed defective TLR7/8 responsiveness, as measured by IL-12 secretion. This result suggests that responsiveness to some TLR stimuli, such as TLR9-triggered IFN-α, can be affected by both types of infection. On the other hand, responses to other TLR stimuli, such as TLR7/8-induced IL-12, only become significantly dysregulated during HIV-1 infection.

In **Paper III**, we found that both HIV-1- and HIV-2-infected individuals with AIDS had higher levels of microbial translocation, measured as concentrations of LPS in the plasma, compared to HIV-negative individuals. The plasma LPS levels were further correlated with CD4+ T cells, viral load, and defective TLR7/8 and TLR9 responsiveness. These results could indicate that either microbial translocation contributes to disease progression in HIV-infected individuals, especially during advanced disease and/or that microbial translocation is triggered by disease progression due to dysregulated immune responses and high viral load. For a better understanding of the role of microbial translocation during HIV infection, longitudinal studies are required where the time of infection is known. Furthermore, results reported in **Papers II** and **III** also imply that men and women might differ in their innate stimuli responsiveness and levels of immune activation during HIV infection, suggesting that additional studies on these topics should consider gender differences.

In **Paper IV**, we showed that short-term TI-associated viremia not only resulted in a decline of CD4+ T cells but also contributed to dysregulation of innate immunity, including loss of mDCs and pDCs in the peripheral blood of HIV-1-infected individuals. Additionally, defective or hyperactivated TLR7/8 and TLR9 responses were noted during TI. The reduced net secretion of TLR9-triggered IFN-α was dependent both on a lower frequency of circulating pDC and a decreased secretion of IFN-α on a per pDC basis. Lowering the viral burden by cART, in the context of HIV-1 infection, has previously been shown to partially revert innate immune dysregulation by restoring the numbers of certain DC populations [268]. However, from our results, it is clear that viremia rebound during short-term TI leads to both dysregulated pDC functions, as well as decreased numbers of pDC, which is also observed during primary HIV-1 infection [192, 213]. Additionally, the study in **Paper IV** reveals that short-term viremia resulted in elevated immune activation, such as increased levels of HLA-DR+ T cells. However, it did not lead to detectable changes in microbial translocation, measured as levels of LPS in plasma, suggesting that T cell activation is an independent marker of immune activation and that prolonged viremia is required for detectable elevation of microbial translocation. These findings add knowledge to the understanding of HIV-1 immunopathogenesis during viral rebound caused by TI or other causes, such as concomitant infections in untreated individuals.

In summary, the work presented in this thesis provides knowledge on the potential role of TLR9 agonists for suppression of HIV infection and how an established HIV infections

can dysregulate innate immune responses that may contribute to the immunopathogenesis observed during an HIV infection.

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