PhD Thesis

DCiphering Complement Receptor-mediated HIV-1 incorporation and effects on DC subset function in search for novel therapeutical targets

by

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Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less. — Marie Curie

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Abstract

Dendritic cells (DCs) are located in peripheral tissues, including oral and vaginal mucosa. They can also be found activated in lymphoid tissues, as tissue-resident DCs. DCs are the first cells encountering a multitude of pathogens. Recognition of pathogens by DCs is essential for the immune system due to their ability to capture, internalize, process and present antigens to naïve T cells in order to initiate a specialized adaptive immune response. DCs play therefore a key role in the defense against pathogens by bridging innate and adaptive immune responses. That is the reason why they were designated sentinels of the immune system.

DCs express numerous pattern recognition receptors (PRRs). Among these, C-type lectin receptors (CLRs) and complement receptors (CRs) interact with pathogen-associated molecular patterns (PAMPs). After entry in the host, invading pathogens are immediately recognized and opsonized by the complement system, which allows interactions with CRs abundantly expressed on DCs surface. The main research focus within this thesis was to unravel the interplay of DCs and DC subsets together with the complement system in shaping the immune system.

Within the first part of the PhD Thesis, interactions of CRs with human immunodeficiency virus-1 (HIV-1) was studied by using a leukemia monocytic cell line (THP-1) as model cell line appropriate to knock out (KO) CRs. Stable KO cell lines for CR3 (CD11b/CD18) and CR4 (CD11c/CD18) in THP-1 cells that can be differentiated into a DC-like phenotype (THP1-DC), were generated. Hence, we used:

- **Complement-opsonized HIV-1 (HIV-C) and non-opsonized HIV-1 (HIV) to study the contribution of CR3 and CR4 in binding, uptake and processing HIV-1 in DCs using the stable CD11b, CD11c and CD18 KO cell lines.** Earlier publications by our group showed that HIV-C is able to efficiently infect DCs in contrast to its HIV counterpart. DC infection in turn made the virus visible to the immune system and resulted in enhanced induction of humoral and cellular immune responses [1]. In this study we focused on the role of CR3 and CR4 in DC infection with HIV-C and HIV and found that WT THP1-DCs exerted a significantly higher infection, phagocytosis and activation using HIV-C compared to HIV. Thus, results using primary DCs were confirmed, which makes THP1-DCs an appropriate model for further experiments using CR3- and CR4-KO cells. According to the data generated during the thesis, CR4 plays a more prominent role regarding these antiviral DC functions than CR3. Also, CD18 KO THP1-DCs, which lack both CR3 and CR4 due to loss of the common beta-chain of both receptors, showed an abrogation of the above mentioned antiviral effects and displayed properties similar to DCs/THP1-DCs infected with HIV.

- **HIV-C and HIV to investigate the role of complement and CRs in sexual transmission.** We studied how complement and CRs are involved in HIV-1 interactions with Langerhans cells (LCs), which are a DC subset found in the skin and foreskin. We also investigated how the virus evades degradation in those cells. For that, we analyzed different steps in the viral life cycle. The data corroborated that CRs play an important role in efficient infection of LCs with complement opsonized HIV-1. Interestingly, cytokine release induced by HIV-C suggested that HIV-C enhanced innate antiviral type I interferon responses as well as pro-inflammatory cytokines compared to non-opsonized HIV-1. These data suggested that complement also affects immune activation at the very beginning of HIV-1 pathogenesis.

Altogether our studies indicate that DCs or DC subsets exert different intracellular mechanisms when incubated with complement-opsonized pathogens compared to their non-opsonized counterparts. Our results may provide novel strategies in the development of vaccines against HIV-1 by enhancing the role of complement, since it also seems to help and/or improve the adaptive immune response.

1. Introduction

Dendritic Cell subsets

Dendritic cells (DCs) are the sentinels of immunity, due to their ideal location throughout the body gateways and their unique ability to prime naïve T cells in lymphoid tissues. They are the most potent antigen-presenting cells (APCs) in the human body and are able to orchestrate and shape adaptive immune responses against a wide variety of pathogens. They are considered major players in both arms of the immune response, innate and adaptive. DCs are exceptionally potent immunogens under inflammatory conditions, yet are also critical for induction and maintenance of self-tolerance in steady state [2, 3]. They are capable of capturing and internalizing foreign particles, but whether they contribute to microbial killing and clearance, is yet unknown [4, 5]. However, DCs contribute for an efficient antigen processing and presenting to T cells. In the periphery, DCs act as sensors undergoing a stimulus-dependent and irreversible differentiation upon microbial exposure, known as maturation. This maturation process varies depending on the stimuli, but it always results in activation of innate and adaptive immune mechanisms. The maturation stimulus influences the induction of high levels of cytokine secretion by DCs. For example, if the cells receive a stimulus with interleukin-12 (IL-12) and type I interferons, they show an antiviral activity. Interferons in turn act in a feedback loop on DCs driving DC-maturation and initiating adaptive immune response by enhancing T and B cell immunity. Thus, DCs display innate immune responses that drive adaptive, pathogen-specific defense mechanisms.

DCs are localized in the blood, epithelia, mucosa and are also a major component of lymphoid tissues, particularly in the T cell areas. Upon pathogen encounter, DCs undergo maturation, being able to leave peripheral organs and migrate to T cell areas within the lymph nodes (LNs) to initiate adaptive immunity [6]. DCs access these areas via afferent lymphatics. DC migration is associated with transition of DCs from ‘antigen-capturing’ to ‘antigen-presenting’ cells. This transition is due to up-regulation of the antigen-presenting machinery comprising, among others, co-stimulatory molecules and MHC class I and II for optimal activation of naïve T cells. DCs form networks of cells along body surfaces, often intimately associated with the epithelium. Langerhans cells (LCs) are found at the basal and supra-basal layers of the stratified squamous epithelium of the skin, oral and ano-genital mucosa. LCs located in the epidermis act as the outermost sentinels of the immune system sampling for invading pathogens [7-9], whereas DCs reside in the dermis. DCs subsets are attracted to the peripheral sites through the action of several chemokine receptors (CCR6, CCR5) and chemokines secreted by inflamed epithelia, such as CCL20/MIP-3α, CCL5/RANTES, CCL3/MIP-1α. The migration process to the lymph nodes

involves up-regulation of CCR7 and an increasing gradient of chemokine ligands CCL19/CCL21 alongside lymphatics and the lymph node [10-14]. Due to their location and their ability to generate 'dendrites' able to capture antigen (Ag), which has been associated with a CXC chemokine receptor, named CXCR3 [15, 16], they act as the first line of defense against mucosal infections. DCs are able to extend their dendrites across tight junctions to detect potential danger signals on the apical side of the epithelial surface.

Pattern Recognition Receptors

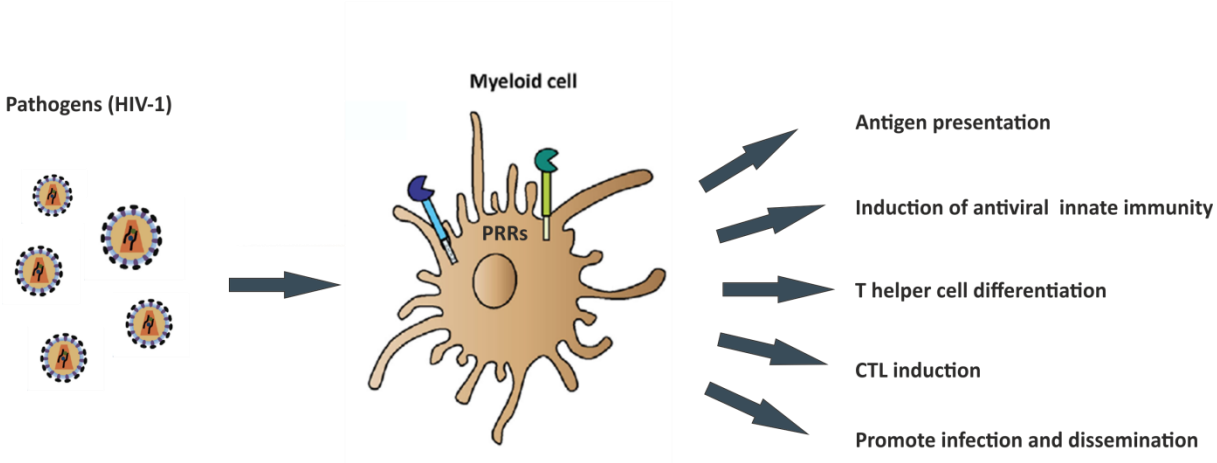


Figure 1. Recognition of invading antigens by PRRs and induction of immune responses. Viral antigens that trigger the CLR can modulate myeloid cell functions, thereby affecting antigen presentation, antiviral innate immune responses, and T helper differentiation.

(Modified from Bermejo-Jambrina *et.al* (2018). C-Type Lectin Receptors in Antiviral Immunity and Viral Escape. Frontiers in Immunology)

Mucosa and skin are targets for invading pathogens and therefore important locations where immunity is initiated. Innate immune cells, such as DCs guard these tissues and are able to sense and capture the invasive pathogens through pattern recognition receptors (PRRs) and lead to initiation of immunity and elimination of the pathogen. DCs express an array of PRRs involved in pathogens capture and their degradation, by binding conserved molecular structures called pathogen-associated molecular patterns (PAMPs) [17]. Different types of PRRs recognize a wide range of PAMPs and induce different transcriptional programs leading to an adjusted immune response. Besides, pathogens often trigger several PRRs simultaneously, leading to a crosstalk between these receptors, which provide immune cells with another important level of control to modify the adaptive immune response against the pathogen. Among PRRs, Toll-like receptors

(TLRs), Rig-I-like receptors (RLRs), C-type lectin receptors (CLRs), Complement receptors (CRs) and NOD-like receptors (NLRs) were described. Innate immune cells, such as DCs and LCs express CLRs that act as PRRs. Most of these CLRs bind carbohydrate moieties in a calcium-dependent manner using conserved carbohydrate recognition domains (CRDs). CLRs are important for recognition and capture of pathogens as these PRRs have a high affinity for their ligands, resulting in the internalization of the pathogens. Internalization often leads to degradation via lysosomes, as it has been shown for the DC-specific ICAM-3 grabbing non integrin (DC-SIGN or CD209) and DEC-205 [18, 19]. However, the binding also induces degradation via autophagy as recently shown for langerin (CD207) [20]. DC-SIGN and langerin are well-studied receptors in DCs and LCs, respectively. For example, DC-SIGN is able to recognize mannose and fucosyl residues expressed on the surface of different human pathogens including human immunodeficiency virus (HIV-1), *Aspergillus fumigatus* (*A. fumigatus*), CMV, Ebola virus, dengue virus and Candida.

Many entry receptors interplay with other receptors or signaling molecules on and in APCs, thus modulating the immune response, e.g. some CLRs contain immune-receptor tyrosine-based activation motifs (ITAMs), hemi-ITAMs, or immune-receptor tyrosine-based inhibitory motifs (ITIMs), whereas some do not contain any obvious signaling motifs [21]. Dectin-1, which is expressed on DCs and macrophages, belongs to the hemi-ITAM group. It binds yeast, zymosan particles and β-1,3- and β-1,6-glucan on fungi and associates upon activation with TLR2 initiating signaling resulting in TNF-α and IL-12 production [22, 23]. ITAM motif, following phosphorylation, mediates IL-2 and IL-10 production through CARD9/NF-κB signaling cascade in macrophages [24]. Other CLRs such as DC-SIGN, Mannose Receptor (MR) and DEC-205 do not contain any known ITAMs or ITIMs [21]. DC-SIGN is an important CLR on DCs involved in transmission of pathogens, such as viruses, to susceptible target cells [25]. DC-SIGN functions as attachment receptor for many different pathogens, in particular viruses as HIV-1, Cytomegalovirus (CMV) and Ebola virus (EBOV), and facilitates dissemination throughout the host. The transmission ability is not exclusive for DC-SIGN since, another capture receptor, DCIR, is involved. DCIR is expressed by DCs and macrophages, and contains an ITIM motif, that mediates inhibitory signals by recruiting the phosphatases SH2-domain-containing protein tyrosine phosphatase 1 (SHP1) or SHP2 after receptor ligation. DCIR inhibits TLR8-dependent IL-12 and TNFα production, whereas TLR2, TLR3 and TLR4-induced cytokine levels are unaffected [26].

Additionally DCs are powerful accessory or co-stimulatory cells for T cell responses including initiation of adaptive immunity *in vivo*. DCs express co-stimulatory molecules, which provide a ‘second signal’, in addition to peptide-MHC or ‘signal one’, directly leading to immunity. The

functional effects of co-stimulatory receptors are therefore intricate and can be both, immune enhancing and regulatory. Co-stimulatory receptors on DCs comprise the B7 receptor family, which are expressed at high levels, together with CD40. CD80 (B7-1) and CD86 (B7-2) molecules belong to the B7 family. Besides activating T cells via CD28, CD80 and CD86 can also interact with the negative regulator CTLA-4 on T cells. They act as a link between innate and adaptive immunity together with PRRs to induce T cell co-stimulation and thereby initiation of adaptive responses [27, 28]. It has been shown that a lack of CD80 and CD86 decreases the efficient capacity of antigen presentation of DCs [29]. Fuji *et al.* published that even though DCs had an increased expression of CD86 and CD80, it is not sufficient for mediating DC maturation. Upon maturation even higher CD80 and CD86 levels are expressed together with aggregates of MHC/peptide complexes, therefore allowing co-stimulation to be effective [30]. DCs also express tumor necrosis factor-receptor (TNF-R) member CD40, which is also, upregulated on mature DCs. CD40 participates in DC development from progenitors, migration of DCs to T cell areas in lymph nodes, antigen presentation on MHC class I molecules and IL-12 production [31].

For many maturation stimuli, TLRs play an important role on DCs. There are 13 known human TLRs, which are involved in pathogen recognition. The prototype response readout for a TLR response is the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the production of inflammatory cytokines, such as TNF-α, IL-1 and IL-6. TLR7 and TLR9 are primarily expressed in DCs, on intracellular vesicular membranes, and are involved in triggering the production of high amounts of type I interferons, increasing innate immunity.

Moreover, DCs and LCs express CRs, which recognize C3 cleavage fragments. DCs express CR3 (C11b/CD18) and CR4 (CD11c/CD18), which belong to the integrin family receptors [32]. CR3, also called Mac-1, is present in numerous innate cells, for instance on neutrophils, mononuclear phagocytes, mast cells and NK cells. The α-chain (CD11b) is non-covalently linked to a β-chain (CD18), thus being a member of the integrin family of cell surface receptors. CR4 is likewise a receptor of the integrin family, which has the common β-chain CD18 but a different α-chain, CD11c. The functions of both, CR3 and CR4, are supposed to be very similar since they both bind to iC3b fragments deposited on the pathogen surface [33, 34]. Both receptors are involved in binding multiple ligands, participating in phagocytosis, synapse formation, leukocyte trafficking and co-stimulation. Although both receptors are thought to react similarly because of their structural homology, possible differences in signaling pathway induction need to be further investigated [34].

The Complement System - a key for surveillance and homeostasis

The immune system comprises cellular and humoral responses which form an interactive network to sense and eliminate invading pathogens. One of the first elements to be activated and inducing innate immune responses is the systemic complement system. It consist of more than 40 heat-labile fluid-phase and cell membrane-bound proteins [35, 36]. The complement system acts as a regulated cascade of enzymatic reactions resulting in lysis, opsonization and inflammation, therefore inducing pathogen clearance and host defense [36]. The complement system can be activated through three different pathways called the classical (CP), the lectin (LP) and the alternative pathway (AP) [36]. The three distinct pathways (CP, LP and AP) are able to recognize foreign pathogens and damaged host cells. Complement activation is tightly regulated by inactivation of complement complexes or cleavage of complement fragments and regulation occurs during all steps of the cascade (see below). Upon infection, the complement system is fully activated. The opsonization process is due to C3b, the active form of C3, which covalently adheres to the pathogen's surface.

The AP is perpetually active in low concentrations to detect non-self surfaces of invading particles. Moreover, complement proteins are activated selectively by specific signals, for instance PAMPs or immune complexes, resulting in the activation of the CP and LP. All pathways, CP, LP and AP, end up in formation of the C3 convertase, which cleaves C3 into C3a and C3b. C3b accumulates covalently linked on the surface of pathogens or infected or damaged cells. Some pathogens are able to avoid C3b deposition using several strategies to overcome complement-opsonization and subsequent lysis.

The CP is activated by antigen/antibody complexes. The complement protein C1q binds to the Fc portion, in particular to the CH2 domain of IgG or the CH3 domain of IgM molecules that are complexed with antigens. The CP can be triggered in an antibody-independent manner when C1q directly binds to pathogens or infected cells. The complement C1q protein is produced mainly by immature DCs, monocytes and macrophages. It is constituted by subunits C1q, C1r and C1s. C1r and C1s subunits act as proteases while C1q is the main player in immune complex recognition. The C1q subunit has a hexamer conformation, thereby allowing the interaction with the Fc region of IgG antibodies, which assemble at the cell surface into hexamers [37, 38]. Each Fc region has a C1q binding site and each C1q binds to a minimum of two Ig heavy chains to be functional. After the binding, C1r and C1s become activated, forming a tetramer. C1r cleaves C1s, which subsequently leads to C4 and C2 cleavage. C4 contains a thioester bond, therefore being able to bind to the immune-complex or to infected cell surfaces. C2 cleavage by C1rC1s into C2a

and C2b, allows formation of the C3 convertase C4b2b. The C3 convertase cleaves the main complement fragment C3 into the anaphylotoxin C3a, which is able to activate nearby immune cells for migration to the sites of the infection, and the opsonin C3b. C3b associates with the C4b2b complex forming the C5 convertase. At this point of the complement cascade, all pathways converge [33, 39].

The AP is constitutively activated during normal physiological conditions, although at very low concentrations. Besides direct activation of the AP, it can also be activated spontaneously as an "amplification loop" when fixed C3b generated by CP and LP binds to factor B and thereby boosts the complement activation initiated by the CP or LP. A conformational change of C3 into C3(H_2O) by spontaneous hydrolysis, recruits factor B and factor D, forming the enzymatic complex C3bBb, known as the alternative C3 convertase. The C3 convertase cleaves the main C3 fragment into the anaphylotoxin C3a and C3b, which undergoes through a conformation change, where the thioester bond is not hydrolyzed, but temporally exposed, allowing it to react to hydroxyl (OH-) or amino (NH_2-) groups around cell/pathogen surfaces [37]. The C3 convertase is degraded after its formation on host cells by the activity of many regulators of complement activation (RCAs). Normally pathogens do not express RCAs, therefore allowing formation of the membrane attack complex (MAC) and subsequent lysis [33, 35].

Lastly, LP becomes activated upon recognition of terminal mannose residues on microbial glycoproteins and glycolipids by mannose-binding lectin (MBL) or ficolins. Lectins are circulating in the plasma and are collagen-like proteins similar to C1q subunit from the CP. MBL interact to mannose remnants on microbial polysaccharides, while ficolins bind N-acteylglucosamine-containing glycans. MBL also associates with serine proteases in a calcium-dependent manner (MASP-1 and MASP-2). The MASPs are homologous to C1r and C1s. To activate MASP-2, MASP-1 is required. MASP-1 and MASP-2 can cleave C2, but only MASP-2 can cleave C4. By these actions, the C3 convertase is formed. This convertase is the same as in the CP, which in turn cleaves C3 into C3a and C3b.

All three pathways converge in a common terminal step of the complement cascade, which consist in the binding of C3b to either the AP C3 convertase consisting of C2bBb3b or to the CP-LP C3 convertase formed by C4b2b3b. Both complexes are known as the C5 convertase, which then cleaves the complement C5 fragment into the anaphylatoxin C5a and the C5b. C5a is similar to C3a, being both anaphylatoxins responsible for the activation and recruitment of immune cells expressing their receptors and, therefore, enhancing inflammation at infection sites. C5b undergoes, as C3b, a conformational change which triggers the formation of MAC. The MAC

consists of C5b together with C6 —which is able to wrap around the TED domain of C5b [40] —, C7 —that is responsible for binding to the membrane— and C8 —which penetrates the lipid bilayer—, resulting in the MAC formation with multiple C9 proteins. The formation of the MAC disrupts the microbial membrane resulting in lysis of the pathogens or infected cells.

Regulators of complement activation (RCAs)

In normal conditions, the complement system has to be tightly regulated to avoid damage on self-tissues. It is controlled by a number of fluid-phase and cell surface proteins, which act as regulators of complement activation (RCAs) preventing spontaneous activation and injury to autologous tissues (Fig. 2, red lettering). This process includes the inhibition of the proteases, such as C1s, restrain and dissociation factors for enzyme complexes, cofactors in proteolytic cleavage processes and proteases which are substrate specific [41].

Fluid phase complement regulators are distributed in human plasma and in body fluid. These regulators are grouped according to their major activity and include regulators of the AP, such as factor H (fH) and the activator protein, properdin. Soluble CP and LP regulators include C1 inhibitor (C1INH), which is able to trap C1r/C1s and MASP-1/MASP-2, and C4BP, which in turn together with factor H block the C3/C5 activation steps. C4BP is able to dissociate the C4b2b convertase and inactivate C4b [36, 42]. Moreover, fH acts as decay accelerator of the normally slow cleavage Bb (catalytic subunit of fB) from C3b of the AP convertase and of Bb from the amplification-loop, also called tick-over process. Besides, it serves as co-factor for fI, to cleave C3b into inactivated C3b (iC3b) [43]. The known soluble inhibitors of the terminal pathway include fH, clusterin and vitronectin, which also have additional functions beyond complement. Factor H diffuses in a dimer or tetramer conformation, which binds to target sites on tissues and cells, thus inhibiting the formation of the C3 convertase on self-tissues and cells [42]. Vitronectin and clusterin have similar functions due their incorporation into C5b-7 transforming the MAC complex into a hydrophilic inactive complex [41].

Membrane-bound complement regulators include CD55 (known as DAF)[44], CD46 (also known as MCP)[45] and CD59 (also known as protectin)[46]. They control the three major complement activation pathways and inactivate both C3 and C4. CD59 binds to C8 of the MAC, blocking the addition of C9 and subsequently stopping the formation of the MAC [41].

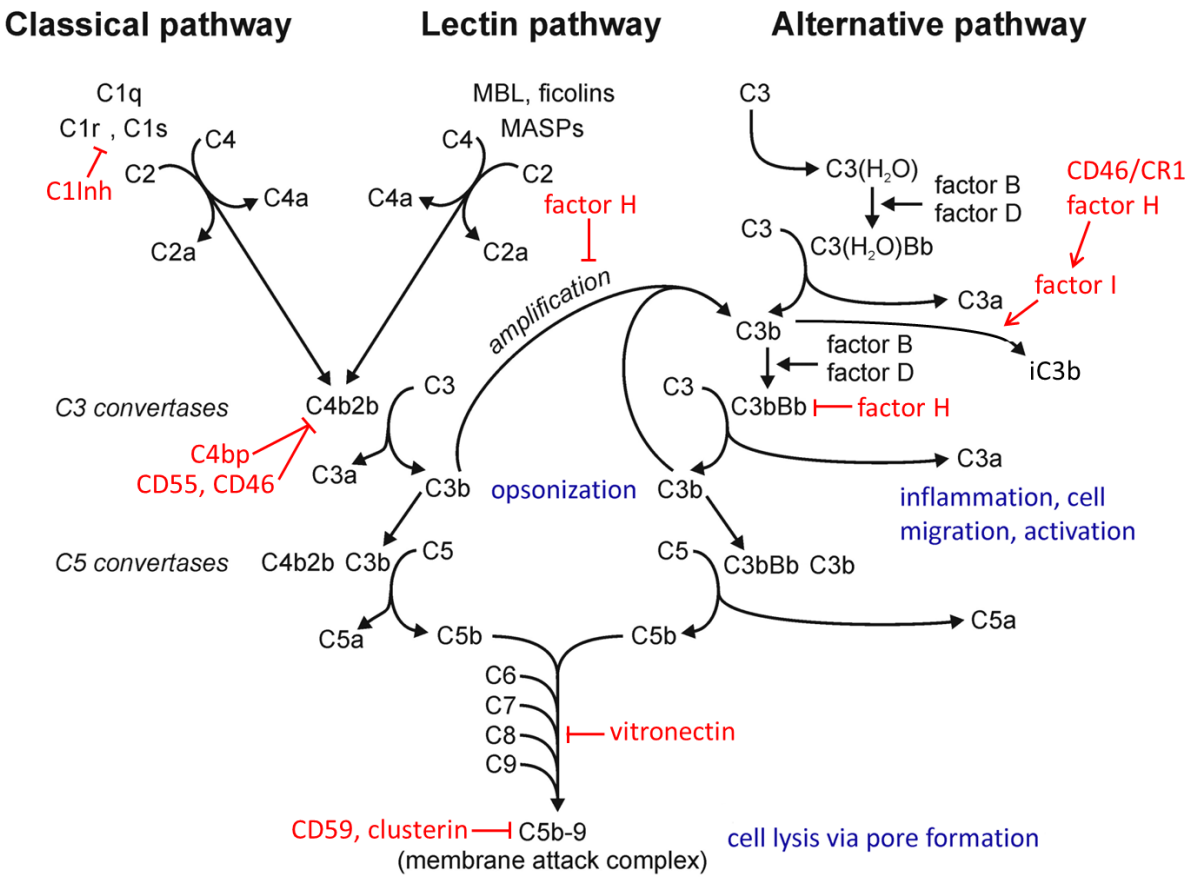


Figure 2. Activation of the complement cascade by the three different pathways. Figure adapted from intechopen.com

Human Immunodeficiency Virus (HIV) & Acquired Immunodeficiency Syndrome (AIDS)

Acquired Immune Deficiency Syndrome (AIDS) was first recognized as a new disease in 1981 when large numbers of young homosexual men succumbed to unusual opportunistic infections and rare malignancies [47, 48]. The retrovirus human immunodeficiency virus type 1, HIV-1, was identified by Barré-Sinoussi and Montagnier in 1983 [49] as the causative agent of what has since become one of the most severe infectious diseases to have emerged in recent history [50, 51]. More than 80% of HIV-1-infected individuals were infected through sexual transmission due to exposure of mucosal surfaces to the virus; most of the remaining 20% were infected by percutaneous or intravenous inoculations [52]. The infection risk is associated with different exposure routes varieties [53], but most of HIV-1 infections occur as a consequence of unprotected sexual contact [54], with the risk of transmission being higher during anal intercourse [54-56]. Interestingly, HIV-1 transmission rates through the oral mucosa varies from 0, 1% to 2% [57, 58] and it could be as high as 5% to 20% during breastfeeding [59, 60].

Since it was discovered and identified, HIV-1 have infected over 60 million people causing more than 25 million deaths [61]. Developing countries has experienced the greatest morbidity and mortality rates in young adults, according to the updated data published in http://www.unaids.org/ and http://who.int/. Recent data from UNAIDS showed that 36.9 million people were globally living with HIV in 2017. From the total number of HIV-1 positive individuals, 35.1 million were adults and 1.8 million children (<15 years). Every year approximately between 1.4 to 2.4 million people become newly infected with HIV-1, whereof 46% are living south of eastern Africa (UNAIDS database with the latest numbers of HIV infections. Available from: http://www.unaids.org/. During HIV-1 infection, three stages can be distinguished. The symptoms vary in type and severity from person to person and some may not get any symptoms at all for many years (Fig. 3).

Stage 1, also called **acute HIV-1 infection**, generally develops within 2 to 4 weeks after HIV-1 infection. During this time, individuals will experience flu-like symptoms, such as fever, headache and rash; followed by a gradual reduction of immune functions. In the acute stage, HIV-1 rapidly multiplies and spreads throughout the body. At this point, HIV-1 infects $CD4^+$ lymphocytes, monocytes, macrophages, DCs and enters the cells via the main receptor, CD4 and the coreceptor CCR5. During this phase virus levels in blood are very high, which greatly increases the risk of transmission.

Stage 2, also called **chronic HIV-1 infection**, occurs once a person has been through the acute primary infection stage and seroconversion process. During this stage of the disease, HIV-1 continues to multiply in the body but at very low levels. People with chronic HIV-1 infection may not have any HIV-1-related symptoms, but – without antiretroviral therapy - they can still spread the virus to others. Often some people start feeling better.

