Towards an in vivo HIV latency model based on humanized mice

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Towards an *in vivo* HIV latency model based on humanized mice

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For my parents Irmgard and Peter
Summary

HIV is the most devastating disease to have emerged in the 20th century. Thanks to enormous preventive efforts and anti-retroviral treatment (ART) the transmission rate, morbidity and mortality decreased dramatically in most countries. While ART is very efficient in suppressing HIV replication, its widespread use revealed substantial disadvantages, such as adverse events, emergence of drug resistant strains, psychological problems, life-long adherence and cost. Even if a successful ART is taken for years it is unable to cure HIV and treatment interruption results in most cases in viral rebound within a short time frame. Therefore novel, more efficacious and simpler treatment strategies are desired. The ultimate goal would be a prophylactic or even therapeutic vaccine, or an adjunct to the existing ART that would be capable of eradicating latently infected cells that are the origin of the viral rebound after ART interruption.

The overall aim of this thesis was to study HIV infection in humanized mice, to utilize the humanized mouse model for testing novel anti-retroviral compounds, as well as for testing different treatment approaches and to verify its potential for studying HIV latency. Humanized mice are immunodeficient mice reconstituted with human CD34+ hematopoietic progenitor/stem cells (HPSCs). Upon HPSC transplantation a lymphoid system of human origin develops and as a consequence humanized mice can be infected with HIV.

We first established and optimized a treatment protocol with conventional ART (cART) for suppressing HIV RNA. An efficacious cART is the prerequisite for testing other anti-retroviral compounds and for studying HIV latency in mice with suppressed viral replication. Since mice demonstrate different pharmacokinetics (PK) compared to humans, we established PK data for all compounds used: Lamivudine (3TC), Tenofovir (TDF), Azidothymidin (AZT), Ritonavir (RTV), and Raltegravir (RGV), as well as for two investigational drugs in a long acting formulation TMC278-LA and TMC181-LA (LA = long-acting). Since daily gavage or injection of drugs over a longer time was hardly tolerated by these mice, we orally administered the drugs by adding them to food pellets. We were able to repetitively show successful suppression of HIV RNA in mice treated with a triple ART consisting of 3TC, TDF and RGV. Furthermore, in all mice HIV RNA rebounded after interruption of ART and for mice with viral breakthrough during therapy we could demonstrate the development of prototype resistance mutations. Supplementary we could demonstrate ex vivo reactivation of HIV from spleen samples of treated mice with suppressed HIV RNA.

We compared the efficacy of two long-acting drugs and of a novel peptide based fusion inhibitor to our standard cART in different treatment schemes, i.e. pre- and post-exposure
prophylaxis, maintenance regimen or initial treatment. Furthermore we tested in a pilot experiment the potential of the histone deacetylase (HDAC) inhibitor (HDAC-i) suberoylanilide hydroxamic acid (SAHA) to reactivate HIV in vivo in mice with suppressed HIV RNA. In addition we wanted to establish a HIV latency model based on lentiviral transduction of CD34+ cells. To this extend we compared and optimized in a first step different transduction protocols using a reporter construct with the gene of the enhanced green fluorescent protein (eGFP) under control of a constitutive endogenous promoter. Overall we found that HIV infected humanized mice recapitulate all hallmarks of HIV infection in humans and permit testing of novel anti-retroviral compounds. We think that the model is suitable to investigate the in vivo efficacy of immune-modulators and other compounds regarding their potential to reactivate HIV from latently infected cells, which is the premise for the development of novel concepts aiming at HIV eradication. In summary humanized mice are a valuable model, bridging the gap between in vitro generated data and potential clinical applications.
Zusammenfassung (German)


Das übergeordnete Ziel dieser Arbeit war es, die HIV-Infektion in humanisierten Mäusen zu analysieren, das Modell zum Testen neuartiger anti-retroviraler Verbindungen in verschiedenen Behandlungsansätzen zu nutzen und das Potenzial des Modells hinsichtlich der Erforschung der HIV-Latenz zu überprüfen.


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1 Introduction

Even though the number of new HIV infections is decreasing worldwide, the number of people living with HIV is still increasing.

Nearly 30 years after the first reports about AIDS, an estimated 35.3 million people were living with HIV globally in 2012 (Source: UNAIDS Report on the global AIDS epidemic 2013, http://www.unaids.org/globalreport/global_report.htm). At the same time there were 2.3 million new HIV infections, which is a 33% reduction in the number of new infections compared to 2001. The number of AIDS deaths dropped down from 2.3 million in 2005 to 1.6 million AIDS deaths in 2012 (Source: UNAIDS Report on the global AIDS epidemic 2013, http://www.unaids.org/globalreport/global_report.htm).

1.1 Origins of the HIV pandemic

The HIV infection is caused by two lentiviruses, namely human immunodeficiency viruses (HIV) type 1 and 2. For the first time in 1981 symptoms, later referred to as acquired immunodeficiency syndrome (AIDS), were described in young homosexual men in Los Angeles and New York, who were succumbing to opportunistic infections and rare malignancies, such as *Pneumocystis carinii* pneumonia (PCP) and Kaposi's sarcoma (KS) caused by depletion of the CD4+ T-cell compartment\(^1\)\(^-\)\(^6\).

In 1983 the identification and isolation of HIV-1 was successfully carried out by the group of Luc Montagnier at the Institute Pasteur\(^7\), followed by the groups of Robert Gallo\(^8\)\(^,\)\(^9\) and Lyndon Oshiro\(^10\). The isolated virus was initially termed human T-lymphotropic virus type III.
Introduction

(HTLV-III) by Gallo's group; whereas Montagnier and colleagues named it lymphadenopathy-associated virus (LAV) as they could demonstrate that their isolate was immunologically different from HTLV-1. In 1986 LAV and HTLV-III were renamed HIV-1. In the same year a closely related virus was found by Montagnier and colleagues in blood samples of West African AIDS patients. The discovered retrovirus was termed human immunodeficiency virus type 2 (HIV-2). By subsequent isolation and characterization of further viruses from various different primate species found in sub-Saharan Africa, referred to as simian immunodeficiency viruses (SIVs), it became clear that HIV-1 and HIV-2 were the results of zoonotic transfers of different ancestor simian immunodeficiency viruses. HIV-1 is closely related to the SIV of chimpanzees (groups M, N and O) or gorilla (group P), whereas HIV-2 is only present in West Africa and is related to SIV from African green monkeys. HIV-1 is therefore categorized into groups M, N, O, and P, of which each represents a distinct transmission of a SIV to humans. Currently it is believed that the relative transmissibility of the different HIV-1 groups depends on various viral and host factors. So far, only the “major” group M underwent pandemic spread, with nine of the HIV-1 group M subtypes (A, B, C, D, F, G, H, J, K) contributing to the global HIV-1 pandemic. HIV-2 is less virulent and pathogenic than HIV-1 and its occurrence is mainly restricted to some countries in West Africa.

Mathematical modeling proposes a common progenitor for group M viruses for the time of 1910-1930 when the virus was spreading endemically in West Central Africa. Probably in the beginning of the 1960s HIV-1 emerged as phylogenetically distinct subtypes most likely due to repeated cross-species transmission.

1.2 Molecular biology of HIV

HIV belongs to the genus Lentiviridae within the family of Retroviridae. The family of Retroviridae consists of seven genera: α-, β-, γ-, δ-, ε-Retroviruses, Lentiviruses and Spumaviruses. Retroviruses are characterized by their unique mode of replication including reverse transcription of their single stranded RNA (ssRNA) genome into double stranded DNA (dsDNA) and its subsequent integration into the host chromosomal DNA. Since HIV infection results in a long asymptomatic phase, albeit HIV replicates and CD4+ T cells decline, HIV belongs to the genus Lentiviruses (Latin: lentus = slow).

The high genetic diversity is an important feature of HIV regarding immune escape and the appearance of resistance mutations to ART. The diversity is caused mainly by the failure
prone HIV reverse transcriptase (RT), by hypermutation of the nascent DNA strand during reverse transcription by APOBEC proteins (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) and by recombination events between distinct HIV strains. The HIV RT, like the RTs of other retroviruses, has no proofreading ability and does not correct errors. Even in comparison with other retroviral RTs the HIV enzyme is remarkably inaccurate with an error rate of 1/1700 nucleotides\textsuperscript{20-22}.

APOBEC3G belongs to the APOBEC proteins, an evolutionary conserved family of inducible cytidine deaminases. APOBEC3G cleaves an amino group of deoxycytidine resulting in deoxyuridine in the nascent (-) DNA strand of the HIV during reverse transcription. Deoxyuridine will be complemented by adenosine in the nascent (+) DNA strand thereby hypermutating the HIV genome. Notably, the APOBEC induced changes in the viral envelope gene were similar in humanized mice that were infected with the HIV JR-CSF strain as compared to humans\textsuperscript{23}. Another exclusive feature of retroviruses is that each virion includes two RNA genomes. Therefore, HIV can be considered to possess “pseudodiploidy” and as a consequence homologous recombination can occur when a cell is co-infected with two different viral strains\textsuperscript{24-26}.

The size of the genome of HIV-1 is ~9.7 kb, consists of (+) ssRNA (single stranded RNA of positive orientation) and comprises 9 genes (gag, pol, env, tat, rev, nef, vif, vpr and vpu), which encode in total fifteen proteins (Fig. 2 and Table I)\textsuperscript{27-29}.

\textbf{Figure 2:} Organization of the HIV-1 genome. The HIV-1 genome contains in total nine open reading frames (ORFs), flanked by two long terminal repeats (LTRs) (grey rectangles). The genes gag (“group-specific antigen”) and env (“envelope”) encode the structural proteins, whereas pol (“polymerase”) encodes the enzymatic proteins of HIV (dark blue rectangles). The two spliced exons encoding the regulatory proteins Tat and Rev, as well as the ORFs for vif, vpr, vpu and nef encoding the accessory proteins are depicted in light blue rectangles.

\textbf{Gag, pol and env}

The genes gag, pol, and env are rather conserved among all retroviruses. HIV-1 additionally encodes two regulatory and four accessory proteins (Table I). In detail, the gag (“group-specific antigen”) gene encodes the matrix protein (MA, p17), the nucleocapsid protein (NC, p7) and the capsid protein (CA, p24); the pol gene encodes the protease (PR), the reverse transcriptase (RT) containing RNase H activity and the integrase (IN); the env gene encodes
the two envelope glycoproteins: transmembrane protein (TM, gp41) and surface protein (SU, gp120).

The Gag and Gag-Pol polyprotein precursors are processed by the viral protease into the various subunits, i.e., matrix (MA), capsid (CA), nucleocapsid (NC), SP2 (spacer peptide 2, p1), p6, protease (PR), reverse transcriptase (RT) and integrase (IN). Env (gp160) is cleaved by cellular proteases, e.g. furin, into surface glycoprotein (SU, gp120) and transmembrane glycoprotein (TM, gp41) moieties.

**Tat and Rev**

The regulatory proteins of HIV-1: Tat\(^{30}\) (p16, transactivator of transcription) and Rev\(^{31}\) (p19, regulator of expression of viral proteins) are translated from multiply spliced mRNA transcripts. They interact with host enzymes and are essential for the successful replication of HIV (Fig. 4). Tat forms together with the positive transcriptional elongation factor B (P-TEFb), consisting of cyclin-dependent kinase 9 (CDK9) and cyclin T1 (CycT1), the Tat/P-TEFb complex. The Tat/P-TEFb complex binds to the transactivation-responsive region (TAR), a regulatory element within the U3 region of the LTR, which is required for transactivation by recruiting other elongation factors to the transcription complex\(^{32-34}\). The Rev protein regulates at a post-transcriptional level the shift between the early phase and the late phase of viral gene expression. This is achieved by its ability to transport unspliced and singly spliced viral mRNA species from the nucleus into the cytoplasm. This Rev-regulated transport requires the binding of Rev to the Rev-responsive element (RRE), which is an elongated RNA stemloop structure of 351 nt within the env gene\(^{35-37}\).

**Table I:** List of HIV-1 encoded viral proteins and their corresponding function.

<table>
<thead>
<tr>
<th>Abb.</th>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>Nucleocapsid</td>
<td>Encapsulates &amp; protects genomic RNA</td>
</tr>
<tr>
<td>CA</td>
<td>Capsid</td>
<td>Forms viral capsid (“core”)</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix</td>
<td>Protein that constitutes the viral matrix</td>
</tr>
<tr>
<td>TM/gp41</td>
<td>Transmembrane protein</td>
<td>HIV receptor protein attached to viral membrane</td>
</tr>
<tr>
<td>SU/gp120</td>
<td>Surface protein</td>
<td>HIV receptor protein that binds to CD4 receptor</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
<td>Proteolytic cleavage of immature HIV proteins</td>
</tr>
<tr>
<td>IN</td>
<td>Integrase</td>
<td>Integration of viral cDNA into host cell DNA</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
<td>Transcribes viral genomic RNA into cDNA</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative replication factor</td>
<td>Down-regulates CD4, prevents apoptosis</td>
</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
<td>Counteracts APOBEC3G</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein U</td>
<td>Down-regulates CD4, counteracts BST-2</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein R</td>
<td>Arrests host cell cycle in G2 phase</td>
</tr>
<tr>
<td>Tat</td>
<td>Transactivator of transcription</td>
<td>Activation and upregulation of HIV transcription</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulator of expression of viral proteins</td>
<td>Exports viral mRNA out of nucleus</td>
</tr>
</tbody>
</table>
**Vif, Vpr, Vpu and Nef**

HIV-1 possesses four accessory proteins: Vif\(^{38}\), Vpr\(^{39}\), Vpu\(^{40}\) and Nef\(^{41}\), which are called “accessory” because they are dispensable for HIV-1 replication in certain cell types. However they are essential for HIV-1 replication in vivo\(^{42-44}\).

**Vif** inhibits the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 G (APOBEC3G) function by binding to APOBEC3G and by recruiting it to a cellular ubiquitin ligase complex, thereby inducing poly-ubiquitination and ultimately proteasomal degradation\(^{45-47}\).

**Vpr** was reported to have many functions including cell-cycle arrest in the G2 phase, activation of transcription, induction of cell death and improvement of reverse transcription activity\(^{42, 48}\).

**Vpu** is an integral membrane protein possessing two key functions. The first function is mediating poly-ubiquitination and proteasomal degradation of CD4 by interacting with freshly synthesized CD4 in the endoplasmatic reticulum (ER)\(^{49, 50}\). The Vpu mediated down regulation of CD4 is possibly helping virus release, preventing superinfection, and enhancing the incorporation of gp160/Env proteins by averting the formation of gp120/CD4 complexes in the cell membrane of productively infected cells. The second function is the requirement of Vpu for the efficient release of new virions in some cell types. Notably, in other cell types Vpu is only required for virus release after stimulation with interferon-α (IFN-α). This observation lead to the discovery of the host restriction factor BST-2 (tetherin)\(^{51, 52}\), which tethers budding virions with membrane anchors penetrating both, virion and the host cell membranes\(^{43, 44, 53}\).

**Nef**, like Vpu, inhibits the expression of the CD4 receptor on the surface of infected cells, but in addition Nef down-modulates MHC-I, and to a lesser degree also CD28 and CXCR4 by redirecting them to lysosomes\(^{44, 54}\). Hence Nef helps virus release, decreases CTL-mediated lysis (CTL = cytotoxic T lymphocyte) of infected cells and modulates signal transduction via the immunological synapse. In addition Nef modulates T cell activation by interacting with cellular kinases, thereby inducing changes in the actin skeleton, activation of NFAT (nuclear factor of activated T-cells), NF-κB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells), and AP-1 (activator protein 1), thus promoting transcription of the HIV-1 LTR promoter. The transcription factors such as NFAT and NF-κB, in turn up regulate the expression of cellular genes encoding inflammatory cytokines, activation markers, and death receptors\(^{54}\).
1.3 HIV virus particle

HIV-1 is an enveloped virus with an approximate virion size about of about 100-130 nm in diameter (Fig. 3). The lipid membrane of the virus is derived from the host cell and contains trimeric complexes of the envelope glycoproteins gp41 (TM) and gp120 (SU). The glycoproteins are necessary for the binding of HIV virus particles to host cells through CD4 and a co-receptor (CXCR4 or CCR5). The myristoylated form of MA (p17) is localized at the inner surface of the membrane, forms the viral matrix and interacts with the cytoplasmic tail of gp41.

The viral matrix ensures the integrity of the virus particle and plays a key role during the budding of virus particles from the cell surface. The conical shaped viral capsid/core consists of capsid proteins p24 (CA) and encloses two molecules of full-length (+) ssRNA copies, which are associated with the nucleocapsid proteins p7 (NC). Additionally, the viral enzymes RT (p64), IN (p32) and PR (p10) are incorporated into the virus particle (Fig. 3).
1.4 HIV replication cycle

1.4.1 Binding and entry

The first step of the HIV replication cycle is the binding to a target cell, followed by entry of the virus. The HIV envelope protein (Env/gp160) binds to the primary cellular receptor CD4 and to a cellular co-receptor, which can be either CXCR4 or CCR5 (Fig. 4 and 5).

![HIV-1 life cycle](image)

Figure 4: HIV-1 life cycle. HIV infection of a host cell starts with the binding of the viral glycoprotein gp120 to the CD4 receptor and chemokine co-receptor (CCR5 or CXCR4). The fusion of the viral membrane with the cell membrane is followed by uncoating of the viral RNA. The viral RNA genome is reverse transcribed into a full-length double stranded DNA, and enters the nucleus as a pre-integration complex. Non-integrated DNA can be present in forms of linear, 1-LTR- and 2-LTR-circles. From un-integrated linear DNA all three classes of viral transcripts: the multiply spliced, singly spliced and full-length transcripts can be transcribed. The multiply spliced, early transcripts such as tat, nef and rev are translated into products. These early viral factors promote the viral replication process. From integrated provirus post-integration transcription is initiated and transcripts translated into the viral proteins. Upon viral protein assembly new HIV virions bud of from the cell membrane and maturation into infectious virus particle is carried out by the viral protease.

The HIV envelope consists of a gp41 and gp120 subunit, which are organized as trimers and form the outer sphere of the virus. The gp41 functions as an anchor and gp120 binds to CD4 via its V3 loop (Table I, Fig. 5). Subsequent to binding of gp120 to CD4 conformational changes in gp120 result in the exposure of the co-receptor binding site, which in turn leads to
the actual fusion process of HIV with the cell, i.e. the insertion of the fusion peptide within gp41 into the host cell membrane.

![Figure 5: Overview of HIV binding process and entry. (1) HIV Env consists of the gp120 and gp41 subunits and attaches in the first step of the viral entry process to the host cell by binding to the CD4 receptor (3). This induces conformational alterations leading to the binding of the respective co-receptor. (3) The V3 loop of Env contributes to the binding to the co-receptor. The fusion peptide of the gp41 subunit immerses into the host cell membrane and upon six-helix bundle formation membrane fusion of viral and target cell membrane takes place (Figure taken from Wilen et al.56).]

1.4.2 Reverse transcription

Following virus entry, the viral core is released into the cytoplasm (Fig. 4 and 5). The reverse transcription of viral (+) ssRNA into dsDNA takes mainly place in the cytoplasm; initial steps may be already initiated in the virus particle57-59. Reverse transcription starts with the binding of the Lys3 tRNA primer to the primer binding site (PBS) that is localized in the U5 region of the LTR (Fig. 6)60. The RNA strand of the RNA–DNA complex resulting from the initial DNA synthesis is a substrate for RNase H intrinsic to the viral RT59. RNase H digests the 5′-end of the (+) ssRNA, thereby liberating the (-) strand DNA. Direct repeats (R) at both ends of the viral genomic RNA enable a transfer of the newly synthesized DNA from 5′ end to 3′ end of the viral RNA (Fig. 6). This process is called first strand transfer (minus strand transfer). As the (-) strand DNA synthesis continues, the RNase H domain of RT degrades the viral RNA template. Only a purin-rich sequence called polypurin tract (PPT) is resistant to RNase H activity and serves as a primer for the (+) strand DNA synthesis61. Plus strand DNA starting from the 3′ end of the PPT synthesis also copies the tRNA sequence, which is then cleaved by RNase H. Nearly all retroviruses remove the complete tRNA, but HIV-1 RT leaves a single adenine ribonucleotide (rA) at the 5 prime end62, 63.

As a result of copying the tRNA primer sequence, the 3′ end of the (+) strand DNA contains 18 nucleotides that are complementary to 18 nucleotides within the PBS sequence at the 3′ end of the (-) strand DNA, thereby allowing the (+) strand DNA to transfer to 3′ end of the (-) strand DNA. This process is called second strand transfer (plus strand transfer). Subsequently the RT completes synthesis of both the (-) and (+) strands. The product of the reverse
transcription is a linear dsDNA that is longer than the RNA template and contains at both ends the same sequence motifs (U3-R-U5) called long terminal repeats (LTRs). The LTRs are essential for the later integration of the dsDNA into the cellular chromosomal DNA and harbor the promoter of HIV. The reverse transcriptase of HIV is one of the main drug targets in anti-retroviral therapy with 13 of the 26 approved drugs being RT inhibitors.

**Figure 6:** Reverse transcription of the viral (+) ssRNA genome into dsDNA. 1) The viral RNA (purple) with a tRNA primer annealed to the primer binding site (PBS). 2) Initiation of reverse transcription by RT producing (-) strand DNA (blue) and degradation of RNA by RNase H activity of RT (dashed). 3) First strand transfer of (-) strand DNA between the two R regions of the viral genome and subsequent elongation of the (-) strand DNA escorted by RNase H activity. 4-5) The poly purine tract (PPT) neighboring to the U3 region is resistant to RNase H activity and serves as a primer for (+) strand DNA synthesis, which carries on until the first 18 nucleotides of the tRNA are transcribed. 6) RNase H cleaves of the tRNA primer leaving rA at the 3' end of the tRNA and is followed by the second strand transfer of (+) strand DNA. 7) Elongation of both of the (+) and (-) strands renders complete viral dsDNA (adapted and modified from Hu and Hughes).

### 1.4.3 Integration

Upon completion of reverse transcription, newly formed viral double stranded cDNA associates with viral and cellular proteins, thereby forming the pre-integration complex (PIC). The PIC has been found to contain the viral proteins: IN, MA, RT, Vpr and NC, as well as the cellular proteins, lens epithelium-derived growth factor (LEDGF/p75), barrier-to-autointegration factor (BAF) and high-mobility group proteins (HMGs).
The PIC possesses nuclear localization signals (NLSs)\textsuperscript{70-72}. NLSs are found in the viral proteins MA, CA, and IN, as well as in a distinct DNA region of the HIV genome, the so called three-stranded DNA flap structure.

The integration of HIV DNA can simplistically be divided into processing the 3′ end of the LTR, strand transfer and DNA repair. Briefly, the IN removes two nucleotides (GT) from each 3′-end of the LTR, which is followed by a highly conserved dinucleotide (CA). Subsequently, the “trimmed” 3′ ends of the viral DNA attack a pair of phosphodiester bonds in the chromosomal DNA causing their breakage. This event is followed by ligation of the CA-3′-OH viral DNA ends to the 5′-O-phosphate ends of the target DNA at the site of integration. Integration is completed by removal of the unpaired GT dinucleotides at the 5′ ends of the viral DNA and repair of the single stranded gaps created between the HIV and host DNA. The gap repair is catalyzed by host-cell enzymes\textsuperscript{59}.

1.4.4 Transcription and RNA export

The integrated HIV DNA also called provirus serves as template for the synthesis of HIV RNA.

Transcription and export of HIV mRNA is a highly structured process. Initially only multiply spliced HIV mRNA is exported to the cytoplasm encoding the accessory and regulatory HIV proteins, Tat and Rev. HIV RNA molecules are spliced in the nucleus producing partially spliced mRNAs, encoding Env, Vif, Vpu, and Vpr, as well as multiply spliced mRNAs that are translated into Rev, Tat, and Nef. Unspliced RNAs serve as mRNAs for the Gag and Gag-Pol polyprotein precursors and are packaged into progeny virions as genomic RNA. After integration levels of Rev are low, therefore export of unspliced and singly spliced RNAs is not possible. Only multiply spliced RNAs are transported from nucleus to cytoplasm leading to the production of Tat, Rev and Nef, which are therefore called “early genes”.

The Rev protein contains a nuclear localization signal (NLS), a nuclear export signal (NES) and an arginine-rich domain (ARD), which binds to the RRE (see 1.2)\textsuperscript{73, 74}.

Export of HIV-1 RNA out of the nucleus happens via the nuclear pore complexes (NPC). Rev export is mediated through interaction with the karyopherin family member Crm1 (exportin 1)\textsuperscript{32, 75, 76}. Rev bound to the RRE of a RNA molecule is interacting with Crm1 via its NES. The export itself is mediated by RanGTPase and Rev is re-imported to the nucleus by the nuclear import factor importin-\(\beta\)\textsuperscript{77, 78}.

Tat and Rev are essential for the control of HIV-1 gene expression. Tat ensures elongation of transcription by directing the cellular factor P-TEFb to nascent RNA polymerases, whereas
Rev is obligatory for the transport of unspliced and singly spliced mRNAs from the nucleus to the cytoplasm\textsuperscript{32} (see 1.2).

Tat and the TAR element within the LTR, where Tat binds to are indispensable for efficient elongation of transcription\textsuperscript{59}. The LTR includes regulatory elements containing binding sites for cellular transcription factors, including a core promoter, which contains three SP1 binding sites, a TATA box, an additional bipartite activator sequence\textsuperscript{79-82}, as well as an enhancer region with two NF-κB binding motifs that are capable of binding members of NF-κB and NFAT families\textsuperscript{83-85}. Mutation of the NF-κB sites has little influence on virus production in transformed cell lines, but an intact NF-κB binding motif is essential for reactivation of latent proviruses\textsuperscript{86, 87}. The HIV transcripts accumulating in the absence of Tat are short and abortive sequences; the initiation of transcription through NF-κB produces enough Tat protein, which is needed for trans-activation and transcriptional elongation\textsuperscript{88}.

Rev is a shuttle protein and thus contains a NLS (nuclear localization signal) as well as a nuclear export signal (NES); in addition Rev has an arginine-rich domain (ARD), which binds to the RRE (see 1.2)\textsuperscript{73, 74} within the env gene sequence. Thus, Rev by binding to RRE is the key HIV protein to assure the export of unspliced and singly HIV mRNA\textsuperscript{32, 75, 76}. Rev bound to the RRE of a RNA molecule is interacting with Crm1 (chromosome region maintenance 1; exportin 1) via its NES. The export itself is mediated by RanGTPase and Rev is re-imported to the nucleus by the nuclear import factor, importin-β\textsuperscript{77, 78}.

\subsection*{1.4.5 Virus assembly and maturation}

The actual assembly brings two copies of genomic viral (+) ssRNA, cellular tRNALys\textsubscript{3} molecule, envelope (Env), Gag polyprotein, protease (PR), reverse transcriptase (RT), and integrase (IN) together. The elements of Gag and Pol are packed as Gag-Pol polyprotein, which is generated by ribosomes shift during translation. The Gag polyprotein is post-translationally myristoylated at the N-terminus, resulting in a myristoylated MA protein. This leads to the targeting of the Gag protein to lipid drafts, which are special domains within the plasma membrane. Gag is essential in recruiting the viral genomic RNA and CA assists the multimerization of Gag\textsuperscript{89}.

HIV is an enveloped virus with the envelope derived from the infected cell. The process of coating needs the interaction of HIV proteins with the cell sorting machinery\textsuperscript{90}. It is thought that the PTAP motif in C-terminal p6 domain of Gag recruits the tumor susceptibility gene 101 (Tsg101), a component of the ESCRT-I complex\textsuperscript{91-93}. The exact mechanism of ESCRT-mediated membrane fission needs still to be solved, but several details have been clarified\textsuperscript{90}.  

\textsuperscript{18}
Viral maturation starts parallel to budding or follows immediately after budding of new virions. Maturation of the viral particle includes the cleavage of the Gag and Gag-Pol polyprotein by the viral protease at ten distinct sites yielding MA, CA, NC, p6, PR, RT, and IN.

1.5 Clinical manifestation & pathogenesis

1.5.1 Transmission
HIV spreads by sexual, percutaneous and perinatal routes. Therefore HIV can be transmitted by having unprotected sex via virus contact to mucosal surfaces, by percutaneous inoculation during transfusion of blood products, by using unsterile injection equipment, needle-stick injuries and during pregnancy, at birth and through breastfeeding. As nearly 80% of adults get infected through exposure at mucosal surfaces, HIV is mainly a sexually transmitted infection.

1.5.2 Natural HIV infection
Subsequent to a productive transmission and irrespective of the way of transmission there is characteristically a well-ordered occurrence of viral and host markers such as viral RNA, p24 antigen and HIV-specific antibodies in the plasma of infected individuals, for which a staging system exists. Natural infection can be divided into eclipse phase, acute phase, chronic phase (phase of clinical latency) and a terminal phase called acquired immunodeficiency syndrome (AIDS) (Fig. 7). The Eclipse phase covers the period between the infection of the first host cell and detectable virus in the plasma. The eclipse phase has an approximate duration of 7-21 days that is grounded on predictions based on reports of high risk exposure incidences and on mathematical modeling. During this phase HIV is propagated mainly in CD4+ T cells in the mucosa, submucosa, draining lymphatic vessels, and potentially to a smaller extend in systemic lymphatic tissues and the gut-associated lymphoid tissue (GALT). Consequently to the fast replication in the GALT and peripheral lymphoid tissue virus can be detected in blood plasma.

