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Dynamics of HIV coreceptors and their utilization by plasma and cerebrospinal fluid HIV-1 isolates

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Lund 2012

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For every complex problem there is an answer that is clear, simple, and wrong.

Henry Louis Mencken (1880-1956)
For every complex problem there is an answer that is clear, simple, and wrong.

Henry Louis Mencken (1880-1956)
The HIV epidemic
The virus
The keyholes
Chemotactic receptor dynamics
The natural course of HIV-1 infection
HIV CNS infection
Anti-HIV treatment
CCR5 antagonists
Materials and Methods
Isolation of human monocytes and stimulation conditions
cDNA preparation and real-time, quantitative PCR analysis
Flow Cytometry
Chemotaxis
Patients and virus isolates
Generation of coreceptor indicator cell lines and phenotypic determination of coreceptor use
Determining in vitro sensitivity to CCR5 antagonists TAK-779 and Maraviroc
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3 Karlsson U, Repits J, Antonsson L, Cederfjäll E, Ljungberg B, Ålenius M, Sabirsh A, Gisslen M, Medstrand M, Esbjörnsson J* and Jansson M*. Late stage R5 HIV-1 isolates exhibit reduced baseline sensitivity to Maraviroc in vitro – correlation with an altered mode of CCR5 use. Submitted. *These authors contributed equally to this study

Abbreviations

AIDS    Acquired Immune Deficiency Syndrome

cART    Combination anti-retroviral treatment

BLT1    Leukotriene B4 receptor 1

CCL3    CC ligand 3 (previously MIP-1α)

CCL4    CC ligand 4 (previously MIP-1β)

CCL5    CC ligand 5 (previously RANTES)

CNS     Central nervous system

CRF     Circulating recombinant form

CSF     Cerebrospinal fluid

C-terminus Carboxyl-terminus

CTL     Cytotoxic T lymphocyte

DNA     Deoxyribonucleic acid

ECL     Extra-cellular loop

ELISA   Enzyme-linked immunosorbent assay

env     Envelope gene

Env     Envelope protein

gag     Group antigen gene

GALT    Gut-associated lymphoid tissue

gp      Glycoprotein

GPCR    G protein-coupled receptor

HAD     HIV associated dementia

HAND    HIV associated neurological disorders

HIV     Human immunodeficiency virus

HIV-1   Human immunodeficiency virus type 1

HIV-2   Human immunodeficiency virus type 2

HTLV-3  Human T-cell lymphotropic virus 3

IFN     Interferon

LAV     Lymphadenopathy associated virus
LPS  Lipopolysaccharide (Endotoxin from gram-negative bacteria)
LTR  Long terminal repeat
MHC  Major histocompatibility complex
MIP  Macrophage inflammatory protein
MVC  Maraviroc
Nef  Negative regulatory factor gene
NK cell  Natural killer cell
N-terminus  Amino-terminus
PBMC  Peripheral blood mononucleat cells
PCR  Polymerase chain reaction
pol  Polymerase gene
qPCR  Quantitative PCR
R5  Uses CCR5
R3R5  Uses CCR5 and CCR3
R5X4  Uses CCR5 and CXCR4
RANTES  Regulated on activation, normal T-cell expressed, and secreted
rev  Regulator of virion protein gene
RNA  Ribonucleic acid
SDF-1  Stromal cell-derived factor 1
SIV  Simian immunodeficiency virus
SMI  Smittskyddsinstitutet
tat  Transactivator gene
TNF  Tumor necrosis factor
TM  Transmembrane
UNAIDS  United Nations program on HIV/AIDS
vif  Virion infectivity factor gene
vpr  Viral protein R gene
vpu  Viral protein U gene
Aims of the study

Paper I

Leukotriene B4 receptor BLT1, chemokine receptor CCR2 and several related receptors, have been found to act as HIV-1 coreceptors \textit{in vitro}. The aim of Paper I was to investigate how immunomodulatory agents regulate the expression of BLT1 and CCR2 in human monocytes.

Paper II

The aim of paper II was to investigate the use of the major HIV coreceptors, CCR5 and CXCR4, by paired plasma and cerebrospinal fluid HIV-1 isolates from individuals with varying degree of immune deficiency. Further, the aim was to dissect CCR5-use in relation to CCR5 antagonist TAK-779 sensitivity through the use of CXCR4/CCR5 chimeric receptors.

Paper III

Paper III expands upon the correlation between CD4+ T-cell count and sensitivity to CCR5 antagonist inhibition revealed in paper II. The aim was to investigate if late stage plasma HIV-1-isolates displayed altered sensitivity to the licensed CCR5 antagonist Maraviroc, and further, if this would relate to previously reported amino acid resistance polymorphisms within the V1-V3 region of gp120.

Paper IV

The aim of paper IV was to investigate the use of alternative HIV coreceptors by paired plasma and cerebrospinal fluid isolates and to correlate receptor use with clinical and virological parameters including HIV-1 subtype.
Summary

In 1996, the discovery of chemokine receptors CCR5 and CXCR4 as major HIV-1 coreceptors paved way for new anti-HIV compounds that target virus-receptor interactions. Several related receptors were subsequently shown to act as HIV coreceptors in vitro, but their relevance in HIV-1 infection in vivo still awaits confirmation (alternative coreceptors). HIV coreceptors are G-protein-coupled chemotactic receptors that mediate immune cell trafficking. This task requires a flexible and dynamic receptor regulation and is guided by immunomodulatory substances during pathophysiological conditions.

In a prototypic study of the regulation of two HIV coreceptors, BLT1 and CCR2, we investigated the effect of pro- and anti-inflammatory substances on receptor expression in human monocytes. We found that both receptors were up-regulated by anti-inflammatory substances, while pro-inflammatory cytokines like IFN-γ induced their down-modulation. Effects of IFN-γ were time-, and concentration-dependent, were exerted by reductions in mRNA transcription, and resulted in diminished chemotactic response to BLT1 ligand Leukotriene B4. Finally, flow cytometry of fresh blood provided in vivo support for our findings as pre-activated monocytes (CD14+CD16+) expressed reduced surface levels of CCR2 and BLT1. The findings provide insights about the regulation of monocyte trafficking during inflammatory conditions such as HIV-1 infection and further suggest that the dynamics of HIV coreceptor expression may be a target for pharmaceutical intervention.

HIV-1 infects the brain and may cause neurological disease such as dementia in the absence of treatment. Evidence point to a compartmentalized infection of the brain where macrophages and microglial cells support HIV replication. Several alternative HIV coreceptors, in addition to CCR5 and CXCR4, are expressed in the central nervous system (CNS) and may contribute to neuropathogenesis. We investigated the use of coreceptors by paired plasma- and cerebrospinal fluid (CSF) HIV-1 isolates from 28 individuals and found that discordant receptor use was common. Isolates that use CCR5 (R5) predominated in CSF also when virus that use CXCR4 (X4/R5X4) were present in plasma. Furthermore, R5 isolates with the ability to use CCR3 (R3R5)
Populärvetenskaplig sammanfattning


I Delarbete I visar vi hur uttrycket av två kemotaktiska receptorer, CCR2 och BLT1, regleras av inflammatoriska och anti-inflammatoriska substanser. Båda dessa receptorer är så kallade HIV coreceptorer och uttrycks bland annat på monocyter som är viktiga målceller för HIV. Arbetet har betydelse för förståelsen av hur trafikeringen av monocyter styrs och kan bidra till ökade kunskaper om deras roll vid HIV infektion. Vidare ger arbetet stöd för att läkemedel som är verksamma mot inflammation även skulle kunna påverka cellers mottaglighet för HIV genom att påverka uttrycket av coreceptorer.

were found in CSF from individuals with late stage disease and correlated with increased CSF viral load. Finally, CSF isolates from individuals with subtype C HIV-1 infection were able to utilize CXCR6 as coreceptor, which also correlated with high CSF viral load. The results are relevant for the treatment of CNS HIV-1 infection with coreceptor antagonists and further highlight CCR3 as a receptor that may be important for neuropathogenesis. Finally subtype specific differences in coreceptor use may remain unnoticed when analyzing plasma virus HIV-1 isolates.

In late stage R5 HIV-1 infection, viral phenotypes with altered CCR5 use and reduced sensitivity to inhibition by receptor ligands may emerge. Through the use of CXCR4/CCR5 chimeric receptors, we found that late stage R5 virus were less dependent upon interactions with the CCR5 N-terminus. Importantly altered CCR5 use correlated with a reduced sensitivity to CCR5 antagonists TAK-779 and Maraviroc (MVC). V3 Env polymorphisms recently found to correlate with blunted virologic response to MVC in clinical trials were found in some of the least susceptible R5 isolates. The findings provide theoretical support for early initiation of CCR5 antagonists in R5 HIV-1 infection. Furthermore, CXCR4/CCR5 chimeric receptors may be useful tools to further investigate the optimal use of CCR5 antagonists.
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HIV viruset invaderar även hjärnan tidigt i förloppet och obehandlad infektion orsakar ofta neurologiska komplikationer och demensutveckling. Målceller för HIV i hjärnan utgörs av immunceller såsom mikroglia och makrofager. Tidigare studier har visat att viruset i hjärnan ofta skiljer sig ifrån blodisolat. I vätskan som omger hjärnan och ryggmärgen (cerebrospinalvätskan) finner man virus som ofta kommer från infekterade hjärnceller. Vi har studerat parade blod- och cerebrospinalvätskevirus från HIV-1 infekterade patienter och har kunnat visa att skillnader i användande av coreceptorer är vanliga.

De viktigaste fynden inkluderar:

1. Virus från hjärnan använde framförallt CCR5 även när det i blodet fanns virus som använder CXCR4. Detta återspeglar sannolikt en fördel att använda CCR5 vid infektion av målceller i hjärnan. Vidare innebär detta att läkemedel som blockerar denna receptor eventuellt kan fungera på hjärninfectionen även när virus i blodet inte påverkas.

2. Coreceptorn CCR3 används i högre grad av isolat från hjärnan och framförallt vid höga virusnivåer i cerebrospinalvätskan. Detta indikerar att CCR3 kan vara viktig för virusets förmåga att replikera i hjärnan. Att högre virusnivåer kunde kopplas till användande av CCR3 ger ett slags bevis för receptorns betydelse i verkligheten som aldrig tidigare visats. Fyndet är av betydelse för förståelsen av hjärninfectionen och öppnar för alternativa behandlingsmöjligheter genom blockad av CCR3.

3. HIV-1 kan indelas i s.k. subtyper varav subtyp C orsakar ca 50 % av den globala epidemin och 80 % av infektioner i Afrika söder om Sahara. Vi fann att hjärnisolaten från flertalet patienter med subtyp C infektion kunde använda receptorn CXCR6 som coreceptor. Även detta sammanföll med oväntat höga virusnivåer i cerebrospinalvätskan. Fyndet gav en hög statistisk säkerhet men bekräftande studier krävs eftersom det rörde sig om få patienter. Resultatet kan ha stor betydelse eftersom höga virusnivåer i cerebrospinalvätska har visat
sig korrelera till neurologiska komplikationer vid HIV-1 infektion

Introduction

This thesis examines different aspects of cellular coreceptors in HIV-1 pathogenesis and is based on four manuscripts included as appendices at the end of this book. In the background section a selection of data needed to understand subsequent results and conclusions are presented.

The first manuscript (Paper 1) investigates how immunomodulatory agents regulate the expression of Leukotriene B4 receptor, BLT1, in human monocytes. The findings are relevant for aspects of Leukotriene B4 biology. However, in this thesis, Paper 1 should be viewed upon as a prototypic study on how inflammation may regulate coreceptor expression, and consequently, immune cell trafficking and pathogenesis in HIV-1 infection.

Papers 2-4 investigate the use of HIV coreceptors by plasma and cerebrospinal fluid clinical HIV-1 isolates. The focus in these manuscripts was to explore how immunological (e.g. CD4 counts) and virological parameters (e.g. viral load, envelope glycoprotein polymorphisms, HIV subtype) may correlate with coreceptor use and sensitivity to inhibition by receptor blocking agents.

Making it simple the main message of this book can be summarized in one sentence: HIV-1-induced inflammation up-regulates virus keyholes on target cells, thereby rendering them susceptible to infection and, as the keyholes normally regulate cell trafficking, these cells now spread the infection, which contributes both to the deterioration of the immune system (which in turn allows for the emergence of virus variants, with altered coreceptor use and reduced sensitivity to antagonists that block the main coreceptor CCR5) and to viral invasion of the brain, where local target cells with a different set of keyholes may select for, possibly subtype specific, virus variants, adapted to the environment and able to induce CNS damage through multiple mechanisms.
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The HIV epidemic

In the summer of 1981 reports from California and New York City described clusters of a rare pneumonia, Pneumocystis Carinii Pneumonia (PCP), and an unusual form of skin cancer, Kaposi Sarcoma, in young homosexual men [1]. These conditions had previously been related to immune deficiency and high age. It was soon suspected that the underlying cause, later to be known as acquired immunodeficiency syndrome (AIDS), was induced by an infectious agent. After subsequent reports of illness in hemophiliacs, injection drug users and in heterosexual women, it was apparent that the new disease spread through contaminated blood and unprotected sex. Reports of related conditions from European countries and of “slim disease” in Africa showed that AIDS was of global concern [2-4]. In 1983 researchers at Pasteur institute in Paris isolated a retrovirus from an enlarged lymph node of a patient with symptoms preceding AIDS [5]. Initially called LAV and HTLV-III, the virus was in 1986 eventually named human immunodeficiency virus (HIV) [6]. The first active anti-HIV drug, AZT, was also launched in 1986 [7]. However, it was not until 1996, with the introduction of protease inhibitors and molecular techniques to assess viral load, that sustained response to treatment was achieved through combination anti-retroviral therapy [8, 9].

The resources invested to fight the global HIV/AIDS epidemic during the first two decades were comparatively modest and anti-retroviral medication was mainly restricted to individuals in high-income countries. Successful HIV prevention campaigns in some low-income countries like Uganda increased the public awareness about routes of infection and had effect on local epidemics [10]. However, it was not until global HIV/AIDS funding increased substantially in the 2000’s and programs to increase access to medication for those in need were launched, that infections and AIDS-related deaths declined (Fig 1).
Background

The HIV epidemic

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The virus HIV belongs to the lentivirus genus within the family of Retroviruses known to cause chronic infections in a variety of vertebrates [12]. HIV-1 is the virus responsible for the global epidemic while the genetically related virus HIV-2 mainly has caused a local epidemic in West African countries [13]. HIV-2 is also less virulent and causes AIDS less frequently and at a slower pace than HIV-1 [14]. Although global spread of HIV-1 accelerated in the early 1980’s, the virus is believed to have crossed over to humans from simian immunodeficiency virus (SIVcpz) infected Pan troglodytes troglodytes chimpanzees in west central African forests around year 1900 [15], while HIV-2 emanated from SIV infected sooty mangabey monkeys (SIVsmm) in West Africa [16]. Three genetically distinct groups of HIV-1 have been found, the major group (M), the “non-M,” non-O group (N), and the “outlier” group (O). Group M is by far the most prevalent group and is further divided into different subtypes (A-D, F-H, J-K). Sub-subtypes and circulating recombinant (CRFs) forms also exist. The different genetic subtypes are mainly confined to certain geographical locations. For instance, subtype B (10% of the global prevalence) dominates in Europe, North America and Australia, and subtype C (50%) in South and East Africa and India. Subtype specific viral characteristics may exist that influence HIV-1 transmission, cellular entry and disease progression [17-20].

Fig 1. Global annual investments to combat the HIV pandemic in relation to new infections and deaths from AIDS. Adopted from UNAIDS with permission.

According to UNAIDS, the annual total funding for combating HIV and AIDS slowly increased in the 1990s, from only $200 million to $1 billion. In contrast, between 2000 and 2009, global AIDS commitments increased dramatically, from $1.4 billion to nearly $16 billion a year. Even though the money spent for the first time decreased estimated deaths from AIDS, the global economic crisis in 2008 caused donor commitments to plateau in 2009 and the curve is now dropping slowly (UNAIDS).

Meanwhile, HIV has caused the death of more than 30 million people, mainly in low and middle-income countries. In 2010, 2.7 million were infected and 1.8 million died of AIDS (UNAIDS). In Sweden, 9879 individuals were reported to be HIV positive and 2 428 were diagnosed with AIDS during 1983-2011. Each year around 450 individuals are diagnosed with HIV in Sweden [11].
The virus

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![Structure of the HIV particle.](image)

*Fig 2.* Schematic view of the virus particle. Kindly provided by Salma Nowroozalizadeh.
The virus particle is spherical and approximately 100 nm in diameter. It is composed of two single stranded RNA molecules located in a conical capsid composed of the viral protein p24. The viral genome consists of approximately 9.7 kilo base pairs, which is divided into three gene segments (pol, gag and env) that encodes enzymes and structural proteins. The HIV-1 genome encodes for additional two regulatory proteins (Tat and Rev) and four accessory proteins (Nef, Vif, Vpr and Vpu). The capsid is surrounded by a matrix of viral p17 proteins, which in turn is covered by an envelope bilayer of phospholipids of cellular origin formed during viral budding from an infected cell. Several spiked knobs consisting of trimers of the viral envelope glycoprotein gp120, which protrude from the envelope, are non-covalently anchored to the phospholipid envelope layer by the transmembrane gp41 protein. The glycosylated Env proteins are responsible for the viral attachment to, and fusion with, cellular target cells.

**Viral replication cycle**

![Viral replication cycle diagram](image)

**Fig 3.** HIV-1 enters target cells through sequential interactions with CD4 and a coreceptor. Inside the cell the viral RNA is reverse transcribed to proviral DNA by viral reverse transcriptase. The viral genome is transported to the nucleus where it is integrated into the host DNA by viral integrase. Cellular enzymes perform subsequent transcription and translation into viral proteins. New virus particles assemble at the
plasma membrane. After budding the viral protease cleaves polyprotein precursors and mature infectious virions are formed. Adopted with permission from NIAID.

**The keyholes – HIV coreceptors**

The primary cellular receptor for HIV entry is the CD4 molecule [21, 22]. However, the CD4 molecule is by itself not sufficient to allow HIV entry. In 1996, the chemokine receptors CCR5 and CXCR4 were found to act as coreceptors that mediated HIV infection of CD4+ immune cells [23-26].

![Fig 4. HIV-1 entry mechanism.](image)

After binding to the CD4 molecule, conformational changes in the gp120 expose epitopes that subsequently bind to a coreceptor. This induces further conformational changes, which allows the fusion peptide to be inserted into the host cell membrane. In the final step a hairpin formation brings the viral envelope and the cellular membrane to fuse. The figure was kindly provided by CSH Press.

Chemokine receptors, like CCR5 and CXCR4, are a group of chemotactic transmembrane (TM) receptors that belong to the superfamily of G-protein coupled receptors (GPCRs) [27, 28]. They consist of a highly acidic N-terminus on the outer surface of the cell, possess seven TM helices, three extra- and intracellular loops and a C-terminus that transmit signals from extracellular ligands to intracellular pathways via G-proteins. Receptor interactions with corresponding chemokines (chemotactic cytokines) mediate important tasks such as immune cell migration, activation and differentiation during pathophysiological conditions [29]. Chemokine receptors and their receptors often display a certain amount of promiscuity [30]. It was actually the discovery that three CCR5 ligands RANTES, MIP-1 α and β (now named CCL5, CCL3 and CCL4) inhibit macrophage tropic HIV-1, that pointed the direction as chemokine receptors subsequently were identified as HIV coreceptors [31]. Shortly
Several other GPCRs have been shown to act as HIV coreceptors in vitro [23, 40-50], but only CCR5 and CXCR4 are undisputedly relevant for HIV-1 infection and pathogenesis in vivo. However, alternative coreceptors may contribute to HIV infection and pathogenesis, e.g., compartmentalized at specific anatomical sites such as within the central nervous system (CNS), and may also aid the infectious process in a co-operative manner with the major coreceptors CCR5 and CXCR4 [51-54].

**Chemotactic receptor dynamics**

The physiological role of chemotactic GPCRs is to act as antennas, which screen the environment for signaling molecules and initiate an appropriate response when these are encountered. This may include cellular activation, differentiation, and importantly, the migration towards increasing concentrations of chemotactic signals [55, 56]. This way, the interplay between chemotactic signals and their corresponding chemotactic receptors regulate immune cell trafficking and function during pathophysiological conditions [57-60]. Fine-tuning of these important tasks requires flexibility in receptor expression [61]. For example, at a site of infection, chemotactic ligands are secreted in order to attract circulating immune cells from the blood stream. Immune cells expressing the corresponding receptors are activated by the signals and transmigrate into the inflamed tissue. At the same time the receptors are internalized and genetically down-regulated to ensure immune cell arrest at the site of inflammation [46]. Following successful engagement with the invading microbe, a new set of receptors is expressed that responds to chemotactic homing signals from nearby lymphoid tissues [62]. Following presentation of the pathogen in lymphoid tissue, a more specific and robust immune response to the invading organism is generated. Hence, a flexible and directed cellular expression of chemotactic GPCRs is a pre-requisite for a functional immune system. In this intricate regulation of chemotactic receptors and their ligands, pro- and anti-inflammatory substances, such as cytokines and microbial products, may play important roles [63-67]. For example, CCR5 expression is mainly restricted to pre-activated subsets of CD4+ T-cells and monocytes, while naïve and resting counterparts display limited expression of the receptor [68-70]. In this way, inflammatory stimuli may increase susceptibility to HIV thereafter, the CXCR4 ligands SDF-1 \( \alpha \) and \( \beta \) were found to inhibit T-cell line tropic HIV-1 isolates [32].