Stage 3, named **AIDS**, is the most severe stage of HIV-1 infection. After HIV-1 has severely damaged the immune system, the body cannot fight off opportunistic infections. At this stage there is a drop of $CD4^+$ T cell numbers, from 800 - 1200mm^3 to <500mm^3, leading to increased appearance of infections, sometimes life-threatening for the patients, which can be monitored. Once the $CD4^+$ T cell counts drop to <200mm^3, the patients become more and more susceptible to serious opportunistic infections and cancer representing AIDS, the end stage of the HIV infection [62].

Ever since HIV-1 was first discovered, the reasons for its sudden emergence, epidemic spread, and unique pathogenicity have been a subject of intense studies. An important finding came out in 1988 when a morphologically similar, but antigenically diverse virus, was found to cause AIDS in patients in western Africa [63]. This new virus strain was called Human Immunodeficiency

Virus type 2 (HIV-2) and it was found to be distantly related to HIV-1 but closely to a simian immunodeficiency virus (SIV), causing immunodeficiency in macaques [64]. Soon thereafter, more viruses were found in various different primates from sub-Saharan Africa, collected and termed as SIVs. Surprisingly, these viruses did not show any signs of pathogenicity in their natural hosts; however they cluster together with human and simian immunodeficiency viruses within the class of lentiviruses. Clavel *et al*. studied on 1986 the polymorphism of HIV-2, indicating that HIV-1 and HIV-2 are substantially different in their genomes [63]. Furthermore, the proteins of HIV-1 and HIV-2 have different sizes and their serological cross-reactivity is restricted to the major core protein, as the envelope glycoproteins of HIV-2. It was illustrated that the envelope glycoproteins of HIV-2 were recognized by the serum of SIV-infected macaques, providing evidence that human and simian immunodeficiency syndrome originated from non-pathogenic SIVs via zoonotic transfer [65]. Over the last 30 years, the HIV-1 retrovirus was intensely studied. HIV-1 samples from all over the world exhibited an extremely high genetic heterogeneity [66]. Phylogenetic analyses of HIV-1 and related viruses from nonhuman primates suggest that three independent transmission events occurred and that is why HIV-1 can be divided into four different subtypes or groups: major (M), outlier (O) and non-major, non-outlier (N) and pending the identification of further human cases (P). Groups N and O are rare and largely restricted to Cameroon and surrounding countries. The majority, approximately 98% of HIV infections worldwide, are caused by group M. Even within group M, there is very high diversity and the epicenter is in Africa, in particular in the Democratic Republic of Congo [67]. Group O was identified in 1990, and represents less than 1% of global infections. In 1998, 13 cases in people from Cameroon were documented and assigned to group N. Group P was discovered in 2009 in a woman from Cameroon living in France and it was so far only found in one other person [68-70]. Anyhow, all the groups differ in their distribution, but all cause $CD4^+$ T cell depletion and AIDS [71]. Same as HIV-1, HIV-2 can be divided into different lineages (group A-H). However, only HIV-2 group A and B have been reported to be spread; group A mostly in people from Western Africa and group B in Cote d'Ivoire [72, 73], whereas all the other groups were found in few persons. Taking into account HIV-1 and HIV-2, it appears that there have been plenty transmission events of SIVs to humans. None of these seems to be older than HIV-1 group M, which was the source of pre-1960 pandemic viruses originated in the 1920s. Group M, the common ancestor of HIV-1 group O, which was identified in 1990 [74], while those of HIV-2 groups A and B may have been more recent [75].

Kinetics of HIV disease progression and treatment

The global HIV pandemic started about 100 years ago. The most important factor that increases the risk of sexual transmission of HIV-1 is the number of copies of HIV-RNA per ml of plasma (viral load), with 2 to 4 times increased risk of sexual transmission for every 1 $\log_{10}$ higher viral load [76]. Acute HIV infection, which causes a very high plasma viral load (pVL) in the first few months, is an important driver of HIV epidemics [52]. Seminal and endocervical viral load independently predict the risk of HIV-1 sexual transmission, after adjustment for plasma viral load [77]. Other factors associated with increased risk of sexual transmission of HIV include sexually transmitted infections such as genital ulcers, herpes simplex type-2 infection, bacterial vaginosis, pregnancy, [78] and receptive anal intercourse [79]. Throughout the clinically latent period associated with HIV infection, the virus continues to actively replicate, usually resulting in symptomatic illness [80-82]. There is a high variability in disease progression between HIV-1-positive individuals, although all are categorized as rapid, typical or intermediate and late or long-term non-progression. The majority of infected individuals (70-80%) experience intermediate disease progression in which they have HIV RNA rise, elevated number of $CD8^+$ T lymphocytes and $CD4^+$ T cell decline, thus developing AIDS related illnesses (Fig. 3). Studies have demonstrated that HIV RNA levels at later time points are better indicators of long term disease progression than levels at seroconversion, with the pVL reaching a stable mean or "set point" after around 6 months or 1 year post-infection. The decline in pVL is supported by several cellular and humoral factors, for instance the secretion of antiviral cytokines. The influence of $CD8^+$T lymphocyte function on HIV disease progression is of considerable interest as cytotoxic T lymphocytes (CTLs) are the main effector cells of the specific cellular immune response, due to the correlation of the initial decline in pVL with the presence of CTLs [83]. Activated by $CD4^+$T helper cells, anti-HIV $CD8^+$ T cells have a crucial role to play in the control of viremia [84], increasing in response to ongoing viral replication [85]. Moreover, the diversity of HIV-specific CTL responses correlates with the control of viral replication and CD4 count, pointing out the need for a response to a wide divergence of antigens to achieve a greatest effect [86].

Chronic immune activation is a characteristic feature of HIV disease progression. Furthermore, HIV-1 becomes genetically diverse due to the selection pressure of the host immune system together with the error-prone viral polymerase, which lead to changes in *env* sequence with a rate of about 1% per year [87]. In this regard, a correlation has been demonstrated between mutations and changes in viral tropism, being the virus able to switch from CCR5 as coreceptor to CXCR4 when mutation occurs in the *env* sequence for the gp120 protein, resulting in infection of a wider range of T cells. Proportionately greater tropism for one or the other of those has been associated with different rates of disease progression. Slow infection development is

associated with a predominance of the "R5 virus strains" that ligate the CCR5 receptor, expressed on activated immune cell surfaces. On the contrary, "X4 virus strains" use CXCR4 coreceptor expressed by naïve CD4+T cells, leading to a drastic depletion of these cells following disease progression [88].

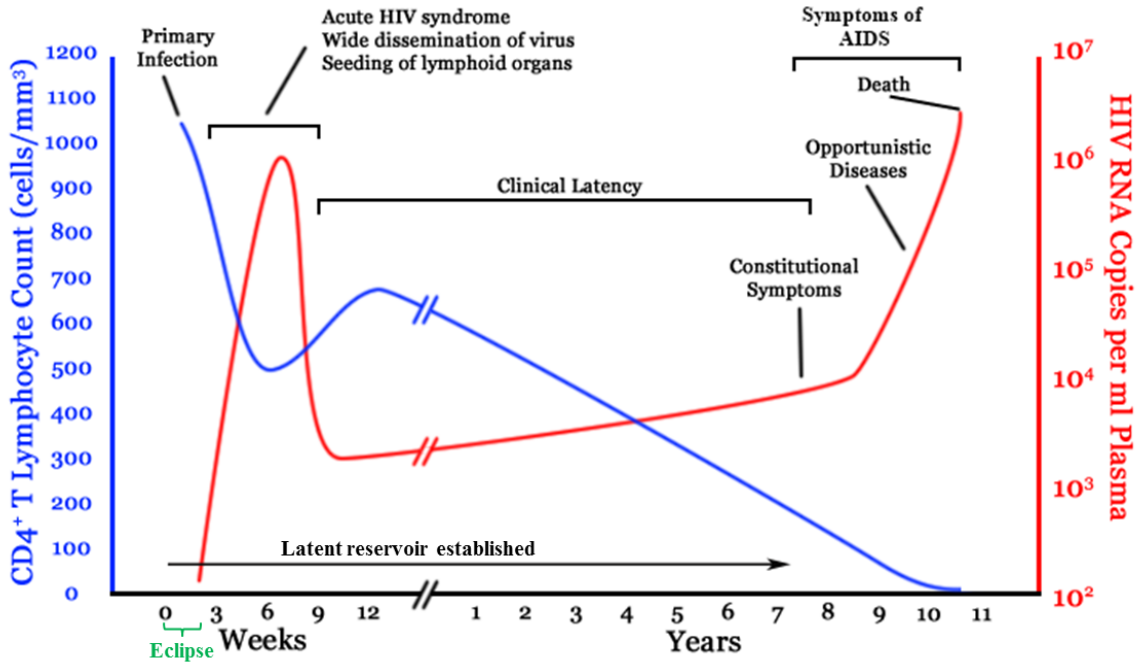


Figure 3. Depiction of a typical course of untreated HIV infection from acquisition to the development of AIDS. The graphic shows clinical signs and symptoms together with the CD4+ T cell counts (blue) which are dropping during untreated infection whereas the pVL rises (red). The Eclipse (green) indicates the day of infection until the first detectable viremia. Figure based on [87].

Treatment options against AIDS existed before 1986, but they mostly consisted of prophylaxis against common opportunistic pathogens, managing some AIDS disease symptoms. In the mid-1990s a revolution on HIV-1 treatment occurred with the development of inhibitors of the reverse transcriptase and protease, which combined with the introduction of drug regimens, enhanced the overall efficiency and therapy durability. At the beginning it was just a monotherapy but, with time, the standard HIV-1 medication was replaced by a combination of antiretroviral agents (ARVs). Combining antiretroviral therapies improves the clinical outcome during Stage 1 or acute phase of HIV-1 infection, due to suppression of viral replication and pVL reduction below detection limits, preserving CD4+ T cells counts in comparison to untreated patients. An improved understanding of the HIV-1 life cycle and infection allowed the establishment of six different therapeutic approaches. Whereas the HIV-1 life cycle presents many potential opportunities for therapeutic intervention, only a few have been exploited. The first step in the HIV-1 life cycle, viral entry, is the target for several classes of antiretroviral agents: attachment inhibitors, chemokine receptor antagonists, inhibitors of viral binding to the host cell membrane —as CCR5 antagonists—, and inhibitors able to hinder fusion and thereby

the entry of the viral capsid. RTIs (reverse transcriptase inhibitors) are able to block reverse transcription of the viral RNA genome into dsDNA. RTIs can be distinguished into nucleoside analogue RTIs (NRTIs) and non-nucleoside RTIs (NNRTIs)[89]. Integrase inhibitors are the most successfully recent HIV-1 drug developed.

In 2010, HIV-1 treatment guidelines in the United States and European Union recommended the initiation of the highly active antiretroviral therapy (HAART) with three fully active antiretroviral agents when $CD4^+$ T cells in peripheral blood decline to 350 per mm^3, a stage at which viral levels can often reach 10.000-100.000 copies/ml (see http://aidsinfo.nih.gov/Guidelines/). With proper adherence, HAART can suppress viral replication for decades, enhancing the life expectancy of the HIV-infected individual. Nowadays, HIV-positive people can be effectively treated in comparison to the early years of the epidemic, when being HIV+ was a death sentence. HIV is a chronic disease but in most cases manageable when treatment starts early during acute infection. A combined antiretroviral therapy (cART) or HAART improves the clinical outcome and AIDS aggravation can be delayed many years.

HIV Structure and Genome

The human immunodeficiency virus (HIV), HIV-1 and HIV-2, are grouped into the genus Lentivirus, from the Latin *lentus*, which means slow, due to the gradual course of the disease progression; within the family of Retroviridae, subfamily Orthoretrovirinae [90]. The name retrovirus comes from the fact that the RNA genome is transcribed by the reverse transcriptase into DNA in the host cell. The DNA is then incorporated into the double-stranded cell genome. Retroviruses are enveloped viruses with two identical positive-sense single-stranded RNA molecules that are enclosed within the core of the virus particle, also called nucleocapsid (NC), which is cone-shaped, formed by the protein p24, and is enclosed in a lipid bilayer, or envelope (Fig. 4A) [62].

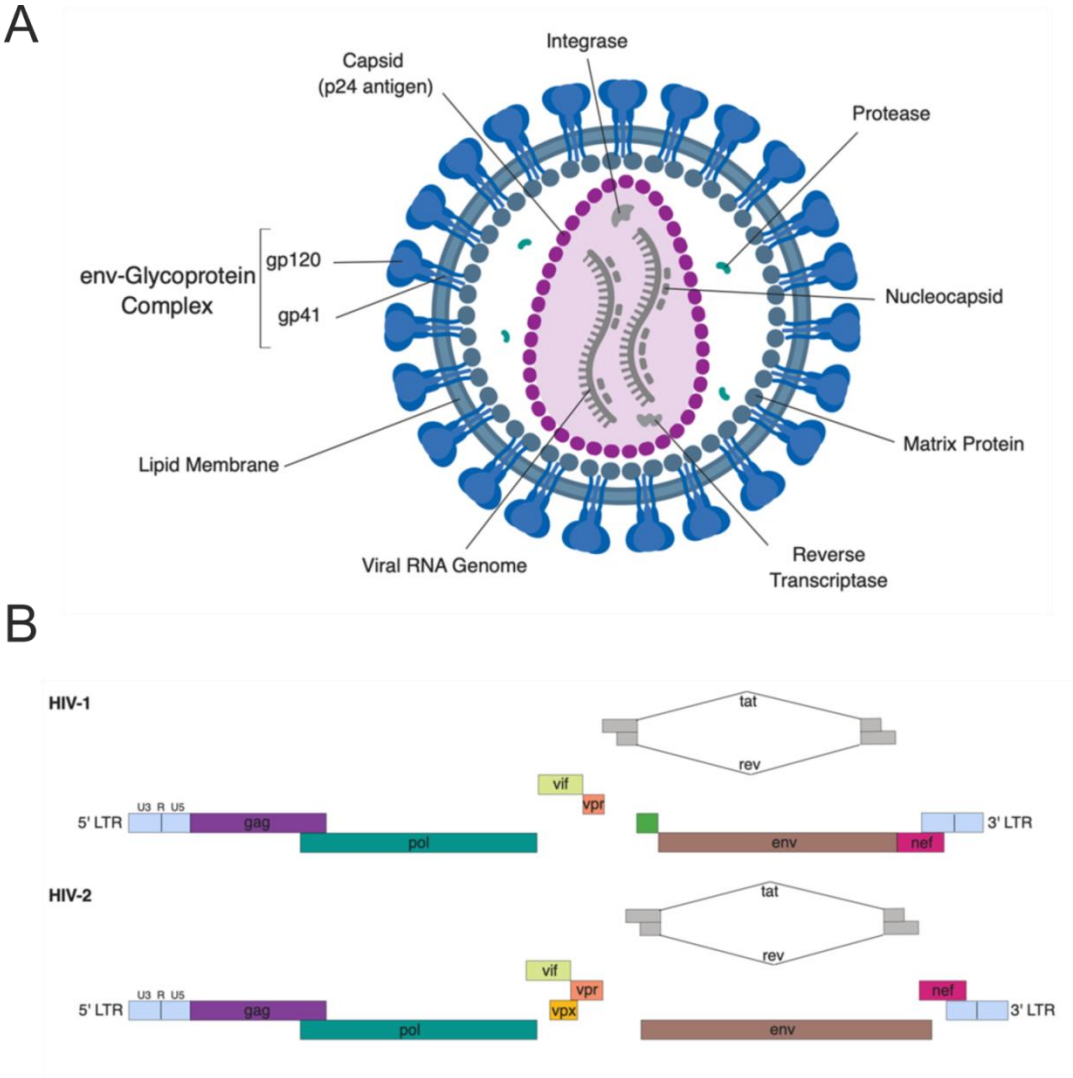


Figure 4. Structure and genome. A) Structure of HIV-1: the ssRNA is surrounded by the capsid protein p24 and the matrix protein p17. Upon budding HIV-1 acquires host cell proteins on its surface as well as the env-glycoprotein complex consisting of gp120 and gp41. B) Genome of HIV-1 and HIV-2: showing the three main genes and the proteins thereof after splicing.

The integrated form of HIV-1 is flanked at both ends by a repeated sequence, the long terminal repeats (LTRs), which are incorporated in the host cell genome (Fig. 4B). The 5´LTR region encodes the transcription promoter of the viral genes. The HIV-1 viral genome encodes 16 viral proteins, with the three major *gag*, *pol* and *env*, coding for structural proteins, viral enzymes, and envelope proteins. The other genes encode regulatory and accessory proteins [91]. Since the genome encodes only 16 proteins, interactions between pairs of proteins are required to accomplish viral replication during the HIV-1 life cycle. After the LTR, gag gene encodes the structural protein Gag or precursor p55. p55 associates with the plasma membrane after translation and simultaneously two copies of viral genomic RNA are recruited along with other viral proteins, causing the budding of the viral particle from the host cell. During a process called viral maturation, the viral protease cleaves p55 into smaller proteins, such as outer core

membrane (MA, p17), the capsid protein (CA, p24), the nucleocapsid (NC, p9 and p6) and the nucleic acid-stabilizing protein. The p24 protein constitutes the conical core of the virus surrounding the viral RNAs. The nucleocapsid p9 is responsible for packaging heterologous RNA into the virions and is important for reverse transcription [92, 93], while p6 is important for the incorporation of Vpr and Vpx, in case of HIV-2 [94].

The *gag* reading frame is followed by the *pol* reading frame coding for the enzymes protease (PR, p12), reverse transcriptase (RT, p51) and RNase H (p15) or RT plus RNase H (together p66) and integrase (IN, p32). The RT acts as RNA-dependent or DNA-dependent polymerase. Therefore RNase H is required to separate the original RNA template from the first DNA strand, promoting the synthesis of the complementary DNA strand. Since the polymerase does not have 5'-3' exonuclease activity replication of the viral genome is error-prone, thus causing several point mutations in each new viral copy [95]. The viral DNA can be synthesized already 6 h after viral entry; however, it can remain unintegrated for longer periods. The protein IN is essential for viral integration into the genomic DNA of an infected cell. It possesses an exonuclease activity, cleaving two nucleotides from the 3´ends of the viral DNA. Thereafter, an endonuclease cuts the host DNA at the integration site and subsequently ligates the viral DNA with the host DNA [96].

Adjacent to the *pol* gene, the env reading frame encodes for the two envelope glycoproteins, gp120 (surface protein) and gp41 (transmembrane protein), derived from the complete 160 kDa protein Env. Env protein is synthesized in the endoplasmic reticulum (ER) and migrates through the Golgi complex, where it is glycosylated. A cellular protease cleaves it into gp120 and gp41.

In addition to the structural proteins, the HIV genome codes for several regulatory proteins. Tat, which stands for trans-activator of transcription, is a protein responsible for the elongation of HIV-1 transcription. The absence of Tat causes the transcription of short HIV transcripts. It has been shown that Tat is also involved in the activation of TNF-β and TGF-β expression, as well as down-regulation of Bcl-2 and MIP-1a, being involved in the inhibition of apoptosis and recruitment of innate immune cells, such as macrophages and monocytes [97, 98]. Rev, which stands for regulator of expression of virion proteins, is responsible to induce the transition from the early to the late phase of HIV gene expression. It accumulates in the nuclei of the cells, binds to a secondary RNA structure, the Rev response element (RRE), facilitating the export of incomplete spliced HIV-RNAs into the cytoplasm [98, 99]. Both are necessary for the initiation of HIV replication, inducing transcription and the transport of the viral RNA transcripts from the nucleus to the cytoplasm. Moreover, the regulatory proteins Nef (negative regulating factor), Vif (Viral infectivity factor), Vpr (Virus protein r) and Vpu (Virus protein unique) are required for viral replication, virus budding and pathogenesis in specific cell types, overcoming host antiviral defense mechanisms. HIV-2 codes for Vpx (Virus protein x) instead of Vpu, which is partially

responsible for the reduced pathogenicity of HIV-2 [100]. Briefly, Nef is responsible for the down-regulation of CD4 and MHC class I expression on the host cell surface and enhances the release of the infectious virus factor tetherin. Vif is expressed late in the viral replication cycle and is critical for viral replication in all primary cells, whereas Vif is dispensable in a small number of human T cell lines, which are therefore referred to as "permissive" cells [101, 102], like $CD4^+$T cells and monocyte-derived macrophages (MDMs). Vif is able to overcome the inhibitory effect of the host cell enzyme APOBEC3G and promotes viral replication. Interestingly, Vif-deficient viruses made in permissive cells are fully competent to undergo one round of virus replication in "non-permissive" primary cells, while Vif-deficient viruses from non-permissive cells remain defective even when they infect permissive cells. In other words, the phenotype of the viral producer cell is essential [70, 103]. APOBEC3 proteins are expressed in many cells types and, specifically, APOBEC3G was described to potently inhibit HIV replication due to cytidine deamination (C to U) of the viral genome [104, 105]. In Vif-deleted virions used in in vitro experiments, APOBEC3G was packaged into virions and induced cytidine deaminations in the viral genome, causing a large number of G to A mutations, making the virus unable to replicate after infecting a new cell [105]. The cells can recognize the reverse transcripts as abnormal and degrade them, but if not, they are integrated into the host genome. However in the last case, due to translational termination codons and missense mutations, cells cannot produce viral progenitors [105]. Vif induces degradation of APOBEC3G before they are packaged into the virion by binding to APOBEC3G and recruiting host E3 ubiquitin ligases, causing the ubiquitination and degradation of APOBEC3G via proteasome [104].

Vpr activates viral replication, promotes infection of non-dividing cells and blocks cell cycle progression in dividing cells. Vpr shares 30% similarity in the amino acid sequence with Vpx (HIV-2 and SIV). Both are virion packaged proteins, which localize in the nucleus of the cells [106, 107]. Vpx is able to provide an enhancement of infection of HIV-1 even though HIV-1 does not encode for Vpx. Vpx is able to form a complex with culin 4A-based E3 ubiquitin ligase, including DCAF1 and DDB1. This complex has been associated with degradation of cellular DNA repair proteins, transcription factors and replication enzymes. For such reason, some groups hypothesized that Vpx was able to counteract another restriction factor in the host, named SAMHD1 (SAM domain-and HD domain-containing protein1). Laguette and colleagues demonstrated that Vpx indeed was able to degrade SAMHD1, when it was incorporated into virions or virus like particles (VLPs). They also showed that knocking down SAMHD1 led to a higher HIV-1 infection in DCs [108]. SAMHD1 is formed by an N-terminal sterile alpha motif (SAM) domain and a C-terminal histine-aspartic domain (HD) [109]. It is known that SAM is able to bind specific DNA sequences, acting as a docking site for kinases, signal transduction, transcription activation or repression [109, 110]. On the other hand, the HD domain is

characteristic of a type of enzymes with phosphohydrolase, phosphatase and nuclease activities [111]. Several groups identified the nuclear localization signal (NLS) of SAMHD1, the site targeted by Vpx that will also bind to DCAF-1-DDB1-E3 ubiquitin ligase, causing SAMHD1 degradation via proteasome [107, 112, 113]. It was demonstrated, that SAMHD1 is a dGTP-regulated deoxynucleotide triphoshydrolase, able to remove the triphosphate from deoxynucleotide triphosphates (dNTPs), blocking HIV infection due to the shortage of dNTPs required for RT to accomplish reverse transcription [114]. HIV-2 and SIV have Vpx which, through SAMHD1 degradation, overcomes restriction and makes viruses able to infect myeloid cells. SAMHD1 is a restriction factor in myeloid and resting $CD4^+$ T cells where dNTP levels are low since those cells do not replicate their DNA. However, SAMHD1 is not a restriction factor in activated T cells, even though they express the protein. Importantly, SAMHD1 restriction activity is negatively regulated by phosphorylation on Thr592. However, this phosphorylation does not regulate its dNTPase activity [109, 115].

Vpu is a type I transmembrane protein, expressed from a bicistronic viral mRNA and localized in the endoplasmic reticulum (ER). Vpu down-regulates, additionally to Nef, CD4 expression, enhances the viral release from the cells and counteracts host restriction [33]. Deletion of Vpu in HIV-1 entails fewer Env glycoprotein spikes and accumulation of the virus in massive clusters on the cell surface, due to failure in detachment [116, 117]. It has been demonstrated that the reduced number of Env spikes was due to the interaction of CD4 and gp160 (gp120 and gp41) in the Golgi, preventing the transport of gp160 to the cell membrane [118]. Vpu-deleted virions fail in being released, which is caused by the bone-marrow stromal antigen BST2, also known as tetherin [119]. Tetherin is an interferon-inducible protein with two membrane anchors, where one pair of membrane anchors is incorporated into the virion envelope while the other remains in the cell membrane. Tetherin has been associated with the activation of NF-κB, causing a production of type I IFN, leading to the antiviral state in the cells [120, 121]. All this is counteracted by Vpu, which is able to sequester tetherin in the perinuclear region, where it is degraded through lysosomal/proteasomal pathways [122].

HIV infectious life cycle

The HIV-1 envelope spikes, which comprise trimers of non-covalently linked heterodimers of the surface gp120 and transmembrane gp41 glycoproteins [123, 124] initiate a cascade of conformational changes and the release of the viral core into the cytoplasm. An initial interaction between gp120 and the surface receptor CD4 initiate the life cycle of the virus. The interaction induces a conformational change in the viral envelope, exposing the binding site for a second cells surface molecule, the CC chemokine receptor 5 (CCR5) or CXC chemokine receptor 4

(CXCR4). Coreceptor engagement leads to insertion of the fusion peptide located at the N-terminus of gp41 into the cell membrane, which in turn triggers significant rearrangements between trimerized N- and C- terminal heptad sequences within gp41, the formation of a six helical hairpin structure, and the apposition and fusion of the viral and host cell membranes [125, 126].

The HIV core, which houses the replication enzymes RT and IN as well as the viral genomic RNA, is encased by a cone-shaped shell [127] composed of the viral capsid protein (CA).

Reverse transcription and integration of the resultant linear viral DNA molecule into a host cell chromosome occurs within the context of the nucleoprotein complex structures that are derived from the viral core. Integration marks the transition from the early to late phase of HIV-1 replication, in which the focus shifts to viral gene expression, followed by the assembly and egress of nascent viral particles. Transcription, which initiates from the U3 promoter within the upstream LTR, requires the viral Tat transactivator protein for efficient elongation. Viral mRNAs are produced as a variety of alternatively spliced species. The smaller messages are exported readily from the nucleus, whereas the unspliced and singly spliced mRNAs require the action of Rev. This small viral protein acts as an adaptor, binding to the Rev-response element (RRE) located within the mRNA *env* coding region and the nuclear export factor CRM1. Recent structural biology advances yield insight into the mechanisms of Tat transactivation [128] and Rev-dependent mRNA export [129, 130].

The retroviral structural proteins CA, matrix (MA) and NC are synthesized as parts of the Gag precursor polypeptide, and HIV-1 Gag is sufficient to assemble virus-like particles at the plasma membrane and bud from cells [131]. MA, through an N-terminal myristic acid [132] and conserved basic amino acid residues [133, 134] contributes to Gag membrane association. The differential exposure of the myristate through a process known as the myristyl switch [135] allows Gag to associate preferentially with the plasma membrane, rather than intracellular membranes. The switch can be activated by phosphatidylinositol 4, 5-bisphosphate [136], a phospholipid that is concentrated in the inner leaflet of the plasma membrane and interacts directly with MA [137].

Retroviral budding is orchestrated by interactions between Prolin (Pro)-rich motifs in Gag that are known as late (L) domains and cellular class E vacuolar protein sorting (Vps) proteins, the actions of which are required to form the nascent particle and sever it from the plasma membrane. The intended functions of Vps proteins are in the formation of multi-vesicular bodies (MVBs), a reaction that is topologically identical to virus budding as in each case a membrane-coated vesicle leaves the cytoplasm, and in abscission during cell division [138, 139]. Most class

E Vps proteins function as subunits of endosomal sorting complexes required for transport (ESCRT), which come in four varieties (ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III). ESCRT-I and ESCRT-II function during membrane budding, whereas ESCRT-III is important for membrane scission.