Often the symptoms of an acute HIV infection make individuals seek medical attention since they regularly suffer from disease signs such as fever, malaise, sore throat, lymphadenopathy and rash, commonly referred to as acute retroviral syndrome (ARS). However in many cases symptoms are not linked to a potential HIV infection, but attributed to nonspecific viral infections, and therefore a HIV test is not done. HIV RNA may reach high levels of up to
1x10^7 copies/ml. Upon primary HIV infection the immune response consists of the generation of HIV-specific CD8+ T cells and neutralizing antibodies. This CTL (cytotoxic T lymphocyte) response drives the evolution of the transmitted virus during the acute HIV infection. The acute HIV infection goes along with a sharp decrease of CD4+ T-cells in the peripheral blood as well as in the secondary lymphoid organs; most evident is the prominent damage made in the gastro-intestinal (GI) tract where more than half of the lymphocytes are killed by direct cytopathic effects.

Figure 7: During the natural course of a HIV infection pathogenesis progresses through three disease phases. After primary infection, during the acute phase, HIV expeditiously replicates to a peak plasma viremia (red) accompanied with a concomitant decrease of CD4+ T cells in the blood (blue). In this phase the virus disseminates into various lymphoid organs and establishes the latent reservoir. Subsequently the host immune system manages to stabilize the CD4+ T-cell population and to reduce plasma viremia until a so called viral set point is reached. The second phase is referred to as clinical latency, during which CD4+ T-cell numbers gradually decreases. Eventually, the number of CD4+ T-cells drops to below a required level (~200 cells/mm^3), causing a loss of control of viremia and thus leading to opportunistic infections. This final stage is referred to as AIDS and ends with the death of the patient (adapted and modified from Pantaleo et al.).

Establishment of the latent reservoir of HIV occurs very early during HIV infection (Fig. 7). Around 3 months after infection the immune response is partially able to control HIV and simultaneous with the appearance of HIV specific CD8+ T-cells plasma viral load decreases until a so called “viral set point” is established. CD8+ T-cells may contribute to the control of HIV infection through both innate and adaptive immune responses. Other factors might as well contribute to the rapid decrease in viral load, such as loss of activated CD4+ T-cell as potential target cells of HIV. Moreover neutralizing antibodies develop during acute infection and may as well contribute for plasma virus levels to decrease. In summary the onset of immune responses leads to an equilibrium reflected by the viral set point, which is
one key predictor of long-term disease progression rates$^{121}$. Plasma viral loads measured in patients at the viral set point are varying from below limit of detection, for very rare “elite controllers”, up to levels of about 100'000 copies/ml. An asymptomatic chronic phase referred to as clinical latency follows. During this chronic phase virus levels generally stay around the set point for many months or years, but CD4$^+$ lymphocytes gradually decline over time. Finally, when CD4$^+$ T cells drop below a threshold of $\sim$200 cells/mm$^3$, the patient is at risk of opportunistic infections and distinct cancers as well as HIV-associated wasting – this phase of the disease is known as AIDS. Without treatment HIV-patients with AIDS eventually die (Fig. 7).

1.6 Anti-retroviral therapy

The effectiveness, tolerability and improved accessibility of ART together have led to considerably decreased numbers of opportunistic infections and deaths, and have turned what used to be a lethal disease into a treatable chronic infectious disease$^{122,123}$.

1.6.1 The concept of ART

In 1987 azidothymidine (AZT), also called zidovudine (ZDV), was the first drug approved by the FDA for treating HIV. AZT is a nucleoside analog and inhibits the RT. Already in 1985 AZT had been found to inhibit infectivity and cytopathic effects of HIV$^{124}$ in vitro.

More drugs followed soon after, all belonging to the class of nucleoside RT inhibitors (NRTIs). These compounds were typically administered as mono or dual therapy, therefore having limited efficacy reflected by only a slight improvement on HIV disease progression and mortality.

1996 a breakthrough was achieved with the introduction of the first protease inhibitors (PIs), which were given in combination with two nucleoside analogues. This triple ART greatly increased the effectiveness and robustness of anti-retroviral treatment$^{125-129}$ with consequent substantial drops in morbidity and mortality$^{130}$.

The initial drugs available had a number of severe side effects and toxicities and thus, in the years following 1996, ART was preferentially prescribed to patients with less than 350 cells/mm$^3$. This recommendation has changed over the years as new drugs were approved on the market with much improved toxicity profile.

Until now a total number of 26 drugs were approved by the FDA, of which 3 are not marketed anymore. In addition elvitegravir (EVG), a novel integrase inhibitor, and cobicistat, which is like ritonavir (RTV) of interest for its ability to inhibit liver enzymes, were approved on
August 27th 2012 as part of the quadruple pill stribild (elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate) manufactured by Gilead Sciences (Table II).

Table II: List of anti-retroviral drugs approved by the U.S. Food and Drug Administration (FDA) for the treatment of HIV-1 infection.
(http://www.fda.gov/ForConsumers/ByAudience/ForPatientAdvocates/HIVandAIDSActivities)

<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Abbreviation</th>
<th>Brand</th>
<th>Manufacturer</th>
<th>Time to Approval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleoside Reverse Transcriptase Inhibitors (NRTIs)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lamivudine</td>
<td>3TC</td>
<td>Epivir</td>
<td>GlaxoSmithKline</td>
<td>17. Nov 1995</td>
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<tr>
<td>Abacavir</td>
<td>ABC</td>
<td>Ziajen</td>
<td>GlaxoSmithKline</td>
<td>17. Dec 1998</td>
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<tr>
<td>Emtricitabine</td>
<td>FTC</td>
<td>Emtriva</td>
<td>Gilead Sciences</td>
<td>02. Jul 2003</td>
</tr>
<tr>
<td>Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delavirdine</td>
<td>DLV</td>
<td>Rescriptor</td>
<td>Pfizer</td>
<td>04. Apr 1997</td>
</tr>
<tr>
<td>Efavirenz</td>
<td>EFV</td>
<td>Sustiva</td>
<td>Bristol-Myers Squibb</td>
<td>17. Sep 1998</td>
</tr>
<tr>
<td>Etravirine</td>
<td>ETR (TMC125)</td>
<td>Intelence</td>
<td>Tibotec Therapeutics</td>
<td>18. Jan 2008</td>
</tr>
<tr>
<td>Rilpivirine</td>
<td>RPV (TMC278)</td>
<td>Edurant</td>
<td>Tibotec Therapeutics</td>
<td>20. May 2011</td>
</tr>
<tr>
<td>Protease Inhibitors (PIs)</td>
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</tr>
<tr>
<td>Saquinavir*</td>
<td>SQV</td>
<td>Invirase</td>
<td>Hoffmann-La Roche</td>
<td>06. Dec 1995</td>
</tr>
<tr>
<td>Ritonavir</td>
<td>RTV</td>
<td>Norvir</td>
<td>Abbott Laboratories</td>
<td>01. Mar 1996</td>
</tr>
<tr>
<td>Amprenavir*</td>
<td>APV</td>
<td>Agenerase</td>
<td>GlaxoSmithKline</td>
<td>15. Apr 1999</td>
</tr>
<tr>
<td>Lopinavir + ritonavir</td>
<td>LPV+RTV</td>
<td>Kaletra</td>
<td>Abbott Laboratories</td>
<td>15. Sep 2000</td>
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<tr>
<td>Tipranavir</td>
<td>TPV</td>
<td>Aptivus</td>
<td>Boehringer Ingelheim</td>
<td>22. Jun 2005</td>
</tr>
<tr>
<td>Darunavir</td>
<td>DRV (TMC 114)</td>
<td>Prezista</td>
<td>Tibotec, Inc.</td>
<td>23. Jun 2006</td>
</tr>
<tr>
<td>Fusion Inhibitors</td>
<td></td>
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<tr>
<td>Enfuvirtide</td>
<td>T20</td>
<td>Fuzeon</td>
<td>Hoffmann-La Roche</td>
<td>13. Mar 2003</td>
</tr>
<tr>
<td>Entry Inhibitors - CCR5 co-receptor antagonist</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maraviroc</td>
<td>MVC</td>
<td>Celsentri</td>
<td>Pfizer</td>
<td>18. Sep 2007</td>
</tr>
<tr>
<td>HIV integrase strand transfer inhibitors</td>
<td></td>
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*not on the market anymore

With the availability of a bigger repertoire of anti-retroviral drugs with fewer side effects the recommendations for initiation of ART have changed. Based on new observations from a randomized controlled trial displaying the reduction regarding the likelihood of HIV transmission and proving clinical benefits for an infected individual, ART is recommended to all HIV-1-infected adults regardless of the CD4+ T cell count. Next to this it was shown that
starting ART immediately during the acute phase of infection results in a smaller number of latently infected cells.

The recommendations of the International Antiviral Society for 2012 suggest initial regimens consisting of 2 NRTIs (tenofovir/emtricitabine or abacavir/lamivudine) in combination with either one NNRTI (efavirenz), one ritonavir-boosted PI (atazanavir or darunavir), or one integrase strand transfer inhibitor (raltegravir)\(^\text{132}\) (Table II).

The development of anti-retroviral compounds with lower toxicity, better PK properties and a higher genetic barrier still remains an important task for improving patient’s life quality and overcoming the problem of resistance to existing drugs.

Next to ART for treating patients with established HIV infection anti-retroviral compounds are being used as pre- and post-exposure prophylaxis.

**Pre-exposure prophylaxis (PrEP)** refers to HIV-prevention approaches using antiretroviral drugs to protect individuals that are at high risk of HIV infection. Together with HIV vaccines and microbicides the efficacy of PrEP regarding prevention of HIV infection is assessed in several clinical trials, in which tenofovir or a combination of tenofovir/emtricitabine is tested for their efficacy to prevent transmission. In four trials the risk of HIV infection was significantly reduced by PrEP\(^\text{133-136}\).

**Post-exposure prophylaxis (PEP)** refers to any preventive treatment started immediately after exposure to a pathogen, e.g. rabies, HIV or *clostridium tetani*. In the early days of the HIV pandemic case studies revealed that the seroconversion frequency after needle-stick injury incidences in healthcare workers decreased when AZT was administered directly after HIV exposure leading to changes in the guidelines for PEP\(^\text{137-139}\).

PEP is most effective when the treatment is started directly after exposure to HIV via unprotected sex, needle stick injuries, or by sharing injection equipment\(^\text{140}\). Since starting PEP at 72 hours post exposure does not show a protective effect anymore, PEP should be administered as soon as possible to any person who had been exposed to HIV, and ART should be given for duration of at least four weeks\(^\text{140}\).

### 1.7 HIV latency

HIV integrates into the host cell genome and can establish latent infection at the level of a single CD4+ T cells. Latency can be defined as a state in which an infected cell does not produce new virus particles due to a block of HIV gene transcription.

In the late 90s the group of Anthony Fauci discovered the presence of an inducible HIV-1 latent reservoir in patients that were successfully treated with antiretroviral therapy\(^\text{141}\), as well
as that a pool of latently infected resting CD4+ T cells is already established early during primary HIV-1 infection\textsuperscript{142}. Most strikingly they found that in patients, which had been successfully treated and had undetectable RNA over years, HIV RNA rebounded within a couple of weeks after treatment interruption, indicating the existence of a long lived latent reservoir\textsuperscript{143}.

Purging the latent reservoir is exceptionally difficult, because the latent reservoir is established early during infection\textsuperscript{142} and is long-lasting with an predicted \( t_{1/2} \) of 44 months\textsuperscript{144} in patients with completely suppressed viral load. In addition the latent reservoir can be re-seeded when there is viral rebound for example when interrupting ART or treatment failure\textsuperscript{145, 146}, or via homeostatic proliferation of latently infected cells\textsuperscript{147}. Latent infection is a rare event, and it is believed that latently infected cells originate from activated CD4+ T cells, which become infected and revert back to a resting memory state, which is non-permissive for expression of integrated provirus\textsuperscript{148}.

Due to the presence of latently infected long-lived resting memory CD4+ T cells, ART alone is not able to eradicate HIV-1 and interruption of ART is inevitably resulting in viral rebound\textsuperscript{143, 149, 150}. Notably this rebound is thought to occur from latently infected cells, rather than from possibly ongoing low-level replication\textsuperscript{151}.

The establishment of the latent reservoir happens during the acute phase of HIV-1 infection (see 1.5.2; Fig. 7). ART virtually always succeeds in suppressing plasma virus below the limit of detection (Fig. 7). Notably, when patients are treated initially with a monotherapy HIV can rebound due to preexisting resistant virus subpopulations\textsuperscript{152}.

Since free virus in the plasma declines fast, the initial rapid decrease in plasma viral load upon start of ART is mainly defined by the decay of infected activated CD4+ T cells producing most of the virus present in the plasma\textsuperscript{153} (Fig. 8). After 1–2 weeks of treatment following the rapid initial decay, plasma virus levels decline at a slower rate (Fig. 8), but it remains still uncertain what sort of the cells are responsible for this second-phase decay\textsuperscript{154}.

Next to a protective vaccine, finding a cure for HIV is the ultimate goal. Enormous efforts are made to find strategies and compounds that activate the latently infected cells while uninfected cells are protected against HIV by continuous ART\textsuperscript{155-166}.

Following reverse transcription of the vRNA into cDNA HIV-1 is able to integrate into the genome of the host cell, and subsequently to establish a latent infection. In some cases however factors prevent integration and lead to a condition referred to as pre-integration latency (see 1.3; Fig. 4).
Figure 8: Theoretical dynamics of viral decay in patients on ART. The plasma viral load is fairly stable during chronic infection before start of treatment. Subsequent to ART initiation the plasma viral load shows multiphasic decay. The first phase of rapid decline is reflecting the decay of activated CD4+ T cells responsible for the biggest portion of plasma virus. The second phase reflects virus producing cells with a t_{1/2} of about 2 weeks and viral load declines to below limit of detection (20-50 copies/ml) after 3–6 months. Still low level viremia persists under suppressive treatment reflecting virus activated from latent reservoir, which can be detected occasionally as so called “blips”. The intrinsic decay rate of the latent reservoir is difficult to be determined. (Adapted and modified from Rong and Perelson et al. and Pierson et al.\textsuperscript{167, 168})

Since unintegrated DNA is considered to be rather unstable, it will be either degraded or, upon activation of the cell, will be integrated into the genomic DNA of the host cell\textsuperscript{153, 169-171}. Because of the unstable nature of unintegrated DNA, the clinical relevance of pre-integration latency is rather small and consequently research in the field of HIV latency is focused on studying post-integration latency\textsuperscript{148, 153, 172}.

Post-integration latency defines a state, in which an infected cell is harboring integrated provirus without ongoing replication or production of new virus particles.

HIV-1 latency is thought to be either established when an activated CD4+ T cell upon infection returns to a quiescent memory CD4+ cells\textsuperscript{173}, or by direct infection of resting memory CD4+ T cells\textsuperscript{174-176}. In this thesis the term latency exclusively refers to post-integration latency.

1.7.1 Molecular mechanisms of HIV latency

Latency can be established due to the absence of viral and/or host cell factors that are needed for HIV transcription, by transcriptional interference, or as a result of epigenetic remodeling of the chromatin structure at the integration site of the proviral DNA.
**Influence of host cell factors**

P-TEFb as outlined above is necessary for efficient elongation of HIV transcription. However, in resting T cells only little amounts of Cyclin T1 can be found, which is needed in addition to CDk9 and Tat to form the Tat/P-TEFb complex (see 1.2 and 1.3). P-TEFb is incorporated together with hexamethylene bisacetamide-inducible protein 1 (HEXIM1) and 7SK snRNA into an inactive complex\(^{177, 178}\). Incubation with suberoylanilide hydroxamic acid (SAHA)\(^{179}\) or hexamethylbisacetamide (HMBA)\(^{180, 181}\) is able to reverse inhibition of P-TEFb by activation of the phosphatidyl-inositol 3-kinase/Akt (PI3K/Akt) pathway. Other cellular factors which play a key-role in the balance between latently and productively infected cells are NFAT, NF-κB and PTB (polypyrrimidine tract binding protein) that are constrained in the cytoplasm in resting CD4+ T cells\(^{182}\).

**Transcriptional interference**

Transcriptional interference, which might contribute to HIV latency, occurs when the RNA polymerase complex from an upstream promoter as related to the HIV integration site removes transcription factors from the 5’ LTR, thus resulting in silent HIV. This phenomenon remains controversial and its *in vivo* significance remains unknown\(^{183}\).

**Chromatin structure and histone acetylation**

The chromatin structure affects the transcription of HIV and thus whether HIV is silent or not. Chromatin consists of DNA, proteins and RNAs and is organized into repetitive units referred to as nucleosomes\(^{184}\). A nucleosome is made of 147 bp of dsDNA which is wrapped around an octamer of histones (H2A, H2B, H3, and H4).

We distinguish tightly packed hetero- from lightly packed euchromatin. Heterochromatin is the condensed and transcriptionally silent form of large portions of the cellular DNA, whereas euchromatin refers to a less condensed state of chromatin, which is the favorite state for the active transcription of genes. For the regulation of the chromatin status histones and DNA can be modified with epigenetic markers, which are influencing the chromatin structure. Histones can be altered by methylation, acetylation, phosphorylation, ubiquitination, and sumoylation\(^{185, 186}\) and DNA by methylation of the cytosine bases\(^{187}\).

Major focus was given to the effects of histone acetylation for activating silent HIV. Already in the early 90s the existence of a nucleosome 1 (Nuc-1) next to the start site for HIV-1 transcription was reported, and the removal of this nucleosome was thought to be crucial for transcription\(^{188, 189}\).
Many reports exist about the requirement of histone acetyltransferases (HATs), such as CREB-binding protein (CBP), p300, p/CAF and hGCN5 for transcriptional activation, and that these HATs can be recruited by Tat. In contrast histone deacetylase 1 (HDAC1) deacetylates Nuc-1 and inhibits Tat activation, potentially contributing to the maintenance of HIV latency.

Several HDAC inhibitors (HDAC-i) are able to activate latently infected cells including valproic acid, trapoxin (TPX), and trichostatin A (TSA) in in vitro assays. The HDAC inhibitor suberoylanilide hydroxamic acid (SAHA, vorinostat) was initially approved for treating cutaneous T cell lymphoma (CTCL). SAHA was effective in reactivating latent provirus in a high-throughput screen for latency activating substances. This finding was confirmed by a recent study in which SAHA was able to activate latent virus from patient samples.

Recently a clinical trial was started using SAHA as a reactivating compound with the official title: “A Phase I/II Investigation of the Effect of Vorinostat (VOR) on HIV RNA Expression in the Resting CD4+ T Cells of HIV-Infected Patients Receiving Stable Antiretroviral Therapy” (http://clinicaltrials.gov/ct2/show/NCT01319383).

1.7.2 In vitro latency models: J-Lat, U1, JΔK and ACH- and primary cells

There are several in vitro latency models using either cell lines or primary T cells, which have been manipulated to some extent to reflect HIV latency.

The so-called J-Lat clones are an assortment of Jurkat clones that were infected with replication competent HIV-GFP and became transcriptionally silent. Analyzing these clones the integration was found to be preferentially near heterochromatic sites (e.g. alphoid repeat regions), which does not reflect the in vivo situation where most proviruses are integrated within introns of actively transcribed genes.

Originally isolated from infected U937 cells, the U1 cell line is a promonocytic cell line selected because it has a low level of viral gene expression and is able to be activated by phorbol 12-myristate 13-acetate (PMA). It comprises two integrated copies of HIV. Controversial data exist about whether latency in this cell line is due to the fact that the integrated proviruses encodes for two forms of Tat that are incapable of trans-activation. An earlier publication claims that one Tat cDNA is lacking the necessary start codon ATG, whereas the second Tat harbors a mutation (H13L), which was found to be defective for activation. However a later publication found that this mutation reactivates to the same levels as wild-type Tat, but stops transcription at a higher frequency.

JΔK cells were derived from a population of infected Jurkat cells harboring mutations in the NF-kB binding sites\textsuperscript{205}.

Comparable to U1, the ACH-2 clones were generated by infecting cells of the A3.01 T cell line\textsuperscript{206}. ACH-2 cells were found to have a point mutation in TAR responsible for latency\textsuperscript{207}.

Since an \textit{in vitro} model based on primary cells is believed to be closer mimicking the \textit{in vivo} situation, huge efforts have been made to generate latent primary CD4+ T cells \textit{in vitro}. The main difficulty in this process is to maintain T cells in culture long enough to become latent with the additional challenge, that culturing T cells always includes some degree of stimulation necessary for cell survival. Regardless of this many different attempts have been made so far to render latent primary CD4+ T cells, briefly:

i. Primary CD4+ T cells have been stimulated with anti-CD3 in medium containing IL-2 (interleukin-2) for 2-3 weeks, thereby increasing cell survival. Upon this initial stimulation cells were infected and subsequently co-cultured with a “feeder layer” of H80 cells, a brain tumor derived cell line. Even though the majority of cells still expressed CD69, which is an early activation marker, some T cells produced infectious virus upon activation with prostratin\textsuperscript{208,209}.

ii. Another model tried to increase T cell survival by lentiviral transduction of T cells with cDNA encoding the anti-apoptotic protein Bcl-2. This method was able to prolong the lifespan, rendering T cells that have the phenotypical attributes of resting memory CD4+ T cells and has been used to screen for new molecules able to activate latently infected cells\textsuperscript{210}.

iii. In another model proliferation of non-polarized cells was induced by stimulation with IL-4, IL-12 and TGF-β, before infecting the cells with a modified HIV virus and subsequent maintenance in IL-2 containing medium. Non-polarized cells are phenotypically similar to central memory T cells (T$_{CM}$) and based on the postulation that central memory T cells comprise the majority of latently infected T cells \textit{in vivo}\textsuperscript{147}, the hypothesis was that non-polarized cell are therefore suitable for recapitulating latency \textit{in vitro}. Stimulation using anti- CD3/CD28 antibodies proofed the presence of latent proviruses in these cells\textsuperscript{87}.

Even though models using primary cells are able to establish latent provirus and are advantageous compared to models based on cell lines, they are still missing essential features: e.g. the complex interactions of immune cells \textit{in vivo}.

In all models manipulations are made to maintain the primary cells in culture, thereby possibly causing changes, which makes these cells different from latent cells \textit{in vivo}. In
addition the HIV induced immune responses cannot be reproduced in vitro, thus any in vitro model will be deficient of possible contributions of the immune system to HIV latency. Because of all these disadvantages, we are convinced that HIV latency must be studied using an in vivo model.

1.7.3 In vivo latency models
There are legitimate ethical concerns when using animal models and are cost intensive due to the demanded infrastructure and maintenance costs. Nonetheless animal models offer valuable information when dealing with complex research questions that in vitro models are unable to answer.

A widely used animal model for HIV is rhesus macaques infected either with simian immunodeficiency virus (SIV) or with a chimeric SIV/HIV virus (SHIV). SHIVs usually contain a SIV backbone where some genes are replaced by their HIV counterparts, i.e. Env, Rev, Tat, and Vpu. In 2009 a novel approach using pig-tailed macaques with marginally modified HIV resulted in sustained infection in these primates.

A small animal model for studying HIV has always been desired, mainly because it raises less ethical concerns, lower costs and the ease of maintenance as compared to primates. The lack of permissiveness of murine and other rodent cells to HIV hampered the development of such a small animal model for HIV. However this handicap has been overcome with the development of so called humanized mice. The first report of HIV latency in a humanized mouse model dates back to 2001. At present several groups have presented data about the existence of latently infected cells in the humanized mouse model.

The main limitation to this model is the small numbers of latently infected cells in respect to the smaller number of total cells rendered from peripheral blood or tissues of a mouse as compared to monkeys.

1.8 Humanized mice
Mice have been used as a model species in medical research for over a century. They are the most commonly used mammalian research model, mostly because of the easiness of maintenance and handling, their short reproduction time and their large degree of homology with humans.

Certain viruses, e.g. influenza A virus, lymphocytic choriomeningitis virus (LCMV) and herpes simplex virus-1 and -2 (HSV-1, HSV-2), are able to infect humans and mice, thus making mice a suitable model for research of these diseases.
On the contrary other host-specific pathogens that have exclusive human tropism are unable to infect mice and efforts to study these diseases in a small animal model have led to the development of so called “humanized mice”. The term humanized mice is commonly used for mice transplanted with cells or tissues of human origin and/or expressing one or more human transgenes.

1.8.1 History of humanized mice

In 1983 the discovery of a naturally occurring mutation \(Prkdc^{scid}\) in CB17 mice, that caused a complete lack of T and B cells, therefore rendering mice with severe combined immunodeficiency CB17-Scid (SCID), paved the way for the development of humanized mice. The early advances in the field of humanized mice, as well as the current progress and potential future improvements have been comprehensively reviewed in detail.

1.8.1.1 Hu-PBL-SCID & SCID-hu

In 1988 humanized mice were described for the first time using CB17-Scid (SCID) immunodeficient mice, which were either created by injection with human peripheral blood lymphocytes (Hu-PBL-SCID) or implantation with a human fetal thymus tissue and subsequent injection of human fetal liver cells (SCID-hu). CB17-Scid mice also served as recipients for transplantation of human bone marrow derived HPCs in combination with cytokine stimulation.

Even though the engraftment levels of human lymphoid cells in SCID mice were relatively low and engraftment transient these works supplied a desired small animal model for studying HIV infection.

1.8.1.2 NOD-Scid

By backcrossing of the \(Prkdc^{scid}\) mutation onto different mouse strains it was found that immunodeficient (non-obese diabetic) NOD-scid (NOD/SCID) mice showed increased levels of human engraftment as compared to other strains and a higher susceptibility to HIV infection. A potential causative for this could be the presence of a polymorphism in NOD/SCID mice resulting in a SIRPα protein which cross-reacts with the human CD47, thus creating tolerance for the human donor cells.

1.8.1.3 BRG, NOG, NSG and BLT

Another breakthrough leading to the development of a so called “novel generation” of humanized mice was realized by crossing mutant forms of the interleukin-2 receptor γ-chain (Il2rg) gene onto mice lacking recombinase activating gene 2 (Rag2) Balb/c-Rag2-/- (BRG)
and two different NOD-Scid backgrounds, NOD/Shi-scid (NOG)\textsuperscript{242} and NOD/LtSz-scid (NSG)\textsuperscript{243, 244}. There are many reports of successful engraftment of human hematopoietic progenitor/stem cells (HPSCs) in Balb/c-Rag2-/−-IL2Rγc−/−, NOD/Shi-scid IL2Rγc−/− and NOD/LtSz-scid IL2Rγcnull mice and comprehensive evaluations were made comparing the properties of the various humanized mouse models based on the use of either BRG, NSG or NOG mouse strains\textsuperscript{229, 245-247}.

Several injection routes have been applied for transplantation of human HPSCs, i.e. intravenous (i.v.)\textsuperscript{243, 244}, intrahepatic (i.h.)\textsuperscript{241} and intraperitoneal (i.p.)\textsuperscript{242} injection and the improvement regarding i.h. injection into irradiated newborns resulted in increased engraftment levels, multilineage development, thymic lymphopoiesis, and partial adaptive immunity.

Notably another model exists commonly referred to as BLT (bone marrow-liver-thymus) that was reported in 2006 by two groups\textsuperscript{248, 249}. BLT mice are generated by co-transplantation of human fetal tissues under the renal capsule of mice and subsequent transplantation of human-derived CD34+ hematopoietic progenitor cells.

Since the human tissues serve as a microenvironment for hematopoiesis high levels of multilineage engraftment are detectable in all tissues of BLT mice, with mucosal surfaces like the vagina and gut are efficiently repopulated\textsuperscript{250-252}. T cells can be selected in a human thymus tissue and the HLA-restricted T cells are able to support adaptive immune responses\textsuperscript{249, 253, 254}. BLT mice are often used to study of HIV infection including HIV latency\textsuperscript{213, 255}.

However BLT mice have some disadvantages including the requirement of fetal tissues. Furthermore, due to the selection in human thymic tissue T cells with affinity to mouse MHC are not eliminated resulting in a higher probability for the development of GvHD (graft-versus-host disease) in BLT mice\textsuperscript{230}.

The consecutive improvements in the field of humanized mice yielded higher engraftment levels and multilineage development, including T and B lymphocytes, NK cells, DCs, monocytes, macrophages, erythrocytes, and platelets\textsuperscript{241-243, 256, 257}. The commonly used strains, i.e. NOG/NSG, SRG, and BLT achieve engraftment levels of about 30–60% human CD45+ cells in the blood and are suitable for usage in experiments which last 6–9 months\textsuperscript{230}.

Notably, in this work we used NOG and NSG strains adapting to the progressive advancement in the field. Transplantation of newborn immunodeficient mice has been performed by i.h. injection of CD34+ cells derived from umbilical cord blood (Fig. 9).
Introduction

Figure 9: Schematic overview of the steps involved in the generation of humanized mice. Umbilical cord blood donations may serve as a source for the purification of the CD34+ cell population that contains hematopoietic progenitor/stem cells (HPSCs). Notably, purified CD34+ cells can be stored in liquid nitrogen for longer time periods. Purified CD34+ cells are transplanted into newborn immunodeficient mice, which have been sub-lethally irradiated prior to the intra hepatic (i.h.) injection of CD34+ cells (dark grey arrows). The additional steps for lentivirus (LV) production and transduction of CD34+ cells are indicated by blue arrows.