![Schematic view of an HIV coreceptor](image)

**Fig 5. Schematic view of an HIV coreceptor.** The N-terminus and Extra cellular loops (ECLs), mainly ECL2, are important for the binding of HIV-1.

HIV-1 strains that exclusively utilize CCR5 (R5) predominate in asymptomatic individuals, whereas viruses with the ability to utilize CXCR4 alone (X4), or in addition to CCR5 (R5X4), emerge in 13-76% of individuals during progression to AIDS, depending on HIV subtype [33]. Although R5X4 phenotypes are more common than X4 variants, the acquisition of CXCR4 use is often termed coreceptor switch. In individuals who progress to AIDS without this switch, R5 phenotypes with an altered use of CCR5 and reduced susceptibility to receptor ligands may emerge [34]. The pivotal role of CCR5 in HIV-1 infection is emphasized by a polymorphism comprising a 32-base pair deletion in the receptor gene (CCR5Δ32). This mutation, yielding truncated receptors that are retained in the cytoplasm, is associated with resistance to HIV-1 infection homozygous individuals (~2% of Caucasians) [35-37]. Furthermore, reduced receptor expression in heterozygous individuals confers a slower progression to AIDS [38, 39].
Several other GPCRs have been shown to act as HIV coreceptors *in vitro* [23, 40-50], but only CCR5 and CXCR4 are undisputedly relevant for HIV-1 infection and pathogenesis *in vivo*. However, alternative coreceptors may contribute to HIV infection and pathogenesis, e.g., compartmentalized at specific anatomical sites such as within the central nervous system (CNS), and may also aid the infectious process in a co-operative manner with the major coreceptors CCR5 and CXCR4 [51-54].

**Chemotactic receptor dynamics**

The physiological role of chemotactic GPCRs is to act as antennas, which screen the environment for signaling molecules and initiate an appropriate response when these are encountered. This may include cellular activation, differentiation, and importantly, the migration towards increasing concentrations of chemotactic signals [55, 56]. This way, the interplay between chemotactic signals and their corresponding chemotactic receptors regulate immune cell trafficking and function during pathophysiological conditions [57-60]. Fine-tuning of these important tasks requires flexibility in receptor expression [61]. For example, at a site of infection, chemotactic ligands are secreted in order to attract circulating immune cells from the blood stream. Immune cells expressing the corresponding receptors are activated by the signals and transmigrate into the inflamed tissue. At the same time the receptors are internalized and genetically down-regulated to ensure immune cell arrest at the site of inflammation [46]. Following successful engagement with the invading microbe, a new set of receptors is expressed that responds to chemotactic homing signals from nearby lymphoid tissues [62]. Following presentation of the pathogen in lymphoid tissue, a more specific and robust immune response to the invading organism is generated. Hence, a flexible and directed cellular expression of chemotactic GPCRs is a pre-requisite for a functional immune system. In this intricate regulation of chemotactic receptors and their ligands, pro- and anti-inflammatory substances, such as cytokines and microbial products, may play important roles [63-67]. For example, CCR5 expression is mainly restricted to pre-activated subsets of CD4+ T-cells and monocytes, while naïve and resting counterparts display limited expression of the receptor [68-70]. In this way, inflammatory stimuli may increase susceptibility to HIV
infection through CCR5 up-regulation on activated CD4+ immune cells [67, 68]. Adding complexity to the issue, an enhanced HIV susceptibility induced by inflammatory cytokines may be counteracted by chemotactic ligands released in response to inflammation [71]. These receptor ligands induce CCR5 down-modulation, which may result in protection from viral infection [72].

The natural course of HIV-1 infection

HIV-1 invades the immune system and untreated infection will eventually result in AIDS and death in the vast majority of cases. Within two-three weeks, infected individuals often develop symptoms of a primary infection, which most commonly presents as fever, skin rash, throat infection, and lymphadenopathy [73]. HIV early establishes a virus reservoir within lymphoid tissue where large amounts of virions are produced daily, accounting for the high systemic viral load during primary infection [74]. A few weeks later viral load generally decreases and CD4+ T-cell count increases as the immune system partly has gained control of the infection.

The immune system employs several strategies, involving both innate and adaptive responses, to combat the infection. The innate immune response is immediate and includes phagocytosis by macrophages, NK cells and dendritic cells, release of inflammatory cytokines such as chemokines, and activation of the complement system. The adaptive immune system evolves with time and consists of cell-mediated and humoral responses. The cell-mediated response includes the activation of cytotoxic CD8+ T-cells (CTLs) by CD4+ T helper cells typ-1 (Th1) and subsequent elimination of infected cells. The humoral responses include the activation of B lymphocytes and the generation of antibodies towards different viral epitopes. With the generation of adapted immune responses, partial control of the infection is established.

However, HIV has developed several strategies to evade the immune system. These include mutational escape, genetic recombination, glycosylation of envelope proteins, latency and down-modulation of the class I major histocompatibility complex (MHC-I) on the surface of infected cells [75-79]. Consequently, even during several years of asymptomatic infection, billions of viral particles may be produced daily.
Target cells for HIV include CD4+ T-cells, monocytes and macrophages as well as microglial cells in the brain. During the acute phase of infection CD4+ memory T-lymphocytes in the gut associated lymphoid tissue (GALT) are preferentially infected and massively depleted [80, 81]. This may irreversibly disturb the mucosal integrity, leading to the translocation of microbial products, e.g. lipopolysaccharides (LPS), into the blood stream [82]. Subsequent activation of immune cells may increase their susceptibility to HIV infection [83], stimulate virus replication [84], and contribute to the viral invasion of the central nervous system (CNS) [85] (see below). Furthermore, the depletion of CD4+ T-cells during progressive HIV infection is by large mediated through activation-induced apoptosis of non-infected cells [86]. Taken together, the contribution of a chronic hyperactive immune system during HIV-1 infection and its consequences for AIDS pathogenesis cannot be overemphasized [87-90].

With time the infection causes the immune system to collapse, and the partial control over virus replication is lost. As a result viral load increases and virus variants with enhanced replication capacity and expanded target cell repertoire emerge and disease progression is accelerated [91-93]. Eventually, infected individuals become susceptible to opportunistic infections and certain forms of cancer, defining the stage of AIDS.
HIV CNS infection

HIV-1 infects the brain early in the course of infection and frequently causes neurologic impairment such as HIV associated dementia (HAD) in the absence of anti-retroviral treatment [94, 95]. HAD manifests as a subcortical dementia, characterized mainly by psychomotor slowing, mood changes and memory deficit [96]. Major neuropathological changes include white matter pallor, giant cell formation and gliosis [94, 97]. With the introduction of cART, HAD largely vanished in clinical practice. However, minor forms of HIV associated neurological disorders (HAND) still pose a medical concern even in individuals on efficient cART [98].

In a widely accepted model, HIV invades the brain through a “Trojan horse” mechanism, where infected pre-activated monocytes (CD14+CD16+) cross the blood brain barrier (BBB) and later differentiate into macrophages [85, 99, 100]. The HIV susceptible CD14+CD16+ monocytes constitute 5-15% of blood monocytes in uninfected individuals [101]. However, with increasing systemic inflammation in progressive HIV infection, the CD14+CD16+ monocytes subset is expanded, which may contribute to an increased viral invasion of the brain and accelerated neuropathogenesis during late stage disease [99, 100, 102-105].

Fig 6. The Trojan horse. Activated CD14+CD16+ monocytes up-regulate CCR5 and become susceptible to HIV-1 infection. Infected CD14+CD16+ cells migrate to the brain in response to chemokines and are believed to be key transporters of HIV into the CNS.
Infection of the central nervous system (CNS) is established as the virus infects local target cells. These are mainly comprised of perivascular macrophages and microglial cells, although non-productive infection of astrocytes also has been demonstrated [94, 106, 107]. The mechanisms by which HIV induces CNS injury are probably multiple and include both direct and indirect effects of viral infection and toxic products secreted by infected cells [104, 105, 108-111].

Previous studies have revealed a compartmentalized infection of the brain, most likely reflecting an adaption to replication in available target cells [108, 112-115]. Several HIV coreceptors are expressed in the CNS, where they may mediate infection of target cells and/or contribute to neurologic damage through indirect mechanisms [116-120]. The role of coreceptors other than CCR5 and CXCR4 for HIV infection in CNS has so far received minor attention.

**Anti-HIV treatment**

Combination anti-retroviral treatment (cART) has revolutionized HIV management. It was early recognized that a combination of compounds, preferably acting at different sites of the replication cycle, was necessary to achieve sustained viral suppression and immunologic success. Over the years, knowledge about the efficiency of drug combinations and toxicity issues has increased. Medication has become simplified with the introduction of tablet formulations that combine different compounds. New pharmaceuticals that are less toxic and displaying more robust genetic resistance barriers have emerged from existing classes. Importantly, the recent introduction of new classes of anti-retrovirals, such as integrase inhibitors and CCR5 antagonists, has provided better opportunities to successfully manage HIV-1 infection in individuals with multi-resistant viruses.

In all, the progress made in the area of anti-HIV treatment has substantially improved the prognosis, and recently infected individuals, in countries with modern care, now have similar life expectancy to uninfected [121]. As cART has become simplified, more efficient and less toxic to patients over time, achieving viral suppression, undetectable viral load and sustained increase in CD4+ T-cell count should be
expected in most instances. However, resistant HIV-1 strains may still pose a major obstacle to successful treatment for some.

**CCR5 antagonists**

In addition to being largely protected against HIV-1 infection, individuals homozygous for the CCR5Δ32 mutation displayed an apparently normal immune status and general health. As CCR5 ligands inhibited R5 virus replication, the receptor became a highly attractive target for pharmaceutical intervention [31]. Subsequent studies have shown that the CCR5Δ32 mutation may confer an increased risk for more serious clinical outcomes of certain flavivirus infections, such as West Nile virus infection and tick-born encephalitis [122, 123].

Within a few years, the first small-molecule inhibitors with potential for oral administration were presented [124-126]. So far three compounds have reached evaluation in phase II-III clinical trials. While Aplaviroc was halted due to unusual, but serious, liver toxicity events and Vicriviroc was discontinued due to failure to show non-inferiority, Maraviroc was in 2009 the first CCR5 antagonist to be approved for the treatment of R5 HIV-1 infection [127] New compounds are being developed and are evaluated in early clinical trials [128].

The small-molecule CCR5 inhibitors all bind to a hydrophobic pocket within the trans-membrane regions of the receptor [129]. The binding induces dramatic changes mainly of the N-terminus and ECL2 regions, leading to a receptor conformation that no longer is recognized by the virus (allosteric inhibition). Resistance to CCR5 antagonists in clinical trials were mainly caused by the expansion of pre-existing naturally resistant X4/R5X4 virus, although R5 virus resistance also occurred (see Discussion).
Materials and Methods

Isolation of human monocytes and stimulation conditions

All experiments were conducted under carefully monitored LPS-free conditions. Highly purified human monocytes (90-95%) were isolated from blood donor buffy coats using Ficoll and Percoll separation. Cells were maintained in serum-free macrophage medium and allowed to rest over night before stimulation with various pro- and anti-inflammatory substances (IFN-gamma, TNF-alpha, LPS, IL-4, IL-10 and Dexamethasone) and, for mRNA decay studies, with Actinomycin D. Cells were harvested at different time-points for further analysis.

cDNA preparation and real-time, quantitative PCR analysis

Cells were lysed in lysis/binding buffer and processed further with a QIAshredder to reduce viscosity. PolyA+ RNA was captured from the cell lysate with Oligo dT14 paramagnetic beads. After repeated washing in buffer solutions, polyA+ RNA was eluted in water. cDNA synthesis was carried out using deoxy-unspecified nucleoside 5-triphosphate (dNTP), Oligo (dT), RNaseOUT and reverse transcriptase in a synthesis buffer solution. Real-time, quantitative PCR was performed in a LightCycler system using the Sybr Green I detection method. External standards were generated using specific PCR products for each gene that had been gel-purified using QIAquick gel extraction kit. The copy number was calculated based on the measured concentration at 260 nm, and serial tenfold dilutions were made in ultra-pure water.

Flow Cytometry

Monocytes were incubated with cold (4°C) washing buffer without Ca²⁺ and Mg²⁺, supplemented with 0.5% bovine serum albumin and 0.05% NaN3 before gentle removal with a cell-scaper. Cells were suspended in washing buffer and stained with the appropriate antibodies. The cells were incubated for 30 min at 4°C in the dark, washed, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson) and the CellQuest software.
Chemotaxis

Non-adherent, IFN-gamma treated monocytes, maintained in suspension in polypropylene tubes, were stimulated with LTB4 at increasing concentrations in a chemotaxis chamber for 1 hour. Cells that had migrated across a polycarbonate filter with 5 um pores were counted using an inverted microscope.

Patients and virus isolates

Twenty-eight HIV-1-infected patients with varying CD4+ T-cell counts, varying levels of CSF and plasma viral load, and with or without HAD were retrospectively selected for participation in the study, from a longitudinal study cohort at the Department of Infectious Diseases, Sahlgrenska University Hospital, Gothenburg, Sweden. Informed consent was obtained from all participants. The Research Ethics Committee at The University of Gothenburg approved the study. Peripheral CD4+ T cell counts and HIV-1 RNA levels in plasma and CSF were analyzed for each individual. The patients, 22 males and six females, had CD4+ T-cell counts ranging from 27 to 820 cells/μL (median 190). Fourteen patients were severely immunodeficient, with CD4+ T-cell counts <200 cells/μL. Plasma viral load ranged from 1900 to 682,000 copies/mL (median 52,000) and CSF viral load ranged from 600 to >750,000 copies/mL (median 66,000). Seven patients had HIV associated dementia, as assessed by the criteria defined by the CDC and the American Academy of Neurology AIDS Task Force. Twenty-five patients were antiretroviral treatment naive, and none had received antiretroviral medication for at least 9 months prior to virus isolation. Paired plasma and CSF samples were collected from each individual and virus isolation was performed as previously described [130].

Generation of coreceptor indicator cell lines and phenotypic determination of coreceptor use

U87.CD4 cells, stably expressing CCR5, CXCR4 or one of three CXCR4/CCR5 chimeric receptors were previously constructed by L. Antonsson [131]. NP-2.CD4
and NP-2.CD4.APJ were kindly provided by Professor Hiroo Hoshino (Gunma University School of Medicine, Japan). NP-2.CD4 cells were stably transfected with sequence verified cDNA encoding coreceptors with documented expression within the CNS (CCR5, CXCR4, CCR3, CXCR6, GPR1, ChemR23, RDC1 and BLT1). Expression was verified by flow cytometry or mRNA analysis. The NP-2 cell line is a glioma cell line that was specifically chosen due to its documented lack of endogenous coreceptor expression [132]. Coreceptor indicator cells were inoculated with patient isolates and positive infections were defined by the production of viral protein p24 in infected wells.

**Determining in vitro sensitivity to CCR5 antagonists TAK-779 and Maraviroc**

IL-2- and PHA-stimulated peripheral blood mononuclear cells were inoculated with virus in the presence of increasing concentrations of antagonists. At defined time-points virus p24 antigen production in infected cultures was assessed. Dose-response curves were generated and antagonist concentrations needed to inhibit 50% (IC50) and 90% (IC90) of virus infections were calculated.

**Sequence analysis of plasma virus V1-V3 Env gp120 sequences and subtype determination**

RNA from virus isolates was extracted and reverse transcribed [33]. The HIV-1 env gp120 V1-V3 region was amplified, cloned and sequenced. Sequences were aligned and manually edited in MEGA4 [133] and further examined for mutations previously reported to convey CCR5 antagonist resistance and or poor virologic response to MVC in vivo. For subtype determination, one representative sequence from each patient was aligned with a reference sequence data set of all major subtypes, sub-subtypes and CRFs.
Results and Discussion

Dynamic expression of HIV coreceptors in human monocytes

Following the discovery of CCR5 and CXCR4 as necessary coreceptors for HIV entry, subsequent attention was focused on means of blocking virus-receptor interactions. Less attention was paid to the highly dynamic expression of coreceptors and means of reducing cellular susceptibility to HIV by intervening with receptor regulation. Importantly, target cell susceptibility to HIV may be enhanced by inflammatory stimuli that increase CCR5 expression [63, 67, 134]. Chronic immune activation is also a hallmark in progressive HIV-1 infection. In a study elaborating on the effects of inflammation for HIV-1 pathogenesis, Andrieu et. al. demonstrated a sustained increase in CD4+ T-cell count in HIV-1 infected individuals treated with the corticosteroid prednisolone [135]. This led us to hypothesize that anti-inflammatory agents, such as glucocorticoids, may reduce cellular sensitivity to R5 HIV infection through the down-modulation of CCR5.

In unpublished work preceding Paper I we investigated how the prototypic glucocorticoid Dexamethasone (Dex) affected CCR5 expression in subsets of white blood cells. We found that CCR5 surface expression was scarce on fresh blood monocytes and that the receptor was up regulated within 18 hours after isolation. Dex reduced CCR5 surface expression by 50-80 % under the same conditions.
Results and Discussion

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Fig 8. Dex enhances R5 HIV-1 entry in monocytes.
Monocytes cultured in cell medium (CM) over night were barely infected by R5 virus BaL. Dex at 10^{-7}M (D-7) enhanced viral entry. This was reversed by the glucocorticoid inhibitor Mifepristone (D-7+M-6). IL-10 had little effect on HIV entry but possibly displayed an additive effect together with Dex (IL-10+D-7). Y-axis displays the amount of early HIV 5´LTR copies as quantified by real-time qPCR.

The methodologies applied in this unpublished work, preceding paper I, have some limitations. Monocytes are activated during the isolation process and commence their differentiation into macrophages upon adherence to tissue culture plastic [136]. Stimulating monocytes early after their purification means intercepting a highly dynamic process, which may blur the interpretation of the results. Glucocorticoids may affect monocyte activation and differentiation into macrophages in multiple ways [137-140]. Previous work demonstrated that CCR5 is up-regulated as monocytes differentiate into macrophages in vitro [141]. Accordingly, our results could have been explained by a crude blockage of monocyte differentiation. However, macrophage differentiation markers such as CD16 and the Mannose receptor were up-regulated by dexamethasone in our study. This indicates that some processes linked to monocyte-macrophage differentiation may even be enhanced in the presence of glucocorticoids. Finally, the increase in early HIV LTR transcripts in glucocorticoid treated monocytes may be explained by the presence of these transcripts in virions.

Fig 7. Dex reduces CCR5 surface expression on human monocytes.
Flow cytometry showed that the up-regulation of CCR5 expression after overnight incubation in cell medium (green) was reduced by treatment with Dex (Red). Dotted lines represent negative isotype controls.

Lowered CCR5 surface expression was accompanied by reductions in receptor mRNA transcripts. Levels of the CCR5 ligands CCL3, CCL4 and CCL5 in cell culture supernatants were reduced by dexamethasone and were hence not responsible for lower surface levels through receptor internalization. The effect of Dexamethasone was time- and concentration dependent, and was blocked by the glucocorticoid antagonist mifepristone (RU 486). To investigate if glucocorticoid induced CCR5 down-modulation affected monocyte susceptibility to R5 tropic HIV, we developed a model to quantify viral entry using real-time quantitative PCR with primers amplifying early HIV LTR transcripts. Unexpectedly, monocytes treated with dexamethasone, and expressing low levels of CCR5, became susceptible to HIV entry by the R5 tropic isolate BaL, whereas untreated monocytes, displaying high levels of CCR5, were barely infected.
Fig 8. **Dex enhances R5 HIV-1 entry in monocytes.** Monocytes cultured in cell medium (CM) over night were barely infected by R5 virus BaL. Dex at 10^{-7}M (D-7) enhanced viral entry. This was reversed by the glucocorticoid inhibitor Mifepristone (D-7+M-6). IL-10 had little effect on HIV entry but possibly displayed an additive effect together with Dex (IL-10+D-7). Y- axis displays the amount of early HIV 5´LTR copies as quantified by real-time qPCR.

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prior to cellular entry. Consequently, a non-infectious viral entry through endocytosis might explain the increase in HIV transcripts in glucocorticoid treated monocytes, a process that previously was shown to include the Mannose receptor [142].

The study design in paper I was adjusted in order to reduce the risk for confounding factors. Using a modified monocyte isolation process, highly purified cell populations were achieved to minimize indirect effects exerted by contaminating lymphocytes. Further, cells were allowed to rest prior to stimulation, in order to ensure that the effects from isolation stress were minimal.

The Leukotriene B4 receptor BLT1 participates in the immune response to invading organisms and may contribute to autoimmune inflammatory conditions and atherosclerosis [143-149]. Furthermore BLT1 as well as CCR2 are coreceptors for selected HIV isolates [50, 150]. Given that monocytes are HIV target cells as well as key players in the orchestration of immune responses this led us to conduct study I. In this work we demonstrated that BLT1 and CCR2 are regulated in a similar manner by pro- and anti-inflammatory substances. Both receptors were down-modulated by the inflammatory mediators TNF-alpha, IFN-gamma and LPS, whereas the anti-inflammatory cytokine IL-10 and the glucocorticoid dexamethasone induced their up-regulation.
Fig 9. The effect of pro- and anti-inflammatory mediators on BLT1 (A) and CCR2 (B) mRNA levels. After stimulation for 4 h stimulated cells were lysed and mRNA was extracted. Following cDNA synthesis, receptor transcripts were assessed using real-time qPCR. Results are described as percent gene transcripts in relation to un-stimulated cells (medium).