The final step of the viral life cycle, which is mediated by PR and occurs concomitant with or soon after budding, converts immature particles to infectious virions via the proteolysis of Gag and Gag-Pol precursor polypeptides to yield the structural components MA, CA and NC, and the PR, RT and IN enzymes [140].

The coreceptor usage accounts for the tropism of the virus. If the virus bind to the coreceptor CCR5, it is named R5-tropic virus and if binding is through CXCR4 is termed X4-tropic virus, but when bind to both, is called R5X4-tropic or dual-tropic HIV-1. HIV tropism specifies the types of cells and tissues, where the virus is going to infect and replicate [141]. On one hand, new transmitted virions use to infect via CCR5 coreceptor cells such as macrophages or dendritic cells that reside in the mucosal surface [142]. A group of the Caucasian population, approximately 16% from the total population, present a mutant allele for CCR5 encoding a truncated protein, thereby not functional, which is not able to be transported to the cell surface. In case of homozygotes for this mutation, they become resistant to HIV-1 infection [143]. On the other hand, X4-tropic virus are associated with infection in T cells, since CXCR4 is expressed and only 15-30% express CCR5 [144].

As HIV-1 enters the human body, it has to cross the mucosal epithelia to encounter the most abundant host cell in the lamina propia, which are resting $CD4^+$T cells. These and other immune cells as activated $CD4^+$T cells, DCs, LCs and macrophages are infected with R5-tropic virus variants and start producing new virions.

The primary step causing HIV-1 infection of CCR5-tropic cells is the engagement of CD4 with gp120 on the surface of the virus. The virus also contains host cell molecules, obtained after the budding process. These host molecules can bind to additional cell surface molecules, such as the C-type lectin receptors, DC-SIGN or langerin, on DCs, or LCs, respectively [25]. Previous publications show that complement opsonization of HIV-1 leads to binding and uptake of the virus via complement receptors 3 and 4 (CR3 and CR4), abundantly expressed on dendritic cells [145]. Although CRs are not essential for infection, the binding of HIV-C to CRs could bring the viral envelope in proximity to CD4 and the coreceptor, involving in different signaling pathways upon stimulation of C-coated virus [146]. Upon the engagement of CD4 and gp120 a hydrophobic fusion peptide is inserted into the host cell membrane, facilitating the fusion of the viral membrane with the host cell membrane [123]. The two plus sense single stranded RNA

molecules together with other viral proteins, which comprise the reverse transcriptase complex, are released into the cytosol. Most of the RNA has been already transcribed into dsDNA therefore the complex starts disassembling forming a pre-integration complex (PIC), consisting of the dsDNA, MA, RT, and some host proteins [147, 148]. Once the PIC reaches the nuclear pore complex, the dsDNA is imported into the nucleus and integrated in the host cell genome. Host cell molecules facilitate the viral genome integration and, upon stimulation of the cell, RNA polymerase II is recruited to begin mRNA transcription together with enhancer proteins, which transmit activation signals and thus transcription [148].

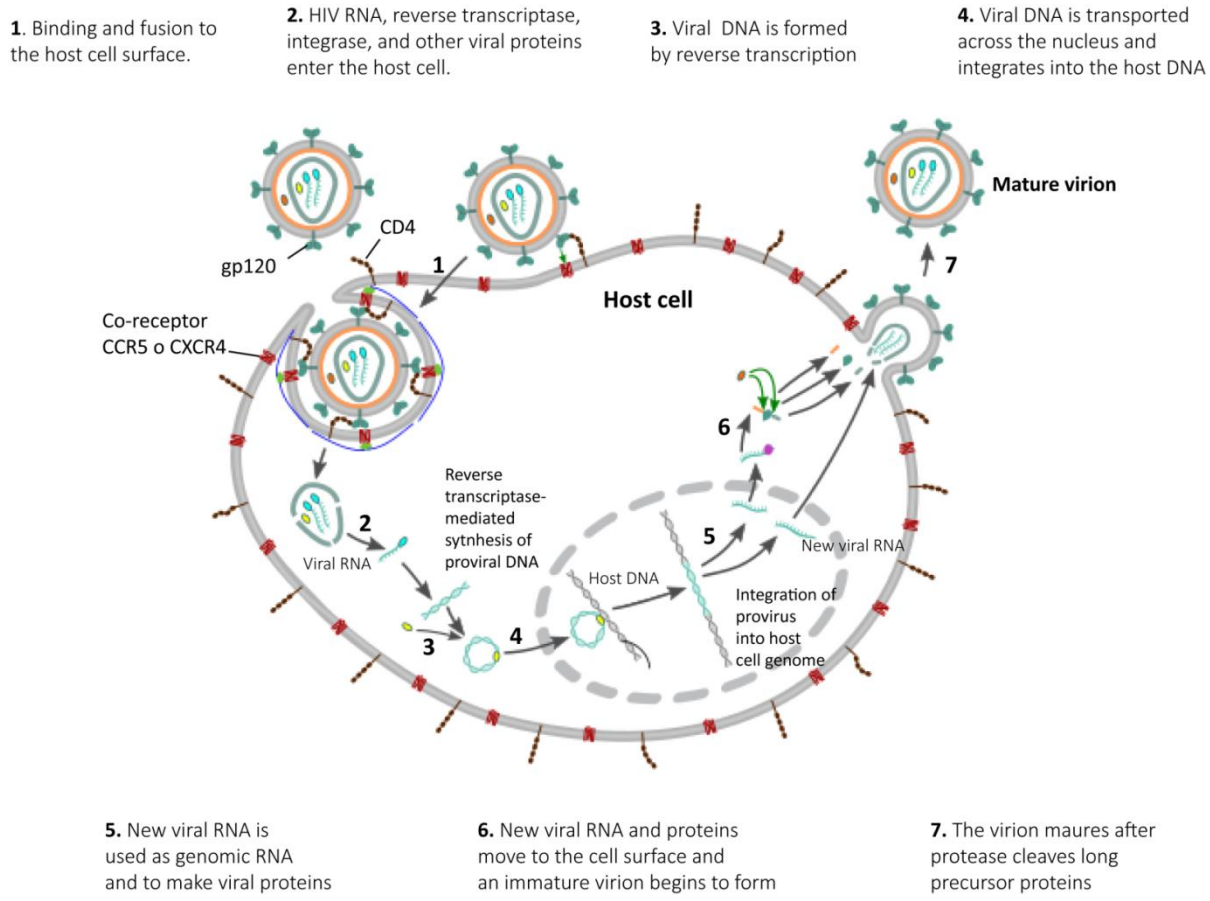


Figure 5. Infectious cycle of HIV-1 in the host cell. HIV-1 fuses with the cell membrane by binding to CD4 and CCR5 or CXCR4. Viral RNA is released into the cytosol and reverse transcribed into DNA, which is integrated in the host cell DNA. After transcription, mRNA is translated into proteins, they assemble next to the cell membrane and the virus buds out of the cells and matures to infect new cells. In the nucleus, efficient elongation of the transcript happened by Tat, allowing mRNA synthesis. Furthermore, for the transport of the mRNA from the nucleus into the cytoplasm and the initiation of mRNA splicing, Rev is required. Once the new viral RNAis in the cytoplasm, the precursor proteins Gag and Gag-Pol are synthesized and processed into mature viral proteins and lastly assembled next to the host cell membrane together with the viral RNA. Gag multimerizes, binds the viral RNA, incorporates Env and mobilizes the ESCRT machinery. As the viable particles bud out of the cell they mature due to final cleavage of the proteins via the protease and are able to infect new cells.

Complement activation by HIV and the role of dendritic cells

Complement and HIV

Immediately upon HIV-1 entry in the body, the virus spontaneously activates the complement system even in the absence of specific antibodies. This is possible due to the binding of HIV-1 surface proteins gp41 and gp120 to C1q and mannose-binding lectin (MBL), respectively [5, 149-152] (Fig. 6). C1q is a component of the C1 complex and is well known to bind immune complexes, triggering the classical pathway (CP) activation and promoting their solubilisation and clearance [153]. Over the past years, several studies have supported the idea that HIV-1 envelope glycoprotein gp41 functions as a viral ligand for gC1qR [154-157], the receptor for the globular heads of C1q complex, which modulates a plethora of immunological functions upon infection and inflammation. A number of studies have demonstrated that MBL binds to HIV-1 Env protein, leading to complement activation [158-160].

The activation of the complement system results in C3 activation and C3b/iC3b deposition on HIV-1, causing their enhanced uptake via complement receptors such as CR3 and CR4 (Fig. 6). Thus, HIV is already coated with complement fragments at the initial stages of infection. It has been shown that complement is already active at the mucosal site or even in the seminal fluid, meaning that the virus is opsonized with C3 cleavage products immediately *in vivo* [161].

The complement system, is a key player of innate immunity, is a first-line defender against HIV-1. On the other hand, also enhances HIV-1 infectivity. The role of complement system in HIV-1 pathogenesis has been debated for many years, but is still not clear if its role is good or bad for the host. It fights against the infection, but the complement-mediated lysis (CoML) of HIV-1 and HIV-1 infected cells is limited by the presence of complement regulators such as CD59 and factor H on the surface of HIV-1 or infected cells [162]. Following seroconversion, in addition to complement fragments, specific antibodies coat the viral surface. Depending on the IgG subtype, formation of immune-complexes strongly enhances the activation of the complement system via the classical pathway and, virus-bound antibodies dramatically increase the deposition of complement fragments (C3b) on virions [161, 163]. Therefore, opsonized infectious viral particles accumulate in HIV-1-positive individuals during the acute and chronic phase of infection. After seroconversion, the adaptive immune system is fully activated due to HIV-specific antibodies and the anti-viral action of T cells. Despite complement clearance and neutralization activity, most HIV particles are not killed and persist in the host covered with C3 fragments, enhancing the virus spread and maintenance through the uptake of RCA by the viral particles

during the budding process. RCA tightly control the complement system to prevent spontaneous destruction of host cells and they also protect HIV-1 from being lysed [164]. Interestingly, complement opsonized HIV-1 accumulates in all compartments tested in HIV-1-positive individuals, such as mucosa and seminal fluid [165]. Thus, complement coated HIV-1 accumulated is able to bind CR-expressing immune cells, e.g. DCs, macrophages, NK cells, B cells or follicular dendritic cells (fDCs). Triggering these receptors leads to cell activation and contributes to inflammation. On the one hand, complement-opsonized HIV-1 (HIV-C) enhances viral infectivity and dissemination *in vitro* [166]. On the other hand, it strengthens cellular immunity as well as type I IFN responses [1, 167, 168]. This highlights the importance of complement-mediated processes during HIV pathogenesis.

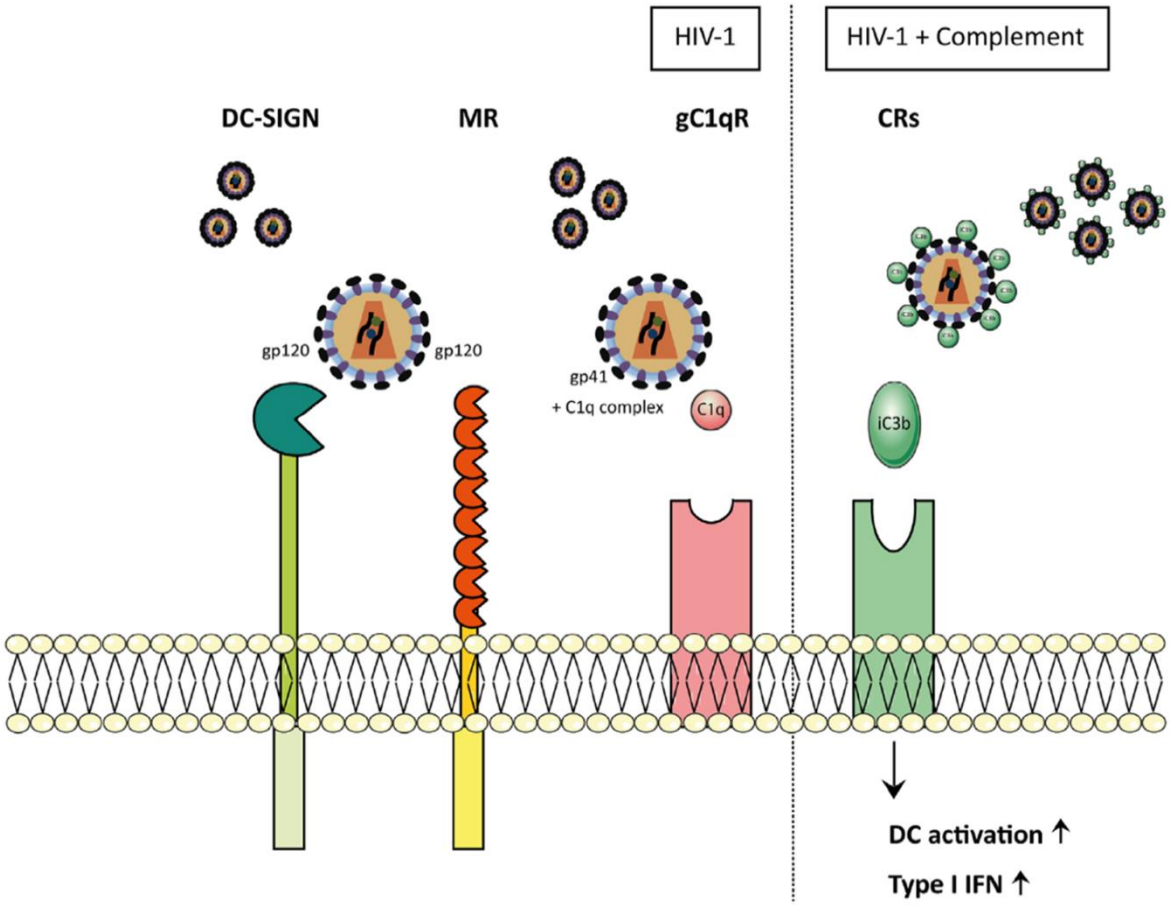


Figure 6. HIV-1 particles opsonization-dependent recognition on myeloid cells. In physiological conditions, when HIV-1 enters the body, it is either non-opsonized (HIV) or complement-opsonized (HIV-C). Depending on the opsonization pattern of the virions, interactions with the receptors differ. Non-opsonized virus (HIV-1) interacts with CLR receptors, such as DC-SIGN and mannose receptor (MR) via the glycoprotein gp120. Furthermore, non-opsonized virus (HIV-1) is able to establish an interaction between gp41 and C1q complex, allowing the final binding to gC1qR. MR and C1qR cause the activation of the complement system via the lectin pathway and the classical pathway, respectively, inducing complement-mediated opsonization of the virions. Once HIV is coated by C3b fragments, it is able to bind to the complement receptors (CRs). Dendritic cells (DCs) exposed to complement-opsonized HIV-1 showed increased activation as well as up-regulation of type I IFNs.

Dendritic cell subsets and HIV

One of the most important cellular components of the innate immune system are DCs. DCs are among others the first cells to encounter the virus when it enters the human body via mucosal sites. Interestingly, they differ in their ability to induce an innate immune response against non-opsonized HIV-1, HIV-C and HIV type 2 (HIV-2) [169, 170]. Recent studies from our group and others showed that opsonization of HIV-1 (HIV-C) strongly modulates DC function through its binding to CR3 and CR4, in contrast to non-opsonized virus which bind to DC-SIGN instead [1, 5, 25, 171]. Usually, most studies have been performed using non-opsonized HIV and DCs, showing a low level productive infection and poor antiviral immune response, although the virus is well transmitted to $CD4^+$ T cells [169, 172]. Our group described that complement acts as an endogenous adjuvant to induce HIV-specific CTLs via DCs, and that this CTL-activating capacity was significantly higher than that of DCs exposed to non-opsonized HIV-1 [167, 168].

The difference in cellular tropism between HIV-1 and HIV-2 comes from Vpx, the HIV-2 accessory protein that leads to SAMHD1 degradation [107, 108, 173], probably one of the most important HIV-1 restriction factors in myeloid cells. Bypassing HIV-1 restriction mechanisms in DCs by degrading SAMHD1 was associated with an increased quality and quantity of virus-specific immune responses (Fig. 7) [169]. This was due to an enhanced infection and co-stimulatory activity of the most-potent antigen-presenting cells [108, 109, 169, 170, 173-175]. SAMHD1 restricts the replication of HIV-1 in non-cycling monocytes, monocyte derived macrophages (MDMs), DCs, and resting T-cells [108, 173, 176]. In myeloid and resting lymphoid cells, where SAMHD1 exists as a mixture of phosphorylated and dephosphorylated forms, the phosphorylation is mediated by CDK2 [177]. Restriction factors expression and their regulation are among the most important factors dictating HIV infection of a specific cell. Importantly, HIV-C negatively regulates SAMHD1 in DCs by inducing its phosphorylation, which makes HIV-C infect DCs more than non-opsonized HIV-1, even though there is no Vpx [1].

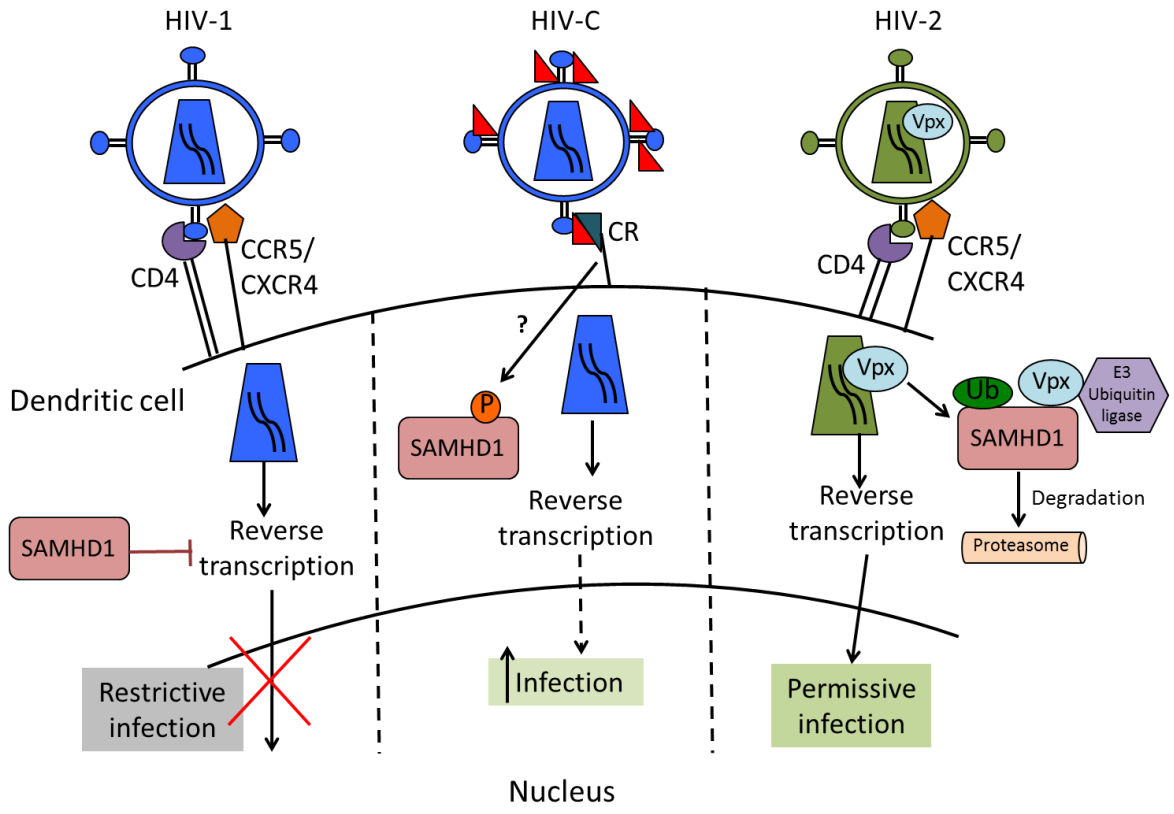


Figure 7. Scheme of HIV-1, HIV-C and HIV-2 restriction by SAMHD1 in DCs. HIV-1 (left) binds to CD4 receptor and CCR5 or CXCR4 coreceptors to enter the cell but due to SAMHD1 action, HIV-1 cannot reverse transcribe and the infection is blocked. HIV-C (middle) binds to complement receptors (CR) (apart from CD4 and CCR5/CXCR4) and that triggers an unknown pathway that leads to SAMHD1 deactivation by phosphorylation, which permits reverse transcription and higher infection than with HIV-1. HIV-2 (right) contains Vpx that mediates SAMHD1 degradation, allowing reverse transcription to occur and productive infection.

Despite the pre- and post-entry restrictions to virus replication, DCs capture HIV or HIV-C, internalizing the virus and migrating to the draining lymph nodes where they are able to prime T cells by presenting HIV-derived peptides to them. However, they can also transmit the virus to T cells. Three mechanisms allow transmission of the virus from DCs to T cells. *De novo* synthesized HIV-1 particles can be transferred to $CD4^+$ T cells in a way similar to the captured virus by DCs, without getting themselves infected. This process is done with high efficiency across virological synapses [178, 179] and is known as *cis* infection [180]. In 2003, McDonald *et al.* used live-cell microscopy to demonstrate recruitment of both captured virus and receptors to the junction between mature DCs and T cells [181], called virological synapse due to the structural similarity to an immunological synapse [182]. The discovery of the virological synapse and the identification of the receptor/ligand interaction responsible for DC-mediated transfer of virus to $CD4^+$ T cells, was named *trans* infection and is considered another way of dissemination. The virological synapse formation between DCs and T cells involves molecules that have not been identified yet. However, DC-SIGN was illustrated to be present at the virological synapse and

HIV-1 binding to DC-SIGN does not lead to its degradation but retention in early endosomes, allowing the delivery to uninfected T cells through formation of the synapse [25]. Additionally, suppression of DC-SIGN has been reported to impair synapse formation and to inhibit trans-infection of X4 virus to T cells [183]. Furthermore, Wiley and colleagues show that *trans*-infection occurs due to internalization of HIV-1 into endosomal multivesicular bodies (MVBs), a third way of virus spreading [184]. In 2012, using different experimental approaches, Gummuluru and Martinez-Picado groups identified glycosphingolipids (GSLs) containing terminal α-2,3 sialic acid residues, such as GM3 (trisialotetrahexosylgangloside). Enhancement of GM3 levels in HIV-1 virions can result in significant increases in virus capture by mature DCs, with subsequent enhancement to mature DC-mediated *trans* infection of T cells. These findings show clear differences in HIV-1 capture and *trans* infection by immature and mature DCs. Immature DCs express high levels of DC-SIGN and other C-type lectin receptors relative to mature DCs. Most of the virus particles captured by immature DCs are targeted to lysosomal compartments for degradation. Some of the HIV particles are then released into exosomes, thereby avoiding immune destruction, and fuse with the target-cell membranes, thus delivering infectious virus [185]. By contrast, mature DCs express high levels of Siglec 1, leading to GM3-dependent HIV-1 capture, which results in virus particle trafficking to non-lysosomal compartments at the cell periphery, remaining contiguous to the extracellular milieu [186, 187].

The most prevalent route of HIV-1 infection is across mucosal tissues after sexual contact (Fig. 8). LCs belong to the subset of DCs that line in the mucosal epithelia of vagina and foreskin. Besides initiating an effective adaptive immune response, they have important functions as well concerning the innate immune response, as was illustrated by De Witte *et al*. [188, 189]. Furthermore, they have a characteristic phenotype. They contain Birbeck granules which are "tennis racket" shaped intracytoplasmic organelles [190]. They also express langerin, a member of the C-type lectin family, which induces the Birbeck granule formation [191]. Langerin interacts with HIV-1, as DC-SIGN in DCs. LCs contain Birbeck granules, which are crucial for their innate activity. HIV-1 degradation take place in the Birbeck granules, thereby HIV-1 dissemination is limited [188]. Geijtenbeek´s laboratory demonstrated that under homeostatic conditions, langerin expressed on LCs act as a restriction factor for HIV-1 [188]. This is possible when the gp120 attaches to langerin, then the viral particle is internalize and degraded in the Birbeck granules, avoiding the dissemination of the virus through the host [188].

While DC-SIGN was originally believed to be the essential DC-specific receptor necessary for capture and trans infection of HIV-1 to $CD4^+$ T cells, subsequent findings suggest that other C-type lectin receptors such as mannose receptor, langerin, and DC immune receptor (DCIR) expressed on dermal DCs, LCs, and blood moDCs could also mediate HIV-1 trans infection [192-

194]. From all of these findings, it is clear that many C-type lectins expressed on varied DC subsets have the capability to bind mannosylated gp120. However, the relative contribution of these lectins to HIV-1 capture and *trans* infection in vivo remains to be determined.

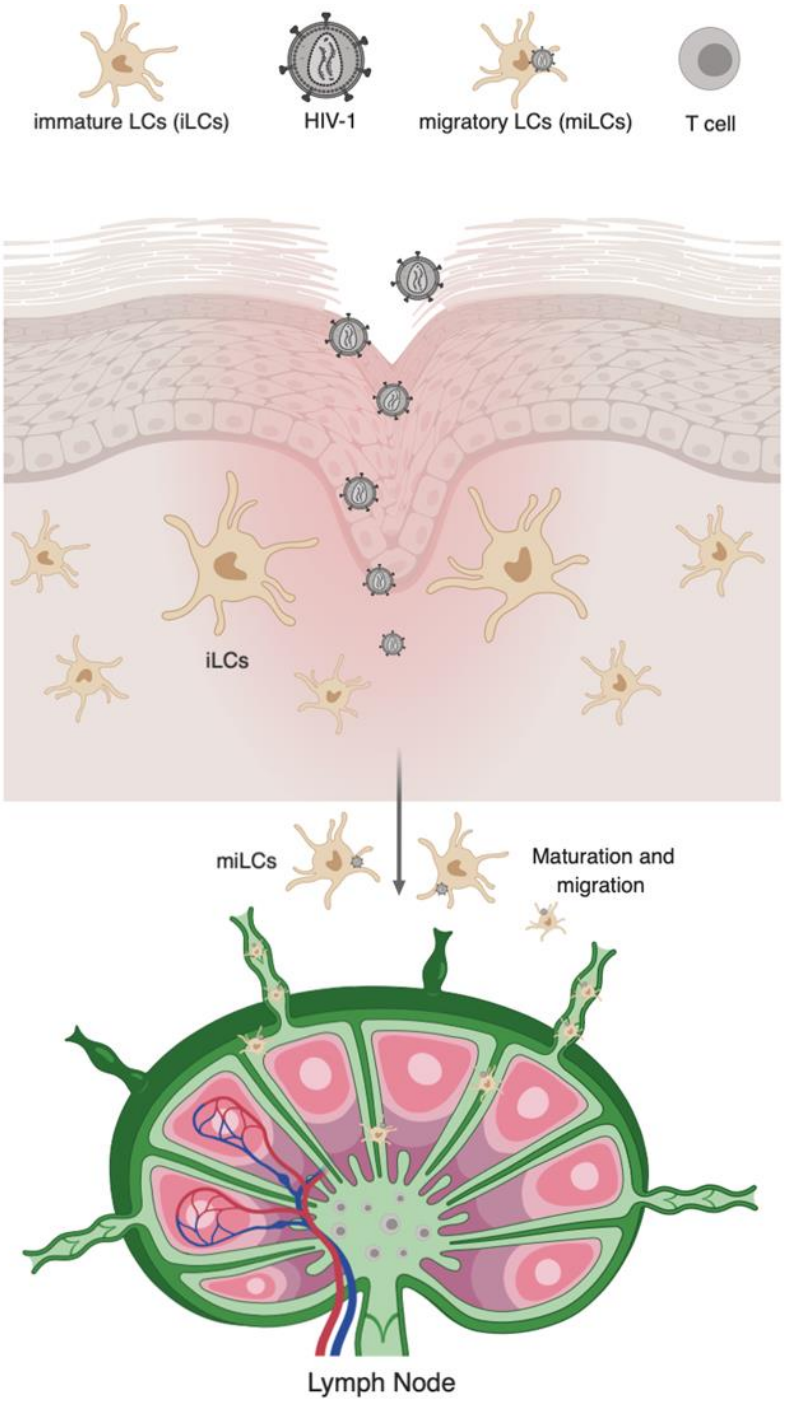


Figure 8. Langerhans cell migration. Following antigenic stimulation, LCs migrates from the epidermis to the lymph node via dermal lymphatics. At the lymph node the LC present the antigen to T lymphocytes. (LC, light orange, virions, light grey; T cell, dark grey).