**HIV & humanized mice**

Next to their exploitation for studying HIV infection humanized mice have been used in research regarding other human pathogens such as Epstein-Barr virus (EBV)\(^{269, 258-261}\), dengue virus (DENV)\(^{262-264}\), Herpes Simplex Virus Type 2 (HSV-2)\(^{265}\), hepatitis C virus (HCV)\(^{266, 267}\), Malaria\(^{268, 269}\) and *Salmonella typhi*\(^{270, 271}\).

A review especially focusing on the diversity of humanized mouse models and their significance for studying HIV infection is included as part of this thesis (Chapter I).

### References

Introduction


Introduction

Introduction


Introduction


Introduction

Introduction

Aims

2 Aims

The overall aim of this thesis was to validate the humanized mouse model for studying HIV infection, for testing novel anti-retroviral compounds in different treatment approaches, and to validate its potential as a HIV *in vivo* latency model.

Specific aims

I. To examine if HIV infection of humanized mice infected with replication competent HIV-1 recapitulates HIV infection in humans, including suppression of HIV replication by ART, viral rebound after treatment interruption and occurrence of resistance mutations.

II. To establish and optimize anti-retroviral treatment protocols and to test novel anti-retroviral compounds and treatment regimens in HIV infected humanized mice.

III. To validate the existence of a latent reservoir in humanized mice and to try to reactivate latently infected cells.

IV. To establish a versatile latency model based on transduction of CD34+ cells. This includes in a first step the evaluation of an efficient and stable transduction protocol using lentiviral vectors.
Chapter I: Modeling HIV infection and therapies in humanized mice

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Declaration of contribution:
Marc Nischang, Gustavo Gers-Huber and Roberto Speck wrote the manuscript.
Marc Nischang and Gustavo Gers-Huber contributed equally.
Chapter I
Modeling HIV infection and therapies in humanized mice
Modelling HIV infection and therapies in humanised mice

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Summary

The human immunodeficiency virus (HIV) type-1 is a human-specific virus. The lack of a widely available small-animal model has seriously hampered HIV research. In 2004, a new humanised mouse model was reported. It was based on the intrahepatic injection of human CD34+ cord blood cells into newborn, highly immunodeficient mice. These mice develop a lymphoid system of human origin and are highly susceptible to HIV infection and showed disseminated infection, persistent viraemia and characteristic helper CD4+ cell loss. Here, we will briefly review the various existing humanised mouse models and highlight their value to the study of HIV infection.

Key words: HIV-1 infection; HIV pathogenesis; humanised mice; BLT mice; SCID; human CD34+ cells; gene therapy; CCR5; antiretroviral treatment; microbicides; HIV pre-exposure prophylaxis (PrEP)

The HIV situation globally

The UNAIDS report on the Global AIDS Epidemic 2010 optimistically announced that the HIV pandemic had peaked in the preceding two years. Nevertheless, the numbers are shocking: an estimated 33.3 million humans – 0.8% of all adults 15–49 years old – are infected, and more than 1.8 million people died in 2009 (http://www.unaids.org/globalreport/Global_report.htm).

More hopefully, the number of people newly infected with HIV declined by nearly one-fifth over the last decade (1999, 3.1 million; 2009, 2.6 million). This decline is based on more widely applied “safer sexual practices” and reductions in mother-child transmission. The UNAIDS vision is Zero New Infections, Zero Discrimination, Zero HIV-associated Deaths. The goal is to halt and reverse the spread of HIV.

A human-specific virus: a challenge for \textit{in vivo} studies

HIV specifically infects human cells. Even cells from chimpanzee, a very close relative of humans, are only somewhat permissive to HIV infection [1]. Human host factors are critical for the virus throughout its entire replication cycle (fig. 1). For example, to enter a cell and begin its replication cycle, HIV engages a receptor complex of CD4 and a chemokine receptor, either CCR5 or CXCR4 [2, 3]. However, expressing human CD4 on murine cells does not make them permissive to HIV. Other human-specific factors, such as the human chemokine receptors, are needed.

Over the last three decades, human transgenes essential for HIV replication were expressed together in rodent cells, but the cells were still not permissive [4]. Furthermore, human transgenes were expressed in rodents in an attempt to generate HIV small-animal models. These models confirmed the human-specific nature of HIV and the \textit{in vitro} data. No replication was observed in mice expressing human CD4 [5], CD4 and CCR5 [6] and Cyclin CDK9 [7], and rats transgenic for human CD4 and CCR5 replicated HIV only at very low levels for limited times [8, 9]. Other \textit{in vivo} studies attempted to create models based on creating chimeric HIV strains. This approach relies on engineering a distinct HIV gene in a species-specific retrovirus, which despite the HIV transgene, replicates vigorously in the original species (e.g., simian-immunodeficiency virus [SIV] engineered to express the HIV envelope [SHIV]) [10]. The use of SHIV in monkeys allowed key questions about immune responses to vaccine constructs expressing various HIV gene encoded proteins to be addressed [10]. However, use of monkeys as animal models is restricted to specific questions with a narrow focus and cannot recapitulate the overall complexity of HIV, since the biological properties between SHIV, SIV and HIV are quite distinct.

Finally, HIV-encoded gene products were expressed entirely as transgenes in mice [11–15]. These studies provided insight into the pathogenic potential of HIV gene products. However, they were expressed universally at high levels, and it is difficult to assess the significance of the resulting data since the dynamic nature of true HIV replication is lacking.
The requirements for a mouse model to study HIV infection

Faithfully modelling any human disease in an animal is difficult. Does the model replicate enough key features of the disease to allow us to conduct experiments? Many of the key features of HIV infection are known. The main route of HIV transmission is vaginal or rectal intercourse. In acute HIV infection, a massive productive infection causes cell death in the lymphatic system, most prominently in the gastrointestinal tract. About 3–5 weeks after acute HIV infection, the levels of HIV RNA decline and the specific anti-HIV CD8+ T-cell response begins. Unlike in acute infection, fewer than 1% of CD4+ T cells are productively infected in the chronic phase [16], a number that cannot fully explain the progressive immunodeficiency. Poorly understood bystander effects seem to contribute to the overall cell loss [17], and sustained immune activation triggers it [18]. Combined antiretroviral treatment (cART) has been very successful in suppressing HIV RNA levels to below the limit of detection in about 90% of treated patients [19] and has resulted in a marked reduction of morbidity and mortality [20]. However, cART does not cure HIV. A small portion of HIV remains silent in long-lived cells, such as the quiescent memory CD4+ T-cells [21]; these cells form a latent reservoir of HIV. Besides finding simpler and more efficient treatment strategies, major efforts are now aimed at eradicating the latently infected cells to eventual cure HIV, and to develop novel gene therapy approaches and vaccination strategies. Other efforts are focused on orally administered pre-exposure prophylactic measures using anti-retroviral drugs and finding effective topical microbicides that can prevent sexual transmission. Thus, the requirements for a HIV mouse model include the following:

- Permissiveness to replication-competent HIV with distinct co-receptor usage (i.e., CCR5- or CXCR4-tropic HIV strains), resulting in high-level viraemia, systemic viral dissemination and histopathology reminiscent of HIV disease in humans.
- Supporting long-term chronic infection, allowing monitoring of HIV infection over time.
- Susceptibility to natural transmission modes of HIV, including vaginal and rectal routes.
- Displaying gradual depletion of CD4+ T-cell numbers during HIV infection.
- Activation of the immune system to lead to HIV-specific immune responses.
- Establishment and maintenance of an HIV latent reservoir.
- Allow development and testing of anti-HIV therapeutic and prevention strategies.

Humanised mice in general

The generation of humanised mice involves either the expression of human transgenes or the transplantation of human tissue into immunodeficient mice. However, as mentioned above, even constitutive expression of multiple human transgenes has not rendered mice fully permissive to HIV infection.

The human-PBL-SCID and foetal thy/liv SCID mouse model

Transplantation of human tissue into immunodeficient mice without rejection was first reported in the early 1980s. This became possible with the identification of a spontaneous mutation of the Prkdc gene in mice, which results in the complete lack of T and B cells and consequently in severe combined immunodeficiency (C.B.-17 SCID/SCID [SCID]; descriptions of the various mouse strains, see box) [22]. The Prkdc gene encodes for the catalytic subunit of a DNA-dependent protein kinase that is needed for V(D)J recombination in developing T and B lymphocytes. The two early humanised (Hu) mouse models were the foetal thymus/liver (thy/liv) SCID-hu mouse [23, 24] and the human-PBL-SCID (PBL, peripheral blood leukocytes) mouse [25]. The foetal thy/liv SCID-hu mouse model is based on surgical placement of foetal thymus/liver tissue under the renal capsule. At 4–6 months post-implantation, foetal thymus/liver tissue forms a conjoint organoid that resembles human thymus and sustains T-cell lymphopoiesis for over a year [23]. The system is susceptible to HIV infection, but in the absence of robust peripheral human leukocyte reconstitution, samplings to analyse the infected human cells are mainly restricted to the engulfed conjoint organoid. Also there is no multilineage human haematopoiesis in this model (table 1).

![Figure 1](image_url)  
HIV-1 needs critical host factors for efficient replication. HIV binds to the HIV receptor complex of the human CD4 cell-surface molecule and a co-receptor, either CCR5 or CXCR4, via the HIV envelope glycoprotein 120 (HIV env gp120). After conformational changes in the HIV env gp41, viral host cell membrane fusion occurs (2). The next steps are the decapsidation (3) and release of the HIV RNA from the virus particle. Reverse transcription generates a viral complementary DNA (cDNA) based on the viral RNA template and using HIV’s own reverse transcriptase (4). Once the cDNA is generated, the preintegration complex (PIC) is assembled, nuclear trafficking and integration of the viral cDNA into the host genomic DNA follow (5). Efficient transcription and elongation require formation of P-TEFb (positive transcription elongation factor b) consisting of Tat (6A), human cyclin-dependent kinase 9 (CDK-9) (6B) and cyclin T1 (6C), which binds to the nascent HIV transcripts. Fully or partially spliced HIV mRNA (7) is used to translate viral proteins. Unspliced HIV RNA is packaged into newly generated virions. Assembly of HIV proteins and RNA and budding takes place at the cellular membrane (8). HIV release is inhibited by murine tetherin at the cellular membrane because murine tetherin is insensitive to the viral protein Vpu, which inhibits human tetherin by directing its proteasomal degradation (blue frame). Human host factors critical for HIV replication are CD4, CCR5, CXCR4 and cyclin T1 (red frame). Additional human specific factors probably exist.
The hu-PBL-SCID mouse model is based on the intraperitoneal injection of human PBL [25] and is susceptible to HIV infection [26]. However, within days, human PBL injected into mice react against the murine disparity with a vigorous activation: their proliferation rate increases, and the CCR5 chemokine receptor and HLA-DR are upregulated [27–29], resulting in xeno-reactive T-cells [30]. Mice with significant blood T-lymphocyte chimerism suffer from high levels of graft-versus-host disease (GVHD) and mortality. Mice with no or transient T-cell chimerism have a low incidence [31]. Use of this model is limited mostly by the lack of de novo development of continuously differentiating human cells, activation status of the xenoreactive T cells and the GVHD (table 1).

New approaches for generating humanised mice

In 2004, a novel humanised mouse model was reported. It was based on transplanting human CD34+ haematopoietic progenitor cells (CD34+ cells) directly into the liver of newborn immunodeficient mice (Rag2γc−/−) [32]. By 10 weeks after transplantation, the mice develop a lymphoid system of human origin with T cells, B cells, NK cells, monocytes and dendritic cells. Notably, the T cells display a pattern of naive and memory cells and a Vβ repertoire similar to that of humans. The mouse mounts a specific antibody response against model antigens, such as pneumococcal and tetanus toxoid antigens, but the response is much weaker than that in humans (table 1).

This model is a significant step toward humanisation. Importantly, the mice lack the γc chain, which results in even more drastic immunodeficiency as compared with SCID mice. The γc chain is an essential component of the IL-2, -4, -7, -9, -15 and -21 receptors. Its absence severely compromises the development of immune cells, including NK-cell development, and thus their rejection potential against transplanted xenogeneic tissue. It also makes the mice less susceptible to lymphoma development. Indeed, the NOD-SCID IL-2Rγ-null mice are much more useful than the NOD-SCID mice for transplanting human tissue [33]. Notably, NOD-SCID IL-2Rγ-null mice show a similar degree of immunodeficiency as Rag1 or 2−/− γc−/− knock-out mice; they have been developed by crossing of SCID mice with non-obese diabetic (NOD) mice and mice deficient in the gamma c (γc) chain of the IL-2 receptor [34].

Table 1: Compilation of the various humanised mouse models.

<table>
<thead>
<tr>
<th>Humanised mouse model</th>
<th>Engraftment</th>
<th>Cellular composition in reconstituted hu mice</th>
<th>Supports HIV infection with</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>hu-PBL SCID [25]</td>
<td>N/A</td>
<td>• T and B cells</td>
<td>• CCR5- and CXCR4-tropic strains5 [26, 28, 62]</td>
<td>• Easy to generate (i.e. good access to PBLs)</td>
<td>• No multilineage haematopoiesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• CCR5 and CXCR4-tropic strains</td>
<td>• Can immediately be used after transfer of PBLs</td>
<td></td>
<td>• Limited time frame for experiments [62]</td>
</tr>
<tr>
<td>Thy/Liv SCID hu [23]</td>
<td>N/A</td>
<td>• T cells</td>
<td>• CCR5- and CXCR4-tropic strains [24, 58, 59]</td>
<td>• Organoid of foetal thymus/liver tissue with sustained T-cell lymphopoiesis</td>
<td>• Strong activation of T-cells [27, 28]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Single positive double positive and double negative Thyomocytes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>• Valuable to study certain pathogenic aspects (see text)</td>
<td></td>
<td>• Emergence of xeno-reactive T-cells (GvHD) [30]</td>
</tr>
<tr>
<td>Rag2−/− γc−/− [32]</td>
<td>++</td>
<td>• T and B cells</td>
<td>• CCR5- and CXCR4-tropic strains [68, 69, 71]</td>
<td>• Long-term multilineage haematopoiesis</td>
<td>• Surgical skills needed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Monocytes</td>
<td>• Specific antibody response to recall antigens [32]</td>
<td>• Suited to study HIV pathogenesis [118, 131, 132], HIV latency [90], gene therapy and novel anti-HIV treatment approaches [90]</td>
<td>• Human foetal tissue needed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Macrophages</td>
<td>• Suited to study HIV pathogenesis [115, 123], HIV treatment [95, 98] and latency and gene therapy approaches [102, 107]</td>
<td></td>
<td>• No multilineage haematopoiesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• NK cells</td>
<td>• Delay between transplantation of human CD34+ cells and development of lymphoid system of ~15 weeks.</td>
<td></td>
<td>• Sampling mainly restricted to the organoid since lack of solid peripheral reconstitution</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• DCs</td>
<td>• Lack of CCR5 expression on intrathymic T progenitor cells</td>
<td></td>
<td>• Surgical skills needed</td>
</tr>
<tr>
<td>NOG [38] or NSG [33]</td>
<td>+++</td>
<td>• T and B cells</td>
<td>• CCR5- and CXCR4-tropic strains [40, 70, 131, 132]</td>
<td>• Higher reconstitution levels as compared to Rag mice [39, 41]</td>
<td>• Sensitive to irradiation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Monocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Macrophage</td>
<td>• Suited for studying HIV pathogenesis [115, 123], HIV treatment [95, 98] and latency and gene therapy approaches [102, 107]</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>• NK cells</td>
<td>• Generation of adaptive immune responses [115]</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>• DCs</td>
<td>• Suited for studying HIV pathogenesis [43, 115], anti-HIV treatment [77, 80, 108], HIV latency [88] as well as novel gene therapies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD/SCID ¶ -hu BLT [42] And NOD/SCID ¶ γc−/− (NSG) BLT [133]</td>
<td>+++</td>
<td>• T and B cells</td>
<td>• CCR5- and CXCR4-tropic strains [43, 77, 80, 88, 108, 115]</td>
<td>• Two step procedure for generating BLT mice</td>
<td>• Two step procedure for generating BLT mice</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Monocytes</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>• Macrophages</td>
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<tr>
<td></td>
<td></td>
<td>• NK cells</td>
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<tr>
<td></td>
<td></td>
<td>• DCs</td>
<td></td>
<td></td>
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<tr>
<td>¶ For simplicity reasons, we put together these two subtly different models; in fact, BLT mouse using NSG background show a superior engraftment as compared to NOD/SCID BLT mice.</td>
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</table>
Modifications for improving the engraftment of human haematopoietic tissue have been investigated, including the use of human fetal liver derived CD34+ cells, cultivating the CD34+ cells with a cytokine cocktail before transplantation [35], pre-conditioning the mice with busulfan instead of irradiation [36, 37], the use of different mouse strains, such as NOD/shi-scid/γc null (NSG) [38] or NOD/SCID/γc null (NSG) mice [39], the transplantation of CD34+ cells intravenously or into the bone marrow or the transplantation of CD34+ cells at older age of the mice [39]. NSG and NOG mice are nearly identical except for the modification of the γc chain receptor; in both strains, triggering through the γc chain receptor is disabled: in NSG mice the receptor is completely knocked down, and in NOG mice the intracytoplasmic tail is truncated. NOG mice are especially vulnerable to developing lymphomas after irradiation; however, they yield similar engraftment results even when not irradiated [40]. Very importantly, the lifespans of humanised mice, except of irradiated NOG mice, appear to be similar to those of wildtype mice; the mice eventually die due to infirmity. NSG mice transplanted at birth with haematopoietic progenitor cells either from human foetal liver or from human cord blood gave the better engraftment than the Balb/c-Rag1-/- and CB-17-SCID/bg mice [41]. Similar data have been reported by Brehm et al. [39]. The BLT mouse deserves special mention [42]. BLT is an acronym for bone marrow liver thymic. In this model, foetal liver/thymus is placed under the renal capsule in 6–8-week-old immunodeficient mice as with standard SCID-hu mice. However, after 3 weeks, the mice are sub-lethally irradiated, and autologous human CD34+ cells are transplanted into the mice. These cells home to the bone marrow and also migrate to the thymus. In the BLT mouse, engraftment of human lymphoid tissue is highly efficient, even to the gastrointestinal tract [43]. The innate and adaptive immune responses appear to be generally more complete in the BLT mice than in humanised mice generated by transplanting human CD34+ cells alone [42]: BLT mice generate a human MHC-restricted T-cell response to Epstein Barr virus (EBV) and activated Vβ2-TCHR+ T-cells when dendritic cells present the superantigen toxic shock syndrome toxin 1 (TSST-1). Notably, TSST-1 specifically activates and induces the TCR Vβ2+ cells to proliferate. Generating an adaptive immune response is facilitated by educating the human T-cells in an autologous thymic microenvironment. This is not the case in the other humanised mouse models, which have xenogeneic mouse thymic environments. To overcome this limitation, immunodeficient mice were generated expressing the human HLA class I genes [44]. Here, mice transplanted with HLA-matched cord blood cells supported the in vivo differentiation of functionally mature human cytotoxic lymphocytes associated with a wide spectrum of functional human T-cell subsets. The mice mounted an EBV-specific immune response upon challenge as quantified by tetramer staining and enzyme-linked immunospot (ELISPOT) assay. Thus, introducing human HLA-class I transgenes significantly improved the humanisation of the mice. Similarly, a new report demonstrated expression of class II (HLA-DR4) in NOD−Rag1−/- /γc−/- mice and consequent improvement in T- and B-cell development and function [45]. Additional human transgenes critical for haematopoiesis have been introduced into the mouse strain backgrounds, and this action should result in a lymphoid system that even more closely approximates the human lymphoid system.

Humanised mice have also been used to study (1) haematopoietic development, (2) a variety of microorganisms, including EBV [42, 46], herpes simplex virus [47], Dengue fever [48, 49], influenza [50] and Salmonella typhi [51, 52], (3) sepsis [53] and iv) virus-induced tumours [54, 55].

Irrespective of the strain, immunodeficient mice are prone to opportunistic infections and must be kept in optimized hygienic animal care facilities. Whether the humanisation protects mice from infections is not known.

### Humanised mice for studying HIV infections

The hu-PBL SCID and foetal liv/thy SCID hu mouse models have been valuable for the study of HIV infection, including immune responses (e.g., the effect of vaccination with vaccinia gp160 and recombinant gp160 [56]), in vivo drug testing [57–59], anti-HIV effects of CD8+ cytotoxic T-cells [60] and neutralising antibodies [61], virulence of HIV isolates [62], and the significance of distinct HIV accessory proteins on virulence [63, 64], and viral latency [65]. However, these models have several limitations. Most importantly, they lack multilineage haematopoiesis and the capacity to generate an effective human immune response (table 1).

The “new generation” of humanised mice has a number of positive aspects, such as multilineage haematopoiesis, no or very rarely graft-vs-host disease, a longer lifespan of the mice, and the generation of some immune responses (table 1). In the next sections, we will focus exclusively on these new humanised mouse models. Reviews comparing the properties of the various humanised mouse models based on the use of either Rag, NSG or NOG mouse strains have recently been published [66, 67]. In this review, we focused primarily on the overall value of humanised mice for studying HIV infection and specified only the mouse strain used when clear differences were described as related to HIV infection or pathogenesis.

### Humanised mice support high-level viraemia

The new humanised mouse models support high levels of HIV infection with either CCR5- or CXCR4-tropic strains [36, 68–71]. Plasma HIV RNA copy numbers of $10^5–10^7$/ml in those mice are similar to the levels found in HIV-infected humans (note, that HIV replication can be easily monitored by repetitive sampling of peripheral blood). HIV-infected cells were detected in the spleen, lymph nodes, thymus and lungs, indicating dissemination of the virus. Unlike hu-PBL SCID mice, humanised mice sustain high-level viraemia for more than a year [35]. Depending on the virulence of the HIV strain used, the mice show distinct CD4+ T-cell depletion rates over time. Initial reports noted either very limited or no HIV-specific humoral immune responses [68, 70]. Importantly, expression of the HIV co-receptors
CXCR4 and CCR5 on engrafted and differentiated human immune cells was similar to that seen in humans [68, 70, 71]. Co-receptor expression in human CD4+ T cells is the major determinant of HIV tropism in vivo [72]. Indeed, as seen in HIV-infected human, disseminated infection in humanised mice with CCR5-tropic strains leads preferentially to infection and depletion of CD4+ memory T lymphocytes [73]. CCR5 is expressed mainly on memory T lymphocytes and is absent from naive T cells.

Humanised mice for studying sexual transmission and its prevention

A prerequisite for studying HIV sexual transmission in humanised mice is the engraftment of the female reproductive tract and/or the gastrointestinal tract with virus susceptible human cells. Both humanised RAg1-/-/- γc-/- mice and Rag2-/-/- γc-/- mice, as well as BLT mice, are well engrafted with human cells in the vagina [74–77], and vaginal HIV transmission is efficient in all these three new mouse models. Like the human gut, the mouse small intestines include abundant Peyer’s patches and the large intestines are populated with lymphoid follicular aggregates with human T and B lymphocytes, macrophages and DC [43, 74]. Here, memory T cells with prominent expression of CCR5 are permissive to CCR5-tropic strains. BLT mice also show human CD4CD8αα cells, a T-cell subset present only in the gut-associated lymphoid tissue [77]. These mice respond with disseminated HIV infection subsequent to either rectal or vaginal infection with cell-free HIV [43, 74, 75, 77]. However, efficient engraftment of the gastrointestinal tract of Rag2-/-/- γc-/- mice with human cells appears to depend on the protocol used: mice transplanted with CD34+ cells derived from human foetal liver and cultured overnight with IL-3, IL-6 and stem cell factor showed human cell engraftment in the gut [74]. This is not the case in mice transplanted with uncultured CD34+ cells derived from cord blood [78]. The latter also differed in their susceptibility to rectal HIV challenge [74, 78]. Humanised mice represent a very significant advancement for evaluating novel microbicides for preventing HIV infection and very nicely complement the much more expensive monkey models. Indeed, several recent studies demonstrated the utility of these models for testing oral and topical pre-exposure prophylaxis strategies with different anti-HIV drugs (e.g., Tenofovir, Maraviroc, Raltegravir) currently on the market [77, 79–82] or with compounds in development [83]. In particular, topical application of the CCR5 antagonist Maraviroc formulated as a gel prevented HIV vaginal transmission [81], and the novel CD4 aptamer-siRNA chimeras [83] showed partial protection.

Studies of anti-retroviral treatment strategies

A few reports have noted the utility of the new humanised mice for evaluating antiretroviral therapies [84–89]. We made a major effort for defining a gold standard for ART in humanised mice by first examining the pharmacokinetic of a number of anti-retroviral compounds [90]. In this work we showed efficacious anti-retroviral treatment when the anti-retroviral compounds were added to food pellets or when long-acting drugs were used [90]. We also demonstrated emergence of resistance in insufficiently treated mice, and viral rebound from previously undetectable levels after ART interruption, confirming a latent reservoir as reported recently [85, 86, 88, 91]. Thus, humanised mice represent a highly valuable model for pre-clinical proof-of-concept studies to evaluate novel anti-retroviral compounds and to study latency that closely approximates the status of HIV-infected humans treated with cART. Several studies also evaluated novel molecules for suppressing HIV in vivo in these new mouse models [89, 92–98]. They involved studies investigating the potential of Tat peptide analogues for inhibiting HIV replication (89) as

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<td>Human host factors</td>
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<td>CD4</td>
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<td>CCR5</td>
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<th>Mouse strains</th>
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<tr>
<td>C.B.-17 SCID/SCID</td>
<td>Severe combined immunodeficiency (SCID)</td>
<td>Mutation in the Pkd1 gene (lack of B and T cells)</td>
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<tr>
<td>human-PBL-SCID</td>
<td>PBL: peripheral blood leucocytes</td>
<td>Intrapertitoneal injection of human PBL into SCID mouse</td>
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<tr>
<td>Rag1-/- γc-/-; Rag2-/- γc-/-</td>
<td>Deficiency in the recombinase activating gene 1 or 2 and the common γ chain of the IL-2 receptor</td>
<td>Lack of B, T and NK cells</td>
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<td>NOD</td>
<td>Non-obese diabetic mouse</td>
<td>Reduction of NK cell activity</td>
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<td>NOD/Scid</td>
<td>NOD and SCID mouse</td>
<td>Lack of B and T cells and NK cell activity</td>
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<td>NSG (NOD/SCIDγc-/-)</td>
<td>NOD/SCID mice with entire knock-out of the common γ chain receptor</td>
<td>Lack of B, T and NK cells and blockade of the maturation and activity of these cells</td>
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<td>NOG (NOD/SCIDγc-/-)</td>
<td>NOD/SCID mice with knockout of the intracytoplasmatic tail of the common γc receptor</td>
<td>Lack of B, T and NK cells and blockade of the maturation and activity of these cells</td>
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<td>BLT</td>
<td>Bone marrow, liver, thymus</td>
<td>NOD/Scid or NOD/Scid/γc-/- mice transplanted with foetal liver, thymus and CD34+ cells</td>
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well as the effects of silencing (si)RNAs directed against viral proteins (e.g., Tat, Rev, Vif) or CCR5 that were delivered either by aptamers binding to the HIV envelope glycoprotein [92], dendrimer nanoparticles [93], single-chain antibodies binding to CD7 [95] or by immunoliposomes targeting the lymphocyte function-associated antigen-1 (LFA-1) [94]. The gp-120-binding aptamers targeted productively infected T cells specifically, and the single-chain antibodies and immunoliposomes targeted the white blood cells independently of HIV infection. In all of these in vivo studies, HIV replication was significantly suppressed. The humanised mice are also useful for studying the protective effect of distinct broadly neutralising antibodies delivered either by antibody-expressing cells administered as “backpacks” [96] or by adeno-associated virus-based vectors [97].

Studying gene therapeutic approaches for HIV/AIDS

Humanised mice represent a unique option to explore haematopoietic stem cell–based gene therapy strategies. Gene manipulation of human CD34+ cells to modulate host factors is also very attractive for HIV pathogenesis studies. Immunodeficient mice have long been used to assess the ability of gene-transduced human CD34+ cells to differentiate into various cellular subsets. In 1994, retroviral vector–transduced human CD34 cells were shown to differentiate into mature T-cell subsets in SCID-hu grafts [99]. Later, long-term engraftment of human CD34+ cells transduced with an HIV vector was demonstrated in NOD/SCID mice; 4–10% of the human cells were transduced [100]. NOD/SCID and the NSG mice seem to engraft similar numbers of transduced human CD34+ cells [101]. However, when using bone marrow from those mice for secondary reconstitutions in mice on the same background, human tissue from NSG mice engrafted far better than that from NOD/SCID mice, pointing to increased numbers of long-term SCID repopulating cells. These features are favourable for studying long-term transgene expression and the analysis of retroviral-insertion sites in primary and secondary transplanted NSG mice. These findings, along with the higher levels of human tissue engraftment, suggest that the “novel generation” of humanised mice will be very useful for studying gene therapy approaches and examining distinct genes for their pathogenic effects in various settings. In addition, zinc-finger nuclease–mediated gene engineering is another very promising tool for exploring gene engineering approaches to treat and/or cure HIV. The major hurdles will not be the identification of targets rendering cells resistant to HIV but to achieve sufficiently high numbers of genetically engineered CD34+ cells, the migration of transduced cells to the niches of haematopoietic stem cells, preventing the insertional risk favouring neoplastic transformation or off-target effects (e.g., activation of the innate immune response). Here, too, the humanised mouse model will be a versatile tool for exploring these questions.