Further investigations focused on IFN-gamma and showed that the effect on BLT1 expression was time- and concentration dependent and was exerted through reductions in mRNA transcription. The down-modulation of BLT1 receptors resulted in diminished chemotactic response to LTB4. Finally, the in vivo relevance of our findings was substantiated through flow cytometry analysis of whole blood where pre-activated CD14+CD16+ monocytes expressed reduced levels of BLT1 and CCR2.
HIV coreceptor use by paired plasma and cerebrospinal fluid HIV-1 isolates

Use of major coreceptors CCR5 and CXCR4 – R5 isolates dominate in CSF

Previous work has shown that discordant HIV-1 variants may reside in CNS and blood/lymphoid tissue, most probably as a consequence of differences in immunologic surveillance and target cell availability between the compartments [112-115]. The few studies that have addressed coreceptor use by HIV-1 isolates from brain or CSF have found a predominance of R5 tropic virus phenotypes [113, 155].

Our work supports this emerging paradigm as R5 CSF isolates predominated also in four out of seven patients that harbored X4/R5X4 plasma isolates. Previous studies have shown that virus phenotypes with high ability to infect macrophages and microglial cells generally are R5 tropic, although exceptions do occur. Consequently, we believe that the predominance of R5 virus in CSF isolates may reflect a limited capacity of most X4/R5X4 virus phenotypes to replicate in CNS target cells [156]. In a clinical perspective, our findings suggest that CCR5 antagonists may potentially suppress HIV CNS infection also in individuals harboring X4/R5X4 plasma isolates.

To further dissect receptor use all isolates from two compartments were tested for their ability to infect U87.CD4 indicator cells expressing CXCR4/CCR5 chimeric receptors (Fig 11). L. Antonsson initially created the chimeras, in order to characterize viral and receptor epitopes of importance for HIV entry [131]. The constructs also turned out to be useful instruments to dissect viral mode of CCR5 use during disease progression [157].

Fig 10. Differential expression of BLT1 and CCR2 surface proteins in subpopulations of human monocytes in vivo. Whole blood was stained with CD14, CD16, and BLT1 (left panel) or CCR2 antibody (right panel). CD14+CD16+ monocytes showed a decreased expression of BLT1 and CCR2 as compared with CD14++CD16– monocytes.

The down-modulation of CCR2 and BLT1 in CD14+CD16+ monocytes may help to explain the abolished recruitment of this monocyte subtype in some in vivo models of inflammation [151].

CD14+CD16+ cells are believed to represent monocytes that have been pre-activated by inflammatory cytokines or microbial products. This is supported by the dramatic increase in CD14+CD16+ monocytes seen in various inflammatory conditions, such as sepsis, HIV-1 infection, and active rheumatoid arthritis [102, 152-154]. CD14+CD16+ monocytes are believed to be key transporters of HIV into the CNS and may therefore contribute the neuropathogenesis of AIDS [85, 99]. In conclusion, our findings could shed further light on the complex mechanisms that regulate monocyte trafficking during pathophysiological conditions including in progressive HIV infection. Although BLT1 and CCR2 previously have been found to act as HIV coreceptors in vitro their role for HIV-1 infection in vivo in this regard still needs to be determined.
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Fig 12. Correlation between CD4+ T-cell count and CSF viral load at the time of virus isolation of CSF HIV-1 isolates with diverse coreceptor use. CSF viral load $> 40000$ copies/mL (grey horizontal line) defines high viral load (CSFhigh). ★=Isolates obtained from individuals with an AIDS defining disease (ADD). Patients with AIDS (defined by CD4+ T-cell counts $\leq 200$ cells/$\mu L$ or ADD) and/or CSFhigh display CSF R5 isolates able to use CCR3 (black circles). In contrast, monotropic R5 phenotypes (grey circles) characterize virus isolates from individuals with higher CD4+ T-cell counts and CSFlow. R5 isolates of subtype C origin (diamonds) that also use CXCR6 (crossed grey diamond) or CXCR6 and CCR3 (crossed black diamond) originated from patients with CSFhigh despite relatively preserved CD4+ T-cell counts. In individuals with X4/R5X4 infection (CCR3-= grey crosses, CCR3+= black crosses), no clear association between low CD4+ T-cell counts and CCR3 use or CSFhigh was seen.

CCR3 and CCR5 were early identified as HIV coreceptors in microglial cells [23]. However, after conflicting subsequent studies, CCR3 attracted less attention as an HIV coreceptor until recently. Using a gene knockout strategy, Agrawal et. al. showed that CCR3 and CCR5 may act in concert to mediate HIV infection of macrophages and microglial cells [51]. Consequently, an enhanced ability by R3R5 viruses to replicate in CNS target cells would explain why their presence in CSF coincide with high CSF viral load in our study. This may provide in vivo support for the role of CCR3 in HIV-1 infection of the brain.

Fig 11. Chimeric CXCR4/CCR5 receptors. L.Antonsson constructed the chimeras by successively exchanging parts of CCR5 with corresponding parts of CXCR4.

Through the use of the CXCR4/CCR5 chimeras we detected a discordant CCR5 use in six out of 21 paired plasma and CSF R5 isolates. However, no specific pattern of receptor use distinguished isolates from the two compartments. Nevertheless, the findings further support a frequent viral compartmentalization in HIV infection.

R3R5 isolates in CSF coincide with AIDS and high CSF viral load

In addition to CCR5 and CXCR4, several alternative chemokine receptors and related GPCRs have demonstrated coreceptor activity for selected HIV strains in vitro. Although no alternative coreceptor has received a defined role for HIV-1 infection in vivo, studies addressing this issue have mainly been performed using blood derived isolates. A number of coreceptors, in addition to CCR5 and CXCR4, are distributed on cells within the CNS where they may contribute to HIV pathogenesis. We constructed NP-2.CD4 cell-lines stably expressing CCR5, CXCR4 and alternative receptors with documented expression in the CNS and tested their ability to mediate viral infection by plasma and CSF isolates. The main findings included an enhanced ability of CSF R5 isolates to use CCR3 (R3R5), specifically in individuals with AIDS. Importantly R3R5 tropism by CSF isolates correlated with high CSF viral load.
Fig 12. Correlation between CD4+ T-cell count and CSF viral load at the time of virus isolation of CSF HIV-1 isolates with diverse coreceptor use. CSF viral load > 40,000 copies/mL (grey horizontal line) defines high viral load (CSF<sup>high</sup>). ★ Isolates obtained from individuals with an AIDS defining disease (ADD). Patients with AIDS (defined by CD4+ T-cell counts ≤ 200 cells/μL or ADD) and/or CSF<sup>high</sup> display CSF isolates able to use CCR3 (black circles). In contrast, monotypic R5 phenotypes (grey circles) characterize virus isolates from individuals with higher CD4+ T-cell counts and CSF<sup>low</sup>. R5 isolates of subtype C origin (diamonds) that also use CXCR6 (crossed grey diamond) or CXCR6 and CCR3 (crossed black diamond) originated from patients with CSF<sup>high</sup> despite relatively preserved CD4+ T-cell counts. In individuals with X<sub>4</sub>/R<sub>5</sub>X<sub>4</sub> infection (CCR3<sup>−</sup> = grey crosses, CCR3<sup>+</sup> = black crosses), no clear association between low CD4+ T-cell counts and CCR3 use or CSF<sup>high</sup> was seen.

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CXCR6 use by CSF isolates from individuals with subtype C HIV-1 infection

Four out of five CSF isolates from individuals with subtype C HIV-1 infection utilized CXCR6 as compared to one out of 23 from non-subtype C infected. CXCR6 use was highly efficient and comparable to CCR5 use in one CSF isolate. This isolate also displayed efficient CCR3 use and emanated from a patient with extremely high CSF viral load despite plasma load being low. Strikingly, CXCR6 use by subtype C isolates coincided with high CSF viral load despite CD4+ T-cell counts being relatively preserved (>400 cells/μL). In fact, all other individuals in the cohort with high CSF viral load had AIDS, as defined by CD4+ T-cell counts <200 cells/μL and/or AIDS defining disease. Minor use of other alternative coreceptors, most often GPR1, was noted for some plasma and CSF isolates. The control subtype B isolate B117, but none of the clinical isolates, could utilize BLT1 for entry.

Further studies are warranted to verify if CXCR6 use is a common feature of subtype C HIV-1 isolates and, if so, if this correlates with increased CSF viral load. The latter is specifically important since this may correlate with the development of neurological morbidity such as HAD [158]. Studies addressing the influence of subtype on HIV-1 related neurological complications are few, but have shown that HAD poses a major health concern, also in areas of the world where subtype C HIV-1 infection dominate. Furthermore, subtype C infections constitute approximately 50% of the global HIV-1 epidemic. Our study, which to our knowledge is the first to have assessed the use of alternative coreceptors in paired plasma and CSF HIV isolates, indicates that important subtype specific differences may exist that remain unnoted when analyzing plasma virus alone.
R5 HIV-1 isolates from early and late stages of infection differ in their use of CCR5

In previous work our group demonstrated that R5 virus in non-switch individuals develop enhanced ability to utilize CXCR4/CCR5 chimeric receptors during disease progression [157]. This also correlated with a decreased viral sensitivity to inhibition by CCR5 ligand CCL5 (RANTES). In paper II late stage R5 viruses from individuals with low CD4 T-cell counts displayed a flexible CCR5 use, as demonstrated by an elevated ability to utilize the CXCR4/CCR5 chimeras, specifically receptor FC-2 (Fig 12).

The chimera FC-2 is basically a CCR5 receptor where the native N-terminus has been replaced by the corresponding part of CXCR4 (Fig 11). Consequently, R5 viruses displaying the ability to utilize FC-2 (FC-2+) are less dependent upon interactions with the native CCR5 N-terminus for infection. However, most late stage R5 viruses utilized FC-2 less efficiently than CCR5, which clearly demonstrates that the N-terminus of the receptor facilitates R5 virus interactions with CCR5 during all stages of HIV infection.

Fig 12. R5 HIV-1 isolates from individuals with low CD4+ T-cell count display an altered mode of CCR5 use. Late stage R5 isolates displayed an increased ability to utilize the chimeric receptor FC-2. As previously shown viral use of CXCR4 also correlated with low CD4+ T-cell counts.

Four out of five CSF isolates from individuals with subtype C HIV-1 infection utilized CXCR6 as compared to one out of 23 from non-subtype C infected. CXCR6 use was highly efficient and comparable to CCR5 use in one CSF isolate. This isolate also displayed efficient CCR3 use and emanated from a patient with extremely high CSF viral load despite plasma load being low. Strikingly, CXCR6 use by subtype C isolates coincided with high CSF viral load despite CD4+ T-cell counts being relatively preserved (>400 cells/µL). In fact, all other individuals in the cohort with high CSF viral load had AIDS, as defined by CD4+ T-cell counts <200 cells/µL and/or AIDS defining disease. Minor use of other alternative coreceptors, most often GPR1, was noted for some plasma and CSF isolates. The control subtype B isolate B117, but none of the clinical isolates, could utilize BLT1 for entry. Further studies are warranted to verify if CXCR6 use is a common feature of subtype C HIV-1 isolates and, if so, if this correlates with increased CSF viral load. The latter is specifically important since this may correlate with the development of neurologic morbidity such as HAD [158]. Studies addressing the influence of subtype on HIV-1 related neurological complications are few, but have shown that HAD poses a major health concern, also in areas of the world where subtype C HIV-1 infection dominate. Furthermore, subtype C infections constitute approximately 50% of the global HIV-1 epidemic. Our study, which to our knowledge is the first to have assessed the use of alternative coreceptors in paired plasma and CSF HIV isolates, indicates that important subtype specific differences may exist that remain unnoted when analyzing plasma virus alone.
Late stage R5 isolates display reduced sensitivity to CCR5 antagonists TAK-779 and Maraviroc 
in vitro.

An important consequence of the discovery of HIV coreceptors was the subsequent development of receptor antagonists as anti-retroviral agents. Several small-molecule CCR5 antagonists have been evaluated in various stages of clinical trials but so far only MVC has been governed worldwide approval for the treatment of R5 HIV-1 infection [127, 164, 165]. Previous work by others and us had shown that CCR5 ligands such as CCL5 as well as CCR5 antagonist TAK-779 inhibited late stage R5 viruses less efficiently than viruses from earlier stages of infection [91, 166, 167]. In paper II selected plasma and CSF R5 isolates, with varying ability to utilize chimeric CXCR4/CCR5 receptors, were evaluated for their sensitivity to TAK-779. For seven isolates with an elevated ability to utilize the chimeras, 90% inhibition (IC90) of viral p24 production was not achieved, even at the highest concentration of TAK-779 (990 nM). In contrast, varying IC90 values were achieved for all seven isolates with low chimeric receptor use. In conclusion altered CCR5 use by late stage R5 HIV isolates included a reduced dependency upon the native N-terminus and correlated with a reduced sensitivity to TAK-779.

TAK-779 is an investigational compound that never reached clinical use and cross-resistance between various CCR5 antagonists is unpredictable [168-173]. In paper III we therefore investigated if late stage R5 isolates also were less sensitive to the clinically available CCR5 antagonist MVC. While all 17 R5 isolates included in the study were fully inhibited, late stage viruses displayed reduced baseline sensitivity to MVC as displayed by increased inhibitory concentration values (IC50 and IC90) (Fig 14).

So why do late stage R5 viruses develop a more flexible use of CCR5? Firstly, increased flexibility in CCR5 use most likely reflects an enhanced viral ability to interact with the receptor. This facilitates the viral entry process, which may be reflected in studies showing that late stage R5 isolates display enhanced replication capacity [91]. The explanation as to why virus phenotypes with superior replication capacity appear later than sooner is probably merely opportunistic. Now they can. At earlier stages of HIV infection the relatively preserved immune system is able to control virus replication in part. The virus evades the host immune response by mutational escape and by concealing epitopes of Env glycoproteins in layers of carbohydrate molecules. This heavy glycosylation provides protection from host immunity but at the same time may complicate viral fusion with target cells. Over time, HIV gradually wears down host immunity, allowing the emergence of less glycosylated viral phenotypes with enhanced entry kinetics [91, 159]. The fact that these late virus variants are more sensitive to antibody neutralization, strongly support their opportunistic nature [160, 161].

The depletion of CD4+ T-cells in progressive HIV infection leaves macrophages as increasingly important target cells [162]. A viral ability to explore low levels of CD4 and CCR5 may be necessary for efficient HIV-1 infection of macrophages [163]. In our assay this may be reflected by an altered CCR5 use by virus variants that emerge in late stage disease. We also found a correlation between increased viral ability to utilize chimeric receptors and elevated ability to infect in vitro propagated monocyte-derived macrophages (data not shown).
Late stage R5 isolates display reduced sensitivity to CCR5 antagonists TAK-779 and Maraviroc in vitro

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Several single or combined mutations, mainly within the gp120 V3 region of the envelope, have been found to convey CCR5 antagonist resistance. However, mutagenesis experiments have shown that "resistance polymorphisms" generally are Env context dependent, as they often do not induce CCR5 antagonist resistance in unrelated HIV strains \[129, 178\]. For example, in our study MVC sensitive subtype C isolates displayed a subtype specific V3 polymorphism (316T) that previously conveyed high-grade resistance to MVC in subtype B isolate CC1.85 in vitro \[171\].

However, other genetic markers may exist that help to predict clinical response to CCR5 antagonists. Recently, the V3 polymorphisms 4L and 19S were the only polymorphisms that significantly correlated with blunted virologic response in the MVC clinical trials. These rare polymorphisms, occurring in only 1-2 % of HIV-1 isolates \[179\], were in our study displayed in two of the least MVC susceptible isolates. Although intriguing, further studies are warranted to verify the role of 4L and 19S for clinical response to MVC, and also as markers for reduced sensitivity in vitro.

In conclusion paper II and III show that late stage R5 isolates display a reduced susceptibility to CCR5 antagonists, including to the only clinically available compound MVC. Furthermore, CXCR4/CCR5 chimeric receptors may prove to be useful as future tools to dissect CCR5 use and sensitivity to CCR5 antagonists by R5 tropic HIV-1.

Reduced baseline sensitivity to MVC may be clinically relevant. MVC is poorly distributed to the CNS which may lead to inefficient local viral suppression \[174\]. Furthermore reduced baseline sensitivity may precede the development of resistance to CCR5 antagonists as demonstrated in vitro \[171, 172, 175\]. Resistance to CCR5 antagonists in vivo has mainly been caused by the expansion of pre-existing X4/R5X4 variants that were not detected in pre-treatment screening \[129\]. Improved screening methods have now been developed to cope with this problem \[176\]. However, also R5 HIV-1 can display resistance to CCR5 antagonists including isolates from previously treatment-naïve individuals \[164, 177\] The mechanisms behind R5 HIV-1 resistance to CCR5 antagonists include a viral ability to utilize drug-bound CCR5 receptors \[164, 168, 170, 171, 173, 175, 177\]. This non-competitive resistance is manifested as

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**Figure 14. Late stage R5 HIV-1 isolates display reduced baseline sensitivity to Maraviroc inhibition**

- (A) CD4+ T-cell counts correlate with virus baseline sensitivity to Maraviroc inhibition, i.e. increased IC\(_{90}\), in individuals with R5 HIV-1 infection.
- (B) Late stage R5 HIV-1 isolates, originating from individuals with AIDS, had 4-20 times higher median IC\(_{90}\) values for Maraviroc inhibition than isolates from individuals with higher CD4+ T-cell counts. The isolates 13pl and 23pl (depicted by ★ displayed V3 polymorphisms 4L and 19S respectively that recently have been related to blunted virologic response in MVC clinical trials. Figures display one representative experiment of three performed.

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\[174\]
dose-response curves with plateaus of less than 100% inhibition. As all isolates in the study were fully inhibited, at varying concentrations of MVC, none of the isolates in paper III were by definition resistant to MVC.

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In conclusion paper II and III show that late stage R5 isolates display a reduced susceptibility to CCR5 antagonists, including to the only clinically available compound MVC. Furthermore, CXCR4/CCR5 chimeric receptors may prove to be useful as future tools to dissect CCR5 use and sensitivity to CCR5 antagonists by R5 tropic HIV-1.
Take home messages and future perspectives

HIV utilizes chemotactic receptors as coreceptors during infection of CD4+ immune cells. These receptors have become attractive targets for new anti-retroviral compounds and a CCR5 antagonist is now clinically available. Immune cells need to display a flexible and dynamic expression of chemotactic receptors, in order to execute their functions as patrollers and combaters of invading pathogens. Immunomodulatory agents may regulate receptor expression and consequently, also cellular susceptibility to HIV infection.

The first study in this thesis describes the dynamic expression of two HIV coreceptors, CCR2 and BLT1. We found that pro- and anti-inflammatory substances regulate monocyte expression of both receptors. While the receptors were down-regulated in response to inflammatory mediators, anti-inflammatory signals induced their up-regulation. The results provide insights to basic mechanisms of chemotactic receptor regulation and monocyte trafficking during pathophysiological conditions. As HIV infected CD14+CD16+ monocytes contribute to the spread of the virus, including to the brain, the results may also be relevant for the pathogenesis of AIDS. Finally, understanding the dynamics of HIV coreceptor expression may pave way for alternative strategies to combat HIV infection through the use of immunomodulatory agents.

Most HIV practitioners and researchers are aware that HIV-1 utilizes CCR5 for cellular entry and that coreceptor use may expand to include CXCR4 during late disease progression. Based on results from previous work and our findings, generated through the use of CXCR4/CCR5 chimeric receptors, solid evidence shows that R5 virus phenotypes with altered coreceptor use emerge in late progressive HIV infection also in the absence of switch to CXCR4 use. These late R5 phenotypes still use CCR5 for cellular entry but in an altered and more efficient fashion. Consequently, our results provide information regarding the pathogenesis of R5 HIV-1 infection. Furthermore, we have demonstrated that altered CCR5 use may correlate with reduced sensitivity to CCR5 antagonists, which may be relevant for the optimization of future HIV-1 treatment strategies that include CCR5 antagonists.
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CCR5 antagonists are unique in the arsenal of anti-retroviral agents, as they prevent HIV entry by interacting with a cellular factor and not the virus itself. Taken together, constraints from a preserved immune system aid CCR5 antagonists in their action by preventing the emergence of naturally resistant isolates. Therefore, early initiation of CCR5 antagonists in monotherapy may effectively inhibit virus replication without the risk of inducing resistance. It would be desirable to test this hypothesis in a future clinical trial.

Investigating coreceptor use by paired plasma- and CSF HIV-1 isolates in the second study, we found that R5 virus isolates predominate in CSF. This probably reflects their adaption to replication in CNS target cells. Even in individuals harbouring X4/R5X4 plasma virus, the corresponding CSF isolates were often CCR5 tropic. This suggests that CCR5 antagonists theoretically may suppress R5 HIV-1 infection in the brain even when naturally resistant virus is present in plasma.

CCR3 use was commonly displayed by CSF R5 HIV-1 isolates (R3R5) and was specifically pronounced when CSF viral load was high. In an area of previously conflicting reports, our findings clearly indicate that CCR3 may act as an important coreceptor for HIV entry of CNS target cells e.g. microglia and macrophages in vivo. The finding is relevant for further studies on HIV pathogenesis in the brain and future HIV control strategies.