2. Aim of the study

The mechanisms by which some individuals are able to control HIV infection and progression in contrast to rapidly progressing individuals are poorly understood. Viral control occurs early during infection and upon entry of viruses via mucosal surfaces, immediate non-specific innate immune responses are spontaneously triggered within a short time the innate immune system is completely activated. The complement system plays a crucial role during viral infection in regard to both innate and adaptive immune responses, being one of the first line of defense against invading pathogens. Importantly, complement coats the surface of HIV-1 at the beginning stages of infection due to a C1q binding site at the HIV-1 glycoprotein gp41. Thus, HIV can interact with complement receptors expressed on cell surface, for instance on DCs.

DCs possess intrinsic cellular defense mechanisms to specifically inhibit HIV-1 replication. Thus, DCs are productively infected only at very low levels with HIV-1 and this non-permissiveness of DCs is suggested to go along with viral evasion. We previously showed that complement-opsonized HIV (HIV-C) was able to productively infect various DC subsets significantly higher than non-opsonized HIV (HIV) or antibody-opsonized HIV (HIV-Ig). The higher infection is associated with higher type I interferon (IFN) and adaptive immune responses. Only HIV-C, which bypassed restriction in DCs, but not HIV or HIV-Ig, induced efficient virus-specific innate and adaptive immune responses. Additionally, Prunster *et al.* demonstrated that complement opsonization of HIV-1 caused a significantly higher productive infection of DCs, which was abrogated when blocking antibodies for CR3 were used. Thereby CR3 and CR4 were identified as receptors for binding complement-opsonize HIV-1 whereas non-opsonized virions used to bind to other PRRs, as DC-SIGN or langerin [34, 145, 188].

Studies involving the opsonization patterns of pathogens are important since they mimic the real infection in physiological conditions. Nowadays, HIV-1 infection has become a chronic disease with a lifelong treatment that can present secondary effects. Therefore, in this study we focused on two important questions:

- which roles are the distinct CRs playing with regard to infection with HIV-1?
- how does entry of complement-opsonized HIV-1 affect signaling and antiviral response of various DC subsets?

In this study the exact role of CR3 and CR4 together with a detailed characterization of the signaling pathways induced upon HIV-C infection should be unraveled in various DC subsets.

Specifically, the role of the distinct complement receptors in infectivity and sensing of HIV-1 and whether type I IFN expression is caused by CR3 and CR4 in concert or alone were addressed.

Results from the study should clarify, if complement opsonization or signaling via CRs act as cell-intrinsic sensors mediating an efficient antiviral immune response. Lastly, results from this study might offer new discoveries, new targets and novel strategies in HIV-1 research to efficiently stimulate antiviral immune responses on cellular and humoral levels. The understanding in detail the differences in infectivity and pathways activated opens new avenues of treatment, shock-and-kill and prevention.

3. Results

3.1 THP-1 cell line: an in vitro cell model to specifically down-modulate CRs on myeloid cells

In order to unravel the exact role of the CR3 and CR4 upon binding of complement-opsonized pathogens (HIV-1) compared to their non-opsonized counterparts, we developed three Knock-out (KO) THP-1 cell lines using the CRISPR/Cas9 system. THP-1 is a human monocytic cell line derived from an acute monocytic leukemia patient. We used THP-1 cells because they can be differentiated to DCs by adding IL4, GM-CSF and TNFα. The KO cell lines were generated using lentiviral vectors harboring the CRISPR/Cas9 system and the guide RNAs (gRNA) targeting the specific gen for KO. Specifically, gRNA targeting human CR3 and CR4 genes for Cas9-mediated depletion were designed. We were successful knocking-out CD11b (α-chain of CR3), CD11c (α-chain of CR4) and CD18 (β-chain of CR3 and CR4, it belongs to the integrin family) (Fig. 9). Then we obtained single cell clones and chose the ones with the desired phenotype for further experiments.

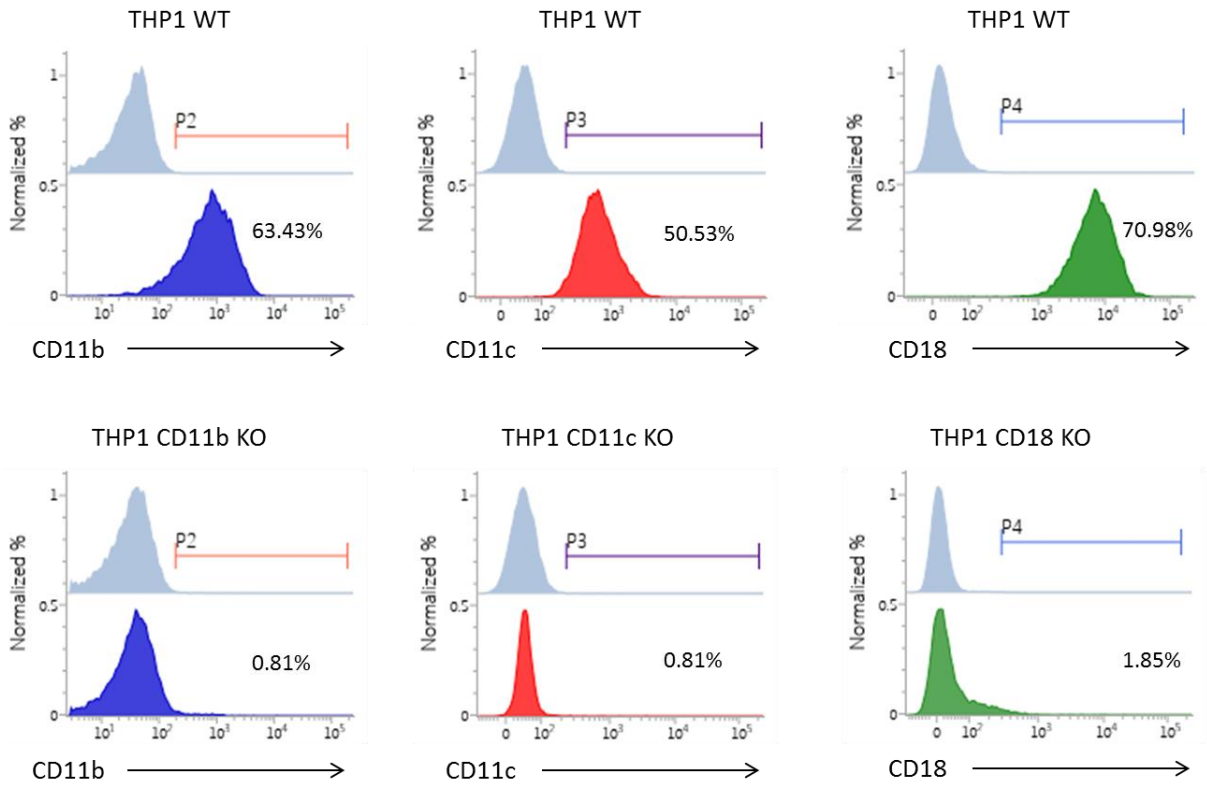


Figure 9. CRs were successfully knocked out on THP1 cells. CR expression measured by FACS in differentiated THP1 WT and the KO for CD11b (left), CD11c (middle) and CD18 (right). As a control, unstained cells are shown on top of each panel.

These three cell lines were used as a tool to study the exact role of each CR and the activated pathways upon complement-opsonized pathogen binding.

Importantly, differentiated WT-THP1 to DCs as well as CR-KO THP1 cells, show a DC-like phenotype with respect to characteristic receptors expressed (Fig. 10) and similar phagocytic activity as their primary counterparts (Fig. 11).

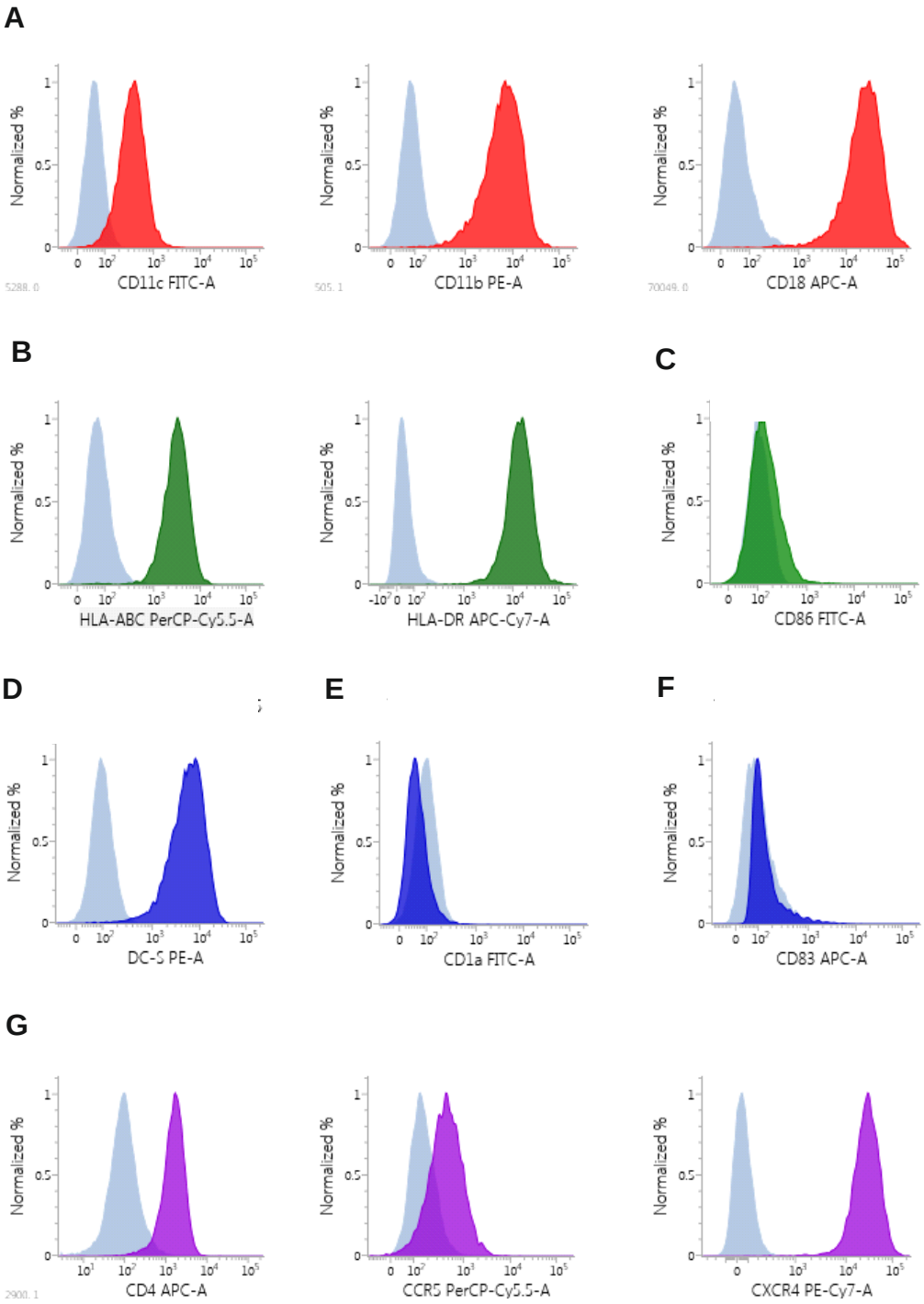


Figure 10. THP1-DCs surface markers expression. Single cell suspensions of THP1-DCs were stained with antibodies against (A) CRs markers (CD11b, CD11c, and CD18), (B) antigen-presentation (HLA-ABC, HLA-DR), (C) co-stimulatory (CD86), (D) DC-SIGN, (E) CD1a, (F) CD83, (G) infection (CD4, CCR5 and CXCR4) and analyzed by flow cytometry. Unstained cells are used as negative control for the flow cytometry analysis, depicted in light grey in each graph.

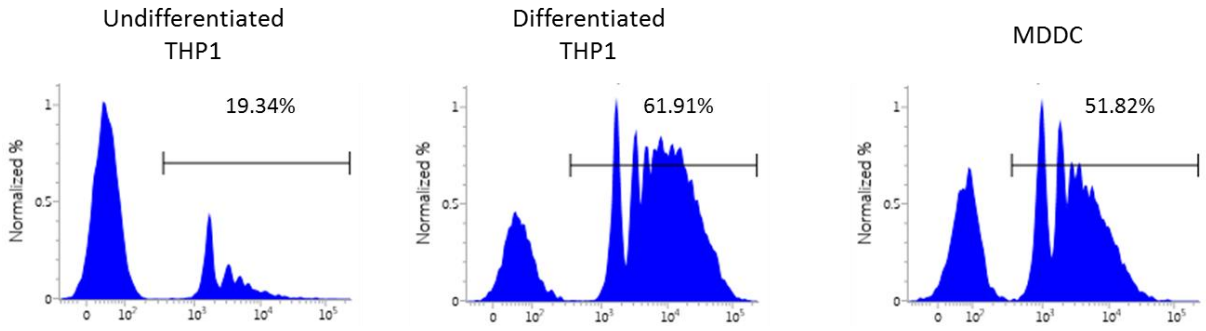


Figure 11. THP1 cells functionally behave as DCs. Undifferentiated THP1 cells, THP1-DCs and primary monocyte-derived dendritic cell (MDDC) were incubated with PE-fluorescent beads and the beads phagocytosis was analyzed by FACS.

3.2 HIV-1

(I) THP1-DCs

Complement-opsonized HIV-1 (HIV-C) and non-opsonized HIV-1 (HIV) to study the contribution of CR3 and CR4 in binding, uptake and processing in DCs using the stable KOs.

The three CR-KO-THP1 DCs cell lines and WT-THP1 DCs were used as a tool to study the impact of the specific CRs upon HIV-C infection. Our group recently highlighted that C-opsonized HIV-1 overcomes restriction in immature DCs (iDCs), resulting in a superior antiviral response. DC activation also occurs upon bacterial co-infections during STIs (sexually transmitted infections) due to the presence of lipopolysaccharides (LPS) and lipopeptides. We previous demonstrated that DCs exposed to HIV-C harbor more virus in the cytoplasm compared to DCs loaded with non-opsonized HIV [1]. This led to a higher productive infection in DCs that could make the virus more susceptible for sensing and to initiate an innate immune activation, since there are more PAMPs present to be recognized by the PRRs of DCs.

Binding of HIV-C to DCs is mainly dependent on CR4.

Binding and internalization of non- and complement-opsonized HIV-1 (HIV and HIV-C, respectively) into WT-THP1 DCs and KO cells were studied in order to evaluate the contribution of the specific receptors. To characterize binding of HIV and HIV-C, THP1-DCs were incubated with virus particles for 6h at 4°C [145]. At 4°C, THP1-DCs bind but do not internalize viral pathogens [145]. LPS-stimulated THP1-DCs and iDCs served as positive and negative controls,

respectively. HIV or HIV-C (25 ng p24/mL) were added to monocyte-derived iDCs and WT-THP1 DCs or KO-THP1 DCs. Cell-bound virus was determined after thorough washing and lysing THP1 DCs by measuring p24 protein levels. HIV-C showed a higher binding to WT-THP1 DCs than HIV.

However, HIV-C binding to KO-THP1 DCs was significantly lower compared to WT-THP1 DCs, showing similar binding levels to non-opsonized HIV (Fig. 12A). We detected a similar binding pattern for CD11b KO-THP1 DCs(11b-THP1 DCs, Fig. 12A, blue) and CD11c KO-THP1 DCs (11c-THP1 DCs, Fig. 12A, yellow) to HIV, although CD18 KO-THP1 DCs (18-THP1 DCs, Fig. 12A, green) showed a slight decrease in binding to HIV (Fig. 12A). However, binding of HIV-C to KO cells was significantly lower compared to WT-THP1. CD18 KO-THP1 DCs (18-THP1 DCs, Fig. 12A, green) showed the lowest binding of HIV-C, followed by CD11c KO-THP1 DCs (11c-THP1 DCs, Fig. 12A, yellow) and CD11b KO-THP1 DCs (11b-THP1 DCs, Fig. 12A, blue). These experiments revealed that CRs, CR3 and CR4 play an important role in the binding of HIV-C to DCs, confirming previous results.

Internalization of HIV-C into DCs is dependent on CR3 and CR4.

In a next step, the internalization efficacy of HIV and HIV-C into WT- and KO-THP1 DCs cells was analyzed. For this, HIV or HIV-C were added at 25 ng p24/ml for 6 h at 37°C. Bound/Internalized HIV or HIV-C were determined by p24 ELISA after washing and lysing the cells. These analyses revealed in accordance to the binding data (Fig. 12A), that the increase of attachment and/or internalization by HIV-C was again stronger in WT-THP1 DCs compared to HIV (Fig. 12B, HIV vs. HIV-C, red). Internalization of non-opsonized HIV into CD11b KO-THP1 DCs (Fig. 12B, blue) was significantly higher than in WT-THP1 DCs (Fig. 12B, red). In contrast, CD11c KO- and CD18 KO-THP1 DCs showed significantly lower internalization of HIV in comparison to WT and CD11b KO cells (Fig. 12B, yellow and green bars). The results regarding HIV uptake with KO-THP1 DCs can be explained by the proximity between receptors. Of note, CD4 has been shown to be the main receptor for DC binding and internalization of HIV-1 [195]. Our data implicate CD4 in binding and internalization together with the close receptors, which in some cases can lead to a higher internalization, as for CD11b KO-THP1 DCs or disruption of the uptake which is the case for CD11c- and CD18 KO-THP1 DCs.

CD11b KO- (Fig. 12B, blue), CD11c KO- (Fig. 12B, yellow) and CD18 KO-THP1 DCs (Fig. 12B, green) internalized significantly lower HIV-C levels than WT cells. The opsonized virus levels, which were internalized, were in the range of their non-opsonized counterparts (Fig. 12B, HIV vs. HIV-C). The increased levels of HIV-C bound and internalized by WT-THP1 DCs were

abolished when the receptors were knocked out (CD11b-, CD11c- and CD18 KO-THP1 DCs), indicating that these receptors, CR3 and CR4 act in concert to uptake HV-C.

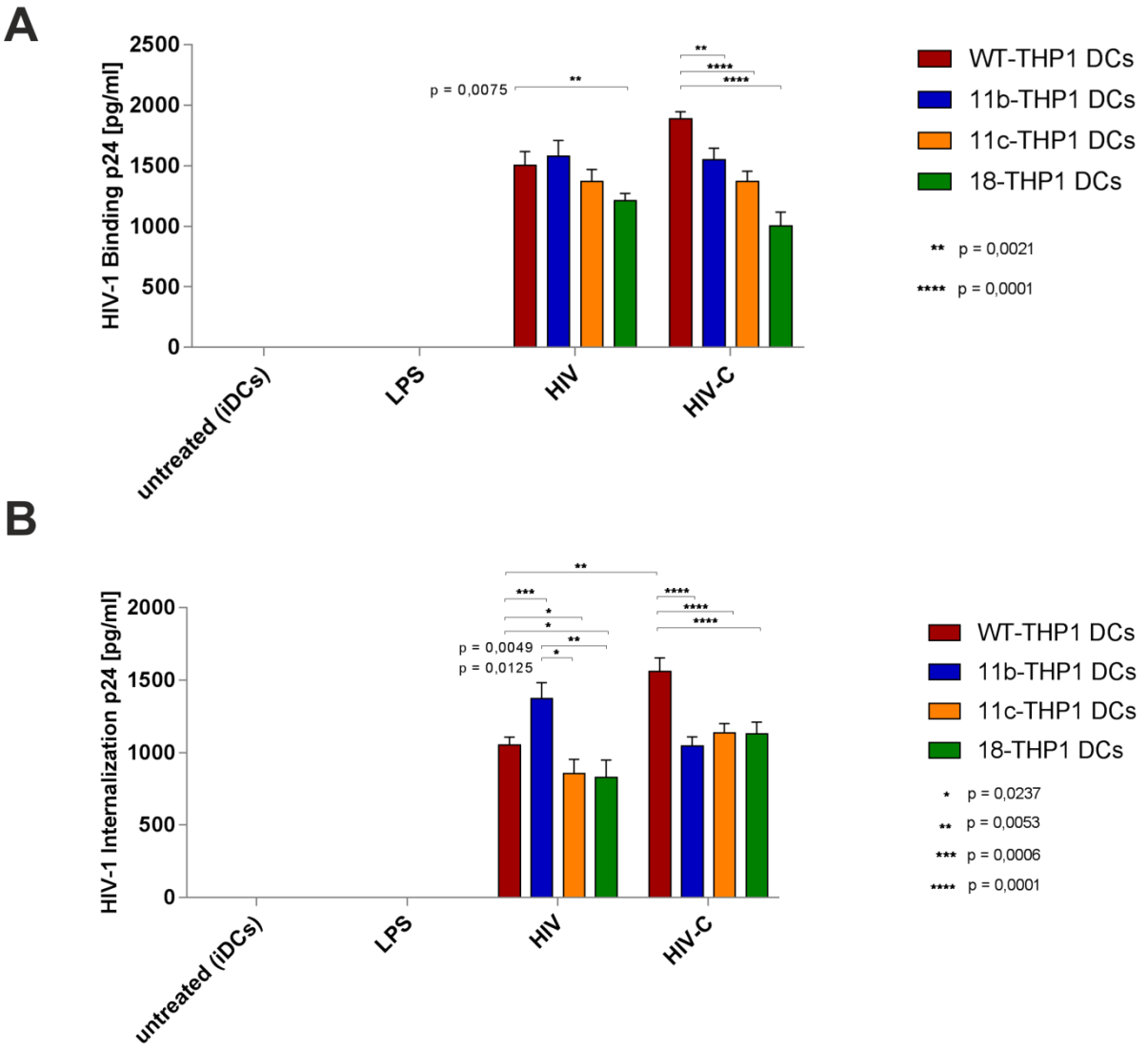


Figure 12. Complement opsonization of HIV-1 enhanced the binding and increased internalization. (A-B) Binding at 4°C (A) and internalization at 37°C (B) were performed in triplicates using 1 x 10^5 WT-THP1 DCs or KO-THP1 DCs cells. Cells were infected with 25 ng p24/ml HIV or HIV-C. Binding and uptake of HIV and HIV-C after 6 h was measured by p24 ELISA. Bar graphs show means ± SEM from three independent experiments. Prior to cell lysate preparation, cells were thoroughly washed to remove unbound virus. Statistical analysis, two way ANOVA with Tukey's multiple comparisons test (*p < 0.05).

CR4 plays a major role with respect to HIV-C infection of DCs

Efficient antiviral T cell responses are initiated when DCs are productively infected by HIV-1 after their resistance to infection is bypassed [167, 169]. In contrast, the inability of DCs to become infected is supposed to be an evasion strategy for HIV-1 survival. As our group previously showed, DCs are efficiently infected by HIV-C, while only low-level productive infection was mediated by HIV [1, 166]. To characterize the specific role of CR3 and CR4 with respect to productive DC infection, WT- and KO-THP1 DCs were exposed to HIV or HIV-C (25 ng p24/ml). We analyzed p24 concentrations in THP1-DCs supernatants several days post-infection.

In WT-THP1 DCs similar results were obtained to what was observed in primary DCs. Thus, while HIV infected WT-THP1 DCs at low levels, the infection was significantly enhanced using HIV-C (Fig. 13, HIV vs. HIV-C, red). In addition, CD11b KO-THP1 DCs showed the same infection kinetics compared to WT, with significantly higher levels of productive infection for HIV-C (Fig. 13, HIV vs. HIV-C, blue).

In contrast, CD11c KO- and CD18 KO-THP1 DCs showed similar p24 levels between HIV and HIV-C (Fig. 13, HIV vs. HIV-C, yellow and green), and productive infection using HIV-C was significantly reduced when compared to CD11b KO- or WT-THP1 DCs (Fig. 13, HIV-C, yellow and green bars vs. blue and red bars). Uninfected iDCs and LPS-exposed THP1-DCs were used as controls.

These experiments revealed that THP1-DCs are a good model for DC infection, not only because similar infection kinetics were observed in WT-THP1 DCs compared to primary DCs but also because complement opsonization of HIV-1 significantly enhanced the productive DC infection. Furthermore, our data revealed that CR3 does not play a major role with respect to infection of DC by HIV-C, since CD11b KO-THP1 DCs cells showed a significant HIV-C-mediated enhancement of DC infection. In contrast, knocking out CR4 resulted in a low-level productive DC infection, comparable to the low-level infection observed using non-opsonized HIV. Knocking out CD18, the common beta chain of CR3 and CR4, did not further reduce productive DC infection compared to CR4 KO-THP1 DCs. To summarize, abrogation of CR3 does not impact productive infection with HIV-C, while CR4 knockout results in low-level DC infection.

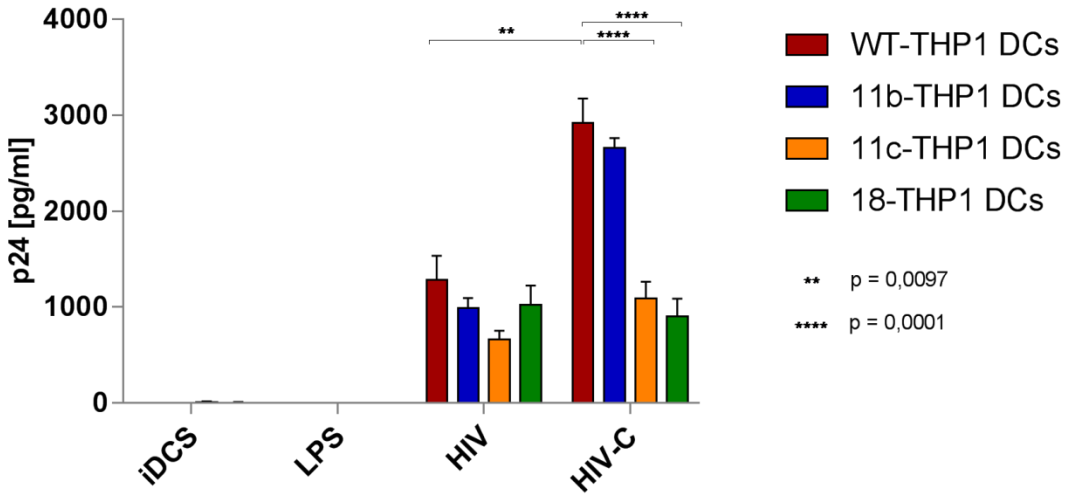


Figure 13. CR4 plays a major role regarding DC infection. THP1-DCs were infected with 25 ng p24/ml of non-opsonized (HIV) and C-opsonized HIV (HIV-C). After 5 days, productive infection was measured in the supernatant by p24 ELISA (means ± SEM). Three representative analyses including technical triplicates are shown. Bar graphs show means ± SEM from three independent experiments. p24 levels within the cell lysates were determined by ELISA. Prior to cell lysate preparation, cells were thoroughly washed to remove unbound virus. Statistical analysis shows 2way ANOVA with Tukey's multiple comparisons test (*$p < 0.05$).

Enhanced DC infection in WT and CD11b KO-THP1 DCs is independent on viral tropism.

To investigate whether the higher productive infection with HIV-C is dependent on the viral tropism, we infected THP1-DCs with differentially opsonized HIV-1. As ascertained flow cytometrically (Fig. 10G) WT- and KO-THP1 DCs express on the cell surface together with the main receptor CD4, both main coreceptors essential for HIV-1 entry. The WT- and KO- THP1 DCs cell lines provide the opportunity to simultaneously evaluate the effect of CCR5 and CXCR4 upon HIV-1 infection. Those cell lines reliably supported HIV-1 infection of diverse laboratory-adapted strains with carrying coreceptor usage (R5, X4 and R5/Xa). R5- (Yu2B and R9Bal molecular clones), R5X4 (93BR020) and X4 (NL4-3)-tropic HIV-1 were used for infection of THP1 WT and CR-KO cells. Importantly, THP1-DCs cells support infection of all evaluated R5 and R5/X4 HIV-1 strains. These analyses revealed that all viruses showed the same infectivity independent on the viral tropism. Most importantly, HIV-1 infection kinetic was always the same, being the enhancement by complement opsonization suppressed by the absence of CD11c and CD18 (Fig. 13).

Vpx-carrying HIV-1 promotes infection of THP1-WT and KO cells.