Humanised mice for generating an HIV-specific immune response

Inducing a robust HIV-specific immune response was reported in NSG mice reconstituted with human CD34+ cells from newborns [114] and in the BLT mouse model [115]. Both papers reported HIV-specific CD4+ and CD8+ T-cell responses with overlapping HIV peptide pools [114, 115] or ELISPOT assays [115]. The relevance of the CD8+ T cells in constraining HIV replication in humanised mice was illustrated by a significantly higher replication rate when CD8+ T cells were depleted [114]. The humanised mice also developed humoral immune responses against HIV [115]. However, antigen-specific immune responses in humanised mice seem to take longer to develop than in adult humans infected with HIV, possibly reflecting the lack of full maturity of the human immune systems of these mice after reconstitution [115]. In previous work, such sol-
id HIV-specific antibody immune responses were not reported. The analysis might have been performed too soon after HIV infection. In view of limited number of studies, we still do not know if the current generation of humanised mice is suited for studying antigen-specific immune responses and, in particular, vaccine approaches. In mice reconstituted directly with human CD34+ cells the selection of T cells is done by murine thymic stromal cells. The subsequent generation of an antigen-specific immune response, however, is based on the processing of antigens by human antigen-presenting cells in the humanised mouse model and therefore might be suboptimal.

**HIV evolution over time**

HIV’s diversity is one of its main features. It is also a key element for immune escape and emergence of resistance to ART. HIV’s diversity is due to the inaccuracy of the HIV reverse transcriptase, hypermutation of the nascent DNA strand during reverse transcription by the members of the APOBEC family, and recombination events between distinct HIV strains. Indeed, the genotypic and phenotypic changes in the viral envelope gene in humanised mice infected with a distinct HIV strain, JR-CSF, showed the mean rate of divergence of viral populations over 44 weeks similar to that in humans [116]. They noted a disproportionate number of guanosine-to-adenosine transitions in the HIV envelope, indicating that APOBEC3G is active in this model. Furthermore, a number of substitutions in the envelope gene were identified.

**HIV immune activation and dysfunction**

Sustained immune activation is the major trigger of HIV-associated immunodeficiency. Various mechanisms, such as disruption of the gastrointestinal tract barrier during acute HIV infection [117] or various HIV accessory gene products, may contribute to the HIV-associated immune activation [18]. Immune activation is also observed on the CD4+ and CD8+ T cells in HIV-infected humanised mice [115,118]. We used the humanised mice to study the role of macrophages in immune activation [118]. We found that HIV infection results in a disturbed phagocytosis by macrophages. Notably, macrophages are essential for clearing bacterial products. We concluded that disruptions of the gastrointestinal tract barrier, together with the macrophage dysfunction, are a main element of higher blood levels of bacterial products and thus in HIV-associated immune activation.

Immune activation affects also the PDL1-PD1 axis (PDL = programmed death ligand). The inhibitory receptor PD-1, which indicates exhaustion of T cells, was increased on the T cells in HIV-infected humanised mice, reminiscent of the findings in humans [115]. Ongoing studies are examining the benefits of blocking the PD-1 pathway.

The presence of various immune cells, such as plasmacytoid dendritic cells (pDC) and T-regulatory cells, in humanised mice presents a unique opportunity to assess their effects on HIV infection and vice-versa (i.e., HIV’s effect on them). For example, rapid infection and activation of pDCs were seen in HIV-challenged humanised mice [119]. Their activation correlated with activation of CD4+ T cells and their apoptosis. While CD4+ T cells were depleted, pDCs were maintained but functionally impaired. The presence of T-regulatory cells in these mice may help to dissect their role in HIV infection. These cells are preferentially targeted by HIV during acute HIV infection in these mice [120].

**CNS invasion by HIV**

AIDS-related dementia occurs in about 30% of HIV-infected patients with advanced immunodeficiency [121]. AIDS dementia is characterised by the immigration of macrophages, formation of microglial nodules, and generation of multi-nucleated giant cells, most likely due to viral induced fusion between microglial cells and/or macrophages. HIV-infected humanised mice show pathologic anomalies in the brain reminiscent of those in HIV-infected patients with AIDS dementia. In particular, activated human blood-borne macrophages migrate into the brain. Human cells enter into the brains more quickly in HIV-infected mice than in control mice. Productively infected macrophages and cells of lymphocyte morphology are found in the meninges and perivascular spaces [122]. Strikingly, CD8+ T-cell depletion aggravated the pathological findings, suggesting that CD8+ T cells could subdue HIV infection to some extent. Using advanced neuroimaging and post-mortem examination, HIV-infected mice show a loss of neuronal integrity [123]. These data are encouraging: humanised mice represent a valuable tool for examining mechanistic and therapeutic aspects of HIV-associated dementia. However, as reiterated by the study authors, additional studies are needed for a more detailed characterisation and validation of the neuronal damage associated with HIV infection in this mouse model [124].

**Future generations of humanised mice**

Despite the advances made in humanised murine models, the reconstituted lymphoid system still lacks a well-elaborated lymphoid architecture. This is partially explained by the lack of human cytokines critical for haematopoiesis and/or by insufficient interactions between cells of the murine stroma and human haematopoietic cells. The less than optimal lymphoid architecture and the education/selection of T-cells on a murine thymic scaffold result in a rather modest adaptive immune response. As outlined above, transplantation of HLA-matched cord blood into a mouse strain transgenic for human HLA gives more robust specific T-cell response. To improve the humanisation of mice, additional human transgenes critical for haematopoiesis are introduced into mice.

The knock-in of human thrombopoietin (TPO) into Rag2−/−, which is essential for the expansion and maintenance of HSC [125], resulted in a higher level of engraftment and an increase in the breadth of multilineage haematopoiesis with higher number of myeloid cells than in control mice expressing the murine TPO [126].

Other knock-in (KI) genes examined for improving the humanisation were human IL-3 in concert with human granulocyte macrophage cytokine stimulating factor (GM-CSF) [127] and colony stimulating factor-1 (CSF-1) [128]. In IL-3/GM-CSF KI mice, transplantation of human pre-gonit cells resulted in a more pronounced inflammatory
reaction in response to intraperitoneal administration of lipopolysaccharide than in controls. In addition, IL-3/GM-CSF KI mice had improved human myeloid immune reconstitution in the lung as exemplified by the presence of alveolar macrophages; the human alveolar macrophages mounted an innate immune response when challenged with influenza virus but could not control it. This model might be especially good for studying pulmonary diseases [127]. The knock-in of CFS-1 enhanced the differentiation of myeloid cells into monocytes and macrophages [128]. As outlined above, differences in the extent the various mouse backgrounds support human cell engraftment exist. Positional cloning identified alleles of the inhibitory receptor signal regulatory protein alpha (SIRPs) as a reason for the difference of engraftment levels between mouse strains [129]. In NOD mice, which have higher engraftment levels than other mice, SIRPs on murine macrophages showed enhanced binding to the human CD47 ligand. This enhanced binding inhibits phagocytosis of the xenograph and secretion of TNF-α by macrophages. Transgenic expression of SIRPs in Rag2−/−/p53−− increased the engraftment level of HSC to a level similar to NSG mice and improved the functionality of the immune system [130]. These next-generation mice will most likely become important assets for the research community. Furthermore, the step-wise progress made in humanisation methods will continue and will create an array of humanised mouse models appropriately suited to address specific research questions.

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Authors' contribution: Authors Marc Nischang and Gustavo Gers-Huber contributed equally.

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HIV-1 needs critical host factors for efficient replication. HIV binds to the HIV receptor complex of the human CD4 cell-surface molecule and a co-receptor, either CCR5 or CXCR4, via the HIV envelope glycoprotein 120 (HIV env gp120). After conformational changes in the HIV env gp41, viral host cell membrane fusion occurs (2). The next steps are the decapsidation (3) and release of the HIV RNA from the virus particle. Reverse transcription generates a viral complementary DNA (cDNA) based on the viral RNA template and using HIV's own reverse transcriptase (4). Once the cDNA is generated, the preintegration complex (PIC) is assembled, nuclear trafficking and integration of the viral cDNA into the host genomic DNA follow (5). Efficient transcription and elongation require formation of P-TEFb (positive transcription elongation factor b) consisting of Tat (6A), human cyclin-dependent kinase 9 (CDK-9) (6B) and cyclin T1 (6C), which binds to the nascent HIV transcripts. Fully or partially spliced HIV mRNA (7) is used to translate viral proteins. Unspliced HIV RNA is packaged into newly generated virions. Assembly of HIV proteins and RNA and budding take place at the cellular membrane (9). HIV release is inhibited by murine tetherin at the cellular membrane because murine tetherin is insensitive to the viral protein Vpu, which inhibits human tetherin by directing its proteasomal degradation (blue frame). Human host factors critical for HIV replication are CD4, CCR5, CXCR4 and cyclin T1 (red frame). Additional human specific factors probably exist.
Chapter II

Humanized Mice recapitulate key features of HIV-1 infection: a novel concept using long-acting anti-retroviral drugs for treating HIV-1

4 Chapter II: Humanized Mice recapitulate key features of HIV-1 infection: a novel concept using long-acting anti-retroviral drugs for treating HIV-1

Marc Nischang1, Roger Sutmuller2, Gustavo Gers-Huber1, Annette Audige1, Li Duo1, Mary-Aude Rochat1, Stefan Baenziger1, Ursula Hofer1, Erika Schlaepfer1, Stephan Regenass3, Katie Amssoms2, Bart Stoops2, Anja Van Cauwenberge2, Daniel Boden2, Guenter Kraus2, Roberto F. Speck1*

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Declaration of contribution:
Marc Nischang performed most of the experiments, except HPLC-MS/MS that was done by Bart Stoops and genotyping method done by Katie Amssoms. Gustavo Gers-Huber, Annette Audige, Li Duo, Mary-Aude Rochat, Stefan Baenziger, Ursula Hofer and Erika Schlaepfer were participating in the common efforts related to the breading and generation of humanized mice. Stefan Regenass supported the work with standard HIV RNA RT-qPCR diagnostic method.
Chapter II

Humanized Mice recapitulate key features of HIV-1 infection: a novel concept using long-acting anti-retroviral drugs for treating HIV-1
Humanized Mice Recapitulate Key Features of HIV-1 Infection: A Novel Concept Using Long-Acting Anti-Retroviral Drugs for Treating HIV-1

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Abstract

**Background:** Humanized mice generate a lymphoid system of human origin subsequent to transplantation of human CD34+ cells and thus are highly susceptible to HIV infection. Here we examined the efficacy of antiretroviral treatment (ART) when added to food pellets, and of long-acting (LA) antiretroviral compounds, either as monotherapy or in combination. These studies shall be inspiring for establishing a gold standard of ART, which is easy to administer and well supported by the mice, and for subsequent studies such as latency. Furthermore, they should disclose whether viral breakthrough and emergence of resistance occurs similar as in HIV-infected patients when ART is insufficient.

**Methods/Principal Findings:** NOD/shi-scid/γc null (NOG) mice were used in all experimentations. We first performed pharmacokinetic studies of the drugs used, either added to food pellets (AZT, TDF, 3TC, RTV) or in a LA formulation that permitted once weekly subcutaneous administration (TMC278: non-nucleoside reverse transcriptase inhibitor, TMC181: protease inhibitor). A combination of 3TC, TDF and TMC278-LA or 3TC, TDF, TMC278-LA and TMC181-LA suppressed the viral load to undetectable levels in 15/19 (79%) and 14/14 (100%) mice, respectively. In successfully treated mice, subsequent monotherapy with TMC278-LA resulted in viral breakthrough; in contrast, the LA compounds together prevented viral breakthrough. Resistance mutations matched the mutations most commonly observed in HIV patients failing therapy. Importantly, viral rebound after interruption of ART, presence of HIV DNA in successfully treated mice and in vitro reactivation of early HIV transcripts point to an existing latent HIV reservoir.

**Conclusions/Significance:** This report is a unique description of multiple aspects of HIV infection in humanized mice that comprised efficacy testing of various treatment regimens, including LA compounds, resistance mutation analysis as well as viral rebound after treatment interruption. Humanized mice will be highly valuable for exploring the antiviral potency of new compounds or compounds targeting the latent HIV reservoir.

Introduction

The HIV pandemic continues to spread. Even in the United States and Europe with their relative universal access to combined anti-retroviral treatment (ART), the prevalence of HIV-infected people is increasing. The 2nd and 3rd generation antivirals are very efficacious. The life expectancy of treated HIV-infected individuals has significantly improved over the last two decades [1] and, in turn, has contributed to the increasing prevalence. However, ART has significant shortcomings, including adverse events, psychological dependence, life-long adherence and cost. Incomplete adherence results in the emergence of drug-resistant HIV strains. Novel and simpler treatment strategies and, in the best-case scenario, a cure are needed.

The HIV pandemic started with an ancestral SIV from a non-human primate crossing into humans [2], and thus, it is not surprising that HIV replication is limited to human and non-human primate cells. Mouse models of HIV infection have been generated by engrafting human lymphoid tissue into SCID mice [3] and are receptive to HIV [4]. For example, SCID mice...
transplanted with fetal liver (liv) and thymus (thy) tissue were very valuable for studying various aspects of HIV pathogenesis, including HIV-induced pathology in the thy/liv implant, screening anti-viral compounds and hematopoietic stem cell-based gene therapy [5]. However, these studies are limited to the thy/liv implant.

Development of a human adaptive immune system in cord blood cell–transplanted mice [6] renewed the interest in humanized mice (hu mice): hematopoietic CD34+ cells were preferentially transplanted into the liver of newborn mice or i.v. at older age. The mice develop a lymphoid-like system of human origin with T and B cells, monocytes, plasmacytoid and conventional DCs, thymus and lymph nodes [7]. Their mature T cells have a broad Vβ repertoire, and more than 40% of T cells display a naive phenotype [6]. This breakthrough was only realized through the development of heavily immunodeficient mice by crossing of SCID mice with non-obese diabetic (NOD) mice by crossing of SCID mice with non-obese diabetic (NOD) mice. The mice develop a lymphoid-like system of human origin with T and B cells, monocytes, plasmacytoid and conventional DCs, thymus and lymph nodes [7]. Their mature T cells have a broad Vβ repertoire, and more than 40% of T cells display a naive phenotype [6]. This breakthrough was only realized through the development of heavily immunodeficient mice by crossing of SCID mice with non-obese diabetic (NOD) mice and mice deficient in the gamma c (T cells) chain of the IL-2 receptor or the generation of Rag1 or 2–/– knockout mice [8].

We and others demonstrated that these hu mice are highly permissive to HIV infection when challenged with CCR5- or CXCR4-tropic HIV strains and show viral dissemination and progressive CD4+ T-cell loss [9,10,11,12,13]. To validate the experimental significance of hu mice for studying HIV pathogenesis and their value for novel interventional approaches, key aspects of HIV infection/pathogenesis must be fulfilled. The model should recapitulate ART of disseminated HIV infection with subsequent recovery of the immune system; interruption of ART should result in a rebound of HIV replication from the latent reservoir. Five studies reported the effects of ART in HIV-infected humanized mice using different drugs and drug combinations [14,15,16,17,18]. While these reports are very promising that humanized mice may be the long awaited small animal model for preclinical proof-of-concept studies, they lack pharmacokinetic (PK) studies for the medicinal compounds used except for the report by Choudhary et al [15] that would help to compare the data obtained in humanized mice to a clinical context.

A "standardized" ART scheme that completely suppresses HIV RNA replication in hu mice would be highly valuable for preclinical proof-of-concept studies for novel anti-retroviral compounds and studies of latency that closely approximate the situation in HIV-infected humans treated with ART.

Thus, we sought to determine if this mouse model is valuable for studying antiretroviral treatment of disseminated HIV infection and if it recapitulates key features, such as viral rebound, breakthrough replication and viral rebound subsequent to interruption of ART. We made a major effort to define the dose of antiretroviral compounds added to the food pellets to compare data to human studies. We also benefited from access to antiretroviral compounds in special formulation that permit once weekly dosing in mice. Thus, we also were able to study a novel concept of anti-HIV therapy based on LA antiretroviral drugs.

**Results**

Engraftment with human cells at around week 15 when hu mice were infected with HIV

Newborn NOG mice were transplanted with CD34+ hematopoietic progenitor cells isolated from umbilical cord blood. At around 15 weeks of age, the engraftment level was 20.7%±13.2 (avg ± std) before HIV infection (Figure S1). Of all human cells, CD4+ T cells were 23.1%±14.5 (avg ± std), CD8+ T cells 12.9%±9, and CD19+ B cells 50.8%±24.2. These engraftment values and their cell subset distributions are similar as reported previously [11,19,20].

**Pharmacokinetics of 3TC, Tenofovir (TDF), TMC278-long-acting (LA) and TMC181-LA**

The easiest and most convenient way for long-term ART in hu mice would be to add the ART to the food pellets. Mice have a higher metabolism than humans, and thus, we converted the dose of the distinct compounds used in humans by a formula as described [21]. The dose calculated was 61.7 mg/kg/day for 3TC and for TDF, considering food uptake of 3–4 g/d for a hu mouse with a body weight of 20–30 g. We generated food pellets containing 0.5 mg of 3TC and TDF per g of food. 3TC and TDF belong to the group of nucleoside resp. nucleotide reverse transcriptase inhibitors (NRTIs).

PK data validated this approach showing plasma levels in the therapeutic range over the entire observation period with fluctuations due to the wake-sleep cycle of the mice (Fig. 1A–D). In contrast, azidothymidine (AZT) at 0.5 mg/g of food and ritonavir (RTV) at 1 mg/g of food gave toxic concentrations clearly above the therapeutic range or sub-inhibitory concentrations, respectively (Figure S2). The plasma concentration of TMC278-LA and TMC181-LA (at the higher dose) was still clearly above the target concentration (C_{target}) even 14 days after s.c. injection (Fig. 1E–F). TMC278 is the recently approved non-nucleoside reverse transcriptase inhibitor (NNRTI) rilpivirine [22], and TMC181 is a pre-clinical-stage protease inhibitor (PI) belonging to the same chemical class as TMC114 (darunavir), but displaying better potency while preserving similarly high resistance coverage. The PK data of the long-acting drugs permitted a once weekly application in mice.

**HIV RNA plasma level is suppressed by ART and promptly rebounds with treatment interruption**

In a pilot experiment, uninfected mice appeared to tolerate AZT, 3TC and RTV well. Thus, while awaiting the PK data of these compounds, we started a first experiment to examine ART in HIV-infected mice (Fig. 2). Mice before ART had an HIV RNA baseline of 10^{4.9}±10^{3.2} copies/ml (avg ± std). Unexpectedly, the treated mice developed wasting within 2 weeks, which we attributed to the ART and, in particular, AZT. We therefore changed the ART immediately to TDF, 3TC and TMC278-LA, and within 1 week, the mice recovered from the wasting disease. Importantly, within 4–8 weeks, 14/21 (66%) mice showed a decline of HIV RNA levels to under the detection limit of 800 copies/ml (Fig. 2A). One mouse with detectable HIV RNA at that time showed suppressed HIV RNA when we bledd it 72 days after start of ART (Fig. 2A). Since we switched the ART in two mice with detectable HIV RNA to monotherapy with TMC278-LA, we are not able to make any statement about their eventual response rate if the ART had been continued. Thus, the overall response rate to the ART (3TC/TDF/TMC278-LA) was 79% (15/19 mice).

In an additional group of mice treated with AZT, 3TC and RTV, we found various laboratory disturbances, most prominent a very significant anemia (Figure S3).

Nearly all mice receiving the 2 weeks of AZT, 3TC and RTV treatment suffered from weight loss (Fig. 2E and F). Remarkably, the mice with viral failure experienced more significant weight loss than the others (Fig. 2G). Mock-treated HIV-infected mice showed...
a very stable weight course with fluctuations of less than 2 g over time.

We subsequently divided the mice with suppressed HIV RNA into two groups, seven mice were maintained on ART for another 5 weeks and, thereafter, treatment was interrupted. The other seven mice were treated by TMC278-LA alone. ART interruption resulted in viral rebound in all mice, indicating the existence of a latent reservoir, such as that in HIV-infected humans (Fig. 2C). Monotherapy with TMC278-LA was insufficiently potent to suppress HIV RNA since 6/7 mice showed a breakthrough of viral replication (Fig. 2D).

While there were no obvious symptoms or signs, we observed a higher mortality in mock-treated HIV infected mice than in ART treated mice. We associated this higher mortality with an unknown HIV-associated phenomenon. This mortality was usually less than 20% and thus when working with small numbers of mice, there

![Figure 1. PK data for 3TC, TDF, TMC278-LA and TMC181-LA.](A and C) Plasma levels of 3TC and TDF, respectively, over a day of mice on food pellets containing 0.5 mg/g food of 3TC or TDF for 2 weeks. (B and D) Decay rate of 3TC (t 1/2 = 5.5 h) and TDF (t 1/2 = 3.5 h), respectively, when replacing the food containing 3TC or TDF with standard food. (E and F) Plasma levels after one dose of either TMC278-LA (160 mg/kg) or TMC181-LA (white dots: 200mg/kg; black dots: 400mg/kg) administered s.c. The data were obtained with mice on ART-containing food pellets for at least 2 weeks to permit PK equilibration. The shaded area in (A–D) indicates the therapeutic range as defined in humans [42,43]. The dashed line in (E and F) indicates the target concentration (C target). Median effective concentration (EC) 50 values of TMC278 and TMC181 are 4.95 ng/ml and 1.29 ng/ml respectively in vitro in MT4 cells cultured with 50% human serum. The different colours indicate the experiments done with the same food batch, and whether we used mice transplanted with human CD34+ cells or not (White, red and yellow dots indicate humanized mice, green and blue dots indicate mice without transplantation of human CD34+ cells).

doi:10.1371/journal.pone.0038853.g001
may be divergence from this estimated mortality rate. In the 2nd set of experiments presented (see below), we had no loss due to “spontaneous” mortality.

Emergence of drug-resistant HIV strains in hu mice

All mice on ART with 3TC, TDF and TMC278-LA which experienced viral failure revealed the consecutive or simultaneous emergence of the prototype 3TC mutation M184I and the TMC278 mutation E138K (Fig. 3 and Table 1). All but one mouse showed viral failure when treated with TMC278-LA monotherapy. Of those mice with viral failure, all but one had the E138K mutation either alone or with the M184I mutation (Table 1). It is unknown if minor drug variants or mutations outside the amplified RT region might explain the lack of any TMC278 resistance mutation detected in the one mouse scored fully susceptible to TMC278 [23].
starting ART. As expected the CD4/CD8 T-cell ratio was significantly higher in the mice with suppressed HIV RNA than in control mice (Fig. 4D).

ART-treated HIV-infected hu mice had HIV RNA under 50 copies/ml but detectable cell-associated HIV DNA

The limited amounts of blood in any running experiment held the sensitivity of the Amplicor Roche® to 400–800 copies/ml. Although it is unlikely, this detection limit does not exclude low viral replication in the ART treated mice. The HIV RNA measurement based on the final bleeding of the mice treated either with the dual ART or the quadruple ART (3TC, TDF, TMC-278LA, TMC181-LA) revealed that 8/10 mice had fewer than 60 copies/ml (Table 2), which emphasizes the efficacy of the ART in the current setting.

In the mice from the second experiment (i.e., mice as shown in Fig. 4), we determined if cell-associated HIV DNA was detectable in splenocytes. Using a real-time PCR specific for YU-2, this was indeed the case in 13/15 mice (Fig. 5A). In all untreated mice, we detected levels of cell-associated HIV DNA higher than in treated ones with the exception of mouse #417. This mouse showed a low-level viremia despite ART that easily explains the relatively high cell-associated HIV DNA. We observed no correlation between cell-associated HIV DNA and engraftment levels or peak viremia.

In vitro reactivation of HIV transcripts in spleen specimens from hu mice in response to re-activating compounds

In order to examine whether latently infected cells exist in hu mice, we examined unspliced HIV Gag mRNA transcripts in spleen specimens from HIV-infected mock- and vero-treated mice prior and after in vitro reactivation. Since we were limited in spleen specimens, we opted for a combination of mitogens in concert with anti-CD3/CD28 and IL-7 to increase our chances of successful reactivation. The short term assay we used as well as the anti-apoptotic effect of IL-7 was certainly beneficial for countering any toxic effects due to the application of such a cocktail. Indeed, we did not observe any toxic effects 18 h after adding this cocktail when we harvested the tissue for quantifying HIV mRNA transcripts. As previously reported [16,17,18], we observed a clear reactivation of HIV Gag transcripts after stimulation (Fig. 5B). We did not isolate the human CD4+ cells to perform in vitro reactivation studies – however, we have no reason to assume that the cell subset harbouring latently HIV will differ from the results published [16,17,18].

**Discussion**

We sought to determine if hu mice recapitulate key features of HIV infection and treatment and to assess the value of long-acting anti-HIV drugs for treating HIV infection. We found that i) conventional ART with two NRTIs and a NNRTI efficiently suppressed HIV viral load and allowed recovery of the immune system; ii) cell-associated HIV DNA was still present in those mice, and interruption of ART resulted in viral rebound; in *in vitro* reactivation of spleen specimens from successfully treated mice yielded increased number of HIV mRNA transcripts as compared to baseline; and iii) simplification of ART with two long-acting drugs kept HIV RNA suppressed. Thus, HIV infection in hu mice mirrors key features of HIV infection in humans, including high titer viremia in untreated mice, suppression of HIV RNA when treated with ART but emergence of resistance when treated with insufficient regimens, viral rebound after treatment interruption, and recovery of CD4+ T cells under ART. Thus, hu mice are a highly valuable animal model to assess the antiviral potency of new compounds or novel strategies to eradicate latent HIV.

Lav/thy SCID hu mice have been used before to investigate novel compounds for their anti-HIV activity [5,24]. However, detailed PK data were mostly lacking, thus making it difficult to interpret the antiviral potency of the compounds. Furthermore, conventional lav/thy SCID hu mice lack peripheral T cells [3]. Thus, SCID-hu Thy/Lav mice were rarely used to study compounds to treat disseminated HIV infection. Most studies administered the drugs either before or immediately after the HIV challenge [25], and that recapitulates post- or pre-exposure prophylaxis where less potent regimens are already effective.

The new generation of hu mice displays an elaborated lymphoid-like system of human origin [6] and is highly susceptible to HIV infection. However, studies with these mice lacked data about their value for standardized pre-clinical testing. Five studies [14,15,16,17,18] reported the efficacy of ART in HIV-infected mice. In these studies, ART was started within 3 weeks after HIV

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**Table 1. Emergence of resistance in the mice under monotherapy with TMC278-LA.**

<table>
<thead>
<tr>
<th>Mouse (Identifier)</th>
<th>day 30 (baseline)</th>
<th>day 73 (under ART with 3TC, TDF, TMC278-LA)</th>
<th>day 128 (38 days under TMC278-LA alone)</th>
<th>day 150 (22 days after interruption of TMC278-LA)</th>
</tr>
</thead>
<tbody>
<tr>
<td># 192**</td>
<td>5*</td>
<td>n.d.*</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td># 242</td>
<td>S</td>
<td>n.d.</td>
<td>n.d.</td>
<td>S</td>
</tr>
<tr>
<td># 232</td>
<td>S</td>
<td>n.d.</td>
<td>E138K</td>
<td>E138K</td>
</tr>
<tr>
<td># 190***</td>
<td>S</td>
<td>M184V</td>
<td>n.d.</td>
<td>M184V/E138K</td>
</tr>
<tr>
<td># 191***</td>
<td>S</td>
<td>M184I</td>
<td>M184V/E138K</td>
<td>M184V/E138K</td>
</tr>
<tr>
<td># 224***</td>
<td>S</td>
<td>M184I</td>
<td>n.d.</td>
<td>M184V/E138K</td>
</tr>
</tbody>
</table>

*#S = susceptible (wildtype strain).

**#192 showed suppressed HIV RNA under TMC278-LA monotherapy.

***#190, 224 showed viral failure under the ART regimen of 3TC, TDF and TMC278-LA. #190 gave a positive signal for HIV RNA but below the limit of detection (<800 copies/ml).

#221, 245 only baseline analyses have been done, and therefore data from these mice were not integrated in the table.

**#n.d. = not done.

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infection (i.e., rather early after HIV challenge). In the first study, HIV was suppressed in 3/6 mice by i.p. ART, and HIV recurred when treatment was interrupted [15]. Surprisingly, a second study did not observe viral rebound after treatment interruption [14]. In subsequent studies, complete response rates were reported with an intensified regimen (i.p. administration of emtricitabine (FTC), TDF, an integrase inhibitor and enfuvirtide) [16] or high doses of ART given i.p [17]. All but one [16] of these studies lack detailed PK data on the administered drugs and data related to long-term administration of ART and to the anti-HIV efficacy of ART in chronically infected mice. Compared to the PK data we generated in mice and considering the therapeutic range in humans, the dosages applied in some of the studies reported are most likely 3–5-fold over the therapeutic range.