Subtype C HIV-1 infections constitute approximately 50% of the global pandemic and 80% of infections in sub-Saharan Africa. The finding that CXCR6 use by large was confined to CSF isolates from individuals with subtype C HIV-1 infection was highly significant despite the low number of subjects in our study. Nevertheless, confirmatory studies using larger cohorts are clearly needed to verify if this holds true. Importantly, CXCR6 use coincided with an unanticipated high CSF viral load in individuals with relatively preserved CD4+ T-cell counts. High CSF viral load has previously been found to correlate with neurologic impairment. Consequently, subtype-specific differences in coreceptor use of relevance for the neuropathogenesis of HIV infection may remain unnoticed when analysing plasma isolates.

Mounting evidence, including our findings, show that viruses with resistance or reduced baseline susceptibility to CCR5 antagonists emerge mainly in immunocompromised HIV-1 infected individuals. A highly plausible explanation for this is that these viruses are not able to replicate in an individual with preserved immune functions. In contrast, virus phenotypes from individuals at earlier stages of infection are highly sensitive to CCR5 antagonists.
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Så här dagen innan avhandlingen ska lämnas till tryck är ”skärpan” sådan att det finns en risk att jag glömt personer som borde vara med här. Så känner ni er träffade av detta så ber jag om ursäkt och framför mitt tack även till alla er.

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classical (CD14−) monocytes express high levels of BLT1 and CCR2 and that both receptors are down-regulated on CD14+ monocytes. Apart from providing insight into the regulation of BLT1 and CCR2, our results reveal a parallel expression and regulation of BLT1 and CCR2, which may help to understand monocyte trafficking during pathophysiological conditions. In this study, we have explored the regulation of BLT1 expression in human monocytes by pro- and anti-inflammatory substances, such as interferon-γ (IFN-γ), tumor necrosis factor (TNF-α), and LPS, down-regulated expression, whereas the anti-inflammatory cytokine, interleukin-10, and dexamethasone up-regulated BLT1 expression, and receptor down-modulation followed alterations in mRNA expression. The effect of IFN-γ stimulation resulted in a diminished chemotactic response to LTB4. The regulation of IFN-γ stimulation and chemotaxis of specific leukocyte subsets [3–5]. For example, several proinflammatory cytokines induce the production of chemotactic signals, such as the leukotriene B4 receptor, BLT1 is needed for the generation of an efficient immune response to invading microbes [21]. Furthermore, LTB4/BLT1 interactions seem to be involved in inflammatory responses. In this study, we focused on the regulation of BLT1 expression in human monocytes by pro- and anti-inflammatory cytokines (chemokines) and the classical chemoattractants, which include complement factors, formyl peptides, and arachidonic acid (AA) metabolites such as leukotriene B4 (LTB4). Directed leukocyte migration is controlled by local production of chemotactic signals and the dynamic expression of chemokine receptors [9]. Two cell-surface receptors for LTB4 have been identified: the high-affinity receptor for LTB4, BLT1, and the low-affinity receptor, BLT2 [12–15]. Like chemokine receptors, they both belong to the G-protein-coupled receptor seven transmembrane domain superfamily. Whereas the role for BLT1 in LTB4-induced chemotaxis through the down-regulation of CCR2 seems to be exerted through a block in transcriptional activity. Alterations in mRNA expression were accompanied by changes in BLT1 surface expression, and concentration-dependent (1–50 ng/ml) and functional activity.
Pro- and anti-inflammatory substances modulate expression of the leukotriene B₄ receptor, BLT1, in human monocytes

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Abstract: The high-affinity leukotriene B₄ (LTB₄) receptor, BLT1, is a chemotactic receptor involved in inflammatory responses. In this study, we have explored the regulation of BLT1 expression in human monocytes by pro- and anti-inflammatory cytokines, lipopolysaccharide (LPS), and dexamethasone. We found that proinflammatory mediators, such as interferon-γ (IFN-γ), tumor necrosis factor-α, and LPS, down-regulated expression, whereas the anti-inflammatory cytokine, interleukin-10, and dexamethasone up-regulated BLT1 mRNA expression. The effect of IFN-γ on BLT1 mRNA expression was rapidly detectable (<4 h) and concentration-dependent (1–50 ng/ml) and seems to be exerted through a block in transcriptional activity. Alterations in mRNA expression were accompanied by changes in BLT1 surface expression, and receptor down-modulation following IFN-γ stimulation resulted in a diminished chemotactic response to LTB₄. The regulation of BLT1 mRNA and receptor protein expression was similar to the regulation of the monocyte chemotactic protein-1 (MCP-1) ligand monocyte chemotactic protein-1 (MCP-1) [3]. This leads to the local recruitment of MCP2-expressing leukocyte subsets, including monocytes, from the bloodstream. As the leukocytes enter the inflammatory site, the same cytokines will prevent further MCP-1-induced chemotaxis through the down-regulation of CCR2 [6, 7]. In this way, the same signals that participate in the mobilization of leukocytes also induce their arrest at the right location. These findings have added important information about how the local cytokine environment can regulate leukocyte trafficking in inflammation.

LTB₄ is a potent lipid inflammatory mediator derived from AA metabolism through the 5-lipoxygenase (5-LO) pathway [8]. Similar to the peptide chemokines, LTB₄ induces activation and chemotaxis of specific leukocyte subsets [9]. Two cell-surface receptors for LTB₄ have been identified: the high-affinity receptor, BLT1 [10, 11], and a low-affinity receptor, BLT2 [12–15]. Like chemokine receptors, they both belong to the G-protein-coupled receptor seven transmembrane domain superfamily. Whereas the role for BLT1 in LTB₄-induced leukocyte migration and activation has been well-characterized, the relevance of BLT2 expression needs further exploration. BLT1 is mainly expressed in granulocytes, monocytes, and to a lesser extent, in subgroups of lymphocytes [16–20]. As the high-affinity receptor for LTB₄, BLT1 is needed for the generation of an efficient immune response to invading microbes [21]. Furthermore, LTB₄/BLT1 interactions seem to be implicated in monocyte trafficking during pathophysiological conditions.

Key Words: mononuclear phagocytes · LTB₄ · IFN-γ · inflammatory mediators

INTRODUCTION

Directed leukocyte migration is controlled by local production of chemotactic signals and the dynamic expression of chemotactic cell-surface receptors [1]. Chemotactic signals, chemoattractants, can be grouped into two families: the chemotactic cytokines (chemokines) and the classical chemoattractants, which include complement factors, formyl peptides, and arachidonic acid (AA) metabolites such as leukotriene B₄ (LTB₄) [2]. The chemical signals are released in tissues in response to inflammatory events and subsequently induce activation and mobilization of specific subsets of immune cells [1].

It is now well established that pro- and anti-inflammatory cytokines as well as microbial products modulate the release of chemotactic factors and the expression of leukocyte chemokine receptors [3–5]. For example, several proinflammatory cytokines induce the production of chemotactic signals, such as the CC chemokine receptor 2 (CCR2) ligand monocyte chemotactant protein-1 (MCP-1) [3]. This leads to the local recruitment of CCR2-expressing leukocyte subsets, including monocytes, from the bloodstream. As the leukocytes enter the inflammatory site, the same cytokines will prevent further MCP-1-induced chemotaxis through the down-regulation of CCR2 [6, 7]. In this way, the same signals that participate in the mobilization of leukocytes also induce their arrest at the right location. These findings have added important information about how the local cytokine environment can regulate leukocyte trafficking in inflammation.
important for the pathogenesis of certain inflammatory diseases [22–25], and BLT1 may act as a coreceptor for human immunity deficiency virus type 1 (HIV-1) [26]. Recently, several investigators, using transgenic mouse models, have also found convincing evidence for a role of BLT1 in the development of atherosclerosis [27, 28].

Previously, we and others have characterized the regulation of BLT1 expression in granulocytes [19, 29–31]. So far, information is scarce regarding the dynamics of BLT1 expression in human mononuclear phagocytes, the key players in several inflammatory conditions, including atherosclerosis. In this study, we show that pro- and anti-inflammatory substances in vitro modulate BLT1 surface expression and mRNA expression in human monocytes as well as chemotactic responsiveness to LTB4. Flow cytometric analysis of fresh peripheral blood monocytes revealed a lower BLT1 surface expression on CD14+, CD16+ monocytes as compared with CD14++, CD16+ monocytes, possibly reflecting the in vivo relevance of our findings. In monocytes, the regulation of BLT1 by inflammatory mediators is similar to what can be seen for the chemokine receptor CCR2. Together with previous work that has revealed a cross-talk between LTB4 and MCP-1 [28, 32, 33], our findings further indicate a functional relationship for these mediators and their corresponding receptors in human monocytes.

MATERIALS AND METHODS

Monocyte isolation

Monocytes were isolated from buffy coats according to Repnik et al. [34]. Briefly,uffy coats were mixed (1:2) with RPMI 1640 (Invitrogen, Copenhagen, Denmark), layered on a Ficoll density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden), and centrifuged for 15 min at 950 g. The mononuclear cells were collected, washed, counted, and diluted to 50–70 × 106 cells/ml before they were placed on a hypotonic Percoll density gradient (Amersham Pharmacia Biotech) and centrifuged for 15 min at 500 g. The monocyte-enriched fraction was collected, washed, and diluted to 2 × 106 cells/ml in macrophage-serum-free medium (SMF) medium (Invitrogen). This is a medium designed for human monocytes and macrophages, which was used to minimize possible stress and activation caused by isolation. Cell viability was >99% as determined by trypan blue exclusion, and monocyte purity was 75–90% as determined by flow cytometric analysis and manual inspection. Cells used for real-time polymerase chain reaction (PCR) were further purified by incubating the monocyte-rich cell suspension during 1 h to let the monocytes adhere. The nonadherent cells were aspirated, and new medium was added. This resulted in a monocyte purity of >95%. All ingredients used during isolation and subsequent stimulation were endotoxin-free as determined by the Limulus amebocyte lysate test.

Stimulation conditions

To give the monocytes time to recover from possible isolation stress and activation induced by plastic adherence, they were incubated overnight to give the monocytes time to recover from possible isolation stress and activation caused by isolation. Cell viability was >95% after an overnight incubation. To assure that the BLT1 mRNA expression in nonstimulated cells did not change over the 4-h incubation, mRNA levels were measured at the end of the incubation period. We next examined whether cytokine-induced alterations of receptor protein levels of control levels) than for BLT1. As for BLT1, IL-10 and TNF-α down-regulated BLT1 mRNA levels in human monocytes were reflected by a reduction in the filters (Fig. 1A). The addition of 5 μg SeraMug Oligo dT14 paramagnetic beads (Sera, Gothenburg, Sweden), or CD3-FITC, or CD5-FITC (Becton Dickinson, Stockholm, Sweden), or CD4-FITC (Becton Dickinson) and the Cells were incubated for 30 min at 4°C in the dark, and washed, and analyzed using a FACS Calibur Flow Cytometer (Becton Dickinson) and the CellQuest software (10,000 cells were counted/sample).

Chemotaxis

Monocytes were stimulated with 50 ng/ml IFN-γ for 6–8 h and then suspended in macrophage–serum-free medium (Invitrogen) to a concentration of 2 × 106 cells/ml in a chemotaxis chamber (Chemtox plate, NeuroProbe, Gaithersburg, MD) together with medium as negative control and cell suspension as positive control. A polycarbonate filter with 5 μm pores (NeuroProbe) was placed over the wells, and 50 μl cell suspension with stimulated or IFN-γ-stimulated cells was placed on the filter and incubated at 37°C, 5% CO2 for 1 h. At the end of the incubation, the filter was removed, and the cells that had migrated into the wells were counted using an inverted microscope (Olympus IX70 equipped with a digital camera (Olympus Cam-erad C-3040ZOOM). The cells in three high magnification fields at the center of each well were counted.

cDNA preparation and real-time, quantitative PCR analysis

All reagents were purchased from Sigma, unless otherwise stated. Cells were lysed in 200 μl lysis/hindbing buffer (100 mM Tris-HCl, pH 8, 500 mM LiCl, 10 mM EDTA, 1% litheric dodecyl sulfate, and 5 mM dithiothreitol (DTT)), and the cell lysate was further processed with a QiAshredder (Qiagen, Stockhol, Sweden) to reduce viscosity. PolyA+ RNA was captured from the cell lysate with 50 μg SeraMug Oligo d(14) paramagnetic beads (Sera, Gothenburg, Sweden). The beads were washed twice with 200 μl l buffer B (100 mM Tris-HCl, pH 8, 150 mM LiCl, 1 mM EDTA, and 0.1% litheric dodecyl sulfate) and once with 100 μl l buffer B (100 mM Tris-HCl, pH 8, 150 mM LiCl, and 1 mM EDTA). PolyA+ RNA was eluted in 10 μl water, and first-strand synthesis was carried out for 30 min at 50°C in a final volume of 20 μl containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 10 mM DTT, 0.5 mM MgCl2, 0.5 μM deoxythymidine triphosphate (dNTP), and 0.5 μg Oligo d(T)12–18, and 20 units RNAseOUT (Invitrogen), and 50 units SuperScript III reverse transcriptase (Invitrogen). PCR primers (MWG, Ebersberg, Germany) were designed with Oligo 4 (National Bioscience, Oslo, Norway), and their sequences are as follows, with expected product size in parenthesis: BLT1 5′-GTTTGG-GAGCTCGCTGGTGGC, 3′-GTGACAGGACGGggTGGTC (216 bp); CCR2 5′-CAAGATATCGACAGAAAGC, 3′-GCGACAGAATAACAGAATC (545 bp).

Real-time, quantitative PCR was performed in a LightCycler system (Roche, Stockhom, Sweden) using the Sybr Green 1 detection method. The reactions were performed in a total volume of 10 μl containing 2 μl diluted (1:20) cDNA or external PCR standard, 50 mM Tris-HCl, pH 8.3, 10 mM KCl, 50 mM (NH4)2SO4, 3 mM MgCl2, 200 μM each dNTP, 0.5 μg/μl BSA, 1.300,000 dilution of SYBR Green I, 0.5 μM each primer, and 0.5 units FastStart Taq DNA polymerase (Roche). After an initial denaturation step at 95°C for 10 min, temperature cycling was initiated. A total of 45 cycles was run, and each cycle consisted of 10 s at 95°C, 10 s at 55°C, and 10 s at 72°C (BLT1) or 15 s at 95°C, 5 s at 54°C, and 22 s at 72°C (CCR2). At the end of each run, melting curve profiles were recorded, and the specificity of the amplification product was further verified by electrophoresis on a Visigel (Stratagene, Stockholm, Sweden) using GelStar (Cambrex, Stockhom, Sweden) DNA staining. To generate external standards, specific PCR products for each gene were gel-purified using QiAquick gel extraction kit (Qiagen). The copy number was calculated based on the measured concentration at 260 nm, and serial tenfold dilutions were made in ultra-pure water.

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mRNA decay studies

Monocytes were incubated with or without 50 ng/ml IFN-γ for 4 h before the addition of 5 μg/ml Actinomycin D (Sigma). At 0, 2, 4, 6, and 8 h, cells were lysed, and mRNA was extracted for subsequent cDNA synthesis and real-time PCR quantification as described previously.

Data analysis

The data obtained from stimulating cells with different IFN-γ concentrations were fitted to an exponential equation (using the Prism software), which was used to calculate an effective concentration (EC₅₀) value.

RESULTS

Pro- and anti-inflammatory agents affect BLT1 mRNA expression

Human monocytes were stimulated for 4 h with various inflammatory mediators, and BLT1 mRNA levels were examined using real-time, quantitative PCR. As shown in Figure 1A, LPS and the proinflammatory cytokines, IFN-γ and TNF-α, induced varying degrees of mRNA down-regulation. This was most pronounced for LPS, which decreased the mRNA levels by almost 50%. IFN-γ and TNF-α down-regulated BLT1 mRNA levels to ~77% of control values, and a combination of the two had a synergistic effect. The anti-inflammatory cytokine, IL-10, and the prototypic glucocorticoid, dexamethasone, up-regulated BLT1 mRNA levels, whereas IL-4 had no obvious effect.

The results from the stimulation with pro- and anti-inflammatory agents on BLT1 mRNA levels were compared with the effect on CCR2 mRNA (Fig. 1B) and were found to be similar: LPS, IFN-γ, TNF-α, and IFN-γ + TNF-α down-regulated CCR2 mRNA expression, although more pronounced (13–15%) than for BLT1. As for BLT1, IL-10 and dexamethasone up-regulated CCR2 expression. IL-4 lowered CCR2 mRNA expression slightly in one of two experiments. To assure that the BLT1 mRNA expression in nonstimulated cells did not change over the 4-h incubation, mRNA levels were compared before and after incubation, and they were found to be stable.

BLT1 mRNA alterations correlate with receptor protein expression at the cell surface

We next examined whether cytokine-induced alterations of BLT1 mRNA levels in human monocytes were reflected by changes in BLT1 surface expression. For this purpose, monocytes were stimulated with IFN-γ, IL-10, TNF-α, or dexamethasone for 8–12 h before flow cytometric analysis. IFN-γ (Fig. 2, A and B) and TNF-α (Fig. 2, E and F) down-regulated BLT1 and CCR2 expression, whereas IL-10 up-regulated both receptors (Fig. 2, C and D). In line with the aforementioned mRNA findings, the cytokine-induced alterations of receptor protein expression were more pronounced for CCR2. Dexamethasone stimulation gave varying results. In three out of five experiments, an up-regulation of BLT1 expression was seen, whereas in the remaining two, stimulated and nonstimulated cells did not differ (data not shown).

Further analysis of IFN-γ-induced effects on BLT1 message levels

Subsequent studies on BLT1 mRNA expression were focused on the down-modulatory effects of IFN-γ.

BLT1 down-regulation by IFN-γ is concentration-dependent

To obtain the optimal stimulation conditions for further analyses, human monocytes were stimulated for 8 h with different concentrations of IFN-γ, ranging from 1 to 50 ng/ml, and BLT1 mRNA was quantified using real-time, quantitative PCR. A decrease of BLT1 mRNA was first seen after stimulation with 2.5 ng/ml (Fig. 3). Increasing IFN-γ concentrations gave a progressive down-regulation of BLT1 mRNA, which was max-
The data yielded an EC50 value of 4 (2–11) ng/ml (mean and 95% confidence interval). Studies of CCR2 mRNA levels following stimulation with different IFN-γ concentrations (data not shown) indicated a similar potency as for BLT1. Our EC50 for CCR2 agreed with previous data [7]. The maximal inhibition, however, was less when measuring BLT1 transcripts (60% reduction) than for CCR2 (82% reduction).

**IFN-γ induced BLT1 mRNA down-regulation over time**

Having defined the IFN-γ concentration that provided the optimal analytical window of BLT1 mRNA down-regulation, the effect over time was studied. Human monocytes were stimulated with 50 ng/ml IFN-γ, and mRNA was extracted at 0, 2, 4, 8, and 16–24 h, followed by real-time PCR quantification. There was a 20% down-regulation of BLT1 mRNA after 2 h (Fig. 4). At 4 h, mRNA levels had decreased by 25%, and the decrease amounted to almost 60% after 8 h. Stimulation for longer time-periods (16–24 h) gave no further decrease.

**mRNA decay studies**

mRNA levels may be altered by shifts in transcript stability or by changes in transcription rate. We evaluated the effects of IFN-γ on mRNA stability by inhibiting transcription with Actinomycin D, which was added to monocytes in the presence or absence of 50 ng/ml IFN-γ, after which mRNA was extracted at the indicated time-points for subsequent PCR quantification (Fig. 5). The half-life of BLT1 mRNA was ~5 h, and IFN-γ was found not to alter transcript stability. In agreement with previous studies [4, 7, 36], the half-life of CCR2 mRNA transcripts was ~2 h, which was reduced upon stimulation with IFN-γ (data not shown).
concentrations (data not shown) indicated a similar potency as (2–11) ng/ml (mean and 95% confidence interval). Studies of independent experiments, and the results are presented as percentage of copy confidence interval). The figure shows triplicates from a representative of three imals at 25–50 ng/ml. The data yielded an EC50 value of 4/H9253 Fig. 3. IFN-

The maximal inhibition, however, was less when measuring ng/ml IFN-

The approximate EC50 value was 4 (2–11) ng/ml (mean and 95% /H9253

The lowest levels of BLT1 mRNA were obtained after stimulation with 25–50

lowers BLT1 mRNA levels in a concentration-dependent Pettersson et al. with IFN-

transcripts was with previous studies [4, 7, 36], the half-life of CCR2 mRNA /H9253

IFN-
tification (Fig. 5). At 4 h, mRNA levels had decreased by 25%, and

induced BLT1 mRNA down-regulation over time. Monocytes stimulated (dotted line) or stimulated with 50 ng/ml IFN-

Fig. 4. IFN-γ regulation of BLT1 mRNA expression over time. Monocytes were stimulated with 50 ng/ml IFN-γ for 0–24 h. A down-regulation was detected at 2 h, reaching lowest levels of BLT1 mRNA at 8 h. After longer incubations (16–24 h), there was no further down-regulation of BLT1 mRNA levels. Figure shows triplicates or more from at least three donors, and the results are presented as percentage of copy number at 0 h.