As shown by Manel *et al*., productive DC infection resulted in an efficient antiviral T cell response [169], while low DC infection was associated with a moderate antiviral immune response. This process was also postulated as 'Trojan horse' strategy [196]. HIV-1 is susceptible to SAMHD1 restriction, as it was explained before (see Introduction). As a result, the virus is limited in its ability to infect DCs. Furthermore, bypassing HIV-1 restriction mechanisms in DCs by Vpx-mediated degradation of SAMHD1, was associated with an increased quality and quantity of virus-specific immune responses [169]. Vpx-containing HIV-1 infects myeloid cells 10- to 100 fold better than WT HIV-1.

The use of a Vpx-carrying virus allow us to see a higher infection, therefore, we wanted to know if the complement opsonization phenotype seen with WT virus was also true for the highly infective Vpx-carrying virus. We infected THP1-DCs with differentially opsonized Vpx-carrying HIV-1 virus preparations (BR322-Vpx, BR322-Vpx-C) [197, 198]. iDCs were used as negative controls. Virus (25 ng p24/ml) was added as described above and p24 concentrations of newly produced HIV or HIV-C were determined by ELISA in THP1-DCs supernatants several days post-infection. As expected, HIV-C-Vpx replication in WT-THP1 DCs was significantly higher than the infection observed with non-opsonized HIV-Vpx (Fig. 14). We observed that complement opsonization was still able to enhance significantly higher infection, even in Vpx-containing virus. The highest productive infection in THP1-DCs was using HIV-C-Vpx (Fig. 14A-E) as also described in primary DCs [1].

The enhanced effect by complement opsonization of HIV-Vpx was abolished in CD18 KO-THP1 DCs (Fig. 14, green bars) reaching the levels of infection with non-opsonized virus. Infection of CD11b- and CD11c KO-THP1 DCs (Fig. 14, blue and yellow bars) by HIV-C-Vpx was also significantly lower compared to WT-THP1 DCs.

The result suggests that the higher infection of HIV-C is due to the engagement of CRs, since when CRs are knocked-out, the levels of infection are similar to the non-opsonized virus, even when SAMHD1 restriction is overcome by Vpx.

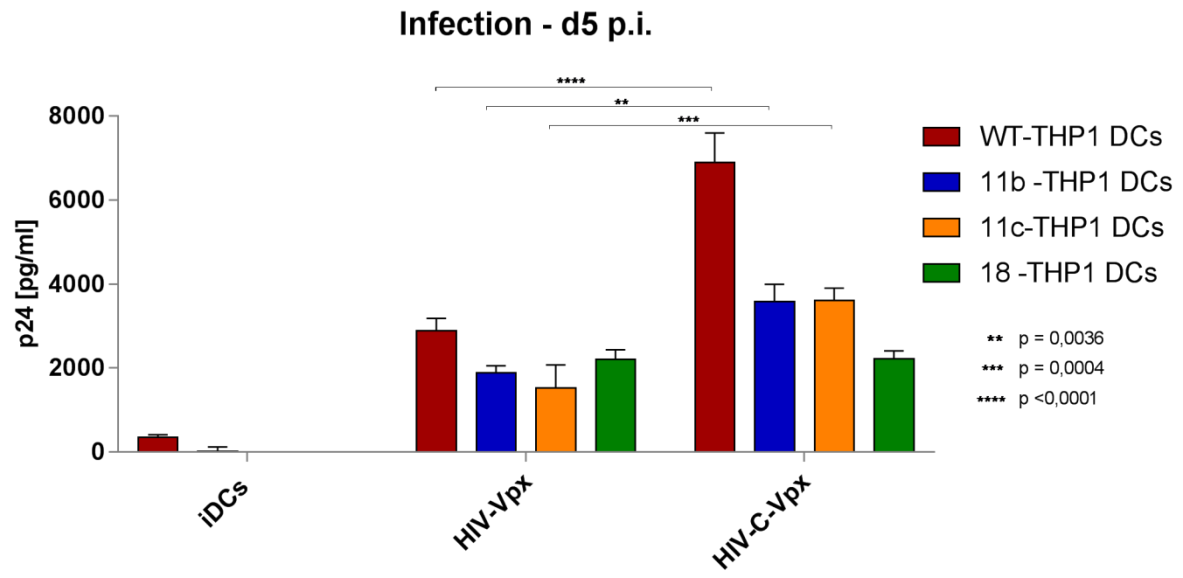


Figure 14. Complement opsonized HIV efficiently infects THP1-DCs. WT- and KO-THP1 DCs were infected with 25ng p24/mL of non-opsonized HIV-Vpx and complement-opsonized HIV-Vpx-C. A-D) Infection kinetics, from day 1 to day 5 of one out of three representative assays including technical triplicates. E) p24 production of THP1-DCs on day 5 p.I, when clear differences are observed. Statistical analysis shows 2way ANOVA with Tukey's multiple comparisons test (*p < 0.05).

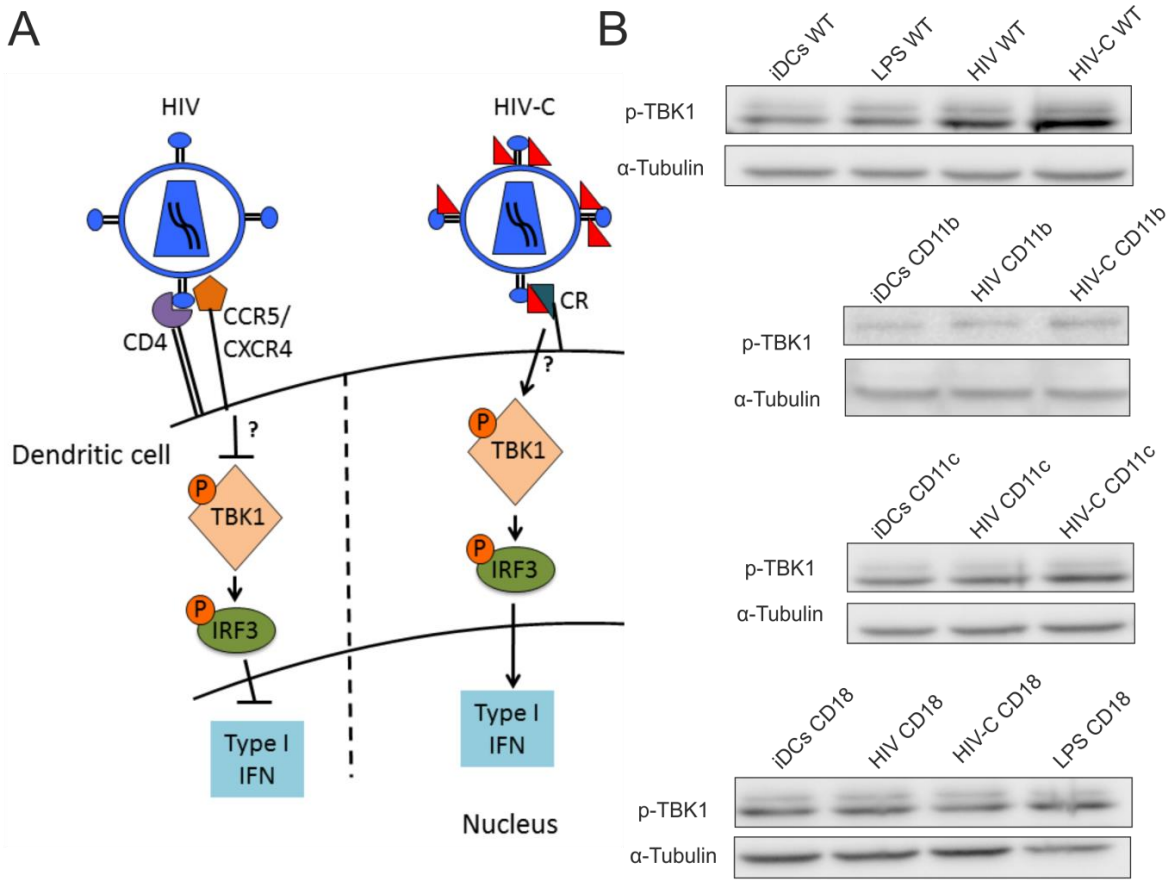
IRF3 and TBK1 are highly activated in HIV-C exposed THP1-DCs and abrogated upon CR4 depletion.

Since the previous results showed that WT-THP1 DCs and CD11b KO-THP1 DCs are highly susceptible to infection using HIV-C, we next studied whether they also sense the virus more efficiently. Virus sensing is associated with enhanced expression of antiviral genes encoding IFN-α and IFN-β and IP-10 (IFN-γ inducible protein 10). To induce these antiviral factors, a coordinate activation of factors, including IFN-regulatory factor 3 (IRF3) or Traf family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1), is required [199, 200] (Fig. 15A).

Recent studies have shown that TBK1 [201, 202] is required for the activation of IRF3 and the induction of IFN-β gene expression in human cell lines in culture [203, 204]. As well, other studies linked the activation of the TBK1-IRF3 signaling axis and the consequently production of type I IFN with viral infection, determining the outcome of host antiviral immune response [205, 206]. To unravel whether IFN-inducing signaling pathways are triggered upon CR engagement, we next studied TBK1 and IRF3 phosphorylation in THP1-DCs. By immunoblotting we found that IRF3 was highly activated in WT-THP1 DCs exposed to HIV-C after 4h pI, while this was not detected in HIV-exposed WT-THP1 DCs (Fig. 15C and E, red bars). In concordance with results shown above, a higher IRF3 phosphorylation was also detected in HIV-C-loaded CD11b KO-THP1 DCs cells (Fig. 15C and E, blue bars).

In CD11c KO-THP1 DCs, IRF3 activation was abrogated using HIV-C, while HIV mediated an enhanced IRF3 activation compared to WT-THP1 DCs (Fig. 15C and E, yellow bars). The lowest IRF3 activation was detected in CD18 KO-THP1 DCs (Fig. 15C and E, green bars).

As expected, a similar phosphorylation pattern as described for IRF3 was shown for TBK1, thereby pointing to the existence of a TBK1-IRF3 axis (Fig. 15B and D). A higher activation of the TBK1-IRF3 signaling axis through HIV-C is induced in WT- THP1 DCs and CD11b KO-THP1 DCs, but knocking out CD11c and CD18 abolish their phosphorylation.


A
HIV
HIV-C
CCR5/
CXCR4
CD4
CR
Dendritic cell
TBK1
IRF3
Type I
IFN
Nucleus
B
iDCs WT
LPS WT
HIV WT
HIV-C WT
p-TBK1
α-Tubulin
iDCs CD11b
HIV CD11b
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HIV CD11c
HIV-C CD11c
iDCs CD18
HIV CD18
HIV-C CD18
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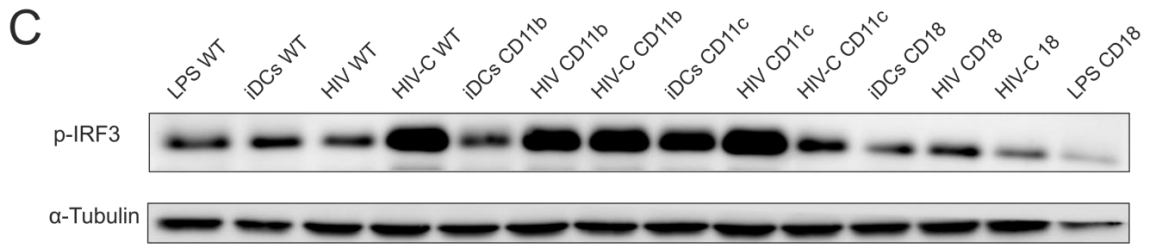
C
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HIV-C 18
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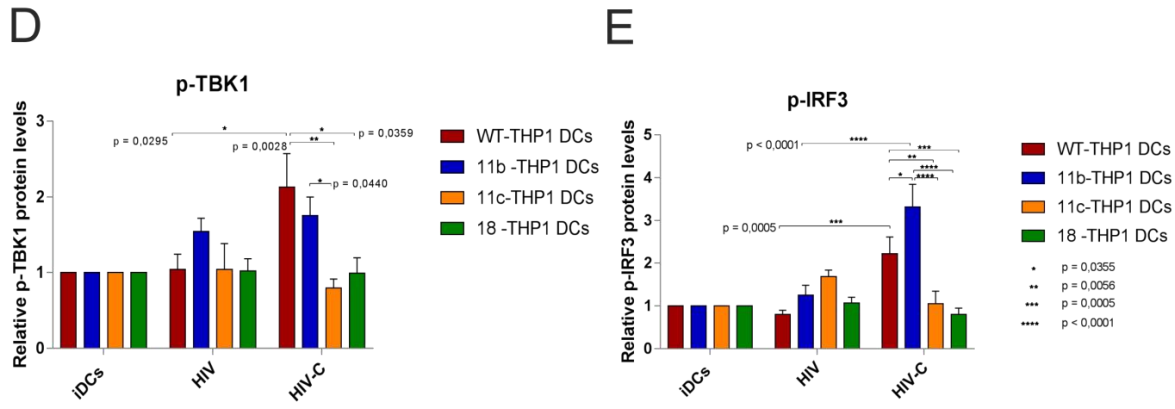
D
p-TBK1
Relative p-TBK1 protein levels
p = 0,0295
p = 0,0028
p = 0,0359
p = 0,0440
WT-THP1 DCs
11b -THP1 DCs
11c-THP1 DCs
18 -THP1 DCs
iDCs
HIV
HIV-C
E
p-IRF3
Relative p-IRF3 protein levels
p < 0,0001
p = 0,0005
WT-THP1 DCs
11b-THP1 DCs
11c-THP1 DCs
18 -THP1 DCs
p = 0,0355
p = 0,0056
p = 0,0005
p < 0,0001

Figure 15. HIV-C mediates TBK1-IRF3 phosphorylation. A) Scheme of HIV-1 and HIV-1-C sensing in THP1-DCs. HIV-1 (left) infection blocks TBK1 phosphorylation and production of type I IFN. HIV-C (middle) triggers a yet unknown pathway that leads to TBK1 activation by phosphorylation and type I IFN production. B) TBK1 phosphorylation was analyzed by immunoblotting. Cells were left untreated (iDCs), or stimulated 4h with LPS, HIV or HIV-C for 4h. Immunoblot assay was performed using phosphorylated TBK1 (pTBK1) and α-Tubulin as loading control. A representative blot out of 7 is depicted. C) IRF3 phosphorylation was analyzed by immunoblotting. Cells were left untreated (iDCs), or stimulated 4h with LPS, HIV or HIV-C for 4h. Immunoblot assay was performed using phosphorylated IRF3 (pIRF3) and α-Tubulin as loading control. A representative blot out of 7 is depicted. D pTBK1 protein expression from 7 independent experiments was summarized using ImageJ quantification. E) p-IRF3 protein expression from 7 independent experiments was summarized using ImageJ quantification.

HIV-C also activates NF-κB and TAK1 in WT-THP1 DCs, while this is abrogated in CD11c- and CD18 KO-THP1 DCs.

Transcriptional activation of the *IFNB* gene requires the activation of IRF3, which might be due to other downstream factors, such as AP-1 and NF-κB [207], indicating that NF-κB binding is essential for IFNβ production. Previous work from our group showed that in contrast to HIV-loaded DCs, IRF3 and NF-κB were translocated to the nucleus in DCs exposed to HIV-C [1]. Thus, we analyzed by immunoblotting the activation of the p65 subunit NF-κB in THP1-DCs upon HIV and HIV-C. Activation of NF-κB was detected in HIV-C but not upon HIV, by using NF-κBp65-Ab direct against the nuclear localization sequence (NLS) of human p65, therefore selectively binding to the activated form of NF-κB. A strong activation of NF-κB was detected in WT- and CD11b KO-THP1 DCs loaded with HIV-C (Fig. 16A-C, red and blue bars). When CR4 was depleted, activation of NF-κB was abrogated; this resulted in a similar signal in HIV or HIV-C exposed cells (Fig. 16A-C, yellow bars). CD18 KO-THP1 DCs data reveal that β_2 integrins can affect the inflammatory cytokine production by fine-tuning NF-κB pathway activation.

Beside NF-κB, transforming growth factor β activated kinase 1 (TAK1) phosphorylation was analyzed, since this molecule was described as an upstream signaling molecule targeted by HIV Vpr [208]. Thereby, we analyzed by immunoblot the activation of TAK1 using an antibody specific to phosphorylated TAK1 (Fig. 16B-D). No significant differences regarding TAK1 phosphorylation were detected independent on the HIV opsonization pattern (Fig. 16B-D). However, slightly lower levels of p-TAK1 were observed in HIV-C-exposed CD11c-THP1 DCs, suggesting that this mutant might fail to activate TAK1 at all (Fig. 16B-D).

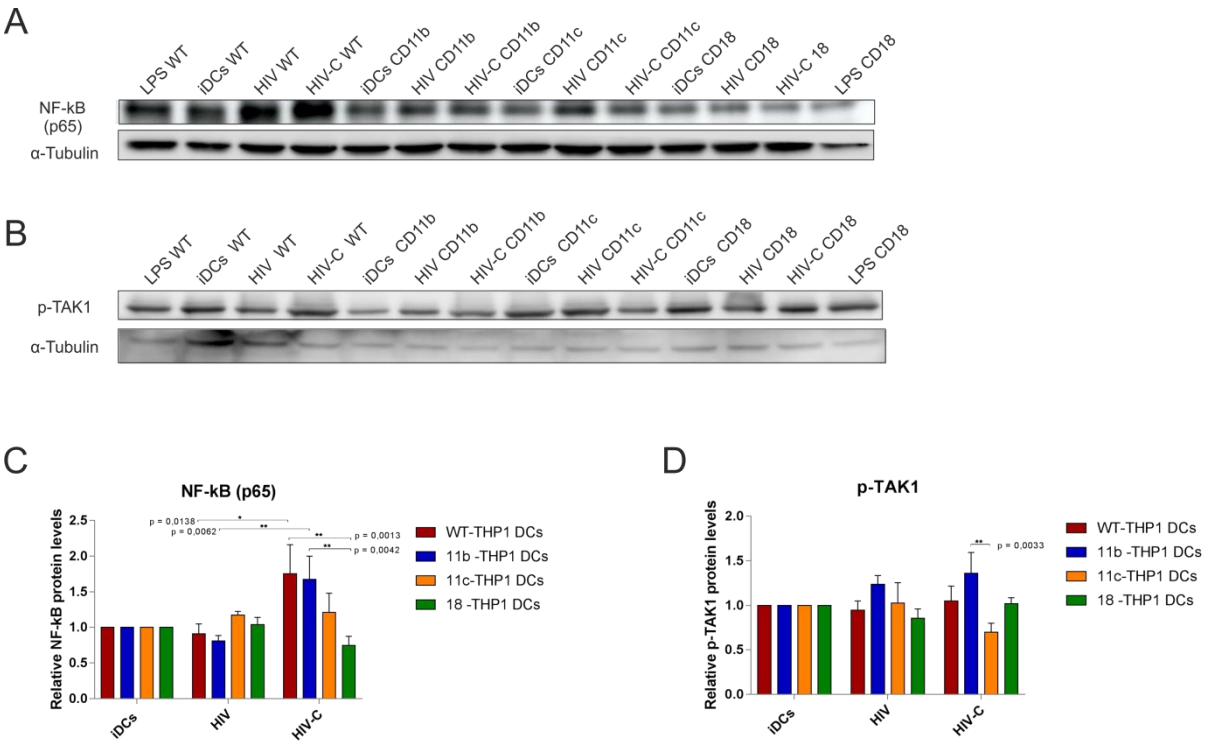

Figure 16. Complement-opsonized HIV-1 enhances NF-κB and TAK1 pathway activation. A) THP1-DCs cells were incubated for 4 hours with the indicated concentration of HIV-1, HIV or HIV-1. At the end of the incubation, cells were harvested and cell lysates containing equal amounts of proteins were subjected to immunoblotting using Abs specific to indicated proteins. B) Data are mean ± SEM of relative NF-κB and p-TAK1 from 7 experiments.

Knocking out CD11c on DCs omits their antiviral capacity after HIV-C exposure

After describing in detail the decreased activation of the antiviral signaling axis TBK1/IRF3/NFkB in CD11c KO-THP1 DCs, we next studied type I IFN expression in the different THP1-DCs. Kinetics of IFNB mRNA expression in WT and KO-THP1 DCs were analyzed from 0 to 12 h after addition of HIV and HIV-C (Fig. 17, 4h time point). We found as described in primary DCs, that complement opsonization of HIV-1 (Fig. 17, HIV-C, dark grey) resulted in a significantly increased IFNB gene expression compared to its non-opsonized counterpart (Fig. 17, HIV, light grey) in WT-THP1 DCs (Fig. 17, WT). Expression of type I IFN levels in HIV-exposed DCs was similar to untreated DCs (Fig. 17, iDCs, black). Knocking out CD11b on DCs resulted in an even significantly enhanced IFNB expression after HIV-C loading compared to HIV-exposed CD11b KO-THP1 DCs (Fig. 17, CD11b, dark vs. light grey bars) or HIV-C-loaded WT-THP1 DCs (Fig. 17, WT vs. CD11b). When CR4 single knock-outs (CD11c KO-THP1 DCs) were incubated with HIV-C, all antiviral capacity of the cells was lost and type I IFN levels were at background levels comparable to iDCs and HIV-DCs (Fig. 17, CD11c). Surprisingly, knocking out CD18 on THP1-DCs, which results in abolishment of CR3 and CR4 as well as the adhesion molecule LFA-1 (CD11a/CD18), triggered an overshooting type I IFN mRNA expression after stimulation HIV

(Fig. 17, CD18, light grey), while type I IFN expression of HIV-C-exposed CD18 KO-THP1 DCs was at background (Fig. 17, CD18, dark grey). These data summarize, while CR3 down-modulates antiviral immune responses, CR4 is the major player with respect to type I IFN induction, when HIV-1 is opsonized. The role of CD18 knock out respecting the significantly up-regulated IFNB expression levels after stimulation with non-opsonized HIV-1 remains to be characterized.

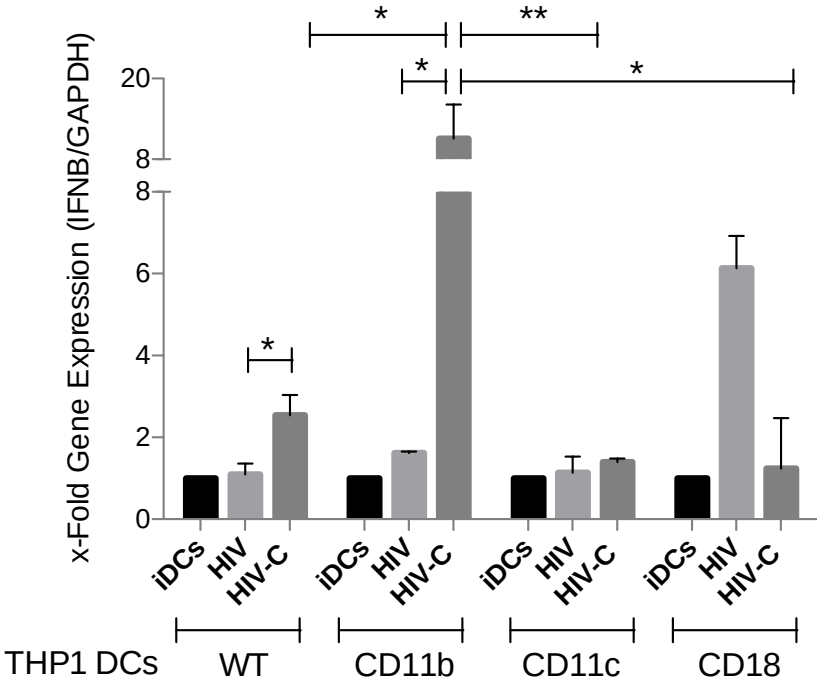

Figure 17. HIV-C induces IFN signaling in WT- and CD11b KO-, but not in CD11c KO- and CD18 KO-THP1 DCs. A-E) mRNA expression was relatively quantified using GAPDH gene expression as reference and the ΔΔCt method. E) Data are mean ± SEM of relative quantification by RT-qPCR was performed by using cells from 4 experiments.

Altogether, these analyses emphasize the important role of complement to efficiently signal the viral presence to THP1-DCs, therefore initiating an efficient antiviral immune response.

HIV-C exposed WT-THP1 DCs enhance production of pro-inflammatory cytokines, while the induction was lost when CR3 and CR4 are knocked out.

Th17 cells produce cytokines important in the maintenance of an intact epithelium and in host defenses against opportunistic pathogens [209]. We earlier illustrated a role of complement in the polarization of Th17 cells via DCs [210]. Therefore, we here analyzed the expression of Th17-polarizing cytokines IL-1β, IL-6 and IL-23 in WT- and KO-THP1 DCs to see in detail whether the Th17-polarizing capacity is mediated via CR3 and/or CR4.

We first studied IL-1β protein levels in supernatants of differentially treated WT and KO-THP1 DCs after 24h. As illustrated in primary DCs, HIV-C mediated significantly higher IL-1β protein levels in WT-THP1 DCs compared to those measured from supernatants of HIV- or untreated WT-THP1 DCs (Fig. 18A, WT). When CR3 was knocked out by CD11b deletion, a significantly higher IL-1β secretion was observed than in HIV WT-THP1 DCs, while protein secretion of HIV-C-exposed CD11b KO-THP1 DCs remained comparable to WT-THP1 DCs (Fig. 18A, WT vs. CD11b). IL-1β secretion from HIV-C-exposed THP1-DCs was significantly down modulated in CD11c KO-THP1 DCs and reduced in CD18 KO-THP1 DCs when compared to WT-THP1 DCs (Fig. 18A, WT vs. CD11c and CD18).

IL6 (Fig. 18B) and IL23 (Fig. 18C) were analyzed on mRNA levels. We observed that HIV-1 induced IL6 mRNA expression levels at 6h pI in WT- and CD11b- and CD11c KO-THP1 DCs, while CD18 KO-THP1 DCs showed basal IL6 mRNA levels comparable to those seen in iDCs. HIV-C up-regulated IL6 in WT- and CD11b KO-THP1 DCs (Fig. 18B, WT and CD11b) in comparison to its non-opsonized counterpart, but the increase was not significant. In CD11c- and CD18 KO-THP1 DCs, IL6 levels were similar between HIV- and HIV-C-treated cells with CD18 KO-THP1 DCs showing the lowest IL6 levels near background (Fig. 18B, CD11c vs. CD18). Though the up-regulation of IL6 in THP1-DCs was not significant as earlier shown in primary DCs [210], increased levels were observed in WT-THP1 DCs.

As illustrated in primary DCs [210], HIV-C infection of WT-THP1 DCs induced a highly significant mRNA expression of IL23 at 8 h pI compared to its non-opsonized counterpart (Fig. 18C, WT, HIV vs. HIV-C). In contrast, IL23 was down-modulated to levels comparable to iDCs or HIV-DCs when exposing CD11b- or CD11c KO THP1-DCs to HIV-C (Fig. 18C, CD11b, CD11c, HIV vs. HIV-C). Notably, CD18 KO-THP1 DCs showed a down-regulation of this cytokine compared to iDCs independent on whether using HIV or HIV-C (Fig. 18C, CD18, iDC vs. HIV and HIV-C).

These data point to the already described Th17-polarizing capacity of complement-opsonized HIV in WT THP1 DCs, while this was lost, when both CR3 and CR4 were knocked out.

Knocking out CR4 induces an anti-inflammatory DC type.

We next studied IL10 cytokine from THP1-DCs stimulated with HIV or HIV-C. We found that IL10 transcript levels were lower in HIV-C exposed WT-THP1 DCs, however this was statistically not significant (Fig 18D, WT, HIV vs. HIV-C). IL10 mRNA expression was not affected upon HIV and HIV-C treatment of CD11b KO-THP1 DCs compared to iDCs (Fig. 18D, CD11b, iDC vs. HIV and HIV-C). On the contrary, we noted that CD11c KO-THP1 DCs exposed to HIV-C up-regulated IL10 mRNA levels (Fig. 18D, CD11c, iDC and HIV vs. HIV-C), while CD18 KO-THP1 DCs showed a

slightly higher induction upon HIV stimulus (Fig. 18D, CD18). We here show, that an anti-inflammatory capacity is mediated in DCs upon CR4 knock-out.