For an effective mouse model, long-term ART must be non-toxic and well tolerated by the mice. This requires solid PK data in

**Table 2.** HIV RNA load at the terminal bleeding in mice on ART or on double long-acting drugs.

<table>
<thead>
<tr>
<th>Mouse identification number</th>
<th>Mice on quadruple ART</th>
<th>Mice on double-long acting drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>#411</td>
<td>#412</td>
<td>#417</td>
</tr>
<tr>
<td>#432</td>
<td>#459</td>
<td>#402</td>
</tr>
<tr>
<td>#413</td>
<td>#415</td>
<td>#457</td>
</tr>
<tr>
<td>#466</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV RNA</td>
<td>n.d.*</td>
<td>n.d.</td>
</tr>
<tr>
<td>Detection limit [copies/ml]</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

* n.d. = non-detectable.
- Humanized mice were sacrificed 151 days after HIV infection and 114 days after starting ART or double long-acting drugs.
- Detection limit: the volume of plasma available was slightly different for the mice euthanized and thus the lower detection limit varied accordingly between 40 and 60 copies/ml.

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the therapeutic range for humans. In our experiments, we added the distinct anti-HIV compounds to the food pellet based upon calculating food intake, weight and metabolic rate. We generated PK data in the therapeutic range for 3TC and TDF. The long-acting drugs TMC278-LA and TMC181-LA were injected s.c. once weekly. We identified dosages that resulted in concentrations clearly above the Ctarget 1 week after its administration. Notably, plasma levels of NRTIs approximate only the concentration of the anti-HIV active intracellular tri-phosphorylated compounds. Since the half-life of the active moiety is longer than from the parental compound [26], we were confident that the dosages would be efficacious for treating disseminated HIV infection in our model. Indeed, this was the case. We observed HIV RNA suppression in 79% in the first and 100% in the second experiment.

From the PK data, the mice in the first experiment were treated for the first 2 weeks with only effective dosages of 3TC and AZT, a dual therapy insufficient to suppress HIV; RTV plasma levels were substantially below the therapeutic range. Furthermore, AZT which was toxic for the mice resulted in decreased food uptake that was reflected by substantial weight loss and sub-therapeutic dose levels. The emergence of drug-resistant HIV was a logical consequence of insufficient ART plasma levels. The observed M184I mutations in our study are the most prevalent among TDF/FTC-treated HIV individuals. Furthermore, we selected the TMC278 E138K resistance–associated mutation in our mouse

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**Figure 5. Recovery of cell-associated HIV DNA (A) and increase of HIV mRNA transcripts in vitro from splenic tissue obtained from HIV-infected mice with suppressed HIV RNA following activation.**

(A) DNA from infected HeLa cells (HeLa inf) and from the spleen of HIV-infected mice served as positive controls, DNA from an uninfected humanized mouse (uninf) served as negative control. The specimens of the treated and HIV-infected mice were from the experiments investigating the antiviral potency of the double long-acting drugs; (MNE = mean normalized expression). B) Splenic tissue specimens from either HIV infected ART naïve hu mice (HIV), ART treated mice (ART) or mice treated with the two long-acting drugs (Double-LA) were subjected to mitogens (PMA, PHA) in concert with anti-CD3/28 and IL-7. 18 hours later RNA was extracted and real-time PCR done for quantifying HIV Gag transcripts. Specimens of two mice which were treated with double LA drugs did not show any HIV transcript at all (data not shown in the graph); in five mice we did not detect any HIV transcripts prior to stimulation. The real-time PCRs were done in duplicates. *this specimen is from a mouse (417) with detectable HIV RNA at the time of euthanization (see Table 2). doi:10.1371/journal.pone.0038853.g005
model. These findings demonstrate that our model consistently reproduced the Phase III trial results of the ECHO & THRIVE studies, which showed that E138K and M104I combination was the most observed resistance associated mutations in patients treated with TMC278 and co-formulated TDF/FTC [27,20]. Indeed, our data indicate that the M104I mutation precedes the E138K mutation; this suggests that patients with archived HIV strains with the M104I are especially prone to viral failure with a subsequent triple compound-based regimen with 3TC or FTC and TMC278. This mimics what happens in patients with poor adherence and emphasizes the preclinical proof-of-concept value of this mouse model for HIV infection.

HIV-infected mice displayed a distinct response rate to ART (i.e., around 50% of all mice under quadruple therapy had an undetectable HIV RNA at day 44, the other 50% at day 99) (Fig. 4A). Since we were limited in blood draws, we have no detailed data about the viral decay in these sets of mice. Notably, we observed a rapid drop of HIV RNA in a majority of HIV-infected mice within 10 days after initiation of ART when we took blood in short intervals (unpublished data). In any case, the response rate to ART may vary in HIV-infected hu mice similar to the variable response observed in HIV-infected individuals starting ART. It would be therefore sensible to perform HIV RNA measurements over at least 2 months to document ART response or failure.

In our experiments, blood draws yielded small amounts, but terminal bleeds yields larger blood volumes, which are a critical determinant for the sensitivity of the Amplicor Roche®. Indeed, the larger blood volumes documented the success of ART with HIV RNA copy numbers below 60/ml in most mice, thus excluding that low-level viremia was still ongoing in the successfully treated animals.

Interrupting ART resulted in prompt viral rebound in all mice. The mice used for that purpose successfully responded to ART and were treated for a longer period of time to assure the decay of potentially low-replicating cells. We also found cell-associated HIV proviral DNA in untreated and successfully treated mice, reinforcing the fact that HIV generates a latent reservoir in HIV-infected hu mice. Besides, cell-associated HIV proviral DNA levels in untreated mice were higher than in treated ones, similar to the case in humans [29]. Similarly to findings reported [16,17,18], we observed a clear increase of HIV transcripts when splenic tissue from HIV infected mice with suppressed HIV RNA was stimulated. These findings are promising for using hu mice for studying the latent reservoir and in particular for studying approaches to eradicate it. We observed no correlation between cellular HIV-associated proviral DNA and engraftment level or peak viremia. These data show that hu mice represent a model to be used for testing novel approaches to eradicate HIV and were treated for a longer period of time to assure the decay of potentially low-replicating cells. We also found cell-associated HIV proviral DNA in untreated and successfully treated mice, reinforcing the fact that HIV generates a latent reservoir in HIV-infected hu mice. Besides, cell-associated HIV proviral DNA levels in untreated mice were higher than in treated ones, similar to the case in humans [29]. Similarly to findings reported [16,17,18], we observed a clear increase of HIV transcripts when splenic tissue from HIV infected mice with suppressed HIV RNA was stimulated. These findings are promising for using hu mice for studying the latent reservoir and in particular for studying approaches to eradicate it. We observed no correlation between cellular HIV-associated proviral DNA and engraftment level or peak viremia. These data show that hu mice represent a model to be used for testing novel approaches to eradicate HIV and were treated for a longer period of time to assure the decay of potentially low-replicating cells. We also found cell-associated HIV proviral DNA in untreated and successfully treated mice, reinforcing the fact that HIV generates a latent reservoir in HIV-infected hu mice. Besides, cell-associated HIV proviral DNA levels in untreated mice were higher than in treated ones, similar to the case in humans [29]. Similarly to findings reported [16,17,18], we observed a clear increase of HIV transcripts when splenic tissue from HIV infected mice with suppressed HIV RNA was stimulated. These findings are promising for using hu mice for studying the latent reservoir and in particular for studying approaches to eradicate it. We observed no correlation between cellular HIV-associated proviral DNA and engraftment level or peak viremia. These data show that hu mice represent a model to be used for testing novel approaches to eradicate HIV.
was subsequently formed to food pellets and sterilized by gamma-irradiation with 25 kGy. All batches of food pellets were analyzed for the correct amount of drugs admixed by HPLC (see below). Food and tap water were given ad libitum. TMC270-LA and TMC181-LA were generated by wet milling the compounds to get nanosizes and their subsequent formulation with non-ionic surfactants [30,31]. They were injected s.c. at 160 and 400 mg/kg, respectively.

HPLC-MS/MS method for measuring levels of TDF and 3TC in plasma and food pellets and TMC278 and TMC181 in plasma

Concentrations of drugs in the plasma and food pellets were determined by a qualified research liquid chromatography and mass spectrometry (LC-MS/MS) method. For the analysis of diet, food pellets were diluted with water (1:10) and homogenized. Aliquots of each homogenate (50 μL) were solubilized with methanol (three volumes) and extracted with an identical volume of acetonitrile. Plasma samples (50 μL) were prepared identically as the food pellet homogenates. Plasma and food pellets were quantified using a specific LC-MS/MS method.

LC-MS/MS analysis was carried out on an API-4000 MS/MS (Applied Biosystems), which was coupled to an HPLC system (Agilent). The MS/MS was operated in the positive ion mode with the TurbolonSpray-interface (electrospray ionization) and optimized for the quantification of the compound (MRM transition for TDF: m/z 504.2→270; for 3TC m/z 288→176; for TMC278 367.2→224 and for TMC181 585.2→429).

The calibration range was flexible and depended on the study design. The limit of quantification was 0.5–10 ng/mL, depending on the compound. The accuracy (intra-batch accuracy for independent QC samples) was 80–120% of the nominal value over the entire concentration range of the samples.

HIV infection and ART

Mice were infected i.p. with HIV YU-2, 1×10⁶ tissue-culture infectious dose 50 (TCID₅₀) per mouse. TCID₅₀ was determined in human CD8+ T cell depleted PBMC from three donors which were stimulated by PHA and anti-CD3 beads (Dynal). HIV RNA plasma levels were measured by RT-qPCR (AmpliPrep/COBAS TaqMan HIV-1 Test, Roche) at various times after infection.

Mice were monitored three times a week for symptoms or signs of adverse events, according to a standard score sheet.

Flow cytometry

Human cells, T cells and B cells were measured by flow cytometry of white blood cells stained for human CD45-APC, CD4-PerCP-Cy5.5, CD8-PB, and CD19-PE-Cy7 (all from BD Biosciences).

qPCR analysis of mouse organ samples

DNA and RNA from half of a spleen were extracted simultaneously with the AllPrep DNA/RNA Kit (Qiagen). DNA qPCR was as described [37], using HotStarTaq Master Mix (Qiagen), 1 μM of each primer and 0.1 μM FAM probe. Experiments were done in duplicate with the real-time thermocycler IQ5 (BioRad) and as cycling profile: 95°C 5 s, 55°C 5 s, 60°C 40 s. The following oligonucleotides were used for HIV gag gene: mf319tq (probe): FAM5'-AGG GGA CTC CAT CTT GGG-3' and mf46 (antisense) 5'-GGA TCT GTC TCT CTC TGG ACC-3' [37], mf289tq (probe): FAM5'-AGG GGA CCC GAC AGG CCC-3'TAMRA [37] and mf83 (antisense): 5'-GGA TCT GTC TCT GTC TCT TTC ACC-3' [37].

Since mice were infected with HIV-1 YU-2, the plasmid encoding YU-2 was used as standard in the PCR reactions with a detection limit down to 5 copies/reaction.

Reverse transcription

RNA was DNase treated using DNA-free kit (Ambion). For reverse transcription random hexamer primers (Operon Technologies) and SuperScript III reverse transcriptase (Invitrogen) were used. Reverse transcription was performed as described earlier [40]; briefly cDNA synthesis was performed using 10 μL DNase treated RNA in the presence of Ribolock Rnase inhibitor (Fermentas) in a total volume of 50 μL as follows: 60 min at 50°C, 60 min at 55°C, 15 min at 70°C and then 1 min on ice. Subsequently 1 μL of RNAseH (NEB) was added to each tube and incubated at 37°C for 20 min. Aliquots were stored at −20°C or used immediately for real-time PCR analysis.

Ex vivo reactivation

Splenic cells of half of a spleen were thawed and split in two equal parts and then incubated in RPMI containing fetal calf serum (10%), IL-2 (10 U/ml), penicillin (5%)/streptomycin (5%) and L-glutamine (5%) at 37°C for 12 hours. Subsequently cells were washed and then cultivated with or without mitogens (PHA at 5 μg/ml, PMA at 10 ng/ml, anti-CD3/CD28 beads (Dynabeads, Invitrogen) and IL-7 at 20 ng/ml. 18 hours later, DNA and RNA were extracted simultaneously with the AllPrep DNA/RNA Kit (Qiagen) which was then used for quantifying the HIV mRNA Gag transcripts.

Genotyping

Dideoxynucleotide-based sequence analysis was performed as described [41]. Briefly, Dideoxy sequencing reactions were performed on the purified amplicon (ABI Prism Big Dye Terminator Cycle Sequencing Kit, Version 3.1, Applied Biosystems) with a set of eight sequence-specific primers distributed over the PR-RT sequence for both strands: F1, 5'-GAGAGCTGAG GGTGTGGG-3'; F2, 5'-AATTGGCCCTGAAAATCC-3'; F3, 5'-CTCCATTTCC TTTGATGGG-3'; F5, 5'-CACCTTTTGG CAAGCACC-3'; R1, 5'-CTCCCACTCAG GAATCC-3'; R3, 5'-CTTCCCAAGAA TCTTTGATGTC-3'; R5, 5'-GGTTCATAAT CAGCTCATG-3'; R6, 5'-GGAA TTGTGCTGGTAGTCC-3'. Reactions were purified with a DyeEx Purification Protocol (Qiagen) and analysed with the ABI3730xl DNA Analyzer (Applied Biosystems). Sequence data files were grouped per sample identifier (ID) and aligned against the reference HXB2 reference sequence by means of the Sequencer TM Program V 4.1.4 (Gene Codes Corp.). A 25% mixture scoring rule (similar to 20% mixture identification by 454 deep sequencing) was used for the electropherogram analysis.
Supporting Information

Figure S1 Engraftment levels of hu mice before HIV infection. The mice were checked for engraftment levels at a median age of 132 days (25-75% percentiles: 103-136) as quantified by staining peripheral blood for the panhuman marker CD45. In addition, the percentage of CD4+, CD8+ and CD19+ cells were determined by flowcytometry.

Figure S2 PK data of AZT and RTV. (A and B) Plasma levels of AZT and RTV, respectively, over a day of mice on food pellets containing 0.5 mg/g or 1 mg/g food of AZT or RTV, respectively. The mice used for analysis of PK data have been on food pellets containing drugs for around 2 weeks for PK equilibration. The shaded area indicates the therapeutic range as defined in humans. The different colours indicate the experiments done with the same food batch.

Acknowledgments
We thank M. Ito (CEA) for providing the NOD/shi-scid/γc/null (NOG) mice, the staff of the Maternité at the Triemli Hospital Zurich, of the Clinical Immunology Laboratory at the University Hospital Zurich, and staff of the Animal Facilities at the University Zurich for their support. We thank S. Opendra (NIH) for providing anti-HIV compounds and Roche for providing RT-PCR reagents.

Author Contributions
Conceived and designed the experiments: MN GGH AA DL MAR SB UH ES SR KA BS. Analyzed the data: MN BS GK DB RS RFS. Wrote the paper: MN RFS. Provided key reagents: KA AvC.

References
Chapter II

Humanized Mice recapitulate key features of HIV-1 infection: a novel concept using long-acting anti-retroviral drugs for treating HIV-1

Supplementary figures

**Figure S1.** Engraftment levels of hu mice before HIV infection. The mice were checked for engraftment levels at a median age of 132 days (25–75% percentiles: 103–136) as quantified by staining peripheral blood for the panhuman marker CD45. In addition, the percentage of CD4+, CD8+ and CD19+ cells were determined by flowcytometry. Black lines are indicating the mean value for each group.

**Figure S2.** PK data of AZT and RTV. (A and B) Plasma levels of AZT and RTV, respectively, over a day of mice on food pellets containing 0.5 mg/g (white open circles) or 1 mg/g food of AZT or RTV (green open circles). The mice used for analysis of PK data have been kept on food pellets containing drugs for around 2 weeks for PK equilibration. The shaded area indicates the therapeutic range as defined in human.

**Figure S3.** AZT at the dose applied was highly toxic. Mice were 2 weeks on a regimen with AZT as added at 0.5 mg/kg to the food pellets and were subsequently euthanized. Extensive laboratory chemistry and hematology work-up was done by the Institute of Clinical Chemistry and the Division of Hematology, USZ. Black lines are indicating the mean value for each group.
Chapter II
Humanized Mice recapitulate key features of HIV-1 infection: a novel concept using long-acting anti-retroviral drugs for treating HIV-1
5 Chapter III: Humanized mice for ART testing and a pilot study to reactivate latently infected cells in vivo using SAHA (vorinostat)

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Declaration of contribution:

Marc Nischang performed most of the experiments. Gustavo Gers-Huber, Annette Audige, Mary-Aude Rochat and Renier Myburgh were participating in the common efforts related to the breading and generation of humanized mice. Stefan Regenass supported the work with standard HIV RNA RT-qPCR diagnostic method.
5.1 Outline

We have confirmed that humanized mice recapitulate central steps in HIV infection and fulfill key aspects such as high-titer viral dissemination, response to ART, viral failure and emergence of prototype resistance mutations in the case of non-adherence, and very importantly viral rebound after ART interruption (Chapter 2)\(^1\)\(^,\)\(^2\). Furthermore we and others have presented data showing the existence of HIV latency in humanized mice\(^2\)-\(^5\), and various studies have analyzed the effects of ART in HIV-infected humanized mice using different anti-retroviral drugs/drug combinations and several different routes of administration\(^4\)-\(^8\). Notably, only limited data exists on pharmacokinetics of retroviral drugs in humanized mice\(^2\), \(^7\), \(^9\)-\(^11\).

In this chapter I will summarize a number of experiments that we have performed regarding the utilization of the humanized mouse model for testing the efficacy of different ART regimens, including investigational drugs in different treatment settings.

This included i) efficacy testing of a long-acting compound (TMC278-LA) in Pre- and Post-exposure prophylaxis scheme, ii) generation of pharmacokinetic data for Raltegravir (RGV), iii) efficacy testing of a combination ART consisting of 3TC, TDF and RGV admixed to the food of HIV infected humanize mice and of two different investigational anti-retroviral drugs, as initial monotherapy or mono-maintenance therapy compared to the triple ART, and finally iv) a pilot study in which we tested the potential of vorinostat (suberoylanilide hydroxamic acid, SAHA) to reactivate latently infected cells in HIV in infected humanized mice under suppressive ART.

5.2 Results

Engraftment levels of human cells in recipient NSG mice.

Previously, we have been using RAG mice (Rag2\(^{-/-}\) γc\(^{-/-}\))\(^12\) and NOG mice (NOD/shi-scid/γcnull)\(^13\) for HIV \textit{in vivo} experiments. Since we experienced sporadically wasting in combination with severe hair loss in some NOG mice after transplantation of CD34+ cells, and because of better engraftment levels in NSG (NOD/LtSz-scid/γc\(^{-/-}\))\(^14,\)\(^15\) mice reported by other groups we decided to switch from NOG to NSG mice. In a first effort we compared the engraftment levels of NSG mice as compared to NOG mice (Fig. 1).

Newborn mice were transplanted with isolated CD34+ cells purified form umbilical cord blood. At around 15 weeks of age, the average engraftment levels of CD45+ cells were 53.1\(\%\)\(\pm\)27.47 (avg\(\pm\)std) in NSG mice and 23.84\(\%\)\(\pm\)20.11 (avg\(\pm\)std) in NOG mice (Fig. 1).
Chapter III

Humanized mice for ART testing and a pilot study to reactivate latently infected cells in vivo using SAHA (vorinostat)

Figure 1: A) Engraftment levels of human cells in uninfected NSG mice (n=68) around 15 weeks post transplantation. Percentage of CD45+, CD3+, CD4+, CD8+ and CD19+ cells as determined by flow cytometry. Mean engraftment levels of human CD45+ cells were 53.1%. Mean percentages of CD3+, CD4+, CD8+ and CD19+ cells of all CD45+ were 34.9%, 22.2%, 12.7% and 33.7%, respectively. B) Engraftment levels of human cells in uninfected NOG mice (n=59) around 15 weeks post transplantation. Mean engraftment levels of human CD45+ cells were 24%. Mean percentages of CD3+, CD4+, CD8+ and CD19+ cells of all CD45+ were 35%, 16%, 11% and 59%, respectively.

The average engraftment level of human cells in recipient NSG mice and the distribution of the cellular subsets that we observed in the peripheral blood around 15 weeks post transplantation of CD34+ hematopoietic progenitor cells are comparable to previously published data 15-17. The mean engraftment level of human CD45+ cells is higher for humanized NSG mice as compared to what we and others observed for humanized NOG mice2, 13, 18, 19 (Fig. 1). In addition we also observed a slightly better robustness of NSG mice and in average slightly bigger litters of newborns compared to NOG mice. Notably, because of these findings we have switched to use NSG mice and our lab will use them in future experimentations.

5.2.1 Pre- and post-exposure prophylaxis treatment strategies for assessing the efficacy of TMC278-LA regarding prevention of HIV-1 infection in humanized NOG mice.

In Chapter 2 we have presented PK data of a novel NRTI, TMC278 (rilpivirine), in a long-acting formulation (LA)².

Here we tested TMC278-LA for preventing HIV transmission either when given prior to HIV challenge (pre-exposure prophylaxis (PrEP)) or after HIV challenge (post-exposure prophylaxis (PEP)). As mentioned above, TMC278-LA is a long-acting nanoparticle formulation of the recently approved NNRTI rilpivirine (TMC278) with superior PK features clearly above the EC50 up to 14 days after a single dose injection (Chapter 2). For studying PrEP we s.c. injected mice with a single dose of TMC278-LA at 160 mg/kg 7, 4 or 1 day before HIV challenge and analyzed whether the mice got HIV infected 20 and 38 days post-infection (dpi) by measuring HIV RNA in the plasma of mice. The overall infection rate of
untreated mice was 3/5 (60%) at 38 dpi. All mice treated one day before viral challenge were tested negative, i.e. 0/5 mice infected (0%). When mice were treated 4 and 7 days before infection, HIV RNA was detected in 4/6 (57%) and 5/6 (83%) mice at 38 dpi respectively (Fig. 2 A). Thus, TMC278-LA did not show a pronounced long-term protection against HIV challenge.

For studying PEP mice received a single dose of TMC278-LA immediately after HIV challenge, or 1 or 3 days later, followed by four additional weekly injections of TMC278-LA, at 7, 14, 21 and 28 dpi. We analyzed the HIV plasma viremia of mice at 30 and 60 dpi respectively (Fig. 2 B). The overall infection rate of untreated mice was 10/12 (83%) at 62 dpi. 0/13 (0%) mice receiving the TMC278-LA dose immediately after infection were infected. Of the mice receiving the first dose 1 or 3 days post infection 4/13 (30%) and 5/13 (38%) were infected (Fig. 2 B). Notably, the result for PEP (Fig. 2 B) reflects the outcome of two repetitive experiments that were performed with similar amounts of animals.

PrEP and PEP exposure studies have also been conducted by others in humanized mice\textsuperscript{20-23}. A unique model in this respect is the BLT humanized mouse model, in which human immune cells extensively engraft into mucosal tissues, which allows transmission of HIV across mucosal surfaces\textsuperscript{20}. BLT mice can be infected using a natural route of infection, which permits an adjustment of the HIV challenge resulting in infectivity comparable to that in humans. Thus, this model allows testing of preventive treatments under conditions that are very closely mimicking the reality. Notably, in our PEP and PrEP studies we used high titer HIV stocks and i.p. injection as route of infection, resulting in a higher viral challenge that most likely leads to an underestimation of the true efficacy of the tested drug in a natural infection.
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Figure 2: Pre-exposure prophylaxis (PrEP) and post-exposure prophylaxis (PEP) in humanized NOG mice. (A) Outcome of pre-exposure prophylaxis at day 20 and 38 post infection (dpi) of mice receiving a single application of TMC278-LA [160 mg/kg] 1, 4 or 7 days before HIV infection. (B) Outcome of post-exposure prophylaxis at 30 and 60 days post infection (dpi) of mice receiving a first dose of TMC278-LA [160 mg/kg] at the same time point as the viral challenge, or 1 or 3 days after HIV-1 infection. This first PEP dose was followed by four additional TMC278-LA injections (7, 14, 21 and 28 dpi). Results of post-exposure prophylaxis represent two independent experiments. HIV positive and negative tested mice are represented by black and white columns respectively. Additionally the number of HIV positive tested mice out of the total number of animals challenged is indicated for each group.

The difference in the results obtained regarding number of HIV positive tested animals from early time points, i.e. 20 dpi and 30 dpi for PrEP and PEP respectively, compared to later time points, i.e. 38 dpi and 60 dpi for PrEP and PEP respectively, is explained by the time span of about 4 weeks that HIV requires to establish sustained high titer viremia in humanized mice after challenge with virus. Equally it is possible that TMC278-LA could be efficacious to suppress HIV transcription transiently if the initial HIV infection was not be prevented (Fig. 2). Consequently it is essential to ultimately test animals in PrEP and PEP settings 4-5 weeks after stopping the anti-retroviral treatment.

5.2.2 Pharmacokinetics of Raltegravir (RGG) and successful triple ART admixed to food pellets of HIV infected humanized NSG mice.

ART admixed to food pellets is considerably less stressful as compared to daily gavage or injections over weeks or months. In addition to the PK data we presented in the work
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published (Chapter 2)², we also measured PK of Raltegravir when admixed to the food pellets, and then assessed the efficacy of a triple ART of 3TC, TDF and RGV all admixed to the food pellets. RGV is a HIV integrase strand transfer inhibitor, which has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of HIV-1 infection in October 2007. We calculated the dose of RGV as before based on a formula that incorporates the higher metabolic rate of mice as compared to humans²⁴.

Figure 3: Steady state plasma concentrations for raltegravir (RGV). (A) Plasma levels of RGV measured over the time of day in mice on food pellets containing 1.4 g of RGV per kg food (white open circles) or 4.7 g/kg (grey open circles) respectively. The therapeutic range in humans is indicated by grey shaded area²⁵. (B) Decay of RGV in the plasma of mice subsequent to ART interruption by replacing the food containing RGV, 3TC and TDF with standard food.

The calculated dose was 164.44 mg/kg per day RGV. Taking into account an average daily food intake of 3.5 g for a NSG mouse with a body weight of 20-30 g, we produced food pellets containing 1.4 g RGV, 0.5 g 3TC and 0.5 g TDF per kg of food pellets.

Steady state plasma concentrations of raltegravir (RGV) in mice on this food-pellet-drug regimen were below the intended concentration (Fig. 3 A). Using a higher dose of 4.7g RGV/kg food the measured plasma concentrations were laying within the therapeutic range (Fig. 3 A). In humans receiving a routine daily dose of 400 mg BID the mean peak plasma concentration of RGV was 2.332 ng/mL (538–3.097 ng/ml) and median trough level was 563 ng/mL (204–751 ng/ml)²⁵. The doses of 4.7 g/kg RGV, 0.5 g/kg 3TC and 0.5 g/kg TDF admixed to food pellets were therefore used in all later experimentations.

After adjustment of the of RGV dose resulting in adequate drug plasma concentrations over 24 hours, we went on to test the efficacy of a triple ART regimen consisting of 3TC, TDF and RGV admixed to food pellets, as well as of two investigational drugs: the protease inhibitor TMC181-LA and an inhibitor of viral entry referred to as “peptide inhibitor”.

In a first experiment we initially treated 42 HIV infected humanized NSG mice with the triple ART regimen starting from day 41 post infection. After 57 days on ART (98 dpi) plasma
viremia was successfully suppressed in 38/42 (90%) mice (Fig. 4 A). All mice were then split into groups, which were either kept on the initial ART (n=17) received once weekly s.c. injections of TMC181-LA at a dose of 400 mg/kg (n=8), or daily s.c. injections of a developmental peptide HIV fusion inhibitor, with a mode of action similar to T-20 (enfuvirtide), at a dose of 2 mg/kg in form of a 20% cyclodextrin (CD) solution containing [0.4 mg/kg] (n=8) (Fig. 4 B-D).

Notably we also included a mock-treated group of mice by injecting a 20% cyclodextrin (CD) solution (n=9) and a group of only HIV infected mice (Fig. 4 E and F).

16/17 (94%) mice that were kept on the ART containing food had suppressed viral loads after 93 days of treatment and 11/15 (73%) mice were suppressed at the end of the experiment after 114 days on ART (Fig. 4 B). The 4 mice with detectable viral loads had plasma HIV RNA levels just above the limit of detection of 400 copies/ml (1100, 460, 620 and 500 copies/ml), which could reflect low level viral replication under ART. Notably, two mice died before the end of the experiment (Fig. 4 B).
Figure 4: Efficacy testing in different maintenance schemes after initial triple ART consisting of 3TC, TDF and RGV admixed into the food pellets of the mice. (A) Overall response rate of mice to initial ART after 57 day of treatment (98 dpi) (B) Group of mice which was kept on the initial ART regimen (C) Mice receiving s.c. injections of the long-acting drug TMC181-LA once weekly with the first dose starting from 98 dpi. (D) Group of mice receiving daily s.c. injections of an investigational peptide inhibitor starting 98 dpi. (E) Group of mice receiving daily mock injections (s.c.) using the same vehicle as for the peptide inhibitor. (F) Group of untreated control mice. Notably, 9 mice were superinfected 31 days after the first viral challenge. Number (n) of animals is indicated if needed.