IFN-γ inhibits LTB4-mediated monocyte chemotaxis To determine if the IFN-γ-promoted down-regulation of BLT1 mRNA and cell-surface receptor protein expression affected functional responses to LTB4, we performed a chemotaxis assay. Monocytes were incubated with 50 ng/ml IFN-γ for 6–8 h and were then evaluated for chemotactic responsiveness to a gradient of LTB4 ranging from 10^-11 to 10^-7 M. Chemotactic responses to LTB4 were almost completely abolished in cells pretreated with IFN-γ (Fig. 6). Differential expression of BLT1 surface protein on human monocyte subpopulations The last set of experiments included a three-color flow-cytometric analysis of two subpopulations of monocytes to study BLT1 and CCR2 expression. Whole blood was stained with antibodies recognizing CD14, CD16, and BLT1 or CCR2. During the analysis, a gate was set for monocytes based on forward- and side-scatter in addition to CD14 and CD16 expression. Two monocyte subsets expressing high levels of CD14 (CD14^+^CD16^-^) or low levels of CD14 and high levels of CD16 (CD14^-^CD16^-^) were defined. Figure 7 illustrates that CD14^-^CD16^-^ monocytes express less BLT1 and CCR2 protein than CD14^+^CD16^-^. The differences between CD14^-^CD16^-^ and CD14^-^CD16^-^ were more pronounced for CCR2 than for BLT1. As CD14^-^CD16^-^ monocytes tend to be smaller and less granular than CD14^-^CD16^-^ monocytes, they are located close to the lymphocytes in a forward- and side-scatter diagram. To exclude the presence of CD16^-^ natural killer cells, which express CD56, we analyzed the cells in the currently set gate for CD56 expression, but these cells were found to comprise less than 1% of the analyzed population (data not shown).

DISCUSSION An important feature of the immune system is the directed migration of specific leukocyte subsets during inflammatory...
events. This immune cell trafficking is orchestrated by chemotactic signals and their corresponding surface-bound receptors [1, 2]. The high-affinity receptor for LTB₄, BLT₁, is expressed by subsets of human cells and is needed for an efficient immune response to invading pathogens [16–21]. Furthermore, LTB₄/BLT₁ interactions are emphasized in the pathogenesis of various inflammatory conditions such as rheumatoid arthritis (RA), asthma, psoriasis, inflammatory bowel disease [22–25], and recent, atherosclerosis [27, 29]. Although BLT₁ is highly expressed in human monocytes, information about its regulation in these cells has been lacking.

In the present study, we have analyzed the regulation of BLT₁ mRNA and receptor surface expression in human monocytes by pro- and anti-inflammatory signals. We show that BLT₁ mRNA is down-modulated by the proinflammatory cytokines IFN-γ, TNF-α, and LPS. In contrast, the anti-inflammatory cytokine IL-10 and the prototypic corticosteroid dexamethasone up-regulated BLT₁ mRNA expression. Flow cytometric analyses, using highly specific monoclonal antibodies [35], revealed concomitant, positively correlated changes in BLT₁ surface expression following monocyte exposure to IFN-γ, TNF-α, and IL-10. In line with previous studies [5, 7], chemokine receptor CCR2 mRNA and surface expression were regulated in a similar manner.

IFN-γ is a pleiotropic cytokine, important for T helper cell type 1-oriented inflammatory responses. It acts as a potent activator of mononuclear phagocytes and has well-characterized effects on CCR2 expression [6, 7]. Further exploration of BLT₁ mRNA modulation therefore focused on IFN-γ. In these experiments, we found that IFN-γ lowered BLT₁ mRNA expression in a time- and concentration-dependent manner, reaching maximal responses at 8 h and 25–50 ng/mL, respectively.

Steady-state mRNA levels may be reduced by inhibiting transcription or by decreasing mRNA stability. It has been reported that inflammatory mediators regulate levels of chemokine receptors by changing mRNA stability. For example, CCR2 mRNA half-life is reduced in monocytes treated with IFN-γ or LPS [4, 7, 37]. In the present work, we show that BLT₁ mRNA transcript half-life is unaffected by IFN-γ stimulation and that the transcripts are relatively stable (5–6 h) when compared with those for several chemokine receptors (1–2 h) [36]. We therefore conclude that IFN-γ reduces BLT₁ mRNA levels through an inhibition of BLT₁ mRNA transcription. In addition, BLT₁ mRNA levels were reduced in a similar manner over time in IFN-γ (Fig. 4) and Actinomycin D-treated (Fig. 5) monocytes, which is in agreement with this conclusion. Based on the relative stability of BLT₁ mRNA transcripts, the transcriptional rate of BLT₁ in our experiments is probably low, and this may explain why IFN-γ modulation of BLT₁ seems to be slower than what has previously been shown for CCR2 [7].

The functional relevance of lowered BLT₁ expression was reflected in our chemotaxis experiments, where IFN-γ stimulation rendered monocytes irresponsible to LTB₄ challenge. As concomitant flow cytometric analyses revealed the presence of BLT₁ receptors, although at a lower density, additional mechanisms seemingly contribute to this finding. To exclude the involvement of ligand-induced receptor desensitization as an explanation for this discrepancy, we measured LTB₄ levels in culture supernatants from IFN-γ-stimulated and nonstimulated monocytes. However, LTB₄ levels were barely detectable and were not elevated by IFN-γ stimulation (data not shown), which is in agreement with previous reports [36–40]. It is, however, possible that IFN-γ stimulation, through other mechanisms, render monocytes less responsive to LTB₄ receptors from subsequent signal transduction pathways. In a study by D’Amico et al. [41], uncoupling several chemokine receptors was demonstrated in monocytes and dendritic cells stimulated with a combination of IL-10 and LPS/IFN-γ.

In recent years, the heterogeneity of human blood monocytes has received increasing attention. Two major populations can be detected based on differences in the expression of CD14 and CD16 [42, 43]. In healthy individuals, 85–95% of circulating monocytes express high levels of CD14 and no CD16 (CD14⁺CD16⁻), whereas 5–15% express CD16 and low levels of CD14 (CD14⁻CD16⁻) [44–46]. CD14⁻CD16⁻ cells may represent monocytes that have been preactivated by inflammatory cytokines or microbial products. This is supported by the dramatic increase in CD14⁺CD16⁺ monocytes seen in various inflammatory conditions, such as sepsis, HIV-1 infection, and active RA [45–48]. Recent studies have revealed that these monocyte subsets have different migratory properties and chemokine receptor expression patterns. For example, although CD14⁺⁺CD16⁻ cells are CCR2-positive and readily migrate toward a gradient of MCP-1, CD14⁺⁻CD16⁺ monocytes lack CCR2 expression and display an attenuated chemotactic response to MCP-1 stimulation [44]. In the present study, we show that CD14⁺⁺ CD16⁻ monocytes express high protein levels of BLT₁ in addition to CCR2. In contrast, CD14⁺⁺CD16⁻ monocytes were only weakly positive for BLT₁ and as previously shown, lacked CCR2 expression. Thus, BLT₁ and CCR2 are not only regulated in a similar manner by immunomodulating mediators but also share expression profiles in human monocytes.

It has been suggested that a down-modulation of chemotactic receptors by inflammatory mediators and microbial products may represent a mechanism of retaining immune cells at sites of inflammation [49]. In this way, locally produced cytokines can control leukocyte tissue infiltration through the regulation of chemotactic ligands and by inducing leukocyte arrest at the appropriate location.

The down-regulation of chemotactic receptors by proinflammatory signals may also represent a mechanism for avoiding excessive accumulation of immune cells at inflammatory sites. For instance, generation of high levels of proinflammatory mediators in severely inflamed tissue will leak out into the circulation, where a preactivation of leukocytes and down-modulation of chemotactic receptors will prevent further monocyte recruitment to the site of inflammation. Geissmann et al. [50] demonstrated that the mouse counterpart to the human CD14⁺⁻CD16⁺ monocyte was excluded from inflamed tissues in vivo, and in light of our findings, this may be explained by a reduced expression of CCR2 and BLT₁ on these cells. Furthermore, in a murine peritonitis model, injection with LPS resulted in the down-regulation of monocyte CCR2 expression and loss of macrophage infiltration in the peritoneum [51]. A concomitant down-modulation of BLT₁ may occur and could...
help to explain the completely ablated monocyte recruitment in this model. Finally, the same mechanism may contribute to the seemingly paradoxical, disease-limiting effects caused by systemic IFN-γ that have been observed in models of experimental autoimmune diseases (reviewed in ref. [52]).

It is interesting that MCP-1/CCR2 and LTB4/BLT1 interactions seem to cooperate in the mobilization of immune cells during inflammation. In a study by Matsukawa et al. [33], exogenous MCP-1 stimulation induced the release of LTB4 from mononuclear phagocytes in a murine model of peritonitis, and recently, Huang et al. [32] demonstrated a strong increase in MCP-1 production and release in primary human monocytes stimulated with LTB4. This receptor cross-talk may represent an amplification loop of importance for leukocyte recruitment during inflammatory events, such as atherosclerosis. The striking similarities between BLT1 and CCR2 regulation and expression in monocytes may reflect this functional relationship.

Increased understanding of atherosclerosis as an inflammatory process has highlighted mononuclear phagocytes as well as inflammatory cytokines such as IFN-γ and TNF-α as major participants in this pathology [53, 54]. In recent years, studies of apolipoprotein E (apoE)−/− and low-density lipoprotein receptor (LDLR)−/− transgenic mice have indicated that not only MCP-1 and its receptor CCR2 but also LTB4/BLT1 interactions substantially contribute to the development of atherosclerotic lesions. For example, treatment of LDLR−/− and apoE−/− mice with a BLT1 antagonist significantly inhibits atherosclerotic plaque formation and reduces monocyte infiltration of the lesions [27]. Deletion of BLT1 from the apoE−/− murine genome also attenuated the initial pathogenesis of atherosclerosis [28]. Furthermore, the 5-LO gene, which is involved in the formation leukotrienes including LTBR, contributes profoundly to the development of atherosclerosis susceptibility in mice [55]. Recently, in a population-based study, Helgadottir et al. [56] found that a single nucleotide polymorphism in the gene encoding a 5-LO activating protein was associated with an increased risk of myocardial infarction and found that this correlated with an increased production of LTBR. Hence, mounting evidence points to a critical role for LTBR and BLT1 during different stages of atherosclerotic disease.

In conclusion, we show that BLT1 expression in human monocytes is modulated by inflammatory mediators in a similar manner to the chemokine receptor CCR2. Our findings could shed further light on the complex mechanisms that regulate monocyte trafficking during inflammation. Information about BLT1 regulation in human monocytes may also increase our understanding of inflammatory diseases that involve LTBR/BLT1 interactions.

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REFERENCES


Paper II
Abstract

Through the use of chimeric CXCR4 = CCR5 receptors we have previously shown that CCR5-tropic (R5) HIV-1 isolates acquire a more flexible receptor use over time, and that this links to a reduced viral susceptibility to inhibition by the CCR5 ligand RANTES. These findings may have relevance with regards to the efficacy of antiretroviral compounds that target CCR5 = virus interactions. Compartmentalized discrepancies in coreceptor use may occur, which could also affect the efficacy of these compounds at specific anatomical sites, such as within the CNS. In this cross-sectional study we have used wild-type CCR5 and CXCR4 as well as chimeric CXCR4 = CCR5 receptors to characterize coreceptor use by paired plasma and cerebrospinal fluid (CSF) isolates from 28 HIV-1-infected individuals. Furthermore, selected R5 isolates, with varying chimeric receptor use, were tested for sensitivity to inhibition by the CCR5 antagonist TAK-779. Discordant CSF = plasma virus coreceptor use was found in 10 = 28 patients. Low CD4+ T cell counts correlated strongly with a more flexible mode of R5 virus CCR5 usage, as disclosed by an increased ability to utilize chimeric CXCR4 = CCR5 receptors, specifically receptor FC-2. Importantly, an elevated ability to utilize chimeric receptors correlated with a reduced susceptibility to inhibition by TAK-779. Our findings show that a discordant CSF and plasma virus coreceptor use is not uncommon. Furthermore, we provide support for an emerging paradigm, where the acquisition of a more flexible mode of CCR5 usage is a key event in R5 virus pathogenesis. This may, in turn, negatively impact the efficacy of CCR5 antagonist treatment in late stage HIV-1 disease.

Introduction

The discovery that the chemokine receptors CCR5 and CXCR4 act as essential keyholes for the entry of human immunodeficiency virus type 1 (HIV-1) into CD4-positive immune cells has increased the understanding of AIDS pathogenesis and provided the basis for new antiretroviral treatment strategies. Following viral attachment to CD4, conformational changes in the HIV envelope glycoprotein 120 (gp120) facilitate viral binding to one of these chemokine receptors with subsequent steps of membrane fusion and capsid entry.1,2 CCR5-utilizing strains (R5 viruses) are invariably found in HIV-1-infected individuals during the asymptomatic phase, whereas virus phenotypes with the ability to utilize CXCR4 (X4 or R5X4 virus) emerge in approximately 50% during progression to AIDS. 3–6

We have previously described the use of a set of CXCR4 = CCR5 chimeric receptors for studies on the evolution of coreceptor use of primary HIV-1 isolates over time. 7–9 In these studies we designated R5 isolates that lacked the ability to use any of the chimeras, i.e., they are able to infect only cells expressing the CCR5 wild-type receptor, as R5 narrow phenotype. R5 viruses able to use one or more chimeric receptors were designated R5 broad(1), R5 broad(2), or R5 broad(3), depending on the number of chimeras that could support viral entry. We demonstrated that an enhanced ability of R5 isolates to utilize these chimeras and wild-type CCR5 was linked to disease progression as well as to a reduced viral susceptibility to inhibition by the CCR5 ligand RANTES. These findings imply that an important feature of R5 virus pathogenesis in progressive HIV-1 disease is the acquisition of a more flexible mode of CCR5 usage. The fact that viruses displaying the R5...
Mode of Coreceptor Use by R5 HIV Type 1 Correlates with Disease Stage: A Study of Paired Plasma and Cerebrospinal Fluid Isolates

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Abstract

Through the use of chimeric CXCR4/CCR5 receptors we have previously shown that CCR5-tropic (R5) HIV-1 isolates acquire a more flexible receptor use over time, and that this links to a reduced viral susceptibility to inhibition by the CCR5 ligand RANTES. These findings may have relevance with regards to the efficacy of antiretroviral compounds that target CCR5/virus interactions. Compartmentalized discrepancies in coreceptor use may occur, which could also affect the efficacy of these compounds at specific anatomical sites, such as within the CNS. In this cross-sectional study we have used wild-type CCR5 and CXCR4 as well as chimeric CXCR4/CCR5 receptors to characterize coreceptor use by paired plasma and cerebrospinal fluid (CSF) isolates from 28 HIV-1-infected individuals. Furthermore, selected R5 isolates, with varying chimeric receptor use, were tested for sensitivity to inhibition by the CCR5 antagonist TAK-779. Discordant CSF/plasma virus coreceptor use was found in 10/28 patients. Low CD4+ T cell counts correlated strongly with a more flexible mode of R5 virus CCR5 usage, as disclosed by an increased ability to utilize chimeric CXCR4/CCR5 receptors, specifically receptor FC-2. Importantly, an elevated ability to utilize chimeric receptors correlated with a reduced susceptibility to inhibition by TAK-779. Our findings show that a discordant CSF and plasma virus coreceptor use is not uncommon. Furthermore, we provide support for an emerging paradigm, where the acquisition of a more flexible mode of CCR5 usage is a key event in R5 virus pathogenesis. This may, in turn, negatively impact the efficacy of CCR5 antagonist treatment in late stage HIV-1 disease.

Introduction

The discovery that the chemokine receptors CCR5 and CXCR4 act as essential keyholes for the entry of human immunodeficiency virus type 1 (HIV-1) into CD4-positive immune cells has increased the understanding of AIDS pathogenesis and provided the basis for new antiretroviral treatment strategies. Following viral attachment to CD4, conformational changes in the HIV envelope glycoprotein 120 (gp120) facilitate viral binding to one of these chemokine receptors with subsequent steps of membrane fusion and capsid entry. CCR5-utilizing strains (R5 viruses) are almost invariably found in HIV-1-infected individuals during the asymptomatic phase, whereas virus phenotypes with the ability to utilize CXCR4 (X4 or R5X4 virus) emerge in approximately 50% during progression to AIDS. We have previously described the use of a set of CXCR4/CCR5 chimeric receptors for studies on the evolution of coreceptor use of primary HIV-1 isolates over time. In these studies we designated R5 isolates that lacked the ability to use any of the chimeras, i.e., they are able to infect only cells expressing the CCR5 wild-type receptor, as R5narrow phenotype. R5 viruses able to use one or more chimeric receptors were designated R5broad(1), R5broad(2), or R5broad(3), depending on the number of chimeras that could support viral entry. We demonstrated that an enhanced ability of R5 isolates to utilize these chimeras and wild-type CCR5 was linked to disease progression as well as to a reduced viral susceptibility to inhibition by the CCR5 ligand RANTES. These findings imply that an important feature of R5 virus pathogenesis in progressive HIV-1 disease is the acquisition of a more flexible mode of CCR5 usage. The fact that viruses displaying the R5

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broad phenotypes are less sensitive to inhibition by RANTES may also be of specific relevance in the context of antiretroviral treatment with CCR5 antagonists as it may reflect a reduced virus susceptibility to these agents.

Prior to the initiation of antiretroviral treatment with CCR5 antagonists it is mandatory to exclude the presence of X4 or R5X4 populations in plasma. However, information is scarce regarding possible compartmentalized discrepancies in HIV-1 coreceptor use that could impact the efficacy of CCR5 antagonists at specific anatomical sites. HIV-1 invades the central nervous system (CNS) early in the course of infection and, in the absence of antiretroviral treatment frequently causes neurological morbidity, such as AIDS dementia complex (ADC). Due to the blood–brain barrier, the CNS constitutes a restricted compartment, where the viral evolution may differ from that in peripheral blood. Within the CNS, autonomous viral production is established in local target cells, mainly comprising resident macrophages and microglial cells. A viral adaptation to replication in these target cells may include alterations in coreceptor usage. Furthermore, the mode of coreceptor use may substantially influence the pathogenic processes in the brain that are responsible for the development of neurological impairment, such as ADC.

Although the cerebrospinal fluid (CSF) cannot be considered to be a compartment identical to brain tissue, it is a more readily sampled site that, due to its proximity and shared barriers, provides an important “window” into HIV CNS infection.

In the present study we have characterized the mode of coreceptor use by paired HIV-1 plasma and CSF isolates through the use of CXCR4/CCR5 chimeric and wild-type receptors. Furthermore, the mode of coreceptor use was correlated with clinical parameters linked to disease progression, and, for selected isolates, with sensitivity to the CCR5 antagonist TAK-779.

Materials and Methods

Patients

Twenty-eight HIV-1-infected patients with varying CD4+ T cell counts, varying levels of CSF and plasma viral load, and with or without ADC were retrospectively selected for participation in the study, from a longitudinal study cohort at the Department of Infectious Diseases, Sahlgrenska University Hospital, Gothenburg, Sweden. Informed consent was obtained from all participants. The study was approved by the Research Ethics Committee at The University of Gothenburg, Sweden. Peripheral CD4+ T cell counts and HIV-1 RNA levels in plasma and CSF were analyzed for each patient (FACS, Becton Dickinson, Mountain View, CA and Amplicor HIV Monitor, Version 1.0, Roche Diagnostic Systems, Basel, Switzerland, respectively). The patients, 22 males and six females, had CD4+ T cell counts ranging from 27 to 820 cells/µl (median 190). Fourteen patients were severely immunodeficient, with CD4+ T cell counts <200 cells/µl. Plasma viral load ranged from 1900 to 682,000 copies/ml (median 52,000) and CSF viral load ranged from 600 to >750,000 copies/ml (median 66,000).

Seven patients had ADC, as assessed by the criteria defined by the CDC and the American Academy of Neurology AIDS Task Force. Twenty-five patients were antiretroviral treatment naive, and none had received antiretroviral medication for at least 9 months prior to virus isolation.

Neopterin levels were analyzed by a commercially available radioimmunoassay (Henningtest Neopterin, BRAMS, Germany) with a upper normal reference value of 5.8 nmol/liter in CSF.

Virus isolates

Paired plasma and CSF isolates were obtained as previously described. Briefly, plasma and CSF samples were centrifuged at 996 x g for 20 min in order to pellet the cells. Cell-free supernatant was centrifuged at 180,000 x g for 30 min at 4°C to pellet free virus particles. None of the CSF samples had a red cell count above 30 x 10⁶/liter. Phytohemagglutinin (PHA)-pretreated peripheral blood mononuclear cells (PBMCs) from healthy blood donors were inoculated with the pelleted material. The cultures were grown in RPMI medium with 10% fetal calf serum and 50 units interleukin-2 (Proleukin, EuroCetus, Amsterdam, The Netherlands), in addition to 2 mg/ml polybrene, 5 mg/ml hydrocortisone acetate, and antibiotics. The supernatants of the cultures were assayed once a week for HIV-1 antigen with a p24 capture ELISA [HIVAG(a)-1, Abbott Laboratories, Chicago, IL]. Virus stocks were stored at −70°C. Prior to infection experiments, the virus was passaged once or twice in interleukin (IL)-2- and PHA-stimulated PBMCs according to standard protocols. The virus content was evaluated in terms of p24 assays using the Vironostika HIV-1 Antigen Microelisa system (Biomerieux, Boxtel, The Netherlands). Selected isolates were also evaluated for the concentration of functional viral reverse transcriptase using the Cavidi HS kit (Cavidi Tech AB, Uppsala, Sweden).

Cell lines

Human glioma U87.CD4 cells were maintained in DMEM with sodium pyruvate and Glutamax-I (Invitrogen, Lidingö, Sweden), 10% fetal calf serum (FCS), 1 x MEM nonessential amino acids, 300 µg/ml G-418, and antibiotics. Stably transfected U87.CD4 cells were supplemented with 0.5 µg/ml of puromycin (Sigma, Stockholm, Sweden). All cells were grown at 37°C in 7% CO2.