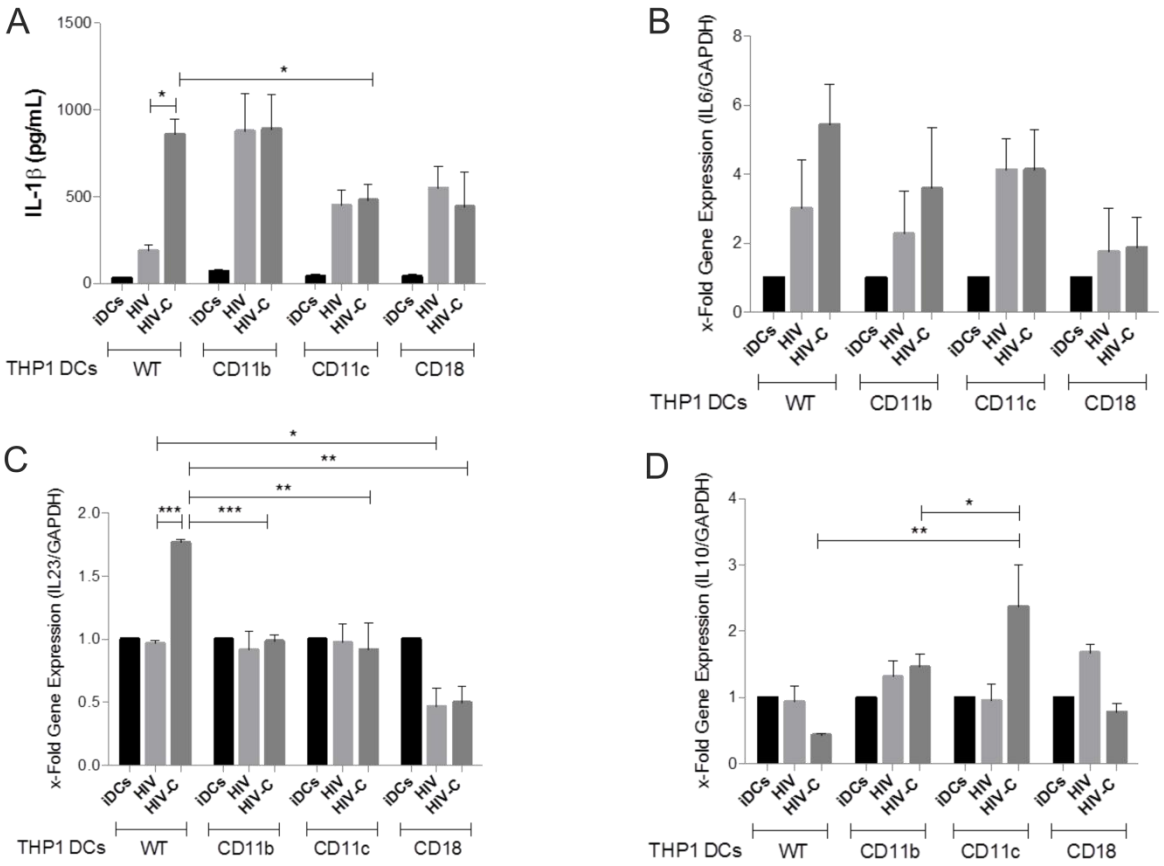

Figure 18. Expression of pro-and anti-inflammatory cytokines in WT- and KO-THP1DCs exposed to non-opsonized and complement-opsonized HIV. A) The concentration of IL-1β in the supernatants was measured by ELISA. THP1-DCs were stimulated with HIV and HIV-C with 25ng p24/mL for 12h, 24h and 48h. Shown the most representative time-point of IL-1β cytokine. The results are displayed as relative cytokine levels, with the values found for iDCs at the basal concentration. Data are mean±SEM of 3 independent experiments. B-D) Summed up quantification of all cell types and analyzed using a 2-Way ANOVA for significant changes between WT- and KO-THP1 DCs upon HIV and HIV-C exposure. LPS-stimulated THP1-DCs were used as positive control (not shown) and untreated THP1-DCs (iDCs) as negative. The target genes were quantified relative to normalization of the reference glyceraldehyde 3-phoisphate dehydrogenase (GAPDH) and analyzed using ΔΔCt method. Real-time quantification was repeated 4 times in duplicate for each cytokine, summarized, and evaluated for significant changes between the 2 groups (HIV and HIV-C) using the unpaired Student t test (2 tailed).

(II) LCs

Complement opsonization of HIV-1 enhances antiviral immunity in LCs.

In vivo, HIV-1 spontaneously activates complement in seminal fluid, plasma and at mucosal surfaces, therefore becomes opsonized and acquires an increased potential to infect LCs or other susceptible cells at mucosal surfaces. However, whether this is involved in viral transmission is unknown. Here we studied the role of complement in HIV-1 infection, transmission and antiviral functions by LCs.

As previously explained, LCs reside in mucosal tissue and are among the first immune cells encountering HIV-1 during sexual intercourse. Immature LCs efficiently degrade HIV-1 via langerin-induced autophagic processes, preventing HIV-1 infection of LCs and subsequent transmission to T cells. Thus, LCs function as a natural barrier to HIV-1. However, activated LCs were shown to be susceptible to HIV-1 infection [188].

We therefore decided to evaluate the role of complement in sexual transmission of HIV-1 using isolated human LCs and an *ex vivo* human tissue transmission model. Seminal fluid (SF) and normal human serum (NHS) were used as sources of complement (C-) to opsonize HIV-1. All the experiments were first performed using NHS (HIV-C), since the opsonization protocol have been widely established and optimized by our group and others. We just corroborate infection and transmission *in vitro* and *ex vivo* using virus preparations opsonized with seminal fluid (HIV-SF).

Receptors for Complement on Langerhans Cells

Membrane receptors for products of complement activation are widely distributed among cells in most mammalian tissues. The specificity of these receptors for certain complement components as well as for their fragments varies among cells, and the same cell may simultaneously express different receptors for different complement components or fragments.

In order to clarify the role of CRs in sexual transmission, the receptor expression of primary LCs, namely CR3, CR4 and langerin, was investigated. Stingl and collaborators demonstrated that human LCs express Fc-IgG and C3 receptors [211]. Based on that, we obtained LCs from the epidermis after a sequential isolation procedure necessary to obtain a pure population. The epidermis was first isolated from patient biopsies, followed by digestion to obtain a single-cell suspension. From the suspension, LCs were positively selected using magnetic anti-CD1a beads. Finally, the isolation procedure was evaluated using flow cytometry staining, confirming the immature state of the cells. For mature LCs, the epidermis was split from the dermis, and LCs were allowed to migrate out of the skin. Immature LCs (iLCs) and mature (Fig. 19), also called

migratory (miLCs), were identified based on expression of CD1a^{high} and CD207/langerin^{high}. iLCs and miLCs are CD1a^{high}, whereas the expression of CD207/langerin varies depending on the activation state. The cells were further characterized by the expression of additional markers such as CD11b and CD11c. iLCs, due to their pathogen uptake capacity, expressed very high levels of langerin, CD11b and CD11c (Fig. 19B, red). However, miLCs, down-regulated the expression of CD11c (Fig. 19B, blue, right panel), while CD11b level remained stale (Fig. 19B, blue, middle panel) and present intermediate expression of langerin [188, 193] (Fig. 19). Therefore, iLCs as well as miLCs express the CR repertoire needed for interactions with HIV-C.

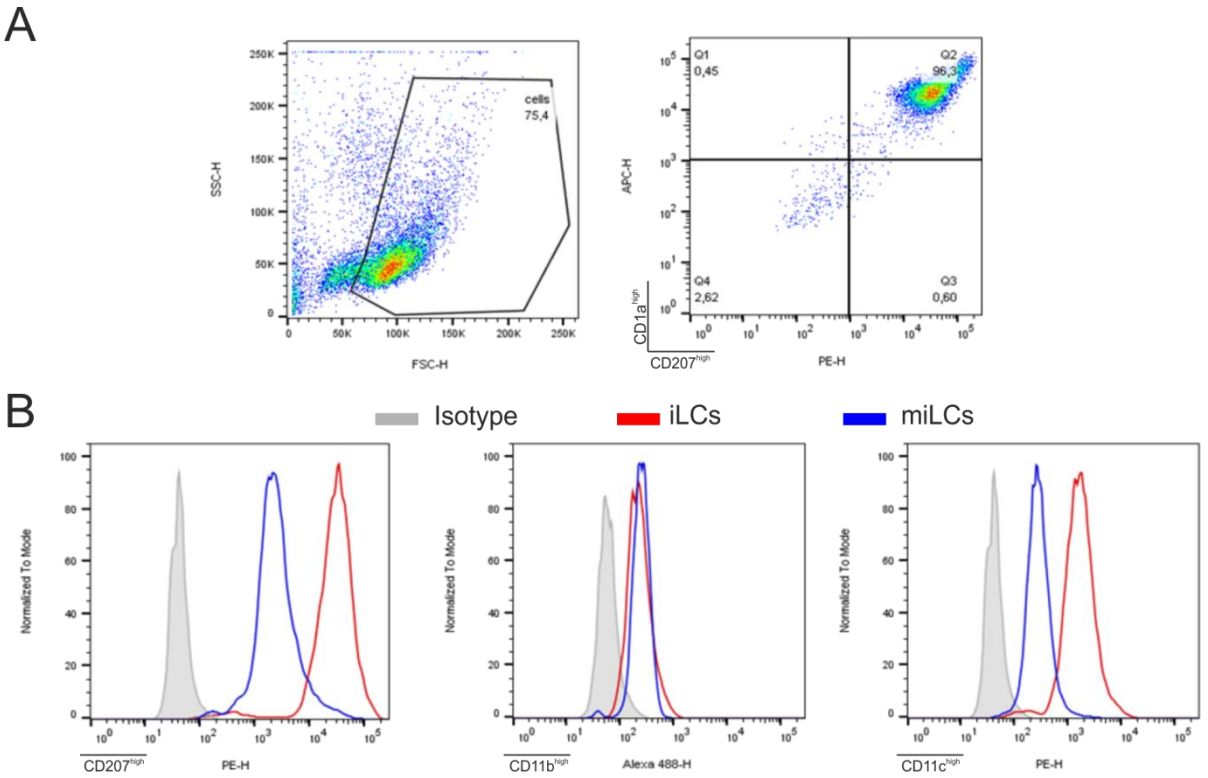

Figure 19. Cytofluorometric analysis of receptors expressed in iLCs and miLCs isolated from the skin. A) Gating strategy. LC population isolated from skin was gated on CD1a^{high} / CD207^{high} cells. B) From the selected LC population, expression of CD207/langerin, CD11b (α-chain of CR3) and CD11c (α-chain of CR4) were analyzed in immature LCs (iLCs) (red) and mature or migratory LCs (miLCs) (blue). Analysis was assessed using a FACSCanto flow cytometer and FlowJo software.

Several studies using *ex vivo* skin explant models have shown that LCs can be infected with HIV-1 and subsequently transmit HIV-1 to T cells [212, 213]. We have used an *ex* vivo tissue transmission model [214] to investigate the role of LCs in transmission of complement-opsonized HIV-1. Human epidermal sheets were exposed to different virus preparations, non-opsonized and C-opsonized. As complement source to opsonize HIV-1, normal human serum (NHS) and seminal fluid (SF) were used. Transmission of HIV-1 may occur following the passage of virus through monostratified mucosal epithelium, such as the endometrial cell monolayer of

the endocervix upon sexual transmission of HIV-1. Semen and cervicovaginal secretions of HIV-1 seropositive individuals contain cell-free HIV-1 particles [215, 216] and soluble complement components [217-221]. Since CRs are expressed on the LCs, as well as on DCs and macrophages in the submucosa, it may be speculated that opsonization of HIV-1 with complement is important in the early events associated with mucosal transmission of HIV-1 [222, 223].

Complement in seminal fluid is activated by HIV-1 particles.

To investigate the ability of HIV-1 to activate complement in seminal fluid (SF), different HIV-1 strains, primary R5-tropic HIV-1 strain 92BR030 and laboratory R5-tropic strain Yu2B, were added to seminal fluid diluted in DPBS (1:1 ratio) and incubated for 1 h at 37°C, resulting in SF-opsonized virus (HIV-SF). The opsonization pattern was determined by virus capture assay (VCA) as describe [166]. Briefly, we used anti-human C3c- (recognizing C3b, iC3b), C3d-, IgG- and mouse IgG Abs as a control for background binding. As a control, opsonization of HIV-1 with NHS (HIV-C) was performed, which results in the covalent deposition of C3 fragments (C3b, iC3b, C3d, C3c) on the viral surface. We incubated HIV-1 (concentration > 1µg p24/ml) for 1 h at 37°C with NHS in a 1:10 dilution to obtain the C-opsonized virus (HIV-C) (Fig. 20).

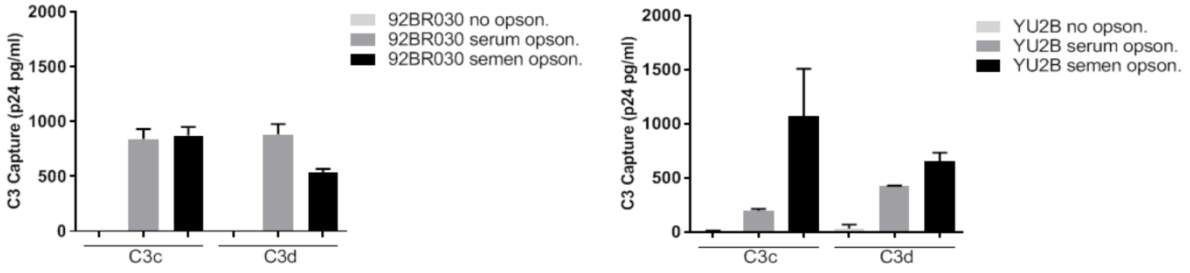

Figure 20. Representative VCA. VCA is performed routinely after opsonization of the viral preps to display C3c, C3d and background IgG deposition (not shown) on viral surface. We saw that opsonization of the virus when using seminal fluid (SF), HIV-SF (92BR030-SF and Yu2B-SF), is comparable to the normal human serum (NHS), HIV-C (92BR030-C and Yu2B-C).

HIV-1 infection and transmission are enhanced in a complement-dependent manner in LCs ex vivo

Several studies using *ex vivo* skin explant models have shown that LCs can be infected with HIV-1 and subsequently transmit the virus to T cells [212, 213]. Therefore, we set up an *ex vivo* model to study whether our LCs were infected with non- and opsonized HIV-1 and were able to promote transmission.

Human epidermal sheets were incubated with 500 ng p24/mL of differentially opsonized HIV-1 particles, HIV, HIV-C and HIV-SF from diverse virus strains (primary R5-tropic HIV-1_{JR-CSF},

HIV-$1_{92BE030}$ and laboratory strain HIV-1_{Yu2B}). After 48 hours, the sheets were washed to remove unbound virus and infected sheets were cultured for another 2 days. miLCs were harvested and co-cultured with U87 cells which are highly permissive independent on the virus strain or the opsonization pattern (Fig. 21C). After 3 days, HIV-C transmission was determined by measuring infection of U87 cells by intracellular p24 staining (Fig. 21A-left panel). Fig. 23-right panel represents the viral transmission from differentially opsonized virus (showed above in Fig. 20). The predominant transmission was due to an efficient infection of the epidermal sheets (Fig. 21B). Infection of *ex vivo* skin explants with pre-opsonized HIV-1 particles, HIV-SF or HIV-C, resulted in an efficient infection and transmission in comparison with the corresponding non-opsonized virus.

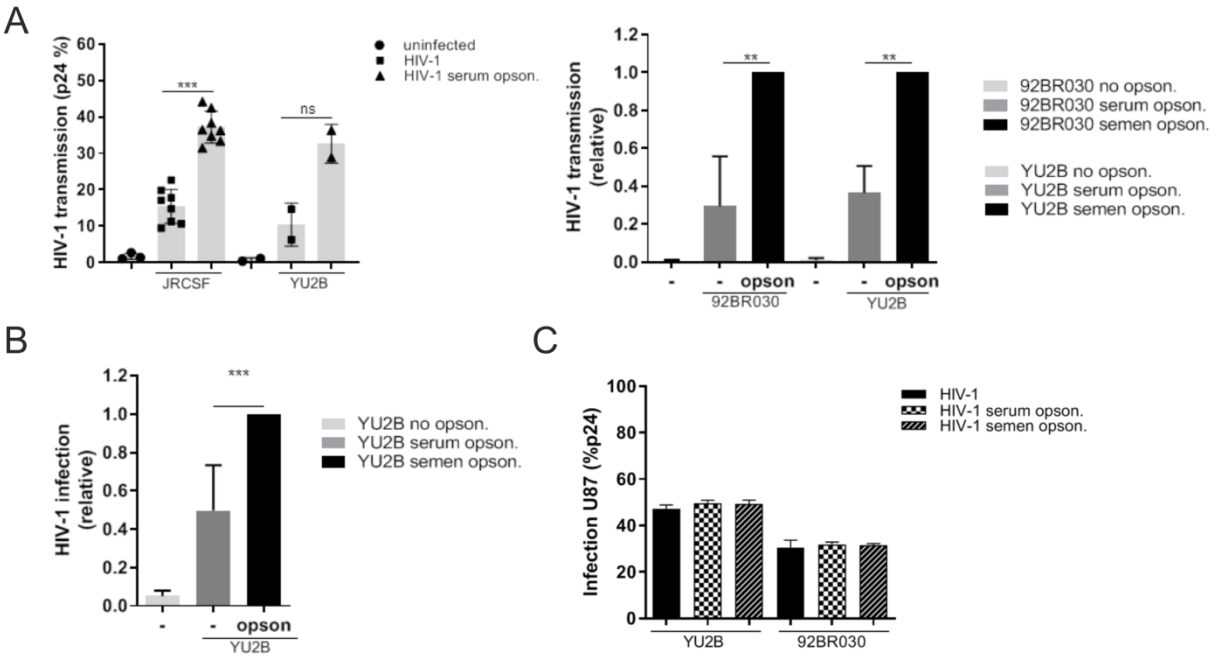

Figure 21. HIV-1 opsonized with seminal fluid or serum enhances *ex vivo* LC infection and transmission to target cells. A-B) Human epidermal sheets were pulsed with differently opsonized (serum opson/ semen opson) virus strains (JRCSF, Yu2B and 92BR030) for 48 h. A) At day 2, migrated LCs were co-cultured with U87 cells and the virus transmission to U87 cells was measured at day 6 by intracellular p24 staining and flow cytometry B) Skin explants were infected with HIV-SF (Yu2B-opson) and infection was determined by intracellular p24 staining at day 2. C) Infection of U87 cells, showing their permissiveness for any kind of virus and opsonization.

We demonstrated that HIV-1 spontaneously activates complement also in semen, which will affect transmission via human vaginal or foreskin epithelial cells and has to be taken into account. Since molecular mechanisms underlying HIV-1 infection and transmission are difficult to identify using the *ex vivo* skin explant models, we used for further experiments immature and migratory LCs isolated from fresh skin to investigate their functions regarding HIV-1 infection and transmission.

Activation of LCs is essential for LC infection and subsequent transmission

After isolation, we obtained over 90% pure LCs, in an immature or mature state, expressing CD4 and CCR5 receptor. We exposed iLCs and miLCs to HIV and HIV-C for 24 h, washed the cells to remove unbound virus and cultured the cells for another 4 days. Infection was analyzed by flow cytometry and intracellular p24 (Fig. 22A-B) in combination with LC-markers CD1a and CD207 were assessed. Additionally, p24 values in culture supernatants were measured.

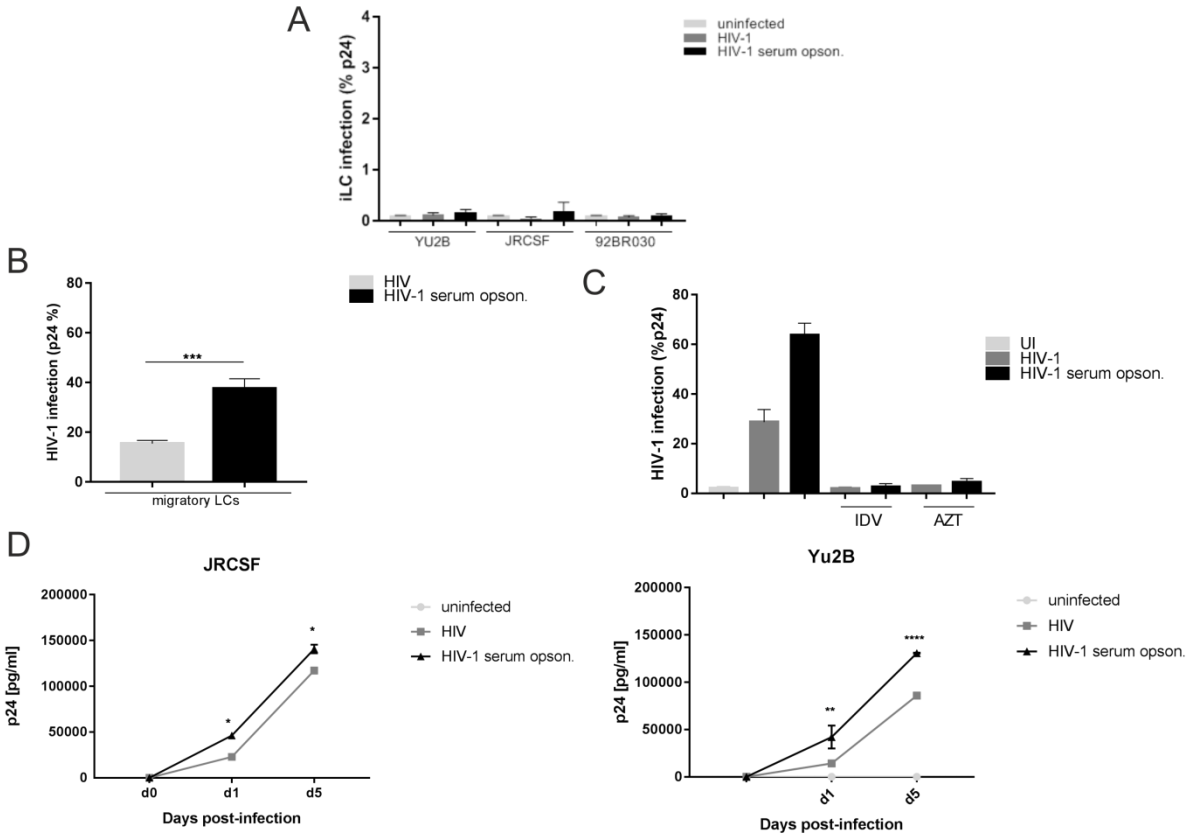

Figure 22. Human primary iLCs block HIV-1 infection whereas miLCS are infected by HIV-1 variants. A) iLCs infection using three different virus strains and opsonization pattern (R5 molecular clone Yu2B and primary isolates JR-CSF and 93BR020). B) miLCs infection using one representative virus strain. A-B) Cells were exposed to virus for 24 h, washed and cultured for 3 days. Infection of iLCs and miLCs was determined by intracellular p24 staining in combination with LC marker CD1a by flow cytometric analyses- The percentage of $CD1a^{high}p24^{high}$ cells are depicted here as % of infected cells. Error bars represent the mean ± SEM of at least 3 independent experiments. C) Effect of AZT and IDV treatment on intracellular p24 levels in LCs. For both experiments, cells were incubated with their respective blocking-Ab or drug for 2h prior to infection. D) Supernatant of cultured miLCs was collected at day 0 (after washing, to remove the unbound virus), day 1, day 5 and day 8 (not shown) post-infection and HIV-1 p24 in the supernatant was measured by ELISA. Error bars represents the mean ± SEM of duplicates.

Surprisingly, independent of the virus strain and opsonization pattern iLCs were resistant to infection (Fig. 22A). However, miLCs showed higher levels of intracellular p24 upon HIV-C infection in comparison to HIV (Fig. 22B). The absence of infection in iLCs is due to the langerin function. It was observed in previous work from the Geijtenbeek Lab [188] a co-localization of the HIV virions with langerin at the cell surface and in the Birbeck granules. Thus langerin on iLCs captures HIV-1 and internalizes it into Birbeck granules. This LC-specific internalization pathway is probably central to the ability of langerin to restrict HIV-1 infection and the subsequent transmission.

To confirm that replication was required to induce productive infection, miLCs were treated with HIV-1 reverse transcriptase inhibitor AZT and protease inhibitor IDV, prior to infection (Fig. 22C). Using AZT and IDV no LC infection was detectable independent on virus strain used or viral opsonization pattern.

As we could show before, HIV-C induced a higher infection in miLCs than HIV, which was proved also by using p24 ELISA (Fig. 22D).

HIV-C infection is mediated via CR3/CD11b and CR4/CD11c while HIV interacts with langerin

We also performed confocal microscopy using miLCs (Fig. 23). The confocal data showed similar results as the infection data obtained by flow cytometry and ELISA. We observed more HIV-C particles inside miLCs than HIV and a co-localization of the C-opsonized virus together with C3b fragments.

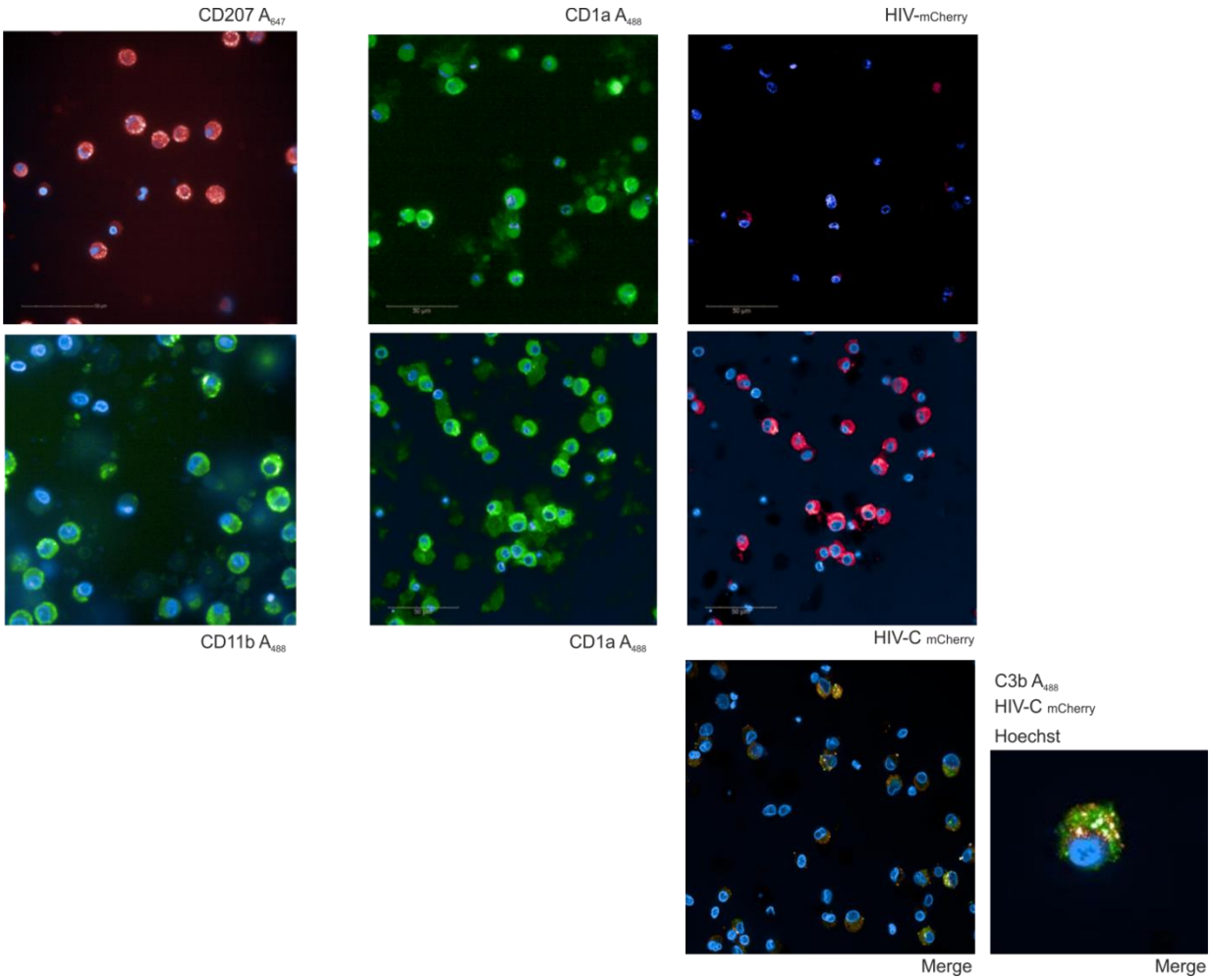

Figure 23. Complement opsonization enhances HIV-1 uptake by miLCs. Confocal microscopic images depicted a higher uptake of HIV-C (HIV-C mCherry) virus compared to the low level of HIV by miLCs (CD1a - CD207 expressing cells). The merge with C3b (C-opsonization) and HIV-C mCherry is depicted. In all images the nucleus is stained using Hoechst. Scale bars indicate 50µm.