Mice (n=8), which were switched from ART to daily injections of TMC181-LA remained aviremic in 80% (4/5). The one mouse (1/5) that showed a detectable viral RNA at the end of the TMC181-LA maintenance regimen had a viral load of 740 copies/ml, just above the limit of detection (400 copies/ml) that could be caused by low level viral replication during ART.
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Notably two other mice under TMC-181-LA maintenance showed transiently low levels of detectable HIV RNA (560 and 1100 copies/ml) during an otherwise suppressive treatment (Fig. 4 C). The picture is totally different for the mice receiving daily injections of the peptide fusion inhibitor: only 25% (2/8) were successfully treated with undetectable HIV in the plasma while 75% (6/8) experienced viral failure (Fig. 4 D). Of the mock-treated animals, which were taken off the ART containing food pellets, all 9/9 (100%) mice had an immediate viral rebound subsequently to the stop of treatment (Fig. 4 E). A control group of n=7 HIV infected untreated mice had a pronounced viremia throughout the whole time of the experiment (Fig. 4 F). The overall HIV infection rate observed in this experiment was 49/52 (94%) (Fig. 4 A and F).

The engraftment level of human lymphoid cells in the peripheral blood of the mice and the cellular subsets were analyzed over the entire time of the experiment by flow cytometry (Fig. 5). Total human cells were defined by using the pan leukocyte marker CD45, T cells by CD3, CD4 and CD8, and B cells by CD19 (Fig. 5).

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

**Figure 5**: Cellular subsets in mice receiving different treatments as measured by flow cytometry over time (mean). (A) Levels of human CD45+ cells. (B) Percentage of CD4+ T cells of CD45+ cells. (C) Ratio of CD4+/CD8+ T cells of all human cells (CD45+ cells). (D) Percentages of CD4+ and CD8+ cells over time. For each group of mice the pooled data is displayed as the mean value +/- standard deviation.

There is a trend that the CD4+ T cell numbers recover after the start of ART (Fig. 5 B) which is also reflected by the ratio of CD4/CD8 T cell (Fig. 5 C). Notably, there seems to be a
We next addressed the question, if an initial mono-therapy consisting of weekly injections of TMC181-LA, or daily injections of the peptide inhibitor would be potent enough to cause a significant decrease, or in the best case a full suppression of HIV plasma viremia. Therefore groups of n=6 infected humanized NSG mice were either put on cART containing food pellets (Fig. 6 A), weekly injected with TMC181-LA (Fig. 6 C), or daily injected with the peptide inhibitor (Fig. 6 D). A control group of n=5 mice was kept without any antiviral treatment (Fig. 6 B). TMC181-LA mono-therapy resulted in 6/6 (100%) mice in viral suppression after 51 days of treatment (Fig. 6 C). Subsequently the regimen was switched to ART containing food pellets for another 51 days (156 dpi). At this time point 5/6 (83%) mice had suppressed plasma viral loads (Fig. 6 C).

In the group of mice, which were daily injected with the peptide inhibitor, 6/6 mice showed an initial one ~log decrease in viral loads after 21 days of treatment (Fig. 6 D). Except of one
mouse with undetectable HIV RNA, all other mice showed rapid plasma viremia rebound comparable to baseline viral RNA levels (Fig. 6 D). In the group of mice initially receiving the ART containing food pellets 5/6 (83%) mice had suppressed viremia after 51 days of treatment, and all of these mice showed an immediate viral rebound after interruption of ART at 105 dpi (Fig. 6 A).

The engraftment level of human lymphoid cells in the peripheral blood of the mice, and the cellular subsets were analyzed over the entire time of the experiment by flow cytometry using antibodies specific for the human epitopes CD45, CD3, CD4, CD8 and CD19 (Fig. 7). After start of the different anti-retroviral treatments the number of CD4 T cells increases as a result of the effective inhibition of HIV replication and virus production (Fig. 7 B). This is also reflected by the increase in the ratios of CD4/CD8 T cells (Fig. 7 C).

Even though besides an initial decrease in viral load the peptide fusion inhibitor was not able to efficiently suppress HIV-1 replication (Fig. 6 D), there seems to be a sustained beneficial effect on the number of CD4 T cells which might be explained by the protective potential of a peptide fusion inhibitor.
5.2.3 **Pilot study using vorinostat to reactivate HIV in latently infected cells.**

As outlined in the introduction the chromatin structure affects the transcription of HIV and thus whether HIV is silent or not. Histone deacetylase 1 (HDAC1) deacetylates Nuc-1 and inhibits Tat activation, potentially contributing to the maintenance of HIV latency\textsuperscript{26}. Major focus is therefore given to the effects of histone acetylation for activating latent HIV. HDAC inhibitors block deacetylation of histone ends, thus causing a decondensation of the chromatin structure and thereby potentially rendering latent provirus accessible to cellular transcription factors such as NFκB, NFAT and AP-1.

Thus, in a pilot study we tried to use the HDAC inhibitor vorinostat (suberoylanilide hydroxamic acid, SAHA) for reactivation of latently infected cells in infected mice with suppressed plasma viremia. Humanized NSG mice (n=23) were therefore put on food pellets containing 3TC, TDF and RGV starting from 38 dpi. A schematic representation of the grouping of mice and the SAHA application scheme is shown in Fig. 8.

![Figure 8](image)

**Figure 8**: Schematic overview of the grouping of mice under suppressive ART. Groups of mice on ART containing food pellets (3TC, TDF and RGV) were either additionally receiving a single dose of SAHA (groups: C, D and E) or solely kept on ART (group: A, B and F). Mice were euthanized 24 hours (A and C) and 48 hours (B and D) after SAHA injection, or bleed after 24 hours and then taken off ART containing food to analyze a potential viral rebound (E and F). Additionally a control group of n=11 mice were not receiving any treatment subsequent to HIV infection.

All HIV infected mice were put on ART consisting of 3TC, TDF and raltegravir. Over all this regimen resulted in a suppression of HIV plasma RNA to below the limit of detection in 22/23 (95%) mice after 88 days on ART (126 dpi) (Fig. 9 A). To assess the effect of a single s.c. injection of SAHA [40 mg/kg] groups of mice were either injected with SAHA on day 124 dpi or received a mock-injection of 20% cyclodextrin solution (Fig. 8).
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At 24 hours (125 dpi) and 48 hours (126 dpi) after SAHA injection some mice (n=15) were sacrificed and blood and organ samples collected for later analyses (Fig. 8). Additionally, some mice were only bled 24 hours after SAHA injection (125 dpi) and ART was stopped subsequently (131 dpi) to assess viral rebound.

**Figure 9**: HIV infected humanized NSG mice receiving anti-retroviral therapy consisting of 3TC, TDF and RGV admixed in the food pellets and HIV infected untreated control mice. **A)** Viral load data of all mice receiving a single injection of SAHA (124 dpi) (grey open circles) or a mock-injection of 20% cyclodextrin (CD) solution (white open circles), with a partial magnification of the graph at the time point of injection (124 dpi) and euthanasia 24 or 48 hours later. **B)** Group of untreated HIV infected mice (control).

HIV plasma RNA was not detected in of any of the SAHA or mock-injected animals 24 or 48 hours after SAHA injection (Fig. 9 A). Subsequent to stop of ART on day 131 post 8/8 animals showed a viral rebound on 153 dpi irrespective whether the mice received SAHA or not (Fig. 9 A). Untreated HIV infected control mice had detectable plasma viremia throughout the entire duration of the experiment (Fig. 9 B).

Mouse spleen samples were analyzed using intracellular staining with an antibody binding to the acetylated form of lysin 9 in histone 3 (Fig. 10). In human CD45+ cell population a slight increase in the acetylation status was observed in mice that were sacrificed 48 hours after SAHA injection (Fig. 10). In contrast to this in mice receiving SAHA that were sacrificed 24
hours after injection, or taken off ART and sacrificed later, no difference in respect to the histone acetylation pattern was observed (Fig. 10).

Figure 10: Acetylation status of lysine 9 in histone 3 (H3K9) of human CD45+ cells from mouse spleen samples of mice receiving a single injection of SAHA or a mock-injection (control) detected by intracellular antibody staining and analyzed by flow cytometry. Mean fluorescence intensity (MFI) (left) and percentage of cells (right) positive for the acetylated form of H3K9.

In addition we performed RT-qPCR analysis of cell-associated HIV RNA from mouse spleen samples using a primer/probe set for detecting HIV gag transcripts (Fig. 11). Mean normalized expression (MNE) values of gag-transcripts normalized to expression of HMBS as a housekeeping gene did not reveal any differences between SAHA treated and mock-treated animals (Fig. 11).

Figure 11: Results of RT-qPCR analysis of cell associated RNA (Gag transcripts) in spleen samples of mice. Original CT values for Gag-RNA transcripts were normalized to CT values for HMBS-RNA as a housekeeping gene. RNA extracted from spleen samples of an infected and an uninfected mouse that were successfully used in a RT-qPCR assay before, were used as positive and negative controls respectively.

To assure the quality of the lot of the batch of SAHA used for the in vivo application in mice, we performed an acetylation assay using human PMBCs incubated for 24 hours with SAHA, or one of the two investigational HDAC inhibitors, Tib.#222 and Tib.#348 respectively (Fig.
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12). In PBMCs from three different donors we could observe good correlation between acetylation status and the concentration of the individual HDAC inhibitors used. Displayed are the mean fluorescence intensity (MFI) and the percentage of H3K9+ cells (Fig. 12).

![Graphs showing acetylation status and concentration of HDAC inhibitors](image)

Figure 12: Incubation of human PBMC from three different donors (208, 209, 210) for 24 hours using different concentrations of the HDAC inhibitor SAHA, and two investigational HDAC inhibitors (#222 and #348). Flow cytometry analysis of PBMCs after intracellular antigen staining using an antibody that specifically binds to the acetylated form of lysine 9 on histone 3 (H3K9). Percentage of CD45+ cells positive for an acetylated lysine 9 on histone 3 (% of cells), as well as the mean fluorescence intensity (MFI) for an acetylated lysine 9 on histone 3 on CD45+ cells are indicated.

All HDACi tested including the batch of SAHA used in the in vivo experiment showed deacetylation of a lysine residue at position 9 in the histone 3. Notably, the investigational HDACi Tib.#222 displayed a somewhat higher activity as compared to Tib.#348 and SAHA.

5.3 Conclusion

In all experimentations we used NOD/shi-scid II2rg/- (NOG) or NOD/LtSz-scid II2rg-/- (NSG) mice transplanted with CD34+ hematopoietic progenitor/stem cells. The average engraftment level of human cells in recipient NSG mice and the distribution of the cellular subsets that we observed in the peripheral blood around 15 weeks post transplantation of
CD34+ hematopoietic progenitor cells are comparable to previously published data\textsuperscript{15-17}. The levels of human CD45+ cells as well as the relative amounts of CD3+ T-cells among all human cells are higher in humanized NSG mice as compared to what we and others observed for humanized NOG mice\textsuperscript{2, 13, 18, 19}. In addition we also observed a slightly better robustness of NSG mice and in average bigger litters of newborns compared to NOG mice. Notably, because of these findings we have switched to use NSG mice and our lab will also use them in future experimentations.

The most convenient way to supply humanized mice with ART is via the oral administration route. Furthermore most of the approved drugs to treat HIV infections in humans are designed for oral uptake. Compared to daily gavage or injections, administration of drugs via the oral route generates less stress for the mice, thereby improving reproducibility of experiments and increasing the overall survival rate. In addition drugs admixed to food pellets and subsequently sterilized by irradiation are stable over a long time.

We complement with this work the pharmacokinetic data necessary for the production of a triple ART consisting of 3TC, TDF and RGV admixed to food pellets. As reported before based on PK data from trials in humans by Wenning et al.\textsuperscript{27}, we did not observe any significant change in mouse plasma concentrations of lamivudine (3TC) and tenofovir (TDF) when co-administered with Raltegravir (RGV) as compared to PK data obtained with food pellets solely containing 3TC and TDF (data presented earlier). The PK data obtained for RGV at the higher dose of 4.7 mg/kg food yielded plasma concentrations in mice laying in the effective therapeutic range as reported for humans\textsuperscript{25}.

This combination ART (cART) of two NRTIs (3TC and TDF) and an integrase inhibitor (RGV) proved to be highly effective in suppressing HIV RNA in the plasma of infected mice to below the limit of detection. The overall success rate in all experimentations presented here was 65/71 (91.5%) mice with suppressed plasma viremia.

In our earlier work we have reported the use of humanized mice for studying TMC278-LA and TMC181-LA in a galenic formulation allowing once weekly administration (long-acting drugs)\textsuperscript{2, 28, 29}. TMC278 is the former compound name of the recently (2011) approved NNRTI, rilpivirine\textsuperscript{30} and TMC181-LA is a prototype protease inhibitor that will most likely not be a candidate for clinical development.

We tested TMC278-LA in PrEP and PEP and found that it completely prevents infection when administered immediately at the same time point as the viral challenge (PEP) or when applied 1 day prior to HIV infection (PrEP). TMC278-LA showed a reasonable preventive
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effect when injected 1 or 3 days after HIV-1 infection, with an infection rate of 4/13 (30%) and 5/13 (38%) respectively. Pre- and post-exposure prophylaxis treatment strategies have been successfully studied in other studies using hu mice\textsuperscript{21,22,31}.

Next we wanted to test TMC181-LA, as well as an investigational peptide inhibitor targeting viral entry for their potency to maintain HIV plasma RNA levels below the limit of detection after initial suppression by cART applied to food pellets. Notably, we have shown in our previous work that TMC278-LA alone was not potent enough, but that a combination of TM278-LA and TMC181-LA was able to keep HIV suppressed\textsuperscript{2}.

Here we show that TMC181-LA mono-therapy is able to keep HIV suppressed in 4/5 (80%) mice. Impressively TMC181-LA mono-therapy was even potent enough to suppress HIV plasma RNA to below the limit of detection when used an initial treatment in 6/6 (100%) mice. The peptide inhibitor mono-therapy was only partially able to keep HIV virus undetectable in the plasma of 2/8 (25%) mice. An initial mono-therapy of daily injections of the peptide inhibitor resulted in a decrease of plasma viremia, and in case of one mouse to undetectable plasma viral load, but in all mice viral failure was observed during the time of treatment.

Since the cART consisting of 3TC TDF and RGV was able to successfully treat a larger cohort of animals we wanted to test the ability of the HDAC inhibitor SAHA for the reactivation of viral transcription. HDAC inhibitors block deacetylation of histone ends, thus causing a decondensation of the chromatin structure and thereby potentially rendering latent provirus accessible to cellular transcription factors such as NFκB, NFAT and AP-1.

In this pilot study mice were injected with a single dose s.c. injection of SAHA. We were not able to detect a “viral blip” of HIV plasma RNA, or a significant difference in cell associated HIV RNA transcripts in mouse spleen samples upon injection of SAHA. However, we could observe a slight change in the acetylation pattern of human CD45+ cells spleen cell samples of mice 48 hours after SAHA injection.

Possibly a single administration of SAHA or a missing additional stimulus of cellular transcription initiation could be possible reasons for the failure of the attempt. In future approaches one could think of combining one or several HDAC inhibitor injections with an additional immunostimulatory substance as for example prostratin. Prostratin signals via the PKC pathway ultimately leading to the release of sequestered NFκB, which could initiate transcription of HIV from integrated provirus.
Our presented standard ART will allow efficacy testing of novel anti-retroviral compounds in vivo in HIV infected humanized mice and would be superbly suited for successfully treating HIV infected humanized mice in order to be able to perform pre-clinical proof-of-concept approaches aiming at eradication of HIV.

In summary, we present PK data of RGV in humanized mice that permits the production of a non-toxic and impressively successful cART admixed to food pellets of mice. This successful non-toxic ART consisting of lamivudine (3TC), tenofovir (TDF) and raltegravir (RGV) can be used as a “gold standard” for testing novel antiretrovirals. In addition this cART allows the suppression of larger cohorts of HIV infected humanized mice in a reasonable timespan, thus enabling to study the potency of compounds for reactivating the HIV latent reservoir in vivo.

We emphasize the value of humanized mice in this respect by presenting data using long-acting formulations of the NNRTI TMC278-LA and the investigational PI TMC181-LA, as well as of an investigational peptide inhibitor in different ART schemes, e.g. PrEP, PEP, mono-therapy and maintenance regimens. Finally, in a proof of concept study we tried to reactivate latent HIV in vivo using the HDAC inhibitor SAHA and we will continue our efforts in this direction.

Overall humanized mice are a most valuable model for studying HIV infection, testing novel anti-retroviral drugs prior to their use in patients and for studying immunomodulatory substances for their ability to reactivate the HIV latent reservoir.

### 5.4 Material & Methods

#### Generation of humanized mice

Immunodeficient NOD/shi-scid Il2rg/- (NOG)\(^\text{13}\) or NOD/LtSz-scid Il2rg/- (NSG)\(^\text{14, 15}\) mice were reconstituted and infected as described previously in this thesis\(^\text{1, 2}\). In all experiments, mice were randomized based on their level of engraftment, date of birth, gender and their HIV RNA load after infection and/or initial suppressive ART into the different groups.

#### Production of food pellets containing anti-retroviral drugs & injectable drug preparations

Food pellets were generated by mixing 2.5 g of 3TC and TDF each, and 7 g or 23.5 g RGV with 5 kg of ground protein-rich, vitamin-fortified food (Nafag 3432, Provimi Kliba AG, Switzerland) which was subsequently formed to food pellets and sterilized by gamma-
irradiation with 25 kGy. Batches of food pellets were analyzed for the correct amount of
drugs admixed by HPLC (see below). Food and tap water were supplied ad libitum.
TMC278-LA and TMC181-LA were generated by wet milling the compounds to get
nanoparticles and subsequent formulation with non-ionic surfactants\textsuperscript{28, 29}. TMC278-LA and
TMC181-LA were injected s.c. at 160 and 400 mg/kg, respectively.
Peptide inhibitor (investigational compound JNJ 53769482; MW = 4666.98 mg/mM) was
injected s.c. at 2 mg/kg in form of a 20% cyclodextrin (CD) solution containing 0.4 mg/ml of
the peptide inhibitor.

**HPLC-MS/MS method for quantifying levels of RGV in plasma**
Concentrations of drugs in the plasma and food pellets were determined by a qualified
research liquid chromatography and mass spectroscopy (LC-MS/MS) method as described
earlier\textsuperscript{2}.

**HIV infection and RT-qPCR**
Mice were infected i.p. with HIV YU-2, 1x10\textsuperscript{6} tissue-culture infectious dose\textsubscript{50} (TCID\textsubscript{50}) per
mouse as described earlier\textsuperscript{2}. HIV RNA plasma levels were measured by RT-PCR
(AmpliPrep/COBAS TaqMan HIV-1 Test v2.0, Roche) at various times after infection\textsuperscript{2}.
Mice were monitored three times a week for symptoms or signs of adverse events, according
to a standard score sheet.

**Administration of SAHA in mice and incubation of human PBMCs with SAHA**
SAHA was applied by a single dose s.c. injection of 0.04 mg/g body weight prepared freshly
in a 20% cyclodextrin solution containing 4mg SAHA/ml.
Human PBMCs of three different donors were purified and subsequently incubated in RPMI
containing fetal calf serum (10%), IL-2 (10U/ml), penicillin (5%)/streptomycin (5%) and L-
glutamine (5%) at 37°C in 96-well plates (250000 cells/well). At the same time PBMCs were
incubated in the described medium containing different amounts of the HDAC inhibitors
(SAHA, 222, 348).

**Flow cytometry**
Human cells, T cells and B cells were measured by flow cytometry of white blood cells
stained for human CD45-APC, CD4-PerCP-Cy5.5, CD8-PB, and CD19-PE-Cy7 (all from BD
Biosciences).
Acetylation status of PBMCs and mouse spleen cells was determined by an intracellular staining using a primary antibody specific for the acetylated form of lysine 9 in histone 3 (H3K9). Surface antigens were stained using CD45-FITC, CD3-APC, CD4-PE-Cy7 and CD8-PB. As primary antibody we used anti-histone H3 (acetyl K9) antibody - ChIP Grade (ab10812, Abcam, United Kingdom) used in a concentration of 2 μg/ml in 50 μl and 100,000 cells. The isotype control antibody rabbit control IgG - ChIP Grade (ab46540, Abcam, United Kingdom) was also used at 2μg/ml in 50μl and 100,000 cells. As secondary antibody we used goat polyclonal antibody to rabbit IgG - Fc (DyLight® 594), pre-adsorbed (ab98464 Abcam, United Kingdom) in a 1:100 dilution in 50μl and 100'000 cells. Intracellular staining procedure was performed using BD Cytofix/Cytoperm wash kit for intracellular staining (Becton, Dickinson and Company, USA) according to the manufacturer instructions.

**qPCR analysis of mouse organ samples**

DNA and RNA of a mouse spleen were extracted simultaneously with the AllPrep DNA/RNA Kit (Qiagen). DNA qPCR was performed as described\(^2\),\(^3\),\(^2\), using HotStarTaq Master Mix (Qiagen), 1 μM of each primer and 0.1 μM FH probe. Experiments were done in duplicate with the real-time thermocycler IQ5 (BioRad) and the cycling profile: 95°C 15 min, 60× (95°C 5 s, 55°C 5 s, 60°C 40 s). The following oligonucleotides were used for HIV gag gene: mf319tq (probe): FAM5’-TGC AGC TTC CTC ATT GAT GGT-3’TAMRA\(^3\), ts5’gag (sense): 5’-CAA GCA GCC ATG CAA ATG TTA AAA GA-3’\(^2\) and skcc (antisense) (5’-TAC TAG TAG TTC CTG CTA TGT CAC TTC C-3’\(^2\)). The following oligonucleotides were used for the reference gene GAPDH: mf70tq (probe): FAM5’-AAG GTC GGA GTC AAC GGA TTT GGT CGT-3’TAMRA, mf45 (sense) 5’-TCG ACA GTC AGC CGC ATC TT-3’ and mf46 (antisense) 5’-GGC AAC AAT ATC CAC TTT ACC AG-3’. Mean normalized expression (MNE) was calculated with qbasePLUS (version 2.0, Biogazelle).

Since mice were infected with HIV-1 YU-2, the plasmid encoding YU-2 was used as standard in the PCR reactions with a detection limit down of about 5 copies/reaction.

**Reverse transcription**

RNA was DNase treated using DNA-free kit (Ambion). For reverse transcription random hexamer primers (Operon Technologies) and SuperScript III reverse transcriptase (Invitrogen) were used. Reverse transcription was performed as described earlier\(^2\),\(^3\); briefly cDNA synthesis was performed using 10 μl DNase treated RNA in the presence of Ribolock Rnase.
inhibitor (Fermentas) in a total volume of 50 μl as follows: 60 min at 50°C, 60 min at 55°C, 15 min at 70°C and then 1 min on ice. Subsequently 1 μl of RNaseH (NEB) was added to each tube and incubated at 37°C for 20 min. Aliquots were stored at -20°C or used immediately for real-time PCR analysis.

Calculations and statistics
Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software). In all figures, points represent values of individual mice or mean values of one group of mice, and lines depict mean values.

5.5 References
Chapter III

Humanized mice for ART testing and a pilot study to reactivate latently infected cells in vivo using SAHA (vorinostat)


6 Chapter IV: Optimization of lentiviral transduction of CD34+ hematopoietic progenitor/stem cells (HPSCs) for a humanized mouse model of HIV latency

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Declaration of contribution:

Marc Nischang, Annette Audige and Li Duo have equally contributed to the generation of the presented data.
6.1 Outline

For the study of HIV latency, the earlier described *in vivo* model based on humanized mice infected with wild type HIV under suppressive ART (Chapter 2 and 3) has some shortcomings, such as: i) the relatively long duration of about 6-7 months, i.e., generation of mice until engraftment (3-4 months), HIV infection and dissemination (1 month) and ART until suppression of HIV RNA (2 months), ii) the need to kill the mouse for performing the analyses, i.e. the one shot option to do analyses. In addition, sensitivity issues exist regarding the detection of HIV cell associated RNA in mice with suppressed viremia, which might be due to the low number of latently infected cells. Notably, the number of latently infected cells is considerably low in HIV-infected patients treated with ART with only 1 out of $10^6$ cells being in a latent state of infection\(^1\) and most likely is about the same in HIV infected mice.

Thus, we aim establishing a model of HIV latency based on a reporter gene under the control of the HIV LTR. For that purpose, we need to transduce CD34+ hematopoietic progenitor/stem cells (HPSCs) using a lentiviral reporter construct prior to their transplantation into mice. Provided that the progeny cells harbor this lentiviral construct, we would be able to monitor latently infected cells by this reporter gene. If the number of transduced cells is sufficiently high, we should be able to monitor the latently HIV infected cells subsequent to stimulating compounds even by bioluminescence imaging. Such a concept requires that transduction of CD34+ cells (gene engineering) is working well.

Gene engineering CD34+ cells would also permit to study the significance of individual genes for HIV pathogenesis, e.g. SOCS and APOBEC, and moreover for studying gene therapy approaches, all of which are ongoing research efforts of our group. Thus, the optimized lentiviral transduction protocol will not only be useful for establishing an alternative HIV latency model, but also for addressing other questions related to HIV infection in a humanized mouse model.

To obtain efficient transduction and engraftment of CD34+ hematopoietic progenitor cells, we compared different protocols focusing on the following read outs: i) transduction efficacy, ii) maintenance of expression of HPSC cell surface markers, and iii) engraftment levels in mice. The efforts made in this regard are described in this Chapter.

6.2 Results

We used a self-inactivating (SIN) lentiviral vector (henceforth referred to as Len-EF1-GFP), which harbors the enhanced green fluorescent protein (eGFP) under the control of the constitutive elongation factor 1-alpha (EF1-\(\alpha\)) promoter for transduction of CD34+ HPSCs
The EF1-α promoter was reported to result in a high and homogenous long-term expression of the transgene in all hematopoietic cell types including CD34+-derived T cells, B cells, NK cells, and myeloid hematopoietic cells, as well as NOD/SCID mouse repopulating cells\textsuperscript{2-4}. Production of lentiviral particles was achieved by co-transfection of the Len-EF1-GFP plasmid with the multiply attenuated packaging plasmid pCMV R8.91 and the vesicular stomatitis virus glycoprotein (VSV-G) envelope-encoding plasmid pMD2.G\textsuperscript{5} (Fig. 1) into 293T cells.

Figure 1: Schematic representation of the plasmids used for the production of lentiviral particles by co-transfection in 293T cells. A) SIN lentiviral reporter construct (Len-EF1-GFP) harboring eGFP under the control of the EF1-α promoter. The 5’-LTR is derived from Rous sarcoma virus (RSV) and the 3’ LTR has a deletion in the U3 region rendering the promoter of the integrated vector defective for transcription. B) Envelope plasmid (pMD2.G) encoding VSV-G. C) Packaging plasmid (pCMVΔR8.91) for the expression of Gag, Pol, Tat and Rev. The HIV env gene in the vector backbone is deleted (Δenv).

Virus supernatant was harvested 48 hours after transfection and subsequently concentrated approximately 100-fold using PEG-it virus precipitation solution (System Biosciences, Mountain View, CA), a polyethylenglycol based precipitation method\textsuperscript{6, 7}. Transduction units (TU) of each lentivirus batch were routinely titrated on SupT1 cells\textsuperscript{8}.

First, we compared two protocols published by van Lent et al.\textsuperscript{8} and Amsellem et al.\textsuperscript{9}. The main differences between these two protocols are the different duration of transduction and the different multiplicity of infection (MOI) used. Whereas in the van Lent protocol CD34+ cells are pre-stimulated in cytokine containing medium for 16 hours followed by 6 hour
transduction using an MOI of about 10-20, in the Amsellem protocol cells are not pre-stimulated but transduction is performed for 72 hours at a higher MOI of about 100-200. In case of the van Lent protocol, the medium used for transduction is supplemented with 20 ng/ml of human interleukin-7 (IL-7), stem cell factor (SCF), and thrombopoietin (TPO). The transduction medium in the Amsellem protocol contains the following human cytokines: SCF (100 ng), Fms-realted tyrosine kinase 3 ligand (Flt3L) (100 ng/ml), TPO (10 ng/ml), and IL-3 (60 ng/ml). We repeatedly achieved higher transduction levels in vitro and in vivo using the protocol published by Amsellem and therefore continued with this protocol with the modification, that we used retronectin coated plates as described by van Lent.

There are numerous commercially available media for culturing and/or expansion of CD34+ HSPCs. Thus, we compared several different media: i) Iscove's Modified Dulbecco's Medium (IMDM) containing 5% FCS and Yssel’s supplement, ii) StemPro medium + nutrients, and iii) HP01 medium, each supplemented with the following cytokines: SCF (100 ng/ml), Flt3L (100 ng/ml), interleukin 3 (II-3) (60 ng/ml) and TPO (10 ng/ml) for their capacity to maintain pluri-potency of the CD34+ cells, facilitate cell growth and yield efficient transduction rates.