Establishment of stably transfected U87.CD4 cells expressing CCR5, CXCR4, or chimeric CXCR4/CCR5 receptors has previously been described (Fig. 1). Briefly, the chimeras FC-1 (CXCR4 Pro-42/Pro-35 CCR5), FC-2 (CXCR4 Asp-74/Ile-67 CCR5), and FC-4b (CXCR4 Ile-185/Cys-178 CCR5) were constructed using the single overlap and extension method. U87.CD4 cells were stably transfected and clones expressing similar levels of receptors as evaluated by flow cytometry were chosen for further experiments.

Virus infections

U87.CD4 cells, stably expressing wild-type or chimeric receptors, were seeded in triplicate in 48-well plates using the U87.CD4 media without G-418 or puromycin. After 3 days, cells at 20–40% confluence were infected for 2 h at 37°C with 30 ng/ml of virus (p24 concentration) in a final volume of 0.13 ml medium. After 2 h, 0.27 ml of medium was added. After incubation for 20–24 h the cells were washed twice and 1 ml of fresh medium was added. Supernatants from the infected cell cultures were collected at day 0 and day 5 of infection and assayed with p24 ELISA. Infection was defined as positive when the p24 content in the supernatant reached normal levels.
CSF viral load ranged from 600 to 1900 to 682,000 copies = treatment naive, and none had received antiretroviral medi-
tation and assayed with p24 ELISA. Infection was defined as
positive when the p24 content in the supernatant reached
100 pg/ml after p24 production by sham-transfected cells had
been deducted. To semiquantitatively assess the efficiency of
chimeric receptor use, we used the following grading sys-
tem: 100–1000 pg p24 antigen production/ml in cell culture
supernatant = low grade usage (+), 1000–10,000 pg/ml =
moderate grade usage (++), and >10,000 pg/ml = high grade
usage (+++).

The criterion used to define discordancy in chimeric re-
ceptor use was a difference in p24 production of at least log_{10}
in parallel infection experiments. To further dissect receptor
use by dual-tropic R5/X4 isolates, biological cloning, as de-
scribed by Mild et al., was performed with minor modifica-
tions. 37 Briefly, U87/CD4/CCR5 and U87/CD4/CXCR4 cells
were inoculated with patient isolates, and infections were
carried out as described above. Isolates with the ability to
utilize both CCR5 and CXCR4 were characterized further
where U87/CD4/CCR5 cells were inoculated with undiluted
virus supernatants from infected U87/CD4/CXCR4 cells and
vice versa. Following the protocol above, supernatants were
analyzed on day 5 for the presence of p24 production. Infec-
tion was defined as positive when the p24 content in the su-
pernatant reached 100 pg/ml.

**TAK-779 inhibition assay**

RT-normalized virus isolated from plasma and CSF was
used for the experiments. PHA-activated PBMCs (10^5), pooled
from three donors, were infected in triplicate with 0.33 ng
RT/ml in the presence of TAK-779 (from Roche, obtained
from the NIH Research and Reference Reagent Program, Di-
vision of AIDS, NIAID, NIH) as previously described. 38 In
brief, TAK-779 was serially diluted in 3-fold steps starting at
the final concentration of 990 nM and simultaneously added
to the cells and virus. Control cultures without inhibitor were
infected in parallel. Infected PBMCs were washed with PBS
on day 1 and inhibitor was added to the cultures at concen-
trations corresponding to the setup. Cell culture supernatants
were harvested on day 7 after infection, and p24 antigen
content was analyzed by Vironostika HIV-1 Antigen Micro-
elisa system (Biomerieux, Boxtel, The Netherlands). The sen-
titivity to TAK-779 was evaluated as 50% or 90% inhibitory
concentration (IC_{50} and IC_{90},) calculated from p24 antigen
release in the control cultures.

**Statistics**

Differences between two independent groups were as-
sumed with the Mann–Whitney U-test. Spearman’s rank cor-
relation coefficient was used to evaluate correlations.

**Results**

**R5 HIV-1 phenotypes predominate in CSF**

Paired plasma and CSF isolates from 28 HIV-1-infected
individuals were tested for their ability to infect U87.CD4 cells
expressing CCR5 or CXCR4. The R5 viral phenotype pre-
dominated both in plasma and in CSF (Table 1). CXCR4 using
viruses were found in plasma samples from seven patients
(six R5X4 and one X4 isolate). In three of the corresponding
CSF isolates only virus with the R5 phenotype was detected,
and p24 production by U87.CD4/CXCR4 cells infected with
one CSF isolate (R5X4) was 30 times lower than for the corre-
sponding (X4) plasma isolate (Patient 25). Paired R5X4 iso-
lates (three plasma and corresponding CSF isolates) were
evaluated for the presence of truly dual-tropic strains and all
contained true R5X4 virus subpopulations (data not shown).
Taken together, HIV-1 with the R5 phenotype is commonly
found in CSF even in the presence of CXCR4 using plasma
virus populations.

**Discordant R5 virus phenotypes in paired R5 plasma/CSF isolates detected through the
use of CXCR4/CCR5 chimeric receptors**

To determine if the R5 phenotypes varied among CCR5
restricted viruses isolated from plasma and CSF, chimeric
receptor use was analyzed. In this analysis we were able to
identify discordant plasma/CSF R5 phenotypes in 6 of 21
patients by significant discrepancies (>10-fold levels of p24 in
culture supernatants) in the use of chimeric receptors (Fig. 2
and Table 1). There were no clear patterns of chimeric receptor
use that could distinguish CSF from plasma isolates, since
both a broader use in four of six CSF isolates and a more
narrow use in two of six CSF isolates were found. Further, R5
phenotypes ranging from R5^discordant to R5^discordant were
represented in both compartments in a nonspecific manner.
Also, in seven patients with ADC, no specific patterns of chimeric
coreceptor use by CSF or plasma isolates were found.

CSF neopterin levels did not correlate with the mode of
coreceptor use. However, as previously shown, 39–43 patients
with ADC had a significantly higher mean CSF neopterin
concentration (95 nmol/liter) than individuals without neuro-
ological complications (28 nmol/liter) (p = 0.03).

**R5 virus ability to utilize CCR5/CXCR4 chimeric
receptor FC-2 is associated with advanced disease
stage and elevated CSF viral load**

Since our previous work indicated a correlation between
chimeric receptor use and degree of immunosuppression, 4 the
mode of CXCR4/CCR5 chimeric receptor use was correlated
with CD4~+~ T cell counts and viral load for each individual.
Table 1. Characteristics of the 28 Subjects Included in the Study^a

<table>
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<th>CDC stage</th>
<th>CD4° T cell count (×10^6 cells/liter)</th>
<th>Plasma-RNA (copies/ml)</th>
<th>CSF-RNA (copies/ml)</th>
<th>CSF-neopterin (umol/liter)</th>
<th>AIDS-related disease</th>
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<th>Coreceptor use in CSF</th>
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^aCDC staging, CD4° T cell counts, HIV-RNA levels, neopterin levels, AIDS-related diseases, and viral coreceptor use. Paired isolates with discordant use of wild-type receptors or chimeric receptors are in bold.

^bADC, AIDS dementia complex; KS, Kaposi sarcoma; MAC, Mycobacterium avium complex; C. esophagitis, Candida esophagitis; PCP, Pneumocystis carinii pneumonia.

FIG. 2. Receptor use of paired plasma and CSF isolates. Diagrams showing results after infection of U87.CD4 cells expressing CCR5, CXCR4, or chimeric CXCR4/CCR5 receptors, with paired plasma (gray bars) and CSF (open bars) virus isolates. (A–F) The six paired isolates with discordant use of chimeric receptors. (G) Paired isolates of concordant R5\_narrow phenotype. (H) Paired isolates of concordant R5\_narrow phenotype. Infections are measured as p24 protein content in the cell culture supernatant. # indicates a p24 value <1. The criterion for discordant use is a >10-fold difference in p24 antigen production between the infections.
Individuals harboring plasma R5\textsuperscript{broad} (2–3) phenotypes had significantly lower CD4\textsuperscript{+} T cell counts as compared to individuals with R5\textsuperscript{trent} or R5\textsuperscript{broad(1)} phenotypes (p = 0.03). The strongest association with immune suppression was found when comparing individuals with FC-2-positive phenotypes (FC-2\textsuperscript{+}) using R5 plasma isolates to those with FC-2-negative phenotypes (FC-2\textsuperscript{-}) (Fig. 3). Nine patients with FC-2\textsuperscript{+} R5 isolates had a median CD4\textsuperscript{+} T cell count of 49 cells/μL, as compared to 495 cells/μL in 12 patients with FC-2\textsuperscript{-} isolates (p = 0.004).

The presence of X4 or R5X4 phenotypes was, as expected, also linked to immunosuppression (median CD4\textsuperscript{+} T cell count of 60) when compared to the FC-2\textsuperscript{-} group (p = 0.005), but the difference was not statistically significant when compared to R5 phenotypes in general. There was no significant correlation between FC-2 usage and plasma viral load even though there was an expected inverse correlation between CD4\textsuperscript{+} T cell counts and viral load (p = 0.01, Spearman). The presence of X4 or R5X4 phenotypes correlated significantly with a higher plasma viral load when compared to the plasma viral load in those harboring viruses with R5 phenotypes (p = 0.035). The presence of FC-2\textsuperscript{-} R5 virus phenotypes within the CSF correlated significantly with an elevated CSF viral load (p = 0.02) (Fig. 3).

**Mode of coreceptor use by R5 isolates correlates with susceptibility to inhibition by the CCR5 antagonist TAK-779**

To evaluate a possible relationship between mode of coreceptor use and susceptibility to inhibition by TAK-779, we selected paired R5 virus isolates from seven patients with varying degrees of immunodeficiency and chimeric receptor use, including three patients with ADC. Whereas a 50% inhibition (IC\textsubscript{50}) was achieved for 13/14 isolates with TAK-779 at varying concentrations, a 90% inhibition (IC\textsubscript{90}) was not achieved for any of the virus isolates with an elevated usage of any chimeric receptor [designated (+) and (+++) in Table 2] even at the highest concentration of TAK-779 (990 nM). In contrast, IC\textsubscript{90} values were achieved for all isolates that were characterized by a lack of or weak ability to utilize any chimeric receptor [designated (−) and (+), respectively, in Table 2]. Plasma and CSF isolates from the three patients with ADC were all incompletely inhibited by TAK-779. There was no correlation between sensitivity to TAK-779 and broadness in chimeric receptor use (data not shown).

**Discussion**

Previous studies have shown that CNS-derived isolates, including those from individuals with ADC, in general are R5-tropic, although exceptions do occur.\textsuperscript{44–48} However, few studies have focused on possible discrepancies in coreceptor use between peripheral virus isolates (plasma derived) and CNS virus isolates (brain or CSF derived).

In line with previous studies we also found a predominance of CCR5-using isolates within CSF in four of the seven patients who harbored X4/R5X4 plasma isolates. We believe that this R5 virus dominance in CSF isolates may be explained by a limited capacity of the studied X4/R5X4 variants to replicate in target cells within the CNS, e.g., brain macrophages and microglial cells.\textsuperscript{49}

In a study of paired plasma and CSF isolates from 46 individuals, Spudich et al.,\textsuperscript{50} using a recombinant phenotypic assay, found discordant CXCR4/CCR5 usage in approximately 10% of subjects, which is similar to our findings. However, whereas they found X4-containing isolates in CSF from two patients harboring only R5 phenotypes in plasma only the opposite discordance was found in our study. Earlier studies by Di Stefano et al.\textsuperscript{51} used cytopathological characterization of MT-2 cells as an index of coreceptor tropism to evaluate blood and CSF isolates. It is now well established that isolates that are syncytia inducing (SI) in the MT-2 cell assay represent CXCR4-tropic isolates and non-syncytia-inducing isolates (NSI) are CCR5-tropic. In their evaluation of 22 individuals with CD4\textsuperscript{+} T cell counts <200 cells/μL, discordant phenotypes were detected in 46% of paired CSF and PBMC isolates.\textsuperscript{52} All discordant isolates were represented by NSI (R5-tropic) strains being detected in CSF in the presence of SI (X4/R5X4) strains isolated from blood, which is in compliance with our
findings. In a clinical perspective, our results on wild-type coreceptor use do not support the necessity to assess CSF virus coreceptor tropism in patients with exclusive R5-tropic plasma virus that are under consideration for CCR5 antagonist treatment. Furthermore, some HIV-1-infected individuals that harbor CXCR4-utilizing viral populations in plasma may theoretically benefit from treatment with CCR5 antagonists, as this could suppress virus replication within the CNS, thereby preventing further HIV-1-induced neurological damage in these patients.

The rationale for including our CXCR4/CCR5 chimeras in this evaluation lies within the results of our previous studies, which emphasize the heterogenic nature of R5 virus coreceptor use, and the possible implications that this may have for the pathogenesis of HIV-1 infection. In six patients, R5-tropic isolates with a discordant use of chimeric receptors displayed no specific patterns of receptor use that could distinguish CSF-derived isolates from plasma isolates. Also, p24 production by infected wild-type CCR5-transfected cells was similar between paired isolates (data not shown). The CSF isolates were not characterized by an increased ability in CCR5 usage.

By assessing coreceptor use in the separate compartments we confirmed that R5 plasma isolates from HIV-infected individuals with low CD4+ T cell counts are more flexible in their use of chimeric receptors. In the present cross-sectional study, the strongest correlation between chimeric receptor use and immunological dysfuncion was found when we specifically assessed viral ability to utilize the chimeric receptor FC-2. We found no correlation between plasma virus chimeric receptor use and plasma viral load, although CD4+ T cell counts and viral load correlated inversely as expected. However, FC-2 usage by CSF R5 isolates correlated significantly with an increased CSF viral load (Fig. 3). We believe that this may be explained by an increased ability of FC-2-utilizing R5 isolates to replicate in target cells within the CNS. This assumption is supported by results from a recent study in which we show that FC-2 usage by R5-tropic viruses correlates with an enhanced ability to infect primary macrophages in vitro (Karlsson et al., unpublished data).

Similar to previous studies, we found a correlation between CXCR4-expression levels and ADC. 39-42 We did not find any significant correlation between CSF viral load and ADC, as has been suggested in some studies. 52 High CSF viral loads is, however, not a uniform finding in ADC. 52 High CSF HIV-RNA levels could also be found in asymptomatic patients and we showed in a previous study that 20% of untreated patients who lack neurological deficits have higher viral loads in CSF than in plasma. 54 The relatively high levels of CSF viral load in the individuals presented here may in part be explained by our selection of participants, as one of the aims was to study patients with varying viral load in the two compartments.

Emerging data suggest that in individuals with AIDS who do not harbor X4/R5X4 virus phenotypes, CCR5-restricted viruses with an altered R5 phenotype may develop at immunodeficiency. 38,55-59 Our study confirms the notion that R5 viruses isolated from severely immunosuppressed individuals are distinct with regards to coreceptor usage. This suggests that alterations in the mode of CCR5 usage may be a key event in R5 virus pathogenesis. In a recent separate study we also showed that evolution of the R5 phenotype can be linked to adaptive molecular changes in the viral envelope glycoproteins, where viruses detected after AIDS onset display gp120 with increased net positive charge. 56 These alterations may be detected in the described assay as an enhanced viral ability to utilize chimeric receptors. HIV-1 infection in CNS may pose a specific obstacle to treatment with antiretroviral agents. Several existing antiretroviral compounds penetrate the blood-brain barrier poorly, and suboptimal CNS drug concentrations may favor the emergence of drug-resistant virus strains. 60-62 A sustained low level inflammation is often detected in CSF samples from patients with undetectable plasma virus levels, even after several years of maintained antiretroviral treatment, 63 and

### Table 2. CD4+ T-Cell Counts, ADC, Sensitivity to Inhibition by TAK-779, and Chimeric Receptor Use by Paired Plasma and CSF HIV-1 R5 Isolates

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aADC, AIDS dementia complex.

bSensitivity to inhibition by TAK-779 for selected R5 HIV-1 isolates was evaluated on PHA- and IL-2-stimulated PBMCs. Maximum concentration of TAK-779 was 990 nM.

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subjects with CSF viral load <2 copies/ml have lower intrathecal immunooactivation than subjects with CSF viral load between 2 and 20 copies/ml irrespective of plasma viral load. Together, this may indicate that full virus suppression of CNS HIV-1 infection is not always achieved with current drug regimens. Whether new compounds, such as CCR5 antagonists, would be more effective in suppressing CNS viral replication is not yet known. This provided the rationale to analyze sensitivity to CCR5 antagonism in selected R5-tropic isolates from the two compartments. For this purpose we selected paired isolates with varying chimeric receptor use, which also allowed us to correlate mode of chimeric coreceptor use with sensitivity to the CCR5 antagonist TAK-779. Although there were no general discrepancies in sensitivity to inhibition by TAK-779 between R5 isolates from the two compartments, it is important to note that the CSF isolates from all three patients with ADC were R5-tropic phenotypes that were incompletely inhibited by TAK-779 in vitro.

Although this evaluation was performed on a limited number of isolates it is striking that 90% inhibition could not be achieved for any of the seven isolates with an elevated usage of any of the chimeric receptors, whereas this was achieved for all other isolates (Table 2). Given that our CXCR4/CCR5 chimeric receptors share a common CCR5 backbone that lacks the N-terminus, it is possible that R5 isolates that are able to utilize these receptors are less dependent on interactions with the N-terminal part of CCR5. In this context it is intriguing that R5 virus resistance to the clinically available CCR5 antagonist maraviroc (Pfizer, Inc., New York, NY), has recently been explained by a reduced viral dependency on interactions with the N-terminus of CCR5. Furthermore, R5 virus resistance to maraviroc is also similarly characterized by a reduced maximum percentage inhibition with no change in IC50. However, the clinical relevance of reduced sensitivity to TAK-779 for the outcome of CCR5 antagonist treatment remains to be determined.

Further studies are warranted to verify the correlation found here between R5 isolate chimeric receptor utilization and viral sensitivity to inhibition by CCR5 antagonism. Nevertheless, chimeric CXCR4/CCR5 receptors may prove to be useful tools, not only in future studies of R5 virus pathogenesis, but also for optimizing antiretroviral treatment with coreceptor antagonists.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

References


et al.


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Late stage R5 HIV-1 isolates exhibit reduced baseline sensitivity to
Maraviroc in vitro – correlation with an altered mode of CCR5 use

Running Head: Late stage R5 HIV-1 is less sensitive to Maraviroc in vitro

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2These authors contributed equally to this work

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Keywords: HIV-1 R5, Maraviroc sensitivity, late stage disease, CCR5 use, V3 mutations
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Abstract

Objective: In our previous studies CCR5-restricted (R5) HIV-1 isolates from individuals in late stage disease displayed reduced sensitivity to inhibition by natural CCR5 ligands and CCR5 antagonist TAK-779. This correlated with an altered mode of CCR5 use. As reduced sensitivity to CCR5 antagonists in vitro may be of clinical concern, the main objective of the present study was to investigate if late stage R5 virus also display reduced baseline sensitivity to the licensed CCR5 antagonist Maraviroc (MVC).

Methods: R5 primary isolates obtained from patients with varying CD4+ T-cell counts were evaluated for sensitivity to inhibition by MVC using an in vitro inhibition assay comprising stimulated peripheral blood mononuclear cells. R5 virus sensitivity to MVC inhibition was related to CD4+ T-cell count and mode of CCR5 use, as determined by the ability to utilize CXCR4/CCR5 chimeric receptors. Furthermore, the gp120 V3 region of the R5 isolates was analysed for amino acid polymorphisms previously associated with resistance to CCR5 antagonists and/or to virological failure in MVC clinical trials.

Results: All isolates were fully inhibited by MVC. However, late stage R5 virus displayed reduced baseline sensitivity to MVC inhibition. Moreover, elevated MVC inhibitory concentration values correlated with a reduced viral dependency upon interactions with the N-terminus of CCR5. V3 amino acid polymorphisms 4L and 19S, previously associated with virological failure in MVC clinical trials, were noted in two of the least susceptible isolates.

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1. Introduction

The discovery that the chemokine receptors CCR5 and CXCR4 act as essential receptors for the entry of human immunodeficiency virus type 1 (HIV-1) into CD4+ target cells has provided the basis for new treatment strategies. While HIV-1 with CCR5 restricted phenotypes (R5) predominate during asymptomatic infection, viruses with the ability to utilize CXCR4 (X4 or R5X4) emerge in 13-76% of individuals during progression to AIDS, depending on HIV-1 subtype (1, 2). A growing bulk of evidence has also revealed that individuals with low CD4+ T-cell counts at late stage disease, where a switch to X4/R5X4 tropism has not occurred, harbour R5 viruses that are distinct from R5 viruses isolated at earlier disease stages (3-11). Importantly, R5 virus isolates from individuals with low CD4+ T-cell counts have been found less sensitive to in vitro inhibition by natural CCR5 ligands, and CCR5 antagonist TAK-779 (4-10). Through the use of CXCR4/CCR5 chimeric receptors we have previously shown that this correlates with an altered use of CCR5, which includes a decreased dependency upon the native N-terminus of CCR5 for target cell entry (7, 8).

Maraviroc (MVC) (Selzentry, Pfizer Inc) is currently the only CCR5 antagonist approved for treatment of patients infected with R5 viruses (12). Prior to the initiation of therapy it is recommended to perform tropism testing, in order to exclude the presence of naturally resistant X4/R5X4 virus variants. However, also R5 HIV-1 can display resistance to CCR5 antagonists, including isolates from treatment naïve individuals (13, 14). Several Env mutations, mainly within, but also outside of the V3 region of the env gene, have been linked to CCR5 antagonist resistance (15-23). However, these mutations have been env context dependent and there is no universal genotypic pattern to distinguish resistant R5 isolates from sensitive strains (15, 24).
Recently, the presence of gp120 V3 amino acid polymorphisms 4L and 19S were found to correlate with poor virological response to MVC treatment (25). Whether these mutations correlate with reduced in vitro susceptibility to MVC has not been investigated.