Blocking CR3 and CR4 significantly decreases HIV-C infection in LCs

To demonstrate that the enhanced infection of HIV-C was mediated by CRs, we treated miLCs with blocking antibodies (Ab) against CD11b (CR3), CD11c (CR4) or both before HIV-C exposure (Fig. 24A). A significant reduction in the intracellular p24 percentage was detected for the cells that were treated with anti-CD11b, anti-CD11c and anti-CD11b/c upon HIV-C infection. In order to evaluate possible side effects of the blocking Ab during the infection, we also exposed treated cells to HIV (Fig. 24B). Upon non-opsonized virus, the amount of intracellular p24 was similar between conditions, suggesting that the blocking Abs against CR3 and CR4 are specific for complement-mediated infection.

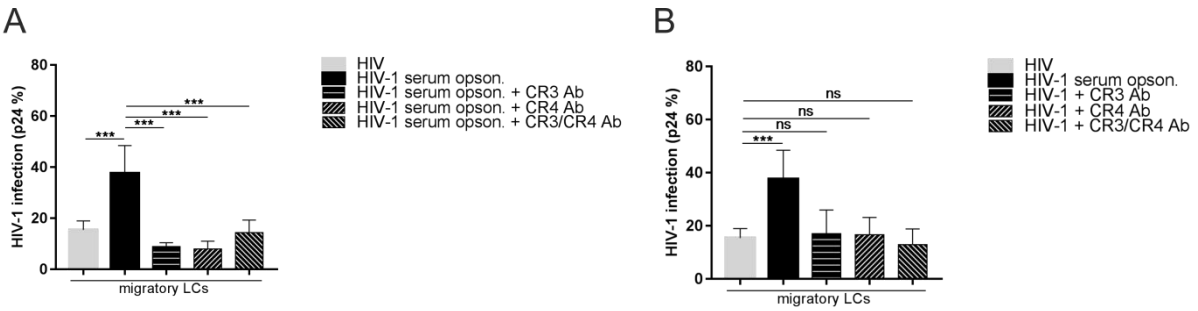

Figure 24. Complement opsonization enhances HIV-1 infection via CR3 and CR4. A-B) Blocking of CR3 and CR4 receptors on LC surface significantly reduced HIV-1 infection when the virus is complement-opsonized.

We could clearly show that HIV-C induced a higher productive infection in miLCs than HIV, by taking advantage of the CR. Hence, by using the blocking Abs against CR3 or CR4 we could detect a significant reduction of the infection (Fig. 24). Therefore, this suggests that if the binding of HIV-C to CRs is abrogated, the effect of C-opsonization is inhibited.

HIV-C enhances binding, fusion and integration in LCs independent of langerin

Moreover, using different approaches, we investigated if langerin plays a role in the fusion of HIV-C. In order to do that, we used the blocking antibodies for CR3 and CR4 together with an isotype as positive control for the binding (Fig. 25A-left panel). In case of langerin we used 10E2, a blocking antibody for langerin (Fig. 25A-right panel) and as control an isotype (data not shown). We observed that the blocking Ab for CR3 and CR4 significantly block the capture of HIV-C but not of HIV. As expected non-opsonized HIV capture is significantly reduced in presence of 10E2 blocking Ab. Therefore, we analyzed whether HIV-1 uptake via langerin or CRs leads to a higher fusion and whether HIV-C can still influence the fusion levels in miLCs. For this, miLCs were infected using an HIV containing Vpr fused to β-lactamase (β-lam). By using this system β-lam bound to Vpr will be released into the cytoplasm after viral fusion. Fusion of HIV and HIV-C with miLCs was measured by the cleavage of the substrate CCF2 by the β-lam in the cytoplasm. Upon diffusion of CCF2 into the cell it is directly de-esterified and thereby not able to enter any cellular compartments. The fusion levels increased when HIV-C was used (Fig. 25B) in comparison to HIV. Next, we again treated the cells using the blocking Ab against CR3, CR4 and langerin to evaluate their effect on the fusion capacity of the virus. As expected, when the cells were treated with CRs-blocking Ab, HIV-C fusion levels were significantly reduced (Fig. 25B). However, in the presence of langerin Ab, HIV-C fusion levels were not reduced. Thus, indicating that langerin does not play a role in HIV-C capture or fusion in LCs (Fig. 25B). HIV viruses pseudotyped with VSV (VSV and VSV-C) were used as a positive control for fusion. No significant

differences were observed between opsonization patterns, being both VSV and VSV-C equally invasive (Fig. 25B).

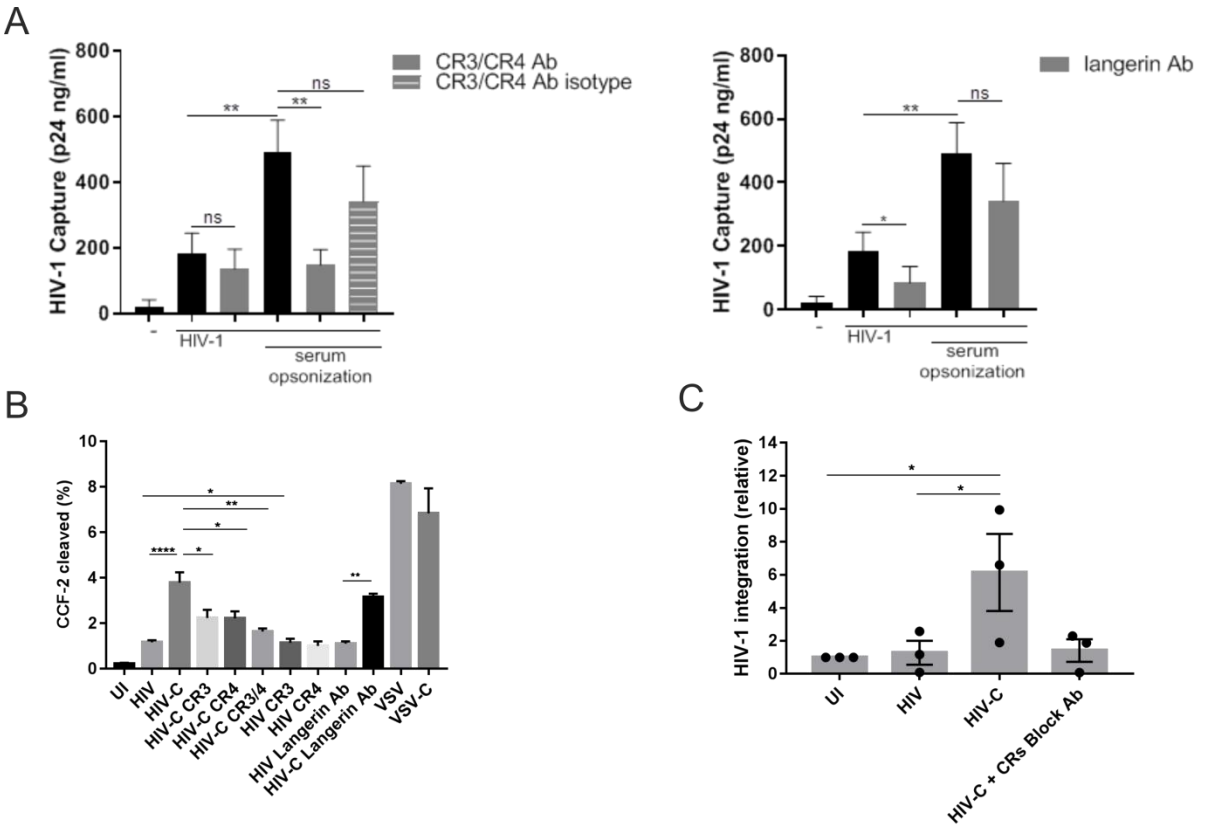

Figure 25. Complement receptors, CR3 and CR4 mediate the capture of HIV-C while HIV interacts with LCs via C-type lectin receptor, langerin. A) Binding of differentially opsonized HIV-1 by LCs co-cultured for 6 h at 4°C. Cells were previously treated with blocking-Ab against CR3, CR4 and langerin for 2 h at 37°C. B) Fusion assays were performed after addition of HIV or HIV-C bearing the fusion protein β-lactamase-Vpr to LCs. Untreated LCs (UI) were used as negative control, while as positive control for infection VSV or VSV-C virus. Cells were incubated with blocking-Ab for CR3 and CR4 for 2 h at 37°C. The Percentages of cleaved CCF2-positive cells was determined by flow cytometry analyses of cleaved CCF2 in the cytoplasm. Percentages of cleaved CCF2-positive cells from three independent donors are depicted. C) Real-time PCR for integrated HIV-1 DNA. Provirus formation in miLCs infected with differentially opsonized HIV. miLCs were exposed to 25 ng of p24/ml HIV, HIV-C. Cells were thoroughly washed after 3 h to remove unbound virus, and DNA was prepared 4-5 days after infection. Integrated DNA was detected in HIV- and HIV-C-exposed cells. This is a representative histogram of three experiments performed with BaL HIV isolates. The gene expression of the target genes (*R/U5*, *2LTR* and integrated HIV-1 DNA) was quantified relatively by normalization with the reference genes GAPDH. Uninfected miLCs were used as control samples to verify the specific amplification of the different HIV-1 DNA forms. A-C) Data are mean ± SEM of at least three different donors.

To further characterize the differences in infection of HIV and HIV-C exposed miLCs, a real time PCR assay for HIV-1 dynamics was applied. This assay shows simultaneously early RT, nuclear

import and integration efficiency of non- and C-opsonized virus preparations into miLCs. Thus, we analysed miLCs 4 or 5 days post-exposure for the presence of HIV and HIV-C associated genes for strong stop (R/U5), 2LTR, and integrated viral DNA (Fig. 25C). Non-opsonized and C-opsonized HIV-1 showed the same amount of R/U5 and 2LTR transcripts, but HIV-C was more efficiently integrated into genomes of miLCs. Measurement of p24 concentrations from supernatants of these miLCs confirmed that HIV and HIV-C loaded cells were productively infected with the virus. These results were verified in different virus strains (data not shown). The experiment was performed using Bal HIV strain.

Seminal fluid opsonization enhances transmission by LCs ex vivo using CR3 and CR4

Furthermore, we wanted to confirm if the C-opsonization from NHS or SF enhance the transmission in the *ex vivo* model (Fig. 26). In order to do that, we performed the same experimental approach used before to evaluate the effect of C-opsonization in transmission ex vivo, but treating now the skin explants with CRs-blocking Ab. The data confirmed that C-opsonization (HIV-C or HIV-SF) productively infect LCs and both are efficiently transmitted to target cells in the *ex vivo* tissue transmission model. That effect was abrogated for C-opsonized HIV and SF-opsonized HIV when CR3 and CR4 were blocked, decreasing LCs infection and subsequent transmission to similar levels to non-opsonized HIV.

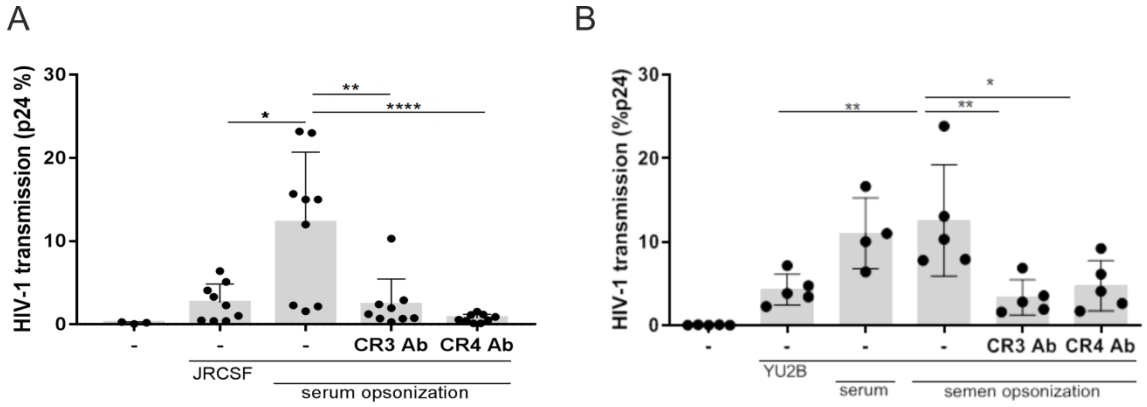

Figure 26. C- and SF-opsonization enhances transmission by LCs *ex vivo* via CR3 and CR4. Human epidermal sheets were exposed to different opsonized virus (HIV, HIV-C and HIV-SF) for 48 hours. A-B) At day 2, emigrated and infected LCs with HIV and HIV-C. Data are mean ± SEM of three different donors. (A) or HIV and HIV-SF (B), were co-cultured with U87 cells and the infection of U87 cells was measured at day 6 by intracellular p24 staining.

HIV-C infection triggers type I IFN responses via CRs

We next investigated whether complement opsonization or signaling via complement receptors expressed on LCs (miLCs) act as cell-intrinsic sensors mediating more efficient antiviral immune responses.

As already mentioned before, HIV-1 is recognized by innate immune sensors that then trigger the production of type I interferons (IFN). Type I IFN cytokines elicit broad antiviral effects by inducing the expression of anti-viral genes called interferon-stimulated genes (ISGs). It is known that certain ISGs such as APOBEC3G, SAMHD1 and MX-dynamic-like GTPase (for instance, *MxA*) exert a robust anti-HIV-1 activity, suggesting that type I IFN responses triggered by HIV-1 infection are detrimental for viral replication and spread.

miLCs express *IFNB* mRNA (which encodes IFN-β) after infection with the molecularly cloned primary strain HIV-$1_{JR\text{-}CSF}$, which peaked at 4h after infection (Fig. 27A). HIV-1 infection induced IFNB expression in miLCs, which was further enhanced by HIV-C and this enhancement was abrogated after treatment with CRs-blocking Ab (Fig. 27A). We next examined whether ISGs were also triggered by HIV-C. HIV-C infection also induced transient expression of ISGs, such as MxA (Fig. 27B); however it was not the case for APOBEC3G (Fig. 27C). The pattern of *IFNB* expression was similar to that of *MxA* induced expression. On the other hand, APOBEC3G seems to be not affected in our case by HIV-1 infection and the opsonization pattern.

These results imply that the viral presence is efficiently recognized by miLCs when HIV-1 is complement opsonized, thereby triggering a potent antiviral immune response.

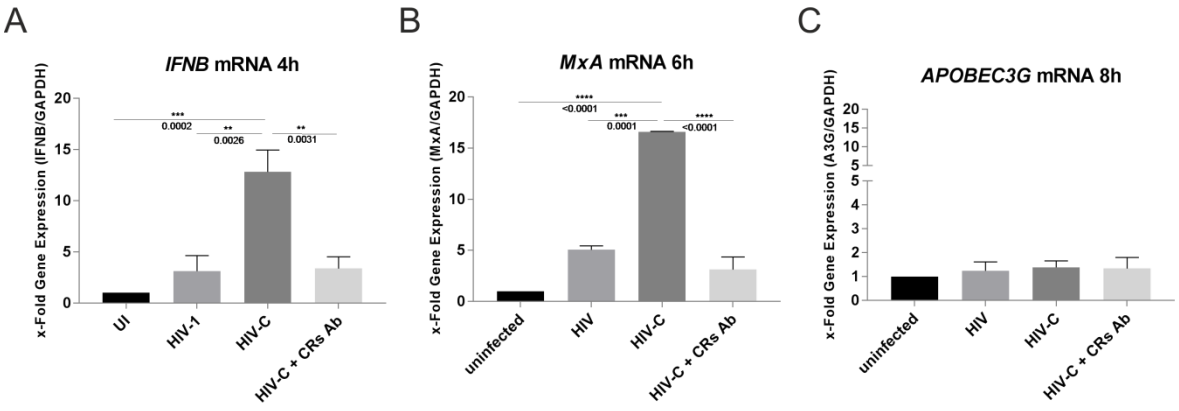

Figure 27. HIV-C-exposed LCs have a higher antiviral potential. A-C) Real-time PCR (RT-PCR) analyses of *IFNB* (A) and of *MxA* (B) and *APOBEC3G* (C) from mRNA in miLCs after infection (time-course from 1h-24h, just 4h is shown) with primary HIV-1 isolate JR-CSF. mRNA expression was relatively quantified using GAPDH expression as reference. Data are mean ± SEM of three different donors.

4. Discussion

Within this thesis we studied interactions of complement and dendritic cell subsets or differentiated THP1-DCs with HIV-1.

THP1-DCs —HIV-1

DCs express a variety of cell surface receptors that use ITAM-containing adapters to relay external signals and enable appropriate cellular changes. Some of those receptors are CR3 (CD11b/CD18) and CR4 (CD11c/CD18), which are integrin receptors, paired with the β2 chain CD18. β2 integrins are important adhesion and signaling molecules able to play important roles in regulating either recruitment of cells to sites of inflammation, cell contact formation and downstream effects on cellular signaling, all resulting in the activation of immune responses. Emerging evidences suggests that the β2 integrin family of adhesion molecules have an important role in immune activation and inflammation. Following that idea, we wanted to clarify whether this is the same regarding CRs and defining the connection between β integrins themselves and inflammatory processes upon HIV-1 infection. Previous studies [224] clarified how β2 integrin CD18 activation influences TLR responses by using macrophages or dendritic cells derived from CD18-KO mice.

Since working in a human context using HIV-1, we aimed to define in more detail the involvement of the specific β integrins and inflammatory processes upon HIV-1 infection.

We studied DC modulation during acute phase of **HIV-1** infection. Using CD11b-, CD11c- and CD18-KO THP1 DCs generated during the thesis, we were able to characterize the contribution of CR3 and CR4 with respect to DC modulation by HIV-C.

Using the KO-THP1 DCs, we identified integrin receptor-dependent signaling when exposed to HIV-C. Binding of HIV-C to CRs expressed on DC might protect the virus from immediate and extensive degradation by internalized particles in intracellular compartments, as shown for non-opsonized HIV [18]. We and others found a significant enhancement of productive DC infection by HIV-C [145, 166, 225, 226], which was associated with a significantly improved antiviral immune response [1] as well as an adjuvant role with respect to induction of HIV-specific CTLs [1, 167, 168]. Significant enhancement of infection was also detected using WT-THP1 DCs, which proves this cell line to be a good model for studying CR functions in relation to HIV-1 infection. Furthermore, this cell line allows us to use the CRISPR/Cas9 system to KO CD11b, CD11c and CD18 genes. While CD11b KO-THP1 DCs showed a similar phenotype to WT-THP1 DCs and primary DCs with respect to enhanced infection when exposed to complement-opsonized HIV-1,

this phenotype was lost, when CD11c was knocked out in the CR4 single (CD11b KO-THP1 DCs) and CR3/CR4 double (CD18 KO-THP1 DCs) KO cells.

This study confirmed that when HIV-1 was opsonized with complement fragments, the virus interacted directly with CRs, however when CRs are abolished because of deletion, binding of HIV and HIV-C to CRs was comparable. Although complement opsonization of the virus did not affect the amount of virus binding to THP1-DCs, internalization was significantly higher for HIV-C for WT-THP1 DCs. Using KO-THP1 DCs the viral binding of HIV-C to CRs and the subsequent internalization by THP1-DCs was nearly abolished, reaching similar levels to HIV. This indicates that HIV-C was more efficiently taken up by THP1-DCs than HIV depended on immobilization of complement fragments on the viral surface, which results in higher cross-linking of endocytic receptors. This provides distinct intracellular handling of HIV-C leading to both increased infection and altered activation of HIV-1 specific immune responses.

Additionally we found a significant enhancement of THP1-DCs infection by HIV-C, which we confirmed using complement-coated Vpx-containing HIV-1. The enhanced infection of THP1-DCs using HIV-C might be due to the phosphorylation of SAMHD1 at Thr592, as shown in primary DCs [1]. We observed that when SAMHD1 is not present or inactivated, there is still a role of the complement regarding higher infection. This demonstrates that the binding of HIV-C to CRs is crucial for the higher infection compared to non-opsonized HIV. Regarding the fact that KO-THP1 DCs exposed to HIV-C showed similar infection levels as with non-opsonized HIV-1, underlines the importance of these results. We hypothesize that HIV-C bound to CRs accumulates in rafts together with CD4 and the chemokine coreceptor CCR5, facilitating fusion and/or the binding via CRs might also trigger activation of a pathway supporting productive infection of DCs.

Opsonized virus can divert the complement system to its advantage by using the CR3/CR4 receptors to infect THP1-DCs. Furthermore, the binding of HIV-C to CR3 and CR4 may induce specific signaling cascades in THP1-DCs, which are favorable for HIV-1 replication or in favour for the host because of a higher virus visibility. Here we demonstrated that HIV-C enhanced the activation of the complex and dynamic regulation of IRF3 via phosphorylation of TBK1 (TBK1/IRF3 axis) and further activation of NF-κB mainly via CR4. Knocking out CR4 in the single or double knockouts resulted in the abolishment of the TBK1/IRF3/NF-κB axis. Still, further experiments are required to fully understand the detailed mechanisms in NF-κB mediated regulation of IRF3 activation during viral infection. Additionally, these activated cascades contribute to the type I IFN positive feedback loop. We observed in WT-THP1 DCs that type I *IFNB* mRNA significantly increased in HIV-C infection, leading to a better antiviral response. These findings are consistent with our other study [1] reporting an enhancement of *IFNB* mRNA

and ISGs in HIV-C exposed DCs. Our findings differ from the results showed in a manuscript published by Ellegård *et al*. [227], who showed that complement opsonization of HIV-1 resulted in a decreased antiviral immune response in DCs. Similar to our infection data, the authors illustrated that DCs are infected to significantly higher levels if the virus was complement-opsonized [227, 228] and they furthermore described an enhanced IRF3 activation in DCs upon HIV-C treatment, which points to an induction of an efficient antiviral immune response by DCs that the authors did not take into account. We also observed enhanced productive infection of HIV-C exposed WT-THP1 DCs and an increased IRF3 activation together with TBK1. But in contrast to Ellegård *et al*. [227], we found an enhanced type I IFN response. Regarding KO-THP1 DCs, we observed that upon HIV-C exposure, CD11b KO-THP1 DCs displayed a significantly higher up-regulation of IFNB mRNA levels compared to WT-THP1 cells, which is also in concordance with the enhanced DC infection within this cell type. In contrast, IFNB mRNA levels were significantly down modulated to background levels in HIV-C-treated CD11c or CD18-KO-THP1 DCs, thereby pointing to the importance of CR4 with respect to efficient antiviral immune responses. Our data demonstrate that while CR3 down-modulates antiviral immune responses, CR4 is the major player with respect to type I IFN induction, when HIV-1 is opsonized. The role of CD18 KO regarding the up-regulation of IFNB expression levels upon stimulation with non-opsonized HIV-1 remains to be characterized.

In previous *in vitro* and *ex vivo* studies, others and we showed that DCs exposed to complement-opsonized retroviruses strongly enhanced viral replication [145, 166, 226] and activation of CTLs [167]. Here, we found that HIV-C affects the induction of T-helper subsets. In attempting to identify the specific role of the integrin-receptor for CR activation, we analyzed the induction and production of inflammatory and anti-inflammatory cytokines related with the different Th subsets activation. All investigated pro-inflammatory cytokines showed a markedly higher mRNA expression or protein level in WT-THP1 DCs exposed to HIV-C compared with non-opsonized HIV, while the anti-inflammatory cytokine IL10 was down-regulated. Gresnigt *et al.* [229] demonstrated that *Aspergillus*-induced Th1 and Th17 responses were significantly decreased by blocking CR3 but not by inhibiting dectin-1 or Toll-like receptor 2 [229]. We observed similar results in our KO-THP1 DCs, resulting in bigger changes for CD11c- and CD18 KO-THP1 DCs. We can confirm that due to complement opsonization of HIV, CR3 and/or CR4 are initiating the induction of Th17-polarizing cytokines.

Immediate polarization of Th17 cells by complement, and THP1-DCs–mediated actions might strengthen the immune defense against opportunistic pathogens especially during early HIV infection.

Our data suggest an important role for cell adhesion events in fine-tuning inflammation. β2 integrins first encounter their ligands within the luminal side of blood vessel. Our results suggest that β2 integrins are required for regulating CR responses and since CD18 is related to cell adhesion, we can speculate that cell adhesion events may control inflammatory cytokine production in the bloodstream and thereby compartmentalize the cytokine production to the site of inflammation. Beyond this initial binding, myeloid cells also encounter ligands within the extracellular matrix while en route to their intended targets. Here, these ligands would be modified by local inflammatory mediators [230], suggesting that distinct β2 integrin ligands may differentially regulate CR responses in a manner that restricts inflammatory cytokine production to the infected tissue and therefore minimizes the damage to the host.

Finally, immune cell signaling, allows for fine-tuned cooperation between a wide variety of immune cells. We consider that β2 integrins are involved in complex immunoregulatory signaling pathways and also have essential immunoregulatory functions.

Our study suggests that the difference in replication efficiency between the non-opsonized and complement-opsonized virus depends directly on the binding to CRs. This binding also leads to a higher antiviral response and proinflammatory cytokine production and altered signaling profile induced by HIV-C. All this could play an important role in virus pathogenesis. In conclusion, the data obtained in this study with THP1-DCs confirms and supports the hypothesis that the host could be able to handle infection as a consequence of enhanced cellular and humoral immune responses. These findings might make new therapeutic options possible, improving antiviral immune responses via complement receptor-mediated signaling in DCs. For example, dysregulated integrin signaling, expression and surface activation is therefore likely to contribute to a variety of inflammatory and autoimmune conditions. Elucidating the function of β2 integrins further, promises to provide novel therapeutic targets for various disorders or chronic infections.

LCs—HIV-1

The initial events of the HIV-1 infection in the genitals are poorly characterized and little is known about the factors influencing initiation of HIV-1 replication in the vaginal tissue. Only low numbers of primary LCs from human genital tissues can be isolated. Therefore, most of the research on the molecular interactions of HIV-1 with LCs has been performed using skin-derived LCs. Several studies using *ex vivo* skin explants models have shown that LCs can be infected with HIV-1 and subsequently transmit HIV-1 to T cells [213, 231, 232]. LCs can be infected *in vitro* and *ex vivo* with HIV-1. However, high concentration of HIV-1 and/or specific infection procedures

have been used to infect LCs, such as spinoculation. This is a non-physiological method that forces HIV-1 to attach to the target cell surface through centrifugation of target cells with the inoculated HIV-1, which increases the infection [192, 213, 232]. This suggests that LC infection is not very efficient. The ability of LCs to inhibit infection is dependent on the C-type lectin receptor, langerin. Langerin captures HIV-1 for rapid degradation. Importantly, this langerin's ability protects LCs from becoming infected *in vivo* and thereby prevents LC-mediated HIV-1 transmission.

Since mucosal secretions contain active complement components [217, 233], one may speculate that virus released in the mucosal lumen becomes opsonized and acquires an enhanced potential for attachment to and infection of LCs, a key target cell for HIV in the early steps of sexual transmission [234]. Complement-coated virus is present already in semen or it is opsonized immediately after entry via mucosal sites [235, 236]. Opsonization with activated C3 fragments will more likely lead to interaction with CR3 and CR4, apart from CD4 and CCR5/CXCR4, instead of other receptors such as C-type lectin receptors, which both are abundantly expressed on DCs subsets, for instance LCs.