The p24 concentration of the precipitated lentivirus supernatant used in the experiment shown in Fig. 2 was 6965 ng/ml and transduction units (TU) titrated on SupT1 cells were 7.88 x 10⁸ TU/ml. CD34+ cells (3 x 10⁶) were transduced at an MOI of 190, incubated for 3 days and subsequently analyzed by flow cytometry. The frequency of GFP+ cells was about 34% in case of IMDM and HP01 medium, but only about 27% with StemPro medium (Fig. 2 A and E). When gating on CD133+ cells, no difference regarding GFP expression existed between the different media and 25% of CD133+ cells were found to express GFP (Fig. 2 E).

CD133 (prominin-1) is a trans-membrane glycoprotein that is expressed on CD34+CD38− HPSCs¹⁰, ¹¹, as well as on the less well characterized rare human CD34− SRCs (severe combined immunodeficiency (SCID)-repopulating cells), a distinct class of primitive HPSCs. CD133 is found to be the best positive markers to increase the degree of purification of CD34− HPSCs¹² and can be generally used to successfully isolate and characterize both CD34+ and CD34− HPSCs¹².

Viability of the cells cultured in IMDM and HP01 medium was similar (~87%) and slightly reduced in the case of StemPro medium (~76%) (Fig. 2 A). We did not observe any differences between untransduced or transduced cells with regard to cell growth over three days (Fig. 2 B and D). Whereas the increase in the total number of cells on day 3 compared to day 0 was 3-fold with HP01 and ~2.5-fold with IMDM, the number of total number of cells decreased in the case of StemPro medium (Fig. 2 A-D). This pattern of expansion was similar
when gating on live cells, CD34+ cells, or CD133+ cells (Fig. 2 C). When analyzing the increase in total cell numbers for GFP+ cells the pattern did not differ (Fig. 2 F). Regarding the primitiveness of the cells as measured by the expression of HSPC markers, we did not find a significant difference in the percentage of cells expressing CD34 or CD133, but we did observe a difference in the mean fluorescence (MFI) intensity of these markers. Primitiveness of the HSPCs was best preserved in the StemPro medium and decrease in MFI was similar for IMDM and HP01 medium (Fig. 2 G).

**Figure 2:** Flow cytometry analysis of HSPCs on day 3 post transduction. Cells cultured and/or transduced in IMDM, StemPro and HP01 medium are represented by grey, black and white colored bars respectively. A) FACS plots showing percentage of live cells and GFP+ cells of HSPCs transduced in IMDM, StemPro and HP01 medium. B) Absolute number of transduced cells 3 days post transduction and of untransduced control cells cultured for 3 days in the same medium. C) Absolute numbers of transduced cells on day 3 post transduction of live cells, CD34+ cells and CD133+ cells. D) Fold increase of cells on day 3 post transduction as compared to the number of cells on day 0. E) Percentage of GFP+ cells of live cells, CD34+ cells and CD133+ cells. F) Absolute numbers of GFP+ cells of live cells, CD34+ cells and CD133+ cells. G) MFI of CD34 and CD133 on day 0 and 3 days post transduction.

Based on these results and because of the fact that HP01 is a completely synthetic medium without the need of supplementation with human or calf serum that might be of concern for phase I clinical trials, we continued our transduction experiments using HP01 medium.
We next wanted to examine the engraftment of transduced CD34+ cells transplanted into immunodeficient mice over time according to our protocol. The lentivirus stock used had $3.5 \times 10^8$ TU/ml, as titrated on SupT1 cells, and a p24 titer of 2.7 μg/ml. In total $2.44 \times 10^6$ CD34+ cells isolated and purified from a single cord blood donation were used for transduction. HSPCs were transduced with lentivirus at an MOI of 147 and after 72 hours cells had expanded 3.1-fold to $6.8 \times 10^6$ cells in total. Two litters of newborn NSG mice of n=12 and n=9 animals were transplanted by i.h. injection with the transduced cells using $3 \times 10^5$ cells per mouse. An aliquot of the transduced CD34+ cells was cultured for three additional days in vitro and analyzed by flow cytometry. Three days post transduction about 31% of all live cells or CD34+ cells were found to express GFP (Fig. 3).

**Figure 3:** Flow cytometry analysis of HSPCs on day 0 and 3 days post transduction. A) Percentages of live cells and CD34+ cells of HSPCs on day 0. B) HSPCs 3 days post transduction. Percentages of live cells and CD34+ cells as well as GFP+ cells of all live cells or of all CD34+ cells.

PBMCs from animals that were transplanted with transduced CD34+ cells were analyzed by flow cytometry for their engraftment levels, cellular subset distribution and GFP expression at 6, 8 and 12 weeks for both groups and in addition at 16, 20 and 24 weeks for the group of n=12 mice (#322-333) (Fig. 5). The gating strategy used in the analyses of GFP+ expression in the different hematopoietic cell subsets for a representative animal (#322) at 24 weeks post transplantation are shown in Figure 4 A and B.
Figure 4: Flow cytometry analysis of PBMCs of a representative mouse (#322) 24 weeks after transplantation of transduced CD34+ HSPCs. A) Gating strategy and cellular subsets. Cells were analyzed for CD45 as a panleucocyte marker, and CD19, CD3, CD14 and NKp46, as markers for B cells, T cells, monocytes and NK cells respectively. CD3+ cells were further analyzed for the ratio of CD4+ and CD8+ cells. B) All cellular subsets were analyzed for the expression of GFP.

Using the panhuman marker CD45, we observed an average engraftment level of human cells of about 51.68% (mice #322-333) and 38.89% (mice #334-342) 24 and 12 weeks post transplantation, respectively (Fig. 5 B and F).

Of all human CD45+ cells analyzed 57.25% were CD19+ B cells, 29.11% CD3+ T cells (20.27% CD4+ and 8.83% CD8+ T cells), 1.76% NKp46+ NK cells and 7.98% CD14+ monocytes for the group of n=12 mice (#322-333) 24 weeks after transplantation (Fig. 5 B).

For the second group of n=9 mice (#334-342) 73.52% were CD19+ B cells, 20.62% CD3+ T cells (11.41% CD4+ and 9.21% CD8+ T cells), 1.32% NKp46+ NK cells and 3.66% were CD14+ monocytes at week 12 after transplantation (Fig. 5 F).
Figure 5: Flow cytometry analysis of PBMCs of n=12 mice (#322-333) and n=9 mice (#334-342) after transplantation of transduced CD34+ HSPCs. A) Engraftment of human CD45+ cells in the peripheral blood of mice starting from week 6 until the end of the experiment (#322-333). B) Subsets of human hematopoietic cells analyzed 24 weeks post transplantation. Cells were analyzed for CD45 as pan leucocyte marker, and CD19, CD3, CD14 and NKP46, as markers for B cells, T cells, monocytes and NK cells respectively. C) Percentage of GFP expressing CD45+ cells over the entire time of the experiment. D) GFP expression of the different subsets of human cells at week 24 post transplantation. E) Engraftment of human CD45+ cells in the peripheral blood of mice starting from week 6 until the end of the experiment (week 12). F) Subsets of human hematopoietic cells analyzed 12 weeks post transplantation. G) Percentage of GFP expressing CD45+ cells over the entire time of the experiment. H) GFP expression of the different subsets of human cells at week 12 post transplantation.
Overall, the levels of CD45+ human cells increased in most of the animals over time (Fig. 5 A and E). All cell populations investigated were present in the transplanted mice, with B cells representing the dominant cell population (Fig. 5 B and F). Similar to the engraftment level, the number of GFP-expressing CD45+ cells increased in most of the animals over the time course of the experiment (Fig. C and G) and GFP+ cells were present in all cell subpopulations (Fig. 5 D and H). The percentage of GFP expressing cells in the different hematopoietic cellular subsets is 27.93%±8.55 of CD45+ cells, 18.26%±7.95 of CD3+ T cells, 17.67%±9.87 of CD4+ T cells, 23.14%±8.20 of CD8+ T cells, 33.52%±10.27 of CD19+ B cells, 50.72%±10.03 of NKp46+ NK cells, 33.92%±8.49 of CD14+ monocytes (mice #322-333) (Fig. 5 D) and 25.63%±3.77 of CD45+ cells, 11.80%±11.03 of CD3+ T cells, 13.50%±14.21 of CD4+ T cells, 11.16%±9.86 of CD8+ T cells, 28.87%±4.86 of CD19+ B cells, 42.54%±6.14 of + NKp46+ NK cells, 35.49%±3.57 of CD14+ monocytes (mice #334-342) (Fig. 5 G).

6.3 Conclusion

The overall aim of our group is to establish a HIV latency mouse model based on the lentiviral transduction of CD34+ cells. For this purpose we thought to ultimately use a functional lentiviral reporter construct in which one or two reporter genes (e.g. eGFP, luciferase, mCD48 receptor) are expressed under the control of the wt HIV LTR promoter, thus allowing screening for compounds that activate LTR driven gene transcription in mice transplanted with transduced CD34+ cells. An alternative option would be to include even a second reporter gene (e.g. mCD48 receptor) driven by a constitutively active promoter. This would allow the tracking and if necessary enrichment of transduced cells by the constitutively expressed transgene.

There is a certain risk that this strategy will not render a model in which the formation of a latency-like state of the integrated lentiviral construct accurately mimics the natural situation. Likewise, it is possible that the close proximity of a constitutively active promoter to the HIV LTR promoter interferes with the formation of a latency-like state.

In addition a reliable transduction protocol is also needed in our laboratory to address questions related to HIV pathology and for gene therapy.

In this chapter I have summarized the efforts made regarding establishment of a standard operation protocol for transduction of CD34+ cells that achieves a reasonably high number of
transduced cells, long term engraftment of the transduced cells in recipient mice and expression of the transgene in all hematopoietic subsets.

Retroviral vectors are commonly used for stable transduction of somatic cells since they have highly evolved mechanisms to perform gene transfer and integration into host cells\(^\text{16}\). However, insertional mutagenesis causing leukemia was documented in 25\% (5/20) of patients suffering from X-linked SCID (severe combined immune deficiency), which were treated using gammaretroviral vectors\(^\text{17, 18}\). Enhancements of retroviral vector safety led to the development of SIN vectors in which the promoter within the LTR is deleted and the transgene is expressed from an internal promoter. Lentiviruses, such as HIV-1, are able to stably transduce proliferating as well as non-proliferating cells\(^\text{19}\) and a further improvement was made when combining the safety of a SIN vector with the capacity of lentiviral vectors, to transduce non-dividing cells\(^\text{20, 21}\). Insertions near oncogenes in transduced human hematopoietic cells were significantly lower when SIN lentiviral vectors were used as compared to LTR-driven gammaretroviral vectors\(^\text{22}\).

We used a so called third generation lentivirus-based SIN vector (Len-EF1-GFP), which encodes GFP under the control of the EF1-\(\alpha\) promoter. Third generation lentiviral vectors contain a truncated version of the HIV 5’LTR, which is fused to a heterologous enhancer/promoter such as CMV or RSV, thereby making the Tat protein in the packaging plasmid dispensable. In the Len-EF1-GFP reporter construct that we used for establishing and optimizing the transduction protocol the HIV 5’LTR was fused to the RSV promoter and the eGFP gene was under the control of an additional, constitutive EF1-\(\alpha\) promoter. Since in the final construct we aim to use a lentiviral vector with wild-type HIV-LTR promoter we choose to use a 2nd generation packaging system that in addition supplies Tat in Trans.

We compared in this work two protocols published by van Lent et al. and Amsellem et al.\(^\text{8, 9}\) which differ in the parameters MOI, media, cytokines and transduction duration. We optimized the protocol regarding transduction efficacy and maintenance of primitiveness of hematopoietic progenitor. Since for some applications, such as gene therapy, a clinically relevant protocol is desired, we aimed to use serum-free culture conditions for transduction of HSPCs. We compared the culture media IMDM, StemPro and HP01 and found that the completely synthetic medium HP01 was the most appropriate medium for our purposes. StemPro medium was best for maintaining the primitiveness of CD34+ cells (as assessed by the cell surface expression of CD34 and CD133), but at the same time no expansion was observed. Absolute numbers of transduced GFP-expressing CD34+ and CD133+ cells were significantly higher in IMDM and HP01 medium as compared to StemPro. One has to make a
compromise between maintaining HSPC status, transduction efficiency and cell proliferation. Extensive proliferation of CD34+ cells has been shown to decrease the rate of primitive HSPCs and negatively influenced the long-term repopulating capacity of these cells.\(^{23-26}\) We analyzed the expression of GFP in various hematopoietic cell subsets in immunodeficient mice transplanted with transduced CD34+ cells over a time course of 24 weeks. We found an engraftment level of about 50% of human CD45+ cells 24 weeks post transplantation. On average 28% of CD45+ cells expressed GFP 24 weeks after transplantation. In all cell subpopulations analyzed, i.e. T cells, B cells, monocytes and NK cell, we found a reasonable percentage of GFP-expressing cells. Notably the lowest number of GFP+ cells was found in the T cell subset (~18-23%).

In summary we have established a reliable and efficient protocol for transduction of CD34+ HSPCs using a third generation lentivirus based SIN vector with an MOI of about 100-200 and completely synthetic transduction medium supplemented with cytokines necessary for the maintenance of CD34+ cells in culture as well as for increasing the transduction efficiency. This protocol is prerequisite before testing different versions of a HIV-based reporter construct harboring reporter genes under the control of the wt HIV LTR promoter. The protocol is also used in our group for addressing different questions related to HIV pathology, HIV latency and to study the effect of lentiviral-mediated gene knock down of the CCR5 co-receptor in CD34+ cells as a gene therapeutic strategy for HIV infection.

6.4 Material & Methods

Isolation of CD34+ cells from umbilical cord blood

Isolation and purification of CD34+ cells from umbilical cord blood was performed using immunomagnetic beads (Direct CD34 Progenitor Cell Isolation Kit, Miltenyi Biotec) as described previously\(^{15}\), with a yield of 0.5–4 x 10\(^6\) CD34+ cells from one donation (purity >90%). Purified CD34+ cells were cryopreserved and stored in liquid nitrogen until use.

Lentivirus production

VSV-G-pseudotyped lentivirus was produced by co-transfection of 293T cells with the SIN vector Len-EF1-GFP plasmid (kindly provided by Daniel Boden, Tibotec; Belgium), the multiply-attenuated packaging plasmids pCMVΔR8.9\(^5\) and the envelope plasmid pMD2.G\(^5\) (kindly provided by Didier Trono, École Polytechnique Fédérale de Lausanne, Switzerland) as previously described\(^8\). Briefly, 293T cells were grown in petri-dishes or cell culture flasks to 50-60% confluency and transfected using CaCl\(_2\) based transfection protocol (ProFection\(^\circledR\))
Mammalian Transfection System, Promega). Plasmids were used in a ratio of 32%, 51% and 17% for the pCMVΔR8.9, the Len-EF1-GFP and the pMD2.G plasmid respectively. 48 hours after transfection cells were analyzed for GFP expression under the microscope and virus containing supernatant filtered using 50 ml Steriflip® Filter Units (Merck Millipore) and concentrated using the PEG-it™ Virus Precipitation Solution (SBI System Biosciences), both according to the manufacturer instructions.

**Determination of transduction units (TU) of lentivirus supernatant**

The titer of lentivirus batches was evaluated by p24 ELISA and by determination of the transduction units (TU) using SupT1 cells as described earlier. For the latter 2 x 10⁵ SupT1 cells were seeded in a 24-well plate in IMDM (10%FCS, 1% Penicillin/Streptomycin). To each well decreasing amounts of lentiviral supernatant were added (100, 30, 10, 3 and 1µl) in a final volume of 100 µl of medium. SupT1 cells were washed 6 hours post transduction, harvested after three days and the percentage of GFP+ cells was measured by flow cytometry. The titer was calculated for each well using the following equation: transduction units (TU)/ml = [%GFP+] × [number of SupT1 cells] × 10/[used volume of lentiviral supernatant in µl]. This method is most accurate if only wells with a GFP+ frequency in a 10–30% range are considered for evaluation.

**Transduction of CD34+ cells**

Transduction of CD34+ HSPCs was performed with modifications as described earlier. Briefly, 24-well plates were coated overnight at 4°C or instead for 1 hour at 37°C with Retronectin (Clonetech, TaKaRa) according to the manufacturer’s instructions and as described by van Lent. CD34+ cells were thawed rapidly at 37°C, washed in 10 ml of PBS and centrifuged at 300 x g for 5 min. Cells were resuspended in 2 ml PBS, counted manually and split into 3 fractions: i) cells for flow cytometry analysis on day 0 (4 x 10⁵ cells in total), ii) cells (untransduced control) for flow cytometry analysis on day 3 (2 x 10⁵ cells in total), and iii) cells for transduction (2.5 x 10⁵ cells per mouse to be transplanted). Subsequently cells were centrifuged at 300 x g for 3 min and after careful aspiration of the supernatant resuspended in the corresponding medium or buffer: i) in 400 µl of FACS buffer, ii) in IMDM (Gibco, Life Technologies Corporation) + YSSEL’s medium (Gene-Probe Diaclone SAS), StemPro®-34 SFM (Gibco, Life Technologies Corporation), or HP01 medium (Macopharma) (1 x 10⁶ of cells/ml), iii) in lentivirus (MOI = 200) containing IMDM, StemPro or HP01 medium (1 x 10⁶ of cells/ml). Staining of cells resuspended in FACS buffer (day 0) were performed as described below. Cells resuspended in IMDM, StemPro or HP01 medium (with
or without virus) were transferred to a 24-well retronectin-coated plate (up to 0.5 ml or 5 x 10^5 cells/well). Cytokines were added to the medium at the following final concentrations: SCF (100 ng/ml), Flt3L (100 ng/ml), IL-3 (60 ng/ml) and TPO (10 ng/ml). Cells were cultured for 3 days. On day 3 after transduction, cells were resuspended, pooled and counted manually. For FACS analysis 5 x 10^4 cells per staining were incubated with antibodies for 20min and washed with FACS buffe. Acquisitions were performed on a CyAn ADP (Beckmann Coulter) and FACS data analysed with FlowJo (Tree Star).

6.5 References


7 Discussion

Despite the success of ART and the efforts made regarding prevention, about 35.3 million people were infected with HIV worldwide in 2012\(^1,2\). One major goal over the past decades has been the search for a prophylactic or even therapeutic vaccine against HIV, but so far no vaccine tested was effective to prevent HIV transmission or to cure HIV infection\(^3,4\). The question on how to purge the HIV latent reservoir in order to eventually approach eradication has become another main focus of the HIV research community\(^5\)-\(^10\).

In the beginning of the HIV pandemic the lack of a small animal model for studying HIV infection has hindered HIV research\(^11\). Such a model is highly needed for studying HIV transmission, for testing novel anti-retroviral compounds, as well as for studying the HIV latent reservoir and its eradication.

With the development of so called humanized mice in 1988 an alternative to SIV-based monkey models became available. The first humanized mouse models were generated by transplantation of human lymphoid tissue (fetal thymus and liver)\(^12\) or injection of human peripheral blood lymphocytes (PBL)\(^13\) into SCID mice and these mice were shown to be permissive to HIV infection\(^14\). These models, while very useful as first small animal models overall display certain characteristics which may be disadvantageous, i.e. limitation of HIV infection to the transplanted conjoint organoid of human origin in the SCID hu-Thy/Liv model, and the risk of graft-vs-host disease (GvHD) resulting in non-physiological immune activation in the hu-PBL-SCID mouse model. In addition, owing to the sole transient presence of human CD4+ and CD8+ T cells in the blood of reconstituted hu-PBL-SCID mice, this model did not permit to perform long term HIV infection experiments.

A novel generation of humanized mice are based on the transplantation of CD34+ cells into different immunodeficient mouse strains\(^15\), Balb/c-Rag2\(^{-/-}\)-IL2R\(\gamma\)c\(^{-/-}\) (BRG)\(^16\), NOD/Shi-scid II2R\(\gamma\)c\(^{-/-}\) (NOG)\(^17\) and NOD/LtSz-scid II2R\(\gamma\)cnull (NSG)\(^18,19\), and various studies compared the properties of the different models\(^20-23\). A sophisticated humanized mouse model using concomitant transplantation of human fetal tissue and CD34+ cells is the so called BLT mouse model (BLT = bone marrow, liver, thymus)\(^24,25\). The BLT mice excel by a thymus of human origin containing human mesenchymal cells. Consequently, T cells are selected in a human environment and are able to mount HLA-restricted immune responses\(^24-26\).

In all of these newer humanized mouse models a lymphoid system of human origin develops with stable multi-lineage hematopoiesis 12-15 weeks after transplantation of CD34+ cells. Favorably, the lymphoid system shows a phenotype with quiescent/activated and naïve/memory cells that is comparable to humans. These novel hu mice are highly susceptible
to high-titer and disseminated HIV infection\textsuperscript{27-32}. With a stable engraftment of human cells and a life span similar to that of wild type mice sustained HIV infection can be monitored for more than a year\textsuperscript{33}. We have reviewed the particular usage of humanized mice for HIV research as part of this thesis (Chapter 1)\textsuperscript{34}.

The overall aim of this work was to validate the humanized mouse model for studying various aspects of HIV infection and for testing novel anti-retroviral treatments. For this we needed to confirm that HIV infection in humanized mice mirrors key aspects of HIV infection in humans, such as suppression of HIV replication by ART, viral rebound after treatment interruption, viral failure in case of occurrence of resistance mutations and the existence of a HIV latent reservoir. We performed extensive PK analyses of anti-retroviral drugs, established and optimized anti-retroviral treatment protocols and tested novel anti-retroviral compounds and treatment regimens in HIV infected humanized mice. The development of a non-toxic ART allowed us to validate the existence of a latent reservoir in humanized mice and we could successfully reactivate latently infected cells \textit{ex vivo}.

Next to the humanized mice HIV infection model we wanted to establish a latency model based on transduction of CD34$^+$ cells. This included in a first step the evaluation of an efficient and stable transduction protocol using lentiviral vectors. The ultimate goal would be to have a lentiviral dual reporter construct in which one reporter is expressed under the control of the HIV wild type LTR promoter and the second reporter driven by a constitutively active promoter. This would allow the tracking and if necessary enrichment of transduced cells by the constitutively expressed transgene and screening for compounds which activate HIV LTR driven transcription \textit{in vitro} and \textit{in vivo} in mice transplanted with transduced HPSC.

In the following paragraphs I will discuss the different aspects of this work separately.

7.1 Humanized mice as a model for testing novel anti-retroviral compounds, treatment strategies and as a model for studying HIV latency

Hu mice are highly susceptible to HIV, characterized by high virus titers and dissemination into all lymphoid organs\textsuperscript{27-32}. Like in humans HIV replication can be examined by measuring HIV RNA copy numbers in the peripheral blood over time, thus humanized mice have become more and more important for studying HIV, for pre-clinical testing of novel anti-retroviral compounds and treatment interventions\textsuperscript{34-36}. In that context, a reference ART when testing novel compounds as well as for studying latency is of utmost importance.
In our work we have been focusing on the validation of humanized for ART testing using conventional anti-HIV compounds (RTV, AZT, 3TC, TDF and RGV) in order to establish a highly efficacious and non-toxic ART, as well as for efficacy studies of novel drugs, such as the recently approved NNRTI (TMC278, rilpivirine) in a long-acting nanoformulated galenic form, a developmental prototype PI (TMC181), related to the approved PI darunavir, also in a nanoparticle-based formulation and a developmental peptide-based HIV fusion inhibitor with a mode of action similar to T-20 (enfuvirtide).

A great effort was made by Stoddart et al. in validating the SCID-hu Thy/Liv mouse model by comparing the efficacy of four different classes of marketed ART drugs, including 3TC, emtricitabine, nevirapine, efavirenz, indinavir, atazanavir and enfuvirtide. In their work, the authors concluded that the 2nd generation of marketed ART drugs is more efficient in suppressing HIV than the first one. As true for many other studies this work is lacking more detailed PK analyses of the various compounds. In a more recent work Stoddart et al. tested an albumin-conjugated C34 peptide HIV-1 fusion inhibitor in the SCID-hu (thy/liv) mouse model in a PrEP setting. The conjugation of the peptide fusion inhibitor to albumin resulted significantly improved PK profile of the otherwise quickly cleared C34 peptide.

We performed PrEP and PEP studies using a NNRTI (TMC278, rilpivirine) in a nanoparticle based galenic form (Chapter 3). As outlined, we did not observe a pronounced protection when the long-acting rilpivirine was administered four or seven days before HIV infection in the PrEP setting. It would be a rational step to investigate what the efficacy of two long-acting drugs in the same setting would be. We assume that the addition of a peptide fusion inhibitor to a NNRTI (e.g. TMC278-LA) and/or PI (e.g. TMC181-LA) would be superior to one drug alone in preventing infection by targeting two subsequent steps in the viral replication cycle, i.e. viral entry and reverse transcription.

PrEP and PEP exposure studies have also been conducted by others in humanized mice. Special mentioning in this respect deserves the BLT humanized mouse model in that human immune cells extensively engraft into mucosal tissues enabling transmission of HIV across mucosal surfaces. BLT mice were successfully used to test: i) orally administered emtricitabine (FTC) and tenofovir (TDF) as PrEP using intravaginal infection; ii) orally administered FTC and TDF as PrEP using rectal and intravenous infection; iii) topical applied TDF as PrEP using vaginal infection, iv) injected CD4 aptamer-siRNA chimeras as PreP using intravaginal infection, and most recently v) vectored immunoprophylaxis (VIP) as PrEP using intravenous as well as vaginal challenge with diverse HIV strains and repeated exposures. The uniqueness of the BLT mice in this respect is the option to use a natural...
route of infection and an adapted HIV challenge resulting in infectivity comparable to that in humans. Thus, this model allows testing of preventive treatments under conditions that are very closely mimicking the reality. Notably, in our PEP and PrEP studies we used high titer HIV stocks and i.p. injection as route of infection, resulting in a much higher viral challenge. This results most likely to an underestimation of the true efficacy of the drug in a natural infection.

Next to BLT mice also RAG mice allow transmission of HIV via vaginal and anal infection routes, but the engraftment of the gastrointestinal tract rendering them susceptible to mucosal transmission only occurs in mice transplanted with CD34+ cells derived from human fetal liver that were subsequently cultured overnight with IL-3, IL-6 and SCF and not in RAG mice transplanted with CBL derived CD34+ cells.⁴⁶, ⁴⁷

Pharmacokinetic (PK) analyses of anti-HIV drugs in humanized mice

Since the PK of a drug is species specific and PK data for each compound a prerequisite in order to find the optimal dosage, we performed extensive pharmacokinetic analyses for the different anti-retroviral compounds used in the HIV infection experiments (Chapter II and III).⁴⁸ With the few exceptions of work from the Goldstein group⁴⁹-⁵¹ and of recently published work from the Gendelman group by Dash⁵², Gautama⁵³ and Roy⁵⁴, we have to realize that thorough PK analyses of the various compounds which were examined for their anti-HIV effects were not done.

There are multiple ways drugs can be administered to mice, i.e. by oral administration of the compounds (drinking water, gavage or supplementation in food), or by injection (i.p. or s.c.). Since ART is to be given for weeks or months daily gavage or daily injections are very stressful for the mice and we therefore preferred adding the drugs to food pellets. Dosing is most likely less precise if drugs are added to the drinking water and in addition stability in aqueous solution is unknown for most compounds. We used the formula published by Reagan-Shaw et al. to convert drug dosages for oral uptake from humans to mice based on measured daily food intake, weight and metabolic rate⁵⁵. We then verified in PK studies whether the food pellets containing the drugs gave the expected PK data. Indeed, the calculated dosages for TDF and 3TC were confirmed by their plasma concentrations over 24 hours that were within the therapeutic range as observed in patients (Chapter 2). In a 2nd step, we performed PK analyses of RGV also added to food pellets. The initially calculated dose for RGV gave concentrations clearly below the therapeutic range and was increased by 3.5 fold in order to yield plasma concentrations in the therapeutic range (Chapter 3).
combination ART consisting of 3TC, TDF and RGV proofed to be very efficacious in suppressing disseminated high titer viremia in HIV infected humanized mice (Chapter 3). Notably the calculated amounts for AZT and RTV did not result in the expected plasma levels, with AZT being in a toxic range and ritonavir being substantially under the inhibitory concentration 50 (IC\textsubscript{50}) (Chapter 2)\textsuperscript{48}. We observed AZT associated toxicity displayed by weight loss and symptoms of wasting in HIV-infected humanized NOG mice dosed with ~66 mg/kg/d AZT admixed to food pellets. Using the same dosage of AZT in a pilot experiment with uninfected NOG mice we did not see any toxicity, which might be explained by the observation that the HIV-1 Tat protein potentiates AZT-induced cellular toxicity in mice\textsuperscript{56}.

Comparable combination ART regimens consisting of two or three drugs have been successfully used by others to suppress HIV viremia in infected humanized mice, despite lacking PK data\textsuperscript{57-59}. Briefly, Marsden et al. used a combination of AZT and indinavir sulfate-didanosine (ddI)\textsuperscript{59}, Denton et al. used FTC, TDF and RGV\textsuperscript{58} and Choudhary et al. used TDF, FTC and T20\textsuperscript{57}.