In our previous study of paired plasma and cerebrospinal fluid HIV-1 isolates, we found that R5 virus, isolated from individuals with low CD4+ T-cell counts, displayed an altered mode of CCR5 use. Some of these isolates were not inhibited by TAK-779 even at the highest concentration used, 990 nM (8). TAK-779 is an investigational CCR5 antagonist that never reached clinical use, and cross-resistance between various CCR5 antagonists is highly unpredictable (16-18, 20, 26, 27). Furthermore, alterations in baseline sensitivity to CCR5 antagonists in vitro may be of relevance for the clinical utilization of MVC. We therefore extended our previous study to investigate if R5 virus sensitivity to MVC was related to CD4+ T-cell counts at the time of virus isolation, and if so, whether or not this would relate to differences in viral CCR5 use. Since some of the isolates included in the study previously were at least partially resistant to TAK-779 (8), Gp120 V1-V3 sequences from each virus isolate were analysed for the presence of mutations previously reported to convey CCR5 antagonist resistance and/or related to virological failure in MVC clinical trials.
2. Materials and Methods

2.1 Patients

Twenty-eight HIV-1-infected patients with varying CD4+ T-cell counts were retrospectively selected from a longitudinally studied cohort for participation in our ongoing characterization of paired plasma- and cerebrospinal fluid isolates (8). Twenty-one of the patients harboured strictly R5-tropic plasma virus and isolates from 17 of these patients were available for further analysis in the present study (Supplementary data Table S1). Informed consent was obtained from all participants, and the study was approved by the Research Ethics Committee at the University of Gothenburg, Sweden. Peripheral CD4+ T cell counts, at the time of virus isolation, were determined with flow cytometry (Becton Dickinson, Mountain View, CA). The patients had CD4+ T-cell counts ranging from 36-820 cells/µL (median 230). Eight individuals had an AIDS defining disease and seven of them had CD4+ T-cell counts < 200 cells/µL. All patients were CCR5 antagonist treatment naïve, and none had received antiretroviral medication for at least nine months prior to virus isolation. None of the patients had active tuberculosis or were under interferon treatment, which could have influenced CD4+ T-Cell counts.

2.2 Virus isolates

HIV-1 plasma isolates were obtained according to standard procedures as previously described (8, 28). R5 virus tropism was phenotypically determined by the ability to infect U87.CD4.CCR5 but not U87.CD4.CXCR4 coreceptor indicator cells (8). Genotypic coreceptor tropism analysis of clonal sequences from each isolate was performed using Geno2pheno (29) and correlated well with the phenotypic assays (data not shown). Virus stocks were stored at -70°C. Prior to infection experiments,
the virus was passaged once or twice in phytohemagglutinin (PHA)-stimulated PBMCs according to standard protocols (30). The virus content was evaluated in terms of p24 antigen using the Vironostika HIV-1 Antigen ELISA kit (Biomerieux, Boxtel, The Netherlands) and for the concentration of functional viral reverse transcriptase (RT) using the Cavidi HS Lenti kit (Cavidi Tech AB, Uppsala, Sweden) according to the manufacturers’ instructions. In addition, a previously characterised primary X4 isolate, J2337 (5, 31) was used as control in MVC inhibition experiments. Late stage R5 HIV-1 were in this study defined as isolates originating from individuals with an AIDS defining disease and/or CD4+ T-cell counts ≤200 cells/µL.

2.3 Cell-lines and characterisation of mode of coreceptor use
Establishment of stably transfected U87.CD4 cells expressing CCR5, CXCR4, or chimeric CXCR4/CCR5 receptors has previously been described (32). Briefly, the chimeras were constructed by successively exchanging regions of CCR5 with corresponding parts of CXCR4 using the single overlap and extension method (33). U87.CD4 cells were stably transfected and clones expressing similar levels of receptors as evaluated by flow cytometry were chosen for further experiments. The chimeras FC-1, FC-2 (Supplementary data Figure S1) and, FC-4b, from our previous study were also included in the present study (8). Stably transfected U87.CD4 cells expressing CCR5, CXCR4, or chimeric CXCR4/CCR5 receptors were used for phenotypic determination of coreceptor tropism and mode of CCR5 use as previously described (8, 32). Briefly, U87.CD4 cells, expressing wild-type CCR5, CXCR4 or chimeric receptors and seeded in 48-well plates in cell culture media, were infected for 24h at 37°C in triplicates (virus concentration corresponding to 30 ng p24 antigen /mL). Infected cells were washed with PBS on day 1. Supernatants from the
infected cell cultures were collected at day 1 and day 6 of infection and p24 content was analysed using the Vironostika Uniform ELISA kit (Biomerieux, Boxtel, The Netherlands), according to the manufacturers protocol. Infection was defined as positive when the p24 content in the supernatant at day 6 reached 100 pg/mL after deduction of p24 content at day 1. In a semi-quantitative assessment of virus ability to utilize chimeric receptors, elevated receptor use was defined as p24 antigen production in cell culture supernatant >1000 pg/mL (8).

### 2.4 Maraviroc inhibition assay

PHA-activated PBMCs (10^7 cells), pooled from two donors, were infected in triplicates with R5 isolates, normalized to 0.33 ng RT/mL, in the presence of MVC (obtained from the NIH Research and Reference Reagent Program, Division of AIDS, NIAID, NIH). In brief, MVC was serially diluted in 5-fold steps starting at the final concentration of 100 nM and added to PBMC and virus. Control cultures for virus replication without MVC were infected in parallel. Infected PBMCs were washed with PBS on day 1 and MVC was added to the cultures at concentrations corresponding to the setup. Cell culture supernatants were harvested on day 7 after infection, and the p24 antigen content was analysed using the Vironostika Uniform ELISA kit (Biomerieux, Boxtel, The Netherlands), according to the manufacturers protocol. Dose-response curves were generated for each isolate and maximum percent inhibition was assessed. MVC sensitivity was also evaluated by determining the 90% and 50% inhibitory concentration (IC_{90} and IC_{50}, respectively), calculated in relation to p24 antigen release in the control cultures. IC_{90} values were regarded as most appropriate to determine differences in dose-response curves as the aim of this study was to assess MVC sensitivity even in minor virus populations, which commonly

exist within heterogeneous virus isolates. The experiments were conducted in a blinded fashion by two different researchers.

### 2.5 Amplification and sequence analysis

RNA from virus isolates was extracted and reverse transcribed as described (1, 34). The HIV-1 env gp120 V1-V3 region was then amplified, cloned and sequenced (six colonies were routinely picked). Sequences were aligned and manually edited in MEGA4 (35). A neighbor-joining tree was reconstructed to control for patient-specific clustering to exclude the possibility of contamination. Next, a translated amino acid alignment of nucleotide sequences was examined for previously reported CCR5 antagonist resistance mutations (17, 20-23, 36), and for mutations 4L, 11R and 19S previously related to poor virological response to MVC in vivo (25).
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2.6 Nucleotide sequence accession numbers

The sequences used in this study were deposited into Genbank under the following accession numbers: The sequences have been deposited to Genbank but the accession numbers were not yet received by the submission of this manuscript

2.7 Statistics

Group comparisons were assessed with the Mann-Whitney U-test and correlations with the non-parametric Spearman Rank test. Statistical analyses were performed in the GraphPad Prism 5 Software, GraphPad software, Inc.
3. Results

3.1 Baseline sensitivity of R5 isolates to inhibition by Maraviroc correlates with CD4+ T-cell counts

Primary R5 isolates derived from plasma of 17 HIV-1 infected patients with varying CD4+ T-cell counts at the time of virus isolation were evaluated for their ability to infect PHA-stimulated PBMC in the presence of increasing MVC concentrations. None of the isolates exhibited plateaus of <100 % inhibition (Supplementary data Figure S2). Hence, all were by definition MVC sensitive. Instead, results revealed an inverse correlation between CD4+ T-cell counts and MVC IC$_{90}$ values, in the original experiment, $r=0.64$, $p=0.006$ (Figure 1a), and in two repeated experiments, $r=0.68$, $p=0.002$, and $r=0.70$, $p=0.002$, respectively. The same correlation was found for IC$_{50}$ values in the original experiment, $r=0.58$, $p=0.01$, and in one of the repeated experiments, $r=0.52$, $p=0.03$. Furthermore, late stage R5 viruses were less sensitive to MVC inhibition, with at least four times increase in median IC$_{90}$ values, as compared to isolates from individuals without AIDS, in the original experiment, $p=0.002$ (Figure 1b), and in two repeated experiments, $p=0.008$ and $p=0.01$, respectively.

Infection with the X4 control isolate remained largely unaffected by MVC (Supplementary data Figure S2). Thus, these results suggest that R5 isolates derived from patients in late stage disease display reduced baseline sensitivity to in vitro inhibition by MVC.

3.2 Mode of coreceptor use by R5 isolates correlates with susceptibility to inhibition by Maraviroc

In order to evaluate if mode of CCR5 use correlated with sensitivity to MVC, the R5 isolates were tested for the ability to enter U87.CD4 cells expressing wild type CCR5

or either of a set of CXCR4/CCR5 chimeric receptors lacking the native CCR5 N-terminus (8). As shown in Figure 2, R5 isolates with elevated ability to utilize either of the chimeric receptors also had significantly increased MVC IC$_{90}$ values as compared to isolates that displayed a weak ability to utilize the chimeric receptors ($5$, $9$- and $4$-fold increased median MVC IC$_{90}$ values in three repeated experiments, $p=0.006$, $p=0.02$, and $p=0.01$, respectively). A similar correlation was found between the use of chimeric receptor FC-2 alone and MVC IC$_{90}$ values (Supplementary data Figure S1). In summary, these findings indicate that R5 isolates that are less dependent upon the native N-terminus of CCR5 generally display decreased sensitivity to in vitro inhibition by MVC.

3.3 Polymorphisms in the Gp120 V3 region related to CCR5 antagonist resistance

To determine if any of the R5 isolates displayed polymorphisms previously related to CCR5 antagonist resistance, reduced sensitivity in vitro, or blunted virological response in vivo, (17, 20, 21, 23, 25, 36, 37) the env gp120 V1-V3 regions of the analysed R5 isolates were amplified, cloned and sequenced. Two isolates (13pl and 23pl) that consistently had among the highest MVC IC$_{90}$ values (Figure 1b), displayed the unusual single amino acid polymorphisms, 4L and 19S, that recently were found to be associated with virological failure in patients enrolled into the phase 3 MVC clinical MOTIVATE trials (25). The two subtype C isolates (10pl and 11pl) carried the A316T polymorphism which previously was sufficient to confer in vitro resistance to MVC in the subtype B isolate CC1/85 (20). However, 316T is commonly found in HIV-1 of subtype C origin (38) and both isolates were fully inhibited by MVC in this study. Taken together, mutations that previously have been
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related to poor virological response in MVC clinical trials were present in some of the least sensitive R5 isolates in our study. Mutations previously described in highly resistant R5 HIV-1 were rare, and did not correlate with reduced MVC sensitivity \textit{in vitro}, again supporting their context dependent nature.
4. Discussion

Previous work by us and others has shown that late stage R5 HIV-1 isolates may display reduced sensitivity to inhibition by natural CCR5 ligands, TAK-779 and enfuvirtide (T20) (4, 6, 8-10). In our recent study of paired plasma- and cerebrospinal fluid isolates, some R5 isolates were found to be at least partially resistant to TAK-779. As cross-resistance between different CCR5 antagonists is highly unpredictable (16-18, 20, 26, 27), and MVC is the only approved compound for clinical use, this prompted us to conduct the current study. With few exceptions, R5 virus resistance to CCR5 antagonists is the result of an acquired ability to utilize drug-bound receptors. This non-competitive resistance is manifested \textit{in vitro} as dose-response curves with plateaus of less than 100% inhibition (16, 17, 20, 21, 27, 39). Since all R5 isolates studied here were fully inhibited by MVC they are by definition considered drug sensitive. Instead, the correlation between CD4+ T-cell counts and virus sensitivity to MVC was ascertained by variations in the amount of drug needed to inhibit 90% of virus replication (IC$_{90}$). Similar results, but less significant, were obtained when correlating MVC IC$_{50}$ values and CD4+ T cell counts. This was expected since the presence of any virus variants with reduced sensitivity within heterogeneous virus isolates is more likely to have a larger effect on the upper part of the response curve. IC$_{50}$ values will therefore more reliably detect the presence of virus variants in clinical samples with reduced sensitivity to MVC, regardless of the relative proportions (Supplementary data Figure S3).

Current guidelines by the WHO state that HIV-1 infected adult individuals with CD4+ T-cell counts ≤350 cells/mL should be offered anti-retroviral treatment (40). It is worth noting that late stage R5 isolates derived from individuals with AIDS in this
Study had significantly lower baseline sensitivity to MVC (4- to 20-fold elevated median IC₅₀ values) than individuals without AIDS. Interestingly, in an in vitro study that preceded the clinical trials, it was reported that R5 Env-recombinant pseudo viruses derived from a group of treatment-experienced individuals had higher MVC IC₅₀ values than those from a group of treatment naïve individuals (41). Even though the authors dismissed the difference as being biologically insignificant, and stated that it was less than the assay-to-assay variation, it was statistically significant. As it is reasonable to speculate that the group of previously treated individuals had a higher proportion of patients with advanced HIV disease, the results may well mirror our findings. In a recent study it was also shown that macrophage tropic R5 Envs displayed reduced sensitivity to MVC (42). As an enhanced macrophage tropism has been noted among late stage R5 viruses, these findings are also in line with the present study (43).

The clinical relevance of shifts in R5 virus sensitivity to MVC in vitro is unclear, but a few possible implications need to be addressed. Reduced levels of MVC in cerebrospinal fluid reflect a relatively poor penetration of the compound to the central nervous system, where modest reductions in MVC sensitivity may result in insufficient viral suppression (44). Furthermore, baseline reductions in sensitivity to CCR5 antagonists may precede the development of highly resistant virus phenotypes. Indeed, the in vitro generation of a virus isolate highly resistant to the CCR5 antagonist AD101 was preceded by a virus with reduced sensitivity and an increased ability to utilize drug-free CCR5 receptors (21, 27). Using a similar approach, Westby et al. reported that two out of five R5 isolates developed high resistance to MVC (20). Notably, in their study, the parental virus of the two resistant clones had 3-100 times
higher baseline MVC IC<sub>90</sub> values than the three isolates that did not develop resistance under the same conditions. Thus, at least in vitro, reduced baseline sensitivity to CCR5 antagonists may favour the development of fully resistant R5 viruses, although there is no data to support that this is applicable in vivo.

Through the use of chimeric CXCR4/CCR5 receptors we have previously shown that reduced R5 virus sensitivity to natural ligands for CCR5, and the small molecule CCR5 antagonist TAK-779, is coupled to an altered mode of CCR5 use (8, 32). In the present work, we also noted that strictly R5 tropic isolates with a reduced viral dependency upon the CCR5 N-terminus, as displayed by an elevated chimeric receptor use, were less sensitive to MVC inhibition. This is in line with recently published data, demonstrating that macrophage tropic isolates where less dependent upon the CCR5 N-terminus, and also less sensitive to inhibition by MVC (42). It is plausible that the deteriorated immune system during late stage R5 HIV-1 infection selects for virus variants with a flexible CCR5 use, which allows for a more efficient macrophage infection (8, 32, 43). We speculate that a reduced dependency upon the N-terminus of CCR5 may enable the virus to better explore relatively low levels of CCR5 found in macrophages (43), which in our assays is manifested as reduced MVC sensitivity. Supporting this theory, a virus variant with reduced sensitivity to CCR5 antagonist AD101 in vitro also displayed an enhanced ability to utilize low levels of CCR5 (27). In contrast, non-competitive, high-grade resistance has most often been attributed to an enhanced ability of the virus to utilize the N-terminus of drug bound CCR5 receptors (22, 37, 45). However, exceptions from this emerging paradigm exist, and clearly underscore the complexity of the mechanisms involved in CCR5 antagonist resistance (22, 45, 46).
Several single or combined mutations, mainly within but also outside the Gp120 V3 region, have been linked to CCR5 antagonist resistance *in vitro* and *in vivo* (17, 20, 21, 24, 36, 47). However, attempts to induce resistance by introducing these mutations in unrelated HIV-1 strains have not been successful, which clearly reflects their context dependent nature (15, 24). For example, both of the subtype C isolates in our study were sensitive to MVC inhibition while displaying the subtype specific gp120 V3 polymorphism 316T, which previously was sufficient to confer high-level resistance to MVC in the subtype B isolate CC1/85 *in vitro* (20).

In a large analysis of pre-treatment V3 sequences from patients enrolled into the MOTIVATE studies, 4L and 19S polymorphisms were recently shown to be the only V3 polymorphisms that were associated with virological failure (25). Whether these polymorphism are related to alterations in susceptibility to MVC *in vitro* has not been investigated. Analysis of our Gp120 V1-V3 sequences revealed that two of the R5 isolates, 13pl and 23pl, displayed the 4L and 19S mutations, respectively. Intriguingly, these polymorphisms are rare, occurring in only 1-2 % of subtype B isolates (48), and were confined to two of the consistently least MVC susceptible isolates in our study.

In conclusion, we believe that the decreased baseline sensitivity to CCR5 antagonists seen in late stage R5 HIV-1 infection may be clinically relevant, and low CD4+ T-cell counts was recently shown to be an independent risk factor for treatment failure with a MVC containing anti-retroviral regimen (49). Consequently, our *in vitro*
observations may provide theoretical support for *in vivo* studies that suggest a benefit of earlier initiation of CCR5 antagonist treatment rather than later (49, 50). Not only because the risk of the development of CXCR4 using virus variants increases, but also due to the emergence of HIV-1 R5 viruses with reduced baseline sensitivity to MVC during late stage disease. Although the unusual V3 polymorphisms 4L and 19S were displayed in some isolates with reduced sensitivity to MVC, their possible role as predictors for blunted clinical virological response and/or reduced sensitivity to MVC *in vitro* needs further confirmation.
Acknowledgements:

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No author have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, the presented study.
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Late stage R5 HIV-1 isolates display reduced baseline sensitivity to Maraviroc inhibition.

- **Figure 1**: CD4+ T-cell counts correlate with virus baseline sensitivity to Maraviroc inhibition, i.e. increased IC90, in individuals with R5 HIV-1 infection ($r=-0.64$, $p=0.006$). Late stage R5 HIV-1 isolates, originating from individuals with AIDS, had 4-20 times higher median IC90 values for Maraviroc inhibition than isolates from individuals with higher CD4+ T-cell counts ($p=0.002$). The isolates 13pl and 23pl (depicted by ★) displayed V3 polymorphisms 4L and 19 S respectively that previously were related to blunted virologic response in MVC clinical trials. Figures display one representative experiment of three performed.

- **Figure 2**: R5 virus ability to utilize chimeric CXCR4/CCR5 receptors plotted versus sensitivity to Maraviroc inhibition. R5 HIV-1 with an elevated ability to utilize the CXCR4/CCR5 chimeric receptors displayed decreased baseline sensitivity to inhibition by Maraviroc, i.e. increased IC90 ($p=0.006$). Grading of chimeric receptor use was based on p24 release after infection of U87.CD4+/chimeric receptor cells. Isolates that yielded p24 content in infected wells ≥1000 pg/mL were considered to have an elevated ability to utilize chimeric receptors, whereas isolates that yielded 0-1000 pg/mL p24 content were designated weak chimeric receptor users. Figure displays one representative experiment of three performed.
Figure 1. Late stage R5 HIV-1 isolates display reduced baseline sensitivity to Maraviroc inhibition

(A) CD4+ T-cell counts correlate with virus baseline sensitivity to Maraviroc inhibition, i.e. increased IC\textsubscript{90}, in individuals with R5 HIV-1 infection (r=-0.64, p=0.006). (B) Late stage R5 HIV-1 isolates, originating from individuals with AIDS, had 4-20 times higher median IC\textsubscript{90} values for Maraviroc inhibition than isolates from individuals with higher CD4+ T-cell counts (p=0.002). The isolates 13pl and 23pl (depicted by ★) displayed V3 polymorphisms 4L and 19 S respectively that previously were related to blunted virologic response in MVC clinical trials. Figures display one representative experiment of three performed.

Figure 2. R5 virus ability to utilize chimeric CXCR4/CCR5 receptors plotted versus sensitivity to Maraviroc inhibition.

R5 HIV-1 with an elevated ability to utilize the CXCR4/CCR5 chimeric receptors displayed decreased baseline sensitivity to inhibition by Maraviroc, i.e. increased IC\textsubscript{90} (p=0.006). Grading of chimeric receptor use was based on p24 release after infection of U87.CD4+/chimeric receptor cells. Isolates that yielded p24 content in infected wells ≥1000 pg/mL were considered to have an elevated ability to utilize chimeric receptors, whereas isolates that yielded 0-1000 pg/mL p24 content were designated weak chimeric receptor users. Figure displays one representative experiment of three performed.
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**Figure S1**

A) 

![Diagram](image)

B) 

![Box plot](image)
Figure S2
Figure S2
Figure S2
Figure S3
Suppl. Table S1. Patient data. Isolates from four of the patients were previously assessed for sensitivity to inhibition by TAK-779 in vitro. ADD = AIDS Defining Disease.

Suppl. Fig. S1. A) Schematic view of receptor FC-2 which was one of the chimeric CXCR4/CCR5 receptors utilized in the study. The N-terminus (Black) of CCR5 has been exchanged for the corresponding N-terminus of CXCR4. B) An elevated ability to utilize receptor FC-2 correlated significantly in all three experiments with a reduced sensitivity to inhibition by MVC in vitro. Figure displays the original experiment.

Suppl. Fig. S2. Individual MVC dose-response curves for the isolates included in the study, and for the X4 tropic control isolate 2337.

Suppl. Fig. S3. Theoretical biphasic curves showing the effect of different proportions of high and low sensitivity virus on isolate sensitivity to an inhibitor. To demonstrate the effect that different amounts of low-sensitivity virus have on biphasic curve shapes, curves were simulated according to the following equation:

\[ Y = b + \frac{(a - b)k}{1 + 10^{\left(\log(IC50) - a\right)/h1}} + \frac{(a - b)(1 - k)}{1 + 10^{\left(\log(IC50) - a\right)/h2}} \]

Where \( a \) is the upper plateau (100%), \( b \) is the lower plateau (0%) and \( k \) is the proportion of the maximal response due to the contribution of the more sensitive viral sub-population (from 0 to 1). Shown here are simulated curves showing inhibitor effects on isolates containing 0 —, 25 —, 50 —, 75 — and 100% — highly sensitive virus. The log(IC50) of the more sensitive virus was set to -9.5 (denoted in eq. 1 as EC501) and the log(IC50) of the less sensitive population was set to -7.5 (denoted in eq. 1 as EC502) and these values are highlighted with dotted lines joined to the 100% and 0% curves, respectively. Hill slopes \( h1 \) and \( h2 \) were set to one in all cases. The simulations demonstrate that the IC90 value is optimal for detecting the presence of any low sensitivity sub-populations because adding even small amounts of virus with low sensitivity has a large effect on IC90 values.
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\[ a \text{ is the upper plateau (100%),} \]

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Dual R3R5 tropism characterizes cerebrospinal fluid (CSF) HIV-1 isolates from individuals with high CSF viral load

Running head: CCR3 use in cerebrospinal fluid HIV-1 isolates

Ulf KARLSSONa,1, Liselotte ANTONSSONb, Bengt LJUNGBERGa, Patrik MEDSTRANDc, Joakim ESBJÖRNSSONb, Marianne JANSSOND,e, and Magnus GISSLENf

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Word count including abstract: 2384
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Word count including abstract: 2384
Abstract

Objective: To study the use of major and alternative coreceptors by HIV-1 isolates obtained from paired plasma and cerebrospinal fluid (CSF) samples.

Design: Paired plasma and CSF isolates from HIV-1 infected individuals with varying clinical, virologic, and immunologic parameters, were assessed for the ability to infect indicator cells expressing a panel of coreceptors with documented expression in the CNS.

Methods: HIV-1 isolates obtained from plasma and CSF in 28 individuals with varying viral load, CD4+ T-cell counts, and with or without AIDS defining disease, were analyzed for the ability to infect NP2.CD4 cells stably expressing a panel of HIV coreceptors (CCR5, CXCR4, CCR3, CXCR6, GPR1, APJ, ChemR23, RDC-1 or BLT1).

Results: All isolates from both plasma and CSF utilized CCR5 and/or CXCR4. However, the ability to use both CCR3 and CCR5 (R3R5) was more pronounced in CSF isolates and correlated with high CSF viral load and low CD4+ T-cell count. Notably, four out of five CSF isolates of subtype C origin exhibited CXCR6 use, which coincided with high CSF viral load despite preserved CD4+ T-cell counts. The use of other alternative coreceptors was less pronounced.

Conclusions: Dual-tropic R3R5 HIV-1 isolates in CSF coincide with high CSF viral load and low CD4+ T-cell counts. Frequent CXCR6 use by CSF derived subtype C isolates indicates that subtype-specific differences in coreceptor use may exist that will not be acknowledged when assessing plasma virus isolates. The findings may
also bare relevance for HIV-1 replication within the CNS, and consequently, for the neuropathogenesis of AIDS.

**Keywords:** HIV-1 R5, CCR3, CSF viral load, late stage disease, Subtype C, CXCR6

**Introduction**

HIV-1 infects the brain early in the course of infection and will, if untreated, cause neurologic disease such as HIV associated dementia (HAD) in a substantial proportion of patients [1]. In recent years also minor neurocognitive disorders in individuals under anti-retroviral medication, have been highlighted [2]. Previous studies have revealed genotypic and phenotypic differences between virus derived from brain compartment and blood [3, 4]. HIV target cells in the central nervous system (CNS) are mainly constituted by mononuclear phagocytes, e.g. perivascular macrophages and microglial cells [5]. Several chemokine receptors, and related orphan receptors, have been shown to mediate HIV entry in CD4 expressing cell lines *in vitro* but only CCR5 and CXCR4 are thought to be relevant as HIV-1 coreceptors *in vivo* [6]. However, this dogma is largely based on studies that have assessed receptor use by blood derived HIV-1 isolates. Also, the relatively few studies that have addressed coreceptor use by virus of CNS origin have mainly assessed CCR5 and CXCR4 tropism [4, 7, 8]. Several alternative coreceptors, in addition to CCR5 and CXCR4, are expressed in the brain, where they may mediate infection of target cells and/or contribute to the neuropathogenesis of AIDS [9]. The Eotaxin receptor CCR3 was early identified as an HIV-1 coreceptor that together with CCR5 could mediate efficient infection of microglial cells by macrophage-tropic isolates [10] and
more recent findings support that both receptors may be necessary for this process [11].

In our previous work we performed a phenotypic characterization of paired plasma and cerebrospinal fluid (CSF) isolates from 28 HIV-1 infected individuals and showed that discordant CCR5 and CXCR4 use between the two compartments was relatively common [4]. In order to investigate if these differences also may be reflected in the use of alternative coreceptors that are expressed within the CNS, we conducted the current study. For this purpose we chose the NP-2 cell line, which is of astroglial cell origin and previously has been shown to lack endogenous expression of minor coreceptors [12]. NP-2.CD4 cells were stably transfected with a panel of coreceptors with documented expression in the brain. Transfected cell-lines were used for the characterization of alternative coreceptor use by paired plasma and CSF isolates and the results were related to virological, immunological and clinical parameters.
Materials and Methods

Patients and virus isolates

The 28 HIV-1 infected individuals included in the study (Table 1) have been previously described [4]. Briefly the cohort was retrospectively selected to include individuals with varying levels of CD4+ T-cell counts (range 27-820, median 190 cells/µL), plasma viral load (range 1900-682000, median 52000 copies/mL) and CSF viral load (range 600->750000, median 66000 copies/mL). Seven patients had HAD, and none had received antiretroviral medication for at least 9 months prior to virus isolation. Virus isolation from plasma and CSF and virus propagation has previously been described [4, 13]. High CSF viral load (CSF\textsuperscript{high}) and low CSF viral load (CSF\textsuperscript{low}) were defined as ≥ or <40000 copies/mL, respectively. This cut off was based on early previous studies and reviews suggesting the initiation of antiretroviral therapy at plasma viral load >30000-50000 copies/mL [14-16]. A subtype B isolate 25 (B117), which previously has been reported to display broad coreceptor use [17], was used as positive control in the infection assays.

HIV-1 subtype determination

RNA from plasma virus isolates was extracted and reverse transcribed as described [18, 19]. The HIV-1 env gp120 V1-V3 region was amplified, cloned and sequenced (six colonies were routinely picked). Sequences were aligned and manually edited in MEGA4 [20]. A neighbor-joining tree was reconstructed to control for patient-specific clustering and to exclude the possibility of contamination. For subtype determination, one representative sequence from each patient was aligned with a reference sequence data set of all major subtypes, sub-subtypes and CRFs.
(downloaded from Los Alamos Sequence Database) in MEGA4 [21]. Finally, a maximum-likelihood phylogenetic tree was reconstructed in Garli 0.951 and bootstrapped 1000 times [22].

**Establishment of NP-2.CD4 cell-lines expressing HIV coreceptors found in the CNS**

NP-2.CD4 and NP-2.CD4.APJ cell-lines were kindly provided by Professor Hiroo Hoshino (Gunma University School of Medicine, Japan). NP-2.CD4 cells were stably transfected with sequence verified c-DNA from either of the following receptors: CCR5, CXCR4, CCR3, CXCR6, GPR1, ChemR23, RDC1, and BLT1 as previously described [23]. The CNS expression of included receptors has previously been documented [9, 24-26]. Receptor expression was verified with flow cytometry (CCR5, CXCR4, CCR3, CXCR6, ChemR23 and BLT1) or mRNA expression (APJ, GPR1 and RDC1). Cells were maintained in transfection medium prior to infection experiments as previously described [23].

**Virus infections**

HIV-1 infections of coreceptor-transfected NP-2.CD4 cell-lines were performed as previously described [4, 23]. In brief, two days before infection, 4×10^3 cells/well were seeded into 48-well plates using medium without antibiotics. At the time of infection, medium was removed and virus, corresponding to 30 ng p24 antigen/mL, was added to duplicate wells in 130 μl medium. Two hours after infection, medium was added to a total volume of 300 μl/well. After overnight incubation, cells were washed three times and one ml fresh medium was added to each well. Seven days after infection, medium was sampled from each well for the detection of viral p24 antigen and
analyzed according to the manufacturer’s description (Vironostika, Biomerieux, Boxtel, The Netherlands). Receptor use was defined as positive when p24 content in the supernatant reached 100 pg/mL (hereafter depicted as + after the indicated coreceptor). In a semi-quantitative grading of receptor use we applied the following system: 100-1000 pg/mL =low-grade use (+), 1000-10000 pg/mL =moderate use (++), >10000 pg/mL =high-grade use (+++) [4].
Results

Dual R3R5 tropism characterizes CSF isolates from HIV-1 infected individuals with high CSF viral load and low CD4+ T-cell count

Paired plasma and CSF isolates from 28 HIV-1 infected individuals were evaluated for their ability to utilize a panel of HIV coreceptors with documented expression in the CNS. The subtypes of the isolates are depicted in Table 1. All isolates were found to utilize CCR5 and/or CXCR4, as previously shown [4]. As depicted in Figure 1, 11 out of 14 CSF R5 virus isolates from individuals with CSFhigh were of dual R3R5 phenotype, while exclusively monotropic R5 CSF isolates were found within the CSFlow group (7/7) (p=0.001, Two-tailed Fisher’s exact test). We also noted that two out of three CCR3- R5 isolates in the CSFhigh group were of subtype C origin and able to use CXCR6. The level of CCR3 use was similar to CCR5 use in some dual tropic CSF isolates (Table 1) and was significantly more pronounced in CSF isolates than in corresponding plasma isolates (p=0.035, Wilcoxon signed rank test). The median CSF viral load in individuals with dual R3R5 CSF isolates was 119000 copies/mL versus 19500 copies/mL in individuals with CCR3- R5 CSF isolates (p=0.001, Mann-Whitney rank sum test). In contrast, plasma viral load did not differ significantly between individuals harboring dual R3R5 plasma virus (median 52000 copies/mL) and those with monotropic R5 plasma isolates (median 23000 copies/mL). However, in patients with R5 HIV-1 infection, the presence of CCR3+ isolates in plasma and/or CSF was associated with lower CD4+ T-cell counts (p=0.03 and p=0.001 respectively, Mann-Whitney rank sum test). Among individuals with R5 HIV-1 infection, there was also an inverse correlation between CD4+ T-cell counts and CSF viral load (p=0.003, Spearman’s rank correlation test), which was lost when including individuals with X4/R5X4 viruses. Accordingly, no correlation was found between
CCR3-use and CSF viral load in patients that harbored either X4 or R5X4 HIV-1 (Figure 1). Dual R3R5 tropism and CSF$^\text{high}$ were not specifically confined to individuals with HAD, but were found also in neuro-asymptomatic individuals with low CD4$^+$ T-cell counts (Table 1). In summary, dualtropic R3R5 CSF isolates are commonly exhibited by individuals with high CSF viral load and coincide with low CD4$^+$ T-cell counts. In the few individuals with X4/R5X4 virus phenotypes, no clear associations between CCR3 use, low CD4$^+$ T-cell counts and CSF$^\text{high}$ were observed.

**HIV-1 CSF isolates of Subtype C origin commonly exhibit CXCR6 use**

CXCR6 use was displayed by four out of five CSF isolates of subtype C origin, as compared to one out of 23 non-subtype C isolates (Two-tailed Fisher’s exact test, p=0.001) (Figure 1 and Table 1). CXCR6 use was highly efficient and equal to CCR5 use in one CSF isolate (patient 11), whereas CSF isolates of patients 3, 9 and 10 had low-grade CXCR6 use and efficient CCR5 use. The CSF isolate of patient 11, also exhibited efficient CCR3 use and was derived from an individual with extremely high CSF viral load (>750,000 copies/mL) while the plasma viral load was low (1450 copies/mL). This patient had a chronic infection and suffered no signs of neurocognitive impairment. It is worth noting that CXCR6 use coincided with CSF$^\text{high}$ in subtype C infected individuals despite relatively preserved CD4$^+$ T-cell counts (Figure 1 and Table 1). Two of the corresponding subtype C plasma isolates displayed low-grade CXCR6 use (patient 9 and 11). Low-grade use of mainly GPR1 but also, RDC-1, APJ and ChemR23 was exhibited by some CSF and/or plasma isolates (Table 1). BLT1 use was only exhibited by the subtype B control isolate, but not by any of the isolates included in the study (data not shown). Taken together, CXCR6 use was displayed by CSF isolates of subtype C origin, which coincided with
To our knowledge, this is the first study to have assessed the use of coreceptors other than CCR5 and CXCR4 in CSF derived HIV-1 isolates. Although CSF is not identical to brain tissue, it is a more readily sampled site that provides an important "window" into HIV CNS infection [28]. In this study we show that CCR3 use characterizes CSF derived R5 HIV-1 isolates in individuals with high CSF viral load, and that this coincides with low CD4+ T-cell counts. CCR3 was early identified as a coreceptor able to promote infection of microglial cells by selected HIV-1 isolates [10] which has support in a more recent study [11]. Using a gene knockout strategy, Agrawal et al. showed that CCR3 and CCR5 co-localize with CD4 molecules at the cell surface and that both coreceptors are required to convey an efficient infection of microglial cells and macrophages by R3R5 HIV-1 isolates [11]. Given that these are the major target cells for HIV-1 in the brain, it is reasonable to speculate that the acquisition of CCR3 use, specifically and more pronounced in CSF isolates, reflects a viral adaption to replication in mononuclear phagocytes within the CNS. This could theoretically explain the correlation between dual R3R5 tropism in CSF derived isolates and elevated CSF viral load found in this study. Consequently, a more efficient CCR3 use in CSF isolates than in plasma isolates may reflect differences in available target cells between the two compartments. Furthermore, increased macrophage tropism is exhibited by HIV-1 during late-stage disease [29], which in our study may be mirrored by the frequent appearance of R3R5 isolates in individuals with low CD4+ T-cell counts.

Unexpectedly, CXCR6 use was exhibited by four out of five CSF derived isolates from individuals with subtype C HIV-1 infection. While efficient CXCR6 use high CSF viral load despite CD4+ T-cell counts well above the level of WHO recommendations for anti-retroviral treatment [27].
Discussion

To our knowledge, this is the first study to have assessed the use of coreceptors other than CCR5 and CXCR4 in CSF derived HIV-1 isolates. Although CSF is not identical to brain tissue, it is a more readily sampled site that provides an important “window” into HIV CNS infection [28]. In this study we show that CCR3 use characterizes CSF derived R5 HIV-1 isolates in individuals with high CSF viral load, and that this coincides with low CD4+ T-cell counts. CCR3 was early identified as a coreceptor able to promote infection of microglial cells by selected HIV-1 isolates [10] which has support in a more recent study [11]. Using a gene knockout strategy, Agrawal et al. showed that CCR3 and CCR5 co-localize with CD4 molecules at the cell surface and that both coreceptors are required to convey an efficient infection of microglial cells and macrophages by R3R5 HIV-1 isolates [11]. Given that these are the major target cells for HIV-1 in the brain, it is reasonable to speculate that the acquisition of CCR3 use, specifically and more pronounced in CSF isolates, reflects a viral adaption to replication in mononuclear phagocytes within the CNS. This could theoretically explain the correlation between dual R3R5 tropism in CSF derived isolates and elevated CSF viral load found in this study. Consequently, a more efficient CCR3 use in CSF isolates than in plasma isolates may reflect differences in available target cells between the two compartments. Furthermore, increased macrophage tropism is exhibited by HIV-1 during late-stage disease [29], which in our study may be mirrored by the frequent appearance of R3R5 isolates in individuals with low CD4+ T-cell counts.

Unexpectedly, CXCR6 use was exhibited by four out of five CSF derived isolates from individuals with subtype C HIV-1 infection. While efficient CXCR6 use
Previously has been shown for the HIV simian counterpart SIV and HIV-2 [30, 31], mainly weak and infrequent receptor use has been exhibited by HIV-1 plasma isolates [32, 33]. Worth noting, CXCR6+ CSF isolates in subtype C infected individuals coincided with CSF^{high} despite relatively preserved CD4+ T-cell counts. In fact, all other individuals in the cohort with CSF^{high} had AIDS, as defined by CD4+ T-cell counts ≤200 cells/μL and/or ADD. Clearly, further studies are warranted to verify if CXCR6 use is more common in HIV-1 CSF isolates of subtype C origin and also if this may correlate with elevated CSF viral load. The latter is specifically important since high viral load may correlate with the development of neurological morbidity such as HAD [34]. Furthermore, subtype C HIV-1 represents approximately 50% of the global epidemic. Studies addressing the influence of subtype on HIV-1 related neurological complications are few, but have shown that HAD poses a major health concern, also in areas of the world where subtype C HIV-1 infection dominate [35].

Our study cohort was retrospectively selected to include individuals with varying clinical, virological and immunological parameters, which may have affected the results. Nevertheless, despite the limited number of individuals included in the cohort, the results presented merit future validation.

In conclusion, increased CSF viral load in late-stage HIV-1 infection correlates with the emergence of dual R3R5 tropic viruses in CSF. In addition, our results indicate that subtype specific differences in HIV coreceptor use may exist and remain unnoticed when analyzing plasma isolates.
Acknowledgements:

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The funding sources had no involvement in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

No author have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, the presented study.

References


Figure 1. Correlation between CD4+ T-cell count and CSF viral load at the time of virus isolation of CSF HIV-1 isolates with diverse coreceptor use. Grey horizontal line denotes CSF viral load = 40000 copies/mL and CSF viral load above this level defines high viral load (CSF_{high}). Grey vertical line defines CD4+ T-cell count = 200 cells/µL. ★ = Isolates obtained from individuals with an AIDS defining disease (ADD). Patients with AIDS (defined by CD4+ T-cell counts ≤ 200 cells/µL or ADD) and/or CSF_{high} display CSF R5 isolates able to use CCR3 (black circles) (p<0.001 and p<0.001, respectively). In contrast, monotropic R5 phenotypes (grey circles) characterize virus isolates from individuals with higher CD4+ T-cell counts and CSF_{low}. R5 isolates of subtype C origin (diamonds) that also use CXCR6 (crossed grey diamond) or CXCR6 and CCR3 (crossed black diamond) originated from patients with CSF_{high} despite relatively preserved CD4+ T-cell counts. In individuals with X4/R5X4 infection (CCR3-= grey crosses, CCR3+= black crosses), no clear association between low CD4+ T-cell counts and CCR3 use or CSF_{high} was seen.
**Figure 1.** Correlation between CD4+ T-cell count and CSF viral load at the time of virus isolation of CSF HIV-1 isolates with diverse coreceptor use. Grey horizontal line denotes CSF viral load =40000 copies/mL and CSF viral load above this level defines high viral load (CSF$^{\text{high}}$). Grey vertical line defines CD4+ T-cell count =200 cells/µL. ★ Isolates obtained from individuals with an AIDS defining disease (ADD). Patients with AIDS (defined by CD4+ T-cell counts ≤ 200 cells/µL or ADD) and/or CSF$^{\text{high}}$ display CSF R5 isolates able to use CCR3 (black circles) (p<0.001 and p<0.001, respectively). In contrast, monotropic R5 phenotypes (grey circles) characterize virus isolates from individuals with higher CD4+ T-cell counts and CSF$^{\text{low}}$. R5 isolates of subtype C origin (diamonds) that also use CXCR6 (crossed grey diamond) or CXCR6 and CCR3 (crossed black diamond) originated from patients with CSF$^{\text{high}}$ despite relatively preserved CD4+ T-cell counts. In individuals with X4/R5X4 infection (CCR3$^-$ = grey crosses, CCR3$^+$ = black crosses), no clear association between low CD4+ T-cell counts and CCR3 use or CSF$^{\text{high}}$ was seen.
Table 1. Characteristics of patients and virus isolates

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CD4+ T-cell counts in declining order, plasma- and CSF viral load, and AIDS defining disease (ADD). HAD= HIV associated dementia, KS= Kaposi Sarcoma, MAC= Mycobacterium Avium Complex, C. esophagitis= Candida esophagitis, PCP= Pneumocystis Jiroveci Pneumonia, at time of virus isolation and HIV subtype. For the assessment of coreceptor use by HIV-1 isolates (plasma/CSF), the following grading system was applied: 0-100 pg p24/mL in culture supernatant =(-), 100-1000 pg/mL =(+), 1000-10.000 pg/mL =++, >10000 pg/mL =+++.

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