Perhaps owned to the fact that AZT was the first approved anti-HIV drug on the market, it is by far the compound most studied in humanized mice\textsuperscript{50, 60-70}. AZT has been administered in dosages ranging from 60 to 480 mg/kg/d by various different routes: i.p injections, orally by gavage or by adding AZT to the food pellets or drinking water or by osmotic minipumps placed under the skin. There is only a limited number of reports studying PK and toxicity of AZT and the degree to which AZT associated toxicity could have an influence on its inhibitory effect remains unknown\textsuperscript{49, 71-73}.

Of particular interest were the PK analyses of the two long-acting drugs TMC278-LA and TMC181-LA which were injected s.c. once weekly. TMC278-LA is the recently approved NNRTI rilpivirine (TMC278)\textsuperscript{37, 38} in a long-acting nanoparticle-based galenic form, TMC181-LA a developmental prototype PI (TMC181), related to the approved PI darunavir, also in a nanoparticle-based formulation. We identified dosages that resulted in concentrations clearly above the EC\textsubscript{50} up to 14 days after its administration confirming their potential to be used in a simplified once weekly application scheme (Chapter 2).

Notably, our group reported the first evaluation of anti-HIV drugs in a nanoformulated galenic form (Chapter 2)\textsuperscript{48}. Studies from the Gendelman lab have also assessed the potency of nanoparticle based drugs in HIV-infected humanized mice in which they carefully checked biodistribution, pharmacodynamics and toxicity of the used compounds\textsuperscript{52-54}. 

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**Humanized mice recapitulate key features of HIV infection in humans**

We demonstrated that hu mice recapitulate key features of HIV infections similar as in HIV-infected patients. The main findings were that hu mice showed sustained and disseminated infection in mice 4 weeks after HIV challenge by the intraperitoneal route. We presented successful suppression of HIV replication treated with different ART regimens and further utilized the model to test different treatment strategies such as pre- and post-exposure prophylaxis, as well as simplified ART schemes using one or two drugs to maintain viral suppression. Importantly, we observed a prompt rebound of plasma viral HIV RNA to levels that were equivalent to pre-treatment viremia after treatment interruption, viral failure in case of occurrence of prototype resistance mutations as observed in clinical trials and we could demonstrate the existence of a HIV latent reservoir, all which are hallmarks of HIV infection in humans (Chapter 2 and 3).

**Emergence of viral resistance mutations in case of poor adherence to ART and viral rebound after ART interruption**

In case of non-adherence to ART we observed treatment failure and by analyzing samples from mice which experienced viral rebound during ART we could detect prototype resistance mutations that are also seen in patients receiving a comparable regimen consisting of TMC278 (rilpivirine), TDF and FTC\(^{37, 38, 74, 75}\). We found the NRTI resistance mutation M184I conferring resistance to 3TC, as well as the NNRTI resistance mutations E138K and K101E conferring resistance to rilpivirine (RPV, TMC278). These were also the most frequently observed mutations in the Phase 3 clinical trials ECHO and THRIVE\(^{37, 38, 74, 75}\). Moreover in our work and in the ECHO and THRIVE trials the M184I mutation preceded the E138K mutation, suggesting that patients with the M184I are particularly prone to viral failure with a triple ART regimen consisting of TDF, TMC278 and 3TC or FTC. Recently it also has been proposed based on *in vitro* experiments and by analyzing patient PBMC samples that the M184I and E138K mutations may pre-exist prior to drug exposure as a result of APOBEC3 editing\(^{76}\). Humanized mice are mimicking accurately what was observed in patients highlighting the preclinical proof-of-concept value of this mouse model for HIV infection.

**Recovery of CD4 T cell population in successfully treated mice**

We repeatedly observed a CD4+ T-cell loss determined by the CD4/CD8 cell ratio in mice upon infection with HIV as compared to uninfected animals. Furthermore we found that ART restored the CD4+ T cell population and we detected a substantial difference in CD4/CD8 cell
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ratio in ART treated animals as compared to infected but untreated control mice (Chapter 2 and 3). In patients increasing CD4+ T cell counts during potent anti-retroviral therapy (ART) is a characteristic of immune recovery\textsuperscript{77, 78}. Very recently it has been shown that an early start of ART results in an enhanced CD4 T-cell recovery in patients\textsuperscript{79}.

**Reactivation of latently infected cells and eradication approaches**

ART interruption resulted in rapid viral rebound to similar plasma titers as before start of treatment in all experiments performed (Chapter 2 and 3). Notably these mice were treated successfully for a longer time period with no detectable viral RNA in the plasma to ensure the decline of cells with low virus replication. In addition we could find in all mice with suppressed viremia cell-associated proviral DNA emphasizing the existence of a stable HIV latent reservoir in HIV-infected hu mice. The levels of cell-associated HIV proviral DNA were higher in untreated as compared to treated mice reflecting the situation in humans\textsuperscript{80}.

HIV infection persists in patients treated with ART. In fact latently infected cells can persist for decades in patients even during effective ART\textsuperscript{81}. In a long-term follow up study conducted by the Siliciano group in patients on suppressive ART with no detectable HIV RNA for as long as 7 years the latent reservoir displayed such a slow decay rate, with a $t_{1/2}$ of about 44 months, that it is unlikely to eradicate HIV solely with ART\textsuperscript{82}. Even though different cellular reservoirs might contribute to the maintenance of HIV during ART the biggest, probably most important and best understood is the reservoir of latently infected memory CD4+ T cells\textsuperscript{81, 83-85}.

In a latent state of infection these long-lived memory CD4+ T cells harbor replication-inactive HIV provirus that is not susceptible to ART or immune effector functions. However, if the infected cell becomes stimulated, the latent virus can be reactivated.

Recent reports about the existence of a HIV latent reservoir in HIV infected humanized mice undergoing suppressive ART have inspired the interest in humanized mouse models for studying the HIV latency, to utilize it for testing reactivating compounds and to ultimately perform potential eradication approaches.

We observed an increase of HIV mRNA transcripts in spleen samples obtained from HIV-infected mice with suppressed HIV RNA upon *ex vivo* activation using a combination of the mitogens PMA and PHA in concert with anti-CD3/28 antibodies and IL-7 (Chapter 2). This clearly underlines the existence of latently infected cells, in which HIV transcription can be triggered by potent mitogens. Similarly, Brooks et al. infected SCID-hu (thy/liv) with a NL4-3 reporter virus in which vpr was replaced by muCD24. Isolation of CD4+/CD8–/muCD24–
thymocytes and subsequent \textit{ex vivo} stimulation using anti CD3/CD28 antibodies, IL-7 and/or the phorbol ester prostratin in the presence of AZT and a viral protease inhibitor induced viral gene expression and rendered the latently infected cells susceptible to treatment with an anti-HIV immunotoxin\textsuperscript{86}. As mentioned before three other reports exist besides our work that demonstrated the successful \textit{ex vivo} stimulation of latently infected cells\textsuperscript{57-59}.

Marsden at al. used a modified version of HIV NL4-3 (NLHSA) in vpr was replaced by the heat stable antigen (HSA) under control of the HIV LTR to infect BLT mice. Five weeks post infection splenocytes were collected, depleted of HSA expressing cells representing productively infected cells, pooled and subsequently stimulated with i) anti CD3 and CD28 antibodies in the presence of IL-2, ii) Prostratin or iii) 12-deoxyphorbol-13-phenylacetate (DPP) in the presence of Raltegravir. Subsequent to either stimulation a significant increase of reactivated latently infected cells was found as assessed by Gag mRNA expression.

Notably, the splenocytes used by Marsden et al. were, other than in our work and the studies by Choudhary et al. and Denton et al., not obtained from mice that were successfully treated over a longer time by suppressive ART. The latter two studies are consistent with our data presenting successful suppression of HIV in infected humanized mice with ART and verification of the existence of HIV latency by \textit{ex vivo} activation of HIV transcription\textsuperscript{58, 87}.

Thus, humanized mice are very promising for studying the latent reservoir and in particular suitable for \textit{in vivo} approaches aiming at reactivation of latently infected cells. In a first pilot experiment we aimed to reactivate HIV transcription \textit{in vivo} in HIV infected mice with suppressed plasma by using the HDAC inhibitor SAHA, but were unable to detect viral replication in the plasma of mice after SAHA injection (Chapter 3). In addition we did not observe an increase in cell-associated HIV RNA levels in spleen samples of mice receiving SAHA. It is possible that multiple doses of SAHA or an additional stimulus are needed to successfully trigger HIV transcription.

In a first study by Archin et al., using isolated resting CD4\textsuperscript{+} T cells of eight patients with fully suppressed HIV RNA by ART, a single dose of vorinostat resulted in an improved cellular acetylation pattern and a mean increase of 4.8 fold in HIV RNA expression\textsuperscript{88}. However these results could not be reproduced in a follow up clinical trial, in which five patients in whom resting CD4\textsuperscript{+} T-cell–associated HIV RNA (rc-RNA) increased after stimulation with vorinostat previously, agreed to receive daily doses of vorinostat three times a week for 8 weekly cycles. Even though the initial dose of vorinostat resulted in an increase in rc-RNA, only in 3/5 participants the measured rc-RNA still increased significantly after recurrent injections suggesting a blunted response after repeated dosing of vorinostat.
In future approaches one could think of combining an HDAC inhibitor with an immunostimulatory substance, e.g. prostratin that signals via the PKC pathway. Such a stimulus would ultimately lead to the release of sequestered NFκB and consequently could be potentially more successful in initiating HIV transcription.

In recent work the Nussenzweig group demonstrated that control of viremia in HIV infected humanized mice can be realized by immunotherapy using combinations of broadly neutralizing antibodies (bNAbs)\textsuperscript{89, 90}, and moreover that prolonged viremic control was achieved by a single bNAb after initial suppressive ART was withdrawn\textsuperscript{91}. Consequently in proof of concept work they confirmed that broadly neutralizing antibodies in concert with stimulating compounds resulted in a decrease in rebound of HIV RNA from latently infected cells humanized mice\textsuperscript{92}, a first step towards eradication of HIV. These data illustrate the value of humanized mice for studying the latent reservoir of HIV and in particular that the humanized mouse model is useful for studying innovative therapeutic approaches targeting the latent reservoir.

**Future perspectives:**

Even though humanized mice are a valuable and well established model for studying HIV and other human infectious agents, there still remain many chances to further optimize the recipient mouse and to improve the engrafted human immune systems.

Advancements regarding murine innate immune responses were achieved by developing human SIRPα transgenic mice\textsuperscript{93}, or by transduction of HPSCs with the SIRPα ligand (CD47)\textsuperscript{94}. As outlined before alleles in SIRPα are the causative for the difference of engraftment levels between mouse strains. The higher engraftment levels observed in NOD mice can be explained by the fact that SIRPα on NOD murine macrophages has enhanced binding to the human CD47 ligand, thereby inhibiting phagocytosis of the xenograft\textsuperscript{95, 96}. Engraftment level of CD34+ cells increased to a level similar to that in NSG mice and the functionality of the immune system was improved by transgenic expression of SIRPα in Rag2-/-γc-/- mice\textsuperscript{93}.

Moreover, human HLA Class I or Class II expression in mice resulted in enhanced adaptive immune responses in hu mice\textsuperscript{97, 98}. Finally, exogenous supplementation of human cytokines and factors, or generation of equivalent transgenic mice further improves human cell engraftment, as reported for GM-CSF, IL-4, M-CSF for monocyte/macrophage lineages, IL-7 for T cells, IL-15 for NK cells, BAFF for B cells and EPO for erythrocytes\textsuperscript{99}.
These “next-generation” mice will most likely become important for studying, pathogenesis of human infectious diseases, for testing therapeutics and vaccines and for studying the immune responses to viral infections in humanized mouse models with improved functionality of the engrafted human immune system\textsuperscript{15,100}.

Irrespective of the benefits achieved with each single change in the humanized mouse models, a standardization for certain purposes such as ART testing is highly desirable in order to achieve comparability of results. Possible parameters for standardization include choice of human mouse model, route and dose of infection, HIV strains used, settings for pre- and post-exposure prophylaxis, level of detection of HIV RNA and dosages of anti-retroviral compounds as well as corresponding PK analyses.

There is a recognizable gap between in vitro generated data and later clinical applications. A recent in-depth comparison of latent HIV-1 reactivation in multiple cell model systems of HIV latency as well as in resting CD4+ T cells from patients revealed substantial differences regarding the response to different stimuli\textsuperscript{101}.

Notably such differences might to a lesser extent also exist when using different humanized mouse model to study HIV infection and in particular HIV latency. A comparative effort as performed for the in vitro models of HIV latency is desirable to evaluate such potential differences.

Humanized mouse models will be essential concerning future eradication approaches and efficacy studies bridging the gap between in vitro latency models and clinical studies.

### 7.2 Towards a HIV latency model based on lentiviral transduction of CD34+ cells

While the HIV model based on HIV infected humanized mice under suppressive ART remains the gold standard to test efficacy of potentially reactivating compounds, a simplified model to screen for compounds that reactivate latent HIV is greatly required.

The overall aim of our group is to establish a versatile HIV latency mouse model based on the lentiviral transduction of CD34+ cells. The ultimate goal of these efforts would be to have a functional lentiviral reporter construct in which one or two reporter genes (e.g. eGFP, luciferase, mCD48 receptor) are expressed under the control of the HIV wild type LTR promoter, thus allowing screening for compounds that activate LTR driven gene transcription, i) in vitro in transduced cells, and ii) in vivo in mice transplanted with transduced CD34+ cells. An alternative option would be to include even a second reporter gene (e.g. mCD48
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receptor) driven by a constitutively active promoter. This would allow the tracking and if necessary enrichment of transduced cells via the constitutively expressed transgene.

It is unpredictable if this strategy will result in the formation of a latency-like state of the integrated lentiviral construct that mimics the natural situation. It is possible that the close proximity of a constitutively active promoter to the HIV LTR promoter could interfere with the formation of a latency-like state.

In addition to the HIV latency model based on lentiviral transduction a reliable transduction protocol is also needed in our laboratory to address questions related to HIV pathology, i.e. knockdown of SOCS (suppressor of cytokine signaling), and for studying the effects of a knockdown of the CCR5 co-receptor in hematopoietic cells regarding their susceptibility to HIV infection.

Consequently our laboratory made a major effort to establish a standard operation protocol for transduction of CD34+ cells that achieves a reasonably high number of transduced cells, as well as long term engraftment of the transduced cells in recipient mice and expression of the transgene in all hematopoietic subsets. To this extend we used a SIN lentiviral vector construct in combination with a second generation packaging system. Third generation lentiviral vectors contain a truncated version of the HIV 5’LTR, which is fused to a heterologous enhancer/promoter such as CMV or RSV, thereby making the Tat protein in the packaging plasmid dispensable. In the Len-EF1-GFP reporter construct that we used for establishing and optimizing the transduction protocol the HIV 5’LTR was fused to the RSV and the eGFP gene was under the control of an additional, constitutive EF1-α promoter. Since in the final construct we aim to use a lentiviral vector with wild-type HIV-LTR promoter we choose to use a 2nd generation packaging system that supplies Tat in Trans. A more detailed discussion on this topic is given in Chapter V.

Essentially we started off by comparing two protocols published by van Lent et al. and Amsellem et al. and by rationally testing and modifying parameters such as MOI, medium, cytokines, pre-stimulation and transduction duration we improved the protocol for our needs concerning efficacy, maintenance of primitiveness of the HPSCs, engraftment of transduced cells in recipient mice and perhaps most importantly transgene expression in all hematopoietic subsets. We obtained the best results regarding expansion and maintenance of progenitor/stem cell markers using HP01 medium, a completely synthetic medium that does not require the addition of human or fetal bovine serum. Because of potential later applications such as gene-therapy we aimed to use parameters suitable for clinical use. All cytokines used in the final transduction medium, i.e. stem cell factor (SCF; 100 ng/ml), Fms-
related tyrosine kinase 3-ligand (Flt3-L; 100 ng/ml), interleukin 3 (IL-3; 60 ng/ml) and thrombopoietin (TPO; 10 ng/ml) are commercially available in clinical grade quality. SCF binds to the c-Kit receptor, which is expressed on HPSCs as well as on different hematopoietic progenitor cells including erythroblasts, myeloblasts, and megakaryocytes\textsuperscript{104-106}. Activation of c-Kit is essential for the survival of HPSCs and induces multiple signaling cascades (RAS/ERK, PI3-Kinase, Src kinase, and JAK/STAT)\textsuperscript{107}. TPO binds to the TPO receptor (TPO-R) that is expressed on platelets, megakaryocytes, and megakaryocytic precursors. TPO is mainly produced by parenchymal cells and sinusoidal endothelial cells in the liver and stromal cells in the bone marrow and is a key cytokine involved in thrombopoiesis\textsuperscript{108, 109}. Flt3-L is structurally homologous to SCF and binds to FLT3/FLK2 receptor tyrosine kinase, which is in turn closely related to the c-Kit receptor\textsuperscript{110}. FLT3 is expressed in various lymphohematopoietic cells and Flt3-L is stimulating the proliferation and differentiation of various blood cell progenitors\textsuperscript{111, 112}. Combinations of the cytokines SCF and TPO\textsuperscript{103} or SCF, Flt-3L and TPO\textsuperscript{102, 113} are standardly included in protocols for culturing or transduction of CD34+ cells. There is a controversy if addition of IL-3 has a pronounced beneficial effect on transduction of HPSCs. While some claim that they observed only little effects when adding IL-3\textsuperscript{114}, others showed that IL-3 had a positive effect on transduction efficiency, in vivo lymphoid reconstitution capacity and long-term engraftment of stem/progenitor cells in patients\textsuperscript{115}. Applying our finalized protocol we were able to repeatedly achieve transduction rates of about 30-40% transduced CD34+ cells (Chapter V; Fig.2 and 3). The total duration of the incubation with lentivirus containing medium was 72 hours and during this period the total number of HPSCs expanded in average 2-4 fold. Massive cell expansion of CD34+ cell populations was proven to be accompanied by a loss of primitiveness and ability to give raise to long-term engraftment\textsuperscript{116-119}. Nevertheless after 72 hours we could not detect a significant difference in the percentage of CD34+ or CD133+ cells, but a decrease in the mean fluorescence intensity of the CD34+ and CD133+ cell populations indicating the start of progressing loss of primitiveness (Chapter V Fig. 2). Thus we believe to have found a good balance between maintaining primitiveness of HPSCs and a reasonable transduction efficiency and proliferation. Moreover, we were able to detect long-term engraftment of transduced cells in recipient NSG mice transplanted with transduced CD34+ up to 24 weeks (168 days) after transplantation and transgene expression in all tested hematopoietic subsets (B, T, NK cells and monocytes). We observed in average a somewhat lower transgene expression in the T cell compartment of about 18% GFP+ cells as compared to B cells, NK...
cells and monocytes, which had in average 33%, 50% and 33% GFP+ cells respectively. This is consistent with observations that transgene expression in T cells is lower as compared to other lymphocyte sub-populations when using an EF1-α promoter and is completely lacking when using the exogenous CMV promoter\textsuperscript{120}.

**Future perspectives:**
In first subsequent experiments performed by members of the Speck group using a lentivirus reporter construct (Len-SGA48) harboring the genes for eGFP and the murine CD48 receptor (mCD48) under control of the wt-HIV LTR promoter we were able to achieve good transduction rates of CD34+ cells in vitro analyzed after 72 hours after transduction. However in PBLs, thymus and spleen cell samples from NSG mice transplanted with transduced CD34+ cells we were not able to detect eGFP/mCD48 expression after ex vivo stimulation (data not presented). Efforts to successfully analyze HIV LTR driven transcription using lentiviral reporter constructs are ongoing and a novel vector construct will be used in this respect, in which luciferase is included as a reporter gene permitting detection of transgene expression by in vivo imaging of a whole mouse using the IVIS platform (PerkinElmer).

Next to this the described transduction protocol is used in the future for other research projects in our laboratory looking at shRNA mediated knockdown of SOCS and CCR5.

### 7.3 References

Discussion

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### List of abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3TC</td>
<td>Lamivudine</td>
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<tr>
<td>Abb.</td>
<td>Abbreviation</td>
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<tr>
<td>ABC</td>
<td>Abacavir</td>
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<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<td>AP-1</td>
<td>Activator Protein 1</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>APOBEC</td>
<td>Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like</td>
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<td>Barrier-to-autointegration factor</td>
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<td>bp</td>
<td>Base pair</td>
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<td>Combination anti-retroviral therapy</td>
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<td>CBP</td>
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<td>CDK9</td>
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<td>Crm1</td>
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<td>Etravirine</td>
</tr>
<tr>
<td>EVG</td>
<td>Elvitegravir</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FOS-APV</td>
<td>Fosamprenavir</td>
</tr>
<tr>
<td>FTC</td>
<td>Emtricitabine</td>
</tr>
<tr>
<td>Gag</td>
<td>Group specific antigen</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GI</td>
<td>Gastro-intestinal</td>
</tr>
<tr>
<td>Gp120</td>
<td>Glycoprotein 120</td>
</tr>
<tr>
<td>Gp41</td>
<td>Glycoprotein 41</td>
</tr>
<tr>
<td>GvHD</td>
<td>Graft-versus-host disease</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyl transferase</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HDAC-i</td>
<td>Histone deacetylase inhibitor</td>
</tr>
<tr>
<td>HEXIM1</td>
<td>Hexamethylene bisacacetamide-inducible protein 1</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency viruses</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HMBA</td>
<td>Hexamethylbisacetamide</td>
</tr>
<tr>
<td>HMGs</td>
<td>High-mobility group proteins</td>
</tr>
<tr>
<td>HPSC</td>
<td>Hematopoietic progenitor/stem cell</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Herpes Simplex Virus Type 2</td>
</tr>
<tr>
<td>HTLV</td>
<td>Human T-lymphotropic virus</td>
</tr>
<tr>
<td>i.h.</td>
<td>Intrahepatic</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IDV</td>
<td>Indinavir</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>ILR2g</td>
<td>Interleukin-2 receptor γ-chain</td>
</tr>
<tr>
<td>IN</td>
<td>Integrase</td>
</tr>
<tr>
<td>kGy</td>
<td>Kilogram</td>
</tr>
<tr>
<td>KS</td>
<td>Kaposi's sarcoma</td>
</tr>
<tr>
<td>LAV</td>
<td>Lymphadenopathy-associated virus</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LEDGF/p75</td>
<td>Lens epithelium-derived growth factor</td>
</tr>
<tr>
<td>LPV</td>
<td>Lopinavir</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MA</td>
<td>Matrix</td>
</tr>
<tr>
<td>MVC</td>
<td>Maraviroc</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleocapsid</td>
</tr>
<tr>
<td>Nef</td>
<td>Negative replication factor</td>
</tr>
<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NFV</td>
<td>Nelfinavir</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation signal</td>
</tr>
<tr>
<td>NNRTI</td>
<td>Non-nucleoside reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibitors</td>
</tr>
<tr>
<td>Nuc-1</td>
<td>Nucleosome 1</td>
</tr>
<tr>
<td>NVP</td>
<td>Nevirapine</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>ORI</td>
<td>Origin of replication initiation</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Primer binding site or Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCP</td>
<td>Pneumocystis carinii pneumonia</td>
</tr>
<tr>
<td>PEP</td>
<td>Post-exposure prophylaxis</td>
</tr>
<tr>
<td>PI</td>
<td>Protease inhibitor</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-integration complex</td>
</tr>
<tr>
<td>Pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>PPT</td>
<td>Polypurin tract</td>
</tr>
<tr>
<td>PR</td>
<td>Protease</td>
</tr>
<tr>
<td>PrEP</td>
<td>Pre-exposure prophylaxis</td>
</tr>
<tr>
<td>PTB</td>
<td>Polypyrimidine tract binding protein</td>
</tr>
<tr>
<td>P-TEFb</td>
<td>Positive transcriptional elongation factor B</td>
</tr>
<tr>
<td>rA</td>
<td>Adenin ribonucleotide</td>
</tr>
<tr>
<td>Rev</td>
<td>Regulator of expression of viral proteins</td>
</tr>
<tr>
<td>RGV</td>
<td>Raltegravir</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAPII</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>RPV</td>
<td>Rilpivirine</td>
</tr>
<tr>
<td>RRE</td>
<td>Rev-responsive element</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RTC</td>
<td>Reverse transcription complex</td>
</tr>
<tr>
<td>RTV</td>
<td>Ritonavir</td>
</tr>
<tr>
<td>SAHA</td>
<td>Suberoylanilide hydroxamic acid</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
</tr>
<tr>
<td>SHIV</td>
<td>Chimeric SIV/HIV virus</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency viruses</td>
</tr>
<tr>
<td>SQV</td>
<td>Saquinavir</td>
</tr>
<tr>
<td>ss</td>
<td>Single stranded</td>
</tr>
<tr>
<td>SU/gp120</td>
<td>Surface protein</td>
</tr>
<tr>
<td>T20</td>
<td>Enfuvirtide</td>
</tr>
<tr>
<td>TAR</td>
<td>Transactivation-responsive region</td>
</tr>
<tr>
<td>Tat</td>
<td>Transactivator of transcription</td>
</tr>
<tr>
<td>T&lt;sub&gt;CM&lt;/sub&gt;</td>
<td>Central memory T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TDF</td>
<td>Tenofovir disoproxil fumarate</td>
</tr>
<tr>
<td>TM/gp41</td>
<td>Transmembrane protein</td>
</tr>
<tr>
<td>TPV</td>
<td>Tipranavir</td>
</tr>
<tr>
<td>TPX</td>
<td>Trapoxin</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer ribonucleic acid</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>TschG</td>
<td>Tierschutz Gesetz (Swiss animal protection law)</td>
</tr>
<tr>
<td>TschV</td>
<td>Tierschutz Verordnung (Swiss animal protection regulation)</td>
</tr>
<tr>
<td>Tsg101</td>
<td>Tumor susceptibility gene 101</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Vif</td>
<td>Viral infectivity factor</td>
</tr>
<tr>
<td>VOR</td>
<td>Vorinostat</td>
</tr>
<tr>
<td>Vpr</td>
<td>Viral protein R</td>
</tr>
<tr>
<td>Vpu</td>
<td>Viral protein U</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>ZDV</td>
<td>Zidovudine</td>
</tr>
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</table>
Acknowledgments

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Curriculum vitae

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Education & work experience:

2013-today  Roche Glycart AG, Schlieren
pRED, Infectious Diseases
Scientist, Infectious Diseases Immunology

2009-2015  Swiss Federal Institute of Technology Zurich (ETHZ), PhD student
University Hospital Zurich, Division of Infectious Diseases and Hospital
Epidemiology
PhD thesis title: “A novel in vivo HIV-1 latency model based on humanized
mice”

2008-2009  University Hospital Freiburg, Germany
Institute for Medical Microbiology, Department of Virology
Project: “Investigating the antiviral effect of Mx1/MxA on Influenza A
replication and gene expression”

2000-2007  University of Stuttgart, Germany, Diploma Biotechnology
Major subjects: Molecular Biology, Technical Biochemistry, Genetics

2007  University of Stuttgart, Germany, Diploma thesis
Department of Molecular Biology
Diploma thesis title: “Post-translational modifications of the movement protein
MP/BC1 of Abutilon mosaic virus AbMV”

2005  Henkel KGaA, Düsseldorf, Germany, Industry internship
3 months internship in the Department VTB-Enzyme Technology
Establishment of a novel screening method and cloning of a PVA-Oxidase

2004-2005  Wageningen University, Netherlands, Student research project
Laboratory of Virology
Research thesis: “Analysis of Influenza A virus cap-snatching in vitro”

1989-1998  Anno Gymnasium Siegburg, University entrance qualification
Major subjects: Biology, English

1995-1996  Gibson City High School, Illinois, USA
Stay abroad as visiting student
Further training:

2009  Course in laboratory animal science (equivalent to FELASA Category B)
2008  Seminar according to § 15 GenTSV for project leaders and biological safety commissaries
2014  Course in laboratory animal science (equivalent to FELASA Category C)

Languages:

<table>
<thead>
<tr>
<th>Language</th>
<th>Skill</th>
</tr>
</thead>
<tbody>
<tr>
<td>German</td>
<td>Mother Tongue</td>
</tr>
<tr>
<td>English</td>
<td>Proficient in spoken and written</td>
</tr>
<tr>
<td>Dutch</td>
<td>Basic knowledge</td>
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</table>

Scientific congresses:

2013  EASL Meeting, Lyon
2011  3rd Swiss Workshop in Fundamental Virology, Thun
2010  2nd Swiss Workshop in Fundamental Virology, Bern
2007  3rd European Congress of Virology, Nuremberg
2005  4th Joint Meeting of Dutch & German Plant Virologists, Wageningen
2005  Dutch Annual Virology Symposium, Amsterdam

Publications:

Kleinow T, Holeiter G, Nischang M, Stein M, Karayavuz M, Wege C and Jeske H
Post-translational modifications of Abutilon mosaic virus movement protein (BC1) in fission yeast.
Virus Research (2008)

Three C-terminal phosphorylation sites in the Abutilon mosaic virus movement protein affect symptom development and viral DNA accumulation.
Virology (2009)

Inadequate clearance of translocated bacterial products in HIV-infected humanized mice.

Humanized mice recapitulate key features of HIV-1 infection: a novel concept using long-acting anti-retroviral drugs for treating HIV-1.

Modeling HIV infection and therapies in humanized mice.
Swiss Med Wkly (2012)