Therefore, we investigated whether LCs capture and transmit HIV and HIV-C to target cells. We show that immature LCs were not infected by HIV or HIV-C, in contrast to migratory LCs (miLCs) which HIV could infect. Both of these behaviors were dependent on C-type lectin receptor, langerin. Langerin is specifically expressed by LCs and induces the formation of Birbeck granules [237]. These are LC-specific cytoplasmic organelles that are involved in antigen processing [238]. Thus langerin on LCs captures HIV-1 and internalizes it into Birbeck granules where it is degraded. This LC-specific internalization pathway is central to the ability of langerin to inhibit HIV-1 infection and transmission.

Several *in vivo* and *ex vivo* data support the idea that HIV-1 can infect LCs and they can mediate transmission [192, 213, 232, 239, 240]. We found that miLCs were sensitive to infection with complement-opsonized HIV-1. Upon culture of miLCs in the presence of R5-tropic HIV-1 particles previously incubated with NHS or semen, we observed a productive infection of miLCs and transmission to target cells, $CD4^+CCR5^+$U87 cell line. We have selected the $CD4^+CCR5^+$U87 cell line as the receptor cell line because of its many advantages over other cell lines. Importantly, $CD4^+CCR5^+$U87 cells support infection of all evaluated R5-tropic strains and clinical isolates, being equally permissive to all opsonization patterns. Furthermore, $CD4^+$U87 cells expressing CCR5 or CXCR4 have been commonly used for HIV-1 replication assays and evaluation of antiviral compounds [241-244]. Thus, activation of LCs might be important to allow HIV-1 entry into mucosal tissues. The major route of transmission by LCs requires productive infection of LCs and production of virus particles, known as *cis* infection [188, 212].

In contrast to *trans* infection where productive infection of the LCs is not required. In line with previous findings, we observed that transmission of HIV-1 by LCs is dependent on productive infection and even enhanced when the virus was complement-opsonized.

miLCs, which have an activated phenotype, have lower expression of antigen capture receptors (langerin and CRs, CR3 and CR4) and as well of coreceptors (CCR5/CXCR4) compared to immature LCs. However, the lower expression did not affect HIV-1 infection of miLCs and the subsequently transmission. miLCs were efficiently taking up HIV-1, especially HIV-C but further studies are required to identify the molecular mechanisms. HIV-1 replication is important for HIV-1 productive infection by LCs. miLCs were treated with different antiretroviral inhibitors, AZT and IDV, as negative controls to show that the p24 detection measured by FACS was real infection. When both inhibitors were added, miLCs infection was abrogated.

The enhanced infection of miLCs using HIV-C was also demonstrated using microscopy. As it was described earlier, we show that the attachment of non-opsonized HIV-1 to miLCs is preferentially associated with the binding to the C-type lectin receptor, langerin. We demonstrate that up-take of HIV-C by miLCs was much higher compared to its non-opsonized counterpart. The enhanced infection in miLCs could be due to an increased binding of opsonized virus by additional interaction of C3 fragments with CR on miLCs, in concordance to published data [245].

In vitro studies indicate that beside the protein backbone of gp120, which directly interacts with langerin [246], the carbohydrate structures on the viral gp120 seem to be crucial for C-type lecin binding [246, 247]. In the present study we wanted to investigate the function of CRs and langerin on primary LCs in HIV-C infection and transmission. As well as to clearly show that the attachment of non-opsonized virus with LCs depends, at least in part, on the interaction to langerin, while the complement-opsonized HIV depends on CRs. We here describe that the binding, the infection and the subsequent transmission of miLCs were significantly reduced by the addition of blocking antibodies to CR3 (anti-CD11b), CR4 (anti-CD11c) and to langerin, 10E2. To exclude enzymatic cleavage of CD4 and coreceptors during the isolation method [248], we used purified migratory LCs. It was published already that conditions in which langerin is blocked by blocking antibodies, saturated by high concentrations of virus or co-infection, abrogate the protection and enable efficient HIV-1 infection. [188]. We here describe that the blocking of CR3 and CR4 inhibited LCs infection and subsequent transmission. Despite the fact that langerin mediates the capture of HIV-1 by LCs, it is interesting that HIV-C was bound by CR3 and CR4 but not by langerin. Bouhlal *et al.* [226] showed, with respect to DCs, that opsonized virus attached more efficiently to C-type lectin receptors, such DC-SIGN, than the corresponding unopsonized virus. We did not observe any significant change in the attachment/fusion when langerin was blocked.

We here demonstrate that different opsonization patterns of HIV-1, detectable in HIV-1 infected individuals, have a profound impact on infection and integration of miLCs. To address whether the binding to CR3 and CR4 also leads to a higher fusion either in the plasma membrane or in the endosomal membrane, we did fusion assays using a Vpr-βlam containing virus, incubation of cells with CCF2 and flow cytometry to assess the number of cells where the viral fusion took place. In accordance to a higher capture and internalization, we here illustrate that both, HIV and HIV-C enter the cytoplasm. The detected higher fusion rate in case of HIV-C might be due to the fact that CR binding facilitates a CD4/CCR5 binding and consequently fusion, therefore allowing the virus to enter in the cytoplasm. This result was confirmed by fusion assay and analysis by confocal microscopy. In contrast to what have been published in DCs [249], activation of LCs enhances their infection capacity, shown as an increase in the viral fusion events. In addition, proviral HIV-1 DNA was detected in miLCs within 4 days of incubation with HIV-C. The amounts of HIV-1 integrated DNA measured by semi-quantitative nested PCR were significantly higher in cells infected with opsonized HIV-1, as compared with cells infected with non-opsonized virus. As reflected by efficient integration into miLCs, complement-opsonized HIV-1, which can be found at the acute phase of HIV-1 pathogenesis, productively infected the cells. In contrast, we observed a no or a significantly impaired infection of HIV and provirus in miLCs.

Enhancement of infection of miLCs observed following pre-incubation of the virus with normal human serum or seminal fluid was complement-dependent and occurred with R5-tropic primary isolates and molecular clones of HIV-1. Using HIV-1 opsonized with one of the complement sources, gave a higher infection than the non-opsonized HIV. Complement opsonization of HIV (HIV-C or HIV-SF) caused the binding to CR-positive cells, such as LCs, and enhanced the infection of these cells. An increased infection due to complement opsonization of HIV-1 was shown for macrophages, monocytes, or DCs and DC-T cell or B cell-T cell co-cultures [145, 225, 247, 250]. Our results show that opsonization of HIV-1 with complement enhances both the infection of migratory LCs and the transmission in *cis* of virus to target cells. These data extend previous observations on the enhancing effect of the complement on infection of monocytes/macrophages [251, 252] and complement receptor-positive epithelial cells [161] and an efficient transmission of HIV-loaded DCs to susceptible $CD4^+$T cells [180].

The exact consequences of type I IFN responses during HIV-1 infection are a topic of debate. Inflammation during infection is predictive of non-AIDS morbidity and death [253, 254]. Type I IFN responses are especially important in limiting viral spread, however, during HIV-1 infection, it might also result in more T cell activation and therefore more HIV-1 target cells available [255, 256]. Here, we examined some interferon-stimulated genes (ISGs) to determine the mechanisms of the rapid inhibition of HIV-1 replication in the acute stage of infection. Restriction factors,

including MxA and APOBEC3G, increase their expression after type I IFN release and have been demonstrated to inhibit HIV-1 replication [257-260]. However, there is paucity of data regarding the interplay between HIV-1 and *APOBEC3G* expression *in vivo*, particularly during primary infection when rapid viral replication occurs, followed by resolution of viremia and establishment of steady state equilibrium between the virus and the body's immune responses. We therefore investigated whether HIV-C infection is associated with changes in *IFNB*, *MxA* and *APOBEC3G*. Taken together, the up-regulation of ISGs like MxA might contribute to inhibit viral replication in the acute phase of infection. However, the viral protein Vif seems to counteract APOBEC3G function [261, 262]. We herein demonstrate that the higher type I IFN observed is active in the cell because it leads to higher IGS.

These presented data suggest that complement opsonization of HIV-1 leads to a different routing of HIV-1 in LCs via CR3 and CR4 binding, evading antiviral function of langerin and increasing HIV-1 infection of LCs and subsequent viral dissemination. Taken together our findings might expand the knowledge about factors contributing to an enhanced HIV-1 susceptibility. This study provides novel insight into the importance of complement in HIV-1 susceptibility and might lead to novel strategies to prevent HIV-1 infection.

Complement activation — Good or Bad in HIV-1 infection?

HIV-1 immediately activates the complement system, which is part of our innate defense. But, will the complement system really do us any good in this setting? Is complement good or bad in HIV-1 infection? There is a big debate in the field according to such question, since the role of the complement system in HIV-1 pathogenesis appears to be multifaceted. For over a decade, it has been proposed that the enhancement of the antibody-dependent complement-mediated effects on HIV-1 and HIV-1 infected cells may be a novel approach for the treatment and prevention of HIV-1 infection.

To answer that question, we need to keep in mind that HIV-1 has developed escape mechanisms that protect it from complement destruction. The complement system is generally a dangerous weapon against microorganisms and is strongly activated during HIV-1 infection, but instead of getting lysed, the virions are opsonized with complement fragments. Usually, when a pathogen is complement opsonized, it is eliminated and cleared from the system by binding to phagocytes expressing CR [162]. However, for HIV-1, the opsonization seems to be rather an advantage, even though a minor fraction of virions are destroyed by the complement system [263]. Through complement deposition, the complement system facilitates the interaction with cells expressing CRs such as monocytes/macrophages and dendritic cells; and with non-infected cells like erythrocytes, follicular DCs and B cells as the delivery and release source for infecting other cells.

C3a and C5a are factors produced by the activated complement cascade; these anaphylotoxins attract DCs and other APCs to the site of infection, which then can be exploited by HIV-1 [263].

Results from our group have shown that HIV-C is more efficiently internalized in DCs compared to HIV via CRs [1]. In vivo, DCs capture and internalize HIV-C and migrate to the lymph nodes where they subsequently transfer infectious virions to the CD4+ T cells. Our studies demonstrated that HIV-C significantly enhanced immature and mature DCs MHC class I presentation of HIV derived antigens. An increased MHC class I presentation and activation of CD8+ T cells is indeed good for the host. Furthermore we showed an increased stimulation of HIV-specific $CD4^+$T cell clones by DCs exposed to HIV-C, which is in contradiction with Tjomsland *et al*. [228]. This discrepancy can be explained by the difference that our group and others [264, 265] perform all experiments using $CD4^+$T cell clones with chemically inactivated virus or in the presence of HIV blockers to prevent viral replication. The higher activation of $CD4^+$ and $CD8^+$ T cells might be at least partially due to higher co-stimulatory capacity of HIV-C loaded DCs and induction of monokine induced by interferon-ɣ (MIG), IFN-inducible protein 10 (IP10) and IFN-inducible protein 9 (IP9). These experiments revealed that HIV-C loaded DCs were prime suppressors of HIV-1 infection, emphasizing the significantly higher antiviral activity induced in DCs by complement-opsonization of HIV. As demonstrated, complement coating of HIV-1 strongly influences intracellular fates or routes of the virus in DCs compared to non-opsonized HIV-1, thereby profoundly affecting DC infection and activation. Since *in vivo* most viruses will be opsonized by complement immediately upon entry via mucosal surfaces or already in seminal fluid, the dogma of non/low-level infection of DCs by HIV associated with a weak T cell activation should be revisited. Although several approaches have been proposed for enhancing complement activation of HIV-1 infection for the treatment and prevention of HIV-1, they still require further evaluation and extensive investigation.

To answer the question we made at the beginning, the virus infects the host and the immune system immediately strikes back. However, in the long run the complement response is limited and the virus seems to success in the battle.

DC based vaccines for HIV infection

Therapeutic vaccination would be the most promising strategy to treat HIV without cART. Earlier classical approaches such as administration of whole inactivated virus or recombinant gp120 protein were evaluated, but the results on controlling viral load were limited [266, 267]. However, DC-based vaccines showed the best results in this field [268-270]. Our results show the importance to study non-opsonized and complement-opsonized HIV-1. HIV-C exists in every compartment in the body and the C3 fragments deposited on the viral surface may alter the interaction with the immune cells. As already explained before, DCs are essential to induce

strong $CD4^+$T cell responses and are crucial to achieve CTL responses able to control HIV replication. Therefore, HIV-C can modulate the DC function and the mechanism behind should be taken in consideration for future studies. On the other hand, DCs contribute to viral dissemination and HIV could be able to avoid correct Ag presentation, overcoming the intrinsic innate immune mechanisms to control viral infection. Therefore it has been implicated to use *ex vivo* generated DCs as vaccines that would overcome some of this problems [271-273]. The purpose of the therapeutic vaccination is to partially reconstitute the patient's immune system boosting antiviral capacities of $CD4^+$ and $CD8^+$ T cell functions, thereby reinforcing also other immune cell functions. As our group have shown until now, complement could be a promising adjuvant for boosting DC functions and could also be implemented in such vaccine designs.

5. Final Conclusion

We here give insight into a substantial novel way of myeloid cell modulation at least during acute HIV-1 infection by triggering integrin receptor signaling.

HIV-1 developed mechanisms to avoid complement-mediated destruction by incorporating regulators of complement-activation during the budding process and additionally binding the fluid phase factor H [171, 274]. Thus, only low levels of virus particles are lysed by action of complement [274]. Additionally, the virus displays a C1q-binding domain in its envelope protein, thereby spontaneously activating the classical complement pathway, even in the absence of HIV-specific antibodies, upon entry into the host [149, 275]. Because of spontaneous activation of the complement cascade and protection against viral lysis, C3-opsononized particles accumulate already in semen or immediately after entry of viruses via mucosal surfaces [235, 236]. Since C3b and its fragments are covalently linked to viral surface, the virus could interact with the CR3 and CR4 expressed in the cell surface of myeloid cells [145, 276]. Opsonization of HIV-1 by complement enhances the binding to CRs, CR3 and CR4, and increases the number of viral particles adsorbed on cells allowing a more efficient fusion and viral entry through CD4 and HIV coreceptors (CCR5/CXCR4).

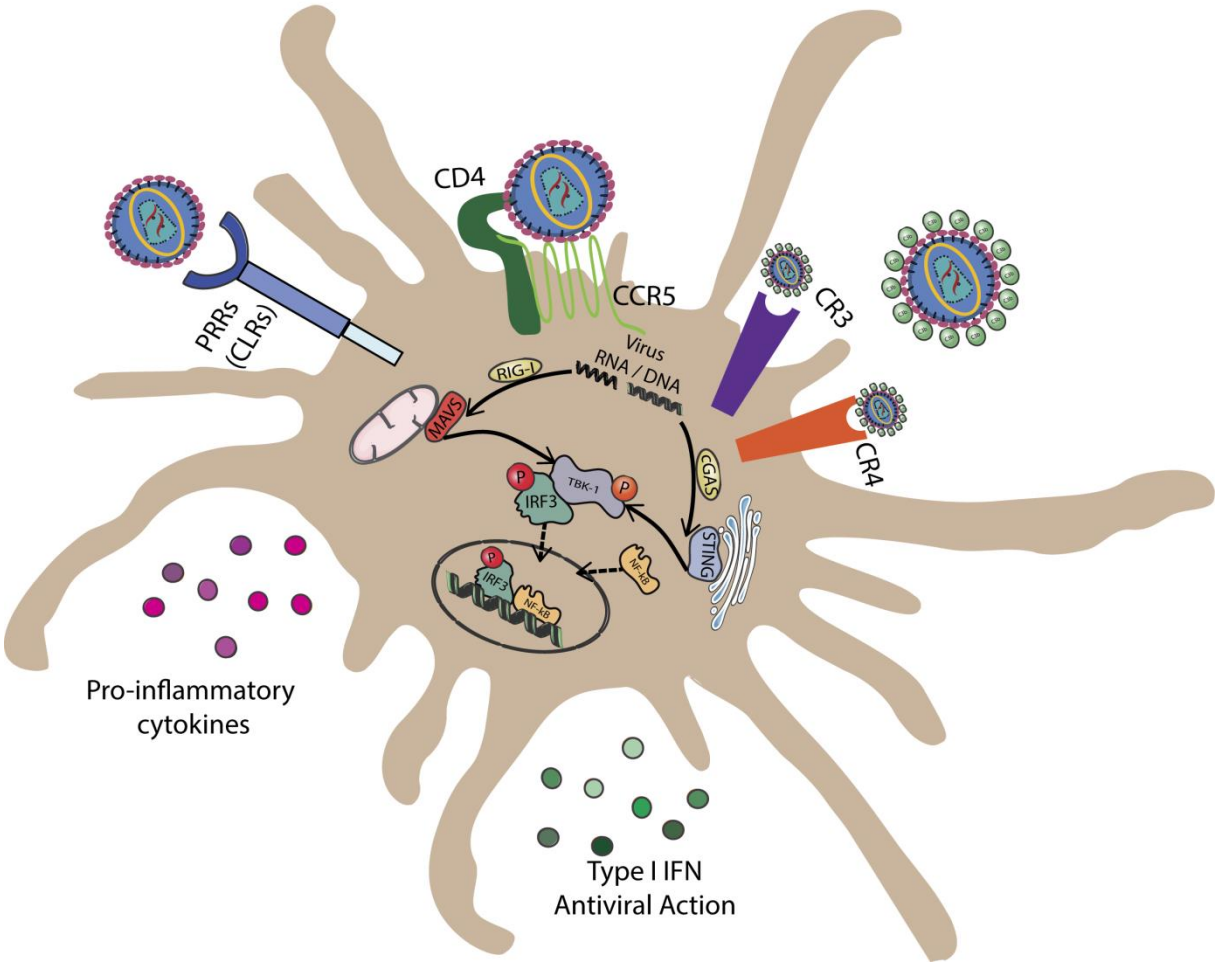

Figure 28. Graphical summary: HIV-C modulates myeloid cells function. In this study, we found that ligation of C3-coated HIV-1 to CR3 and CR4 mediates phosphorylation of TBK1. The increased DC infection was associated with the activation of the TBK1/IRF3 signaling axis. IRF3 is translocated to the nucleus, where it activates promoters containing IRF3 binding sites, such as IFN-β promoters, together with NF-κB, enhancing the up-regulation of type I IFNs and ISGs. Due to these changes, DCs, which were exposed to complement-opsonized HIV-1, mediated significantly higher antiviral immune responses compared to HIV-loaded DCs.

Upon HIV-1 infection, PRRs are involved in the recognition of viral cDNA or RNA, respectively. After HIV-1 infects human cells, cDNA is synthesized by RNA reverse transcription. Moreover, recent studies have shown that DNA sensor cyclic GMP-AMP (cGAMP) synthase (cGAS) and stimulator of interferon gene (STING) are ISGs, and that their induction can enhance type I IFN production via the cGAS/STING/TBK1/IRF3/IFN-β feedback loop [277-280]. cGAS recognizes DNA and subsequently produces cGAMP. cGAMP activates STING that in turn recruits and activates TBK-1 which phosphorylates IRF3. Finally, phosphorylated IRF3 is translocated to the nucleus, where it activates promoters containing IRF3 binding sites, such as IFN-β promoters.

Transcription activation of IFN-β gene requires the assembly of an enhanceosome containing ATF-2/c-Jun, IRF3/IRF7 and NF-κB [207], which indicates that NF-κB binding is essential for IFN-β production.

These studies have expanded the horizon of IFN regulation, suggesting that ISGs might positively modulate type I IFN production at multiple levels during viral infection. Overall, our study underscores the importance of antiviral type I IFN responses in acute HIV infection, during which time LCs and DCs are a prominent target for HIV-1. Also, reveals the identified pathways as important novel targets for early therapeutic intervention to boost endogenous antiviral immunity in the acute phase or even as a prophylactic measure.

6. Future challenges - Ongoing investigations

The results from our studies show the importance of studying both non-opsonized and complement opsonized HIV-1. Complement HIV-1 exist in every compartment in the body and the C3 fragments deposited on the viral surface may alter the interaction with the immune cells. After identifying that HIV-C is able to bypass SAMHD1 restriction and induce more of type I IFN, we further designed an experimental strategy to investigate the downstream pathway underlying the binding of CRs to HIV-C. Some published manuscripts using either non-opsonized or non-enveloped virus gave some ideas to investigate proteins involved in the type I IFN signaling, indicating that the route of HIV infection is different when the virus is non- or complement-opsonized, as we already showed for type I IFN signaling and the use of KO-THP1 DCs. We saw differences in protein activation up-stream type I IFN production, regarding activation of antiviral signaling pathways, for instance TBK1-IRF3 axis. However is still unclear whether type I IFN expression upon HIV-C infection is due to endocytosis or a different way of entry, and if endocytosis is involved, whether the complement receptors are involved in the process. The PAMP that triggers the type-I IFN production upon HIV-C infection could be the viral nucleic acids [281]. The reason to believe that, it is the TBK1 phosphorylation upon HIV-C infection together with the fact that there are more viruses in the cytoplasm of cells infected with HIV-C. Thus, suggesting that are more chances for cytoplasmic sensors to interact with the viral nucleic acids in the cytoplasm. Therefore, we assume that the virus way to entry could affect the exposure of the viral DNA or RNA to the cell sensors for nucleic acids. The definition of the exact mechanism behind CRs signaling events behind HIV-C infection in DCs is right now on-going in our group and will in the future deliver a more detailed picture.

Therefore, the effect HIV-C has on the ability of the DCs subsets to function as potent antigen presenting cells and the mechanisms behind the increased infection, transmission and antiviral response induced by complement-opsonized virions should be taken in consideration for future studies.

7. Methodology

(I) THP1-DCs

Generation of human monocyte-derived DCs and THP1-DCs

Blood for the monocyte isolation was received by the Central Institute for Blood Transfusion & Immunological Department, Innsbruck, Austria. Briefly, PBMCs (peripheral blood mononuclear cells) were isolated from blood of healthy donors [166, 168] obtained by a density gradient centrifugation using a Ficoll Paque Premium (GE Healthcare) gradient. After washing, $CD14^+$ monocytes were isolated from PBMCs using anti-human CD14 Magnetic Beads (BD) – the purity of the isolated cells was at least 98%. Monocytes were stimulated by addition of IL-4 (200U/ml) and GM-CSF (300U/ml) for five days to generate immature DCs (iDCs), which were used for all further experiments. Non-stimulated iDCs were used as controls for all experiments using DCs. THP1-WT and KO DCs were generated from the respective THP1 cells by addition of IL-4 (200U/ml), GM-CSF (300U/ml) and TNF-α (10ng/ml) for five days.

Genome editing using CRISPR/Cas9-mediated depletion of CD11b, CD11c and CD18

For CRISPR/Cas9-mediated depletion, guide RNA (gRNA) targeting sequences for CD11b (5´-GCCGTAGGTTGGATCCAAACAGG-3´), CD11c (5´-GTAGAGGCCACCCGTTTGGTTGG-3´) and CD18 (5´-TGGCCGGTGTCGCSGCGSSTGG-3´) were selected using an online prediction tool—CRISPR Design; Zhang Lab [282]. gRNAs were cloned into a lentiCRISPRv2 vector via BsmBI restriction sites. lentiCRISPRv2 was a gift from F. Zhang (Massachusetts Institute of Technology, Cambridge, MA; Addgene plasmid 52961 [283].

Lentiviral transduction

Lentiviral plasmids were co-transfected with Lipofectamine LTX (Invitrogen, cat 15338100) together with pMDG, psPAX2 and lentiCRISPRv2 into the HEK293LTV producer cell line. Supernatants containing viral particles were harvested 48h and 72h post transfection, filtered using a 0.2µm filter and directly used to transduce target THP1 cells with 5 µg/ml Polybrene (Sigma-Aldrich, cat TR-1003-G). After seven days, transduced cells were selected using puromycin (5 µg/ml, Sigma-Aldrich, cat SBR00017). After selection, the depletion efficiency of CD11b, CD11c and CD18 was analyzed by flow cytometry. Single-cell clones of the specific KO cells were generated after FACS sorting by the Core Facility FACS Sorting at the Medical University of Innsbruck.

Virus production

Primary isolates as 92BR030 (subtype B/B, R5-tropic) or the laboratory strain BaL were obtained by the National Institutes of Health AIDS (available through World Health Organization depositories). Virus was propagated in PHA-L and IL-2 stimulated PBMCs. 93BR020 (subtype B/F, X4/R5-tropic) and the laboratory strain NL43 were produced in M8166 cell line. 293T cells were used as viral producer cells, thereby were transfected with YU2B-, R9- and R9Bal-proviral plasmids using the $CaCl_2$ method. Virus supernatants were collected and cleared by filtration through 0.22 μm pore-size filters and concentrated by ultracentrifugation at 20000 rpm for 90 minutes at 4°C. The virus pellet was resuspended in RPMI 1640 without supplements and stored in small aliquots at -80ºC to avoid multiple thawing. One aliquot was taken to determine the virus concentration by p24-ELISA [284] and the 50% tissue culture infective dose of the viral stock.

Opsonization of HIV-1

Incubation of HIV-1 with active normal human serum (NHS) results in the covalent deposition of C3 fragments (C3b, iC3b, C3d, C3c) on the viral surface. We incubate purified R5-/X4 –tropic HIV-1 (concentration >1 μg p24/mL) for 1h at 37°C with NHS as a complement source (HIV-C) in a 1:10 dilution. Subsequent to opsonization, the virus was washed and pelleted by ultracentrifugation (14000 rpm for 90 minutes at 4°C), and resuspended in 500 μL of RPMI medium without supplements.

Virus Capture Assay (VCA)

The opsonization pattern was determined by using a virus-capture assay (VCA), as previously described [166] with antihuman C3c/C3d, IgG and mouse IgG antibodies as control for background binding. The coated VCA plates were incubated overnight with differentially opsonized viral preparations (1 ng of p24 per well) at 4°C and washed 4 times with RPMI medium to remove unbound virus. Bound virus was lysed (2% Igepal) and transferred to a pre-coated p24 ELISA [284] plate to confirm the opsonization pattern.

Plasmids

The Vpx expression construct pcDNA3.1Vpx SIVmac239-Myc was used to obtain Vpx-carrying HIV virus preparations [285]. To generate chimeric HIV/β-lam pro-viral clones, R9Bal or YU2B and, vpr/βlam expression constructs were used. Vesicular Stomatitis Virus G protein (VSV-G) pseudotyped HIV-1 virus was generated using the corresponding provirus plasmid (HIVΔenv) and the VSV-G expression plasmid pMDG.

p24-ELISA

A sensitive (2000 pg p24/mL) anti HIV-1 p24 antigen ELISA [171] was established, based on two different monoclonal antibodies. The murine antibody IAM-24-M01 was selected as the capture antibody and the second human antibody IAM-24-37G12 was biotinylated (NHS-Biotin) as recommended by the manufacturer. To achieve a high sensitivity Streptavidin*β-galactosidase with resorufin-β-D-galactopyranoside was substrate for detection. Standard p24 protein was derived from an inactivated and clarified cell culture supernatant (MN-isolate).

THP1-DC infection

Cells ($1x10^5$/100µL) were infected in triplicates with 25ng p24/mL of differentially opsonized HIV-1 as described before [145, 166]. To confirm the detection of a productive infection and not cell-associated HIV-1, we thoroughly washed the cells after 12h incubation with differentially virus preps and cultured the cells at 37°C/5% CO_2. By ELISA we measured the p24 concentrations of the supernatants following spinning down the plate on several days post infection.

Capture of HIV-1

Differentially THP1-DCs (WT and KOs) were exposed to 250 ng p24/ml of R5 tropic non-opsonized (HIV) or complement-opsonized (HIV-C) HIV-1. After 6h incubation at either 4°C for binding or 37°C for internalization, cells were washed 4 times to remove unbound virus. Cell pellets were lysed with 2% Igepal and viral amount was assessed by p24 ELISA.

Phagocytosis assay (Latex Beads)

0.8 µm fluorescence (PE) latex beads (Sigma-Aldrich) were incubated with NHS for 2 h at 37°C, then the beads were washed and resuspended in medium. Finally, THP1-DCs ($2x10^5$ cells for each condition) were incubated for 3 h at a 1:10 ratio with non-opsonized or C3-opsonized latex beads to evaluate the phagocytosis capacity. We analyzed the percentage of PE-positive cells by flow cytometry.

Immunoblot analyses of phosphorylated proteins

THP1-DCs were starved in RPMI 1640 containing 0.5% FCS and 1% L-Glutamine for 3h. The starving of cells was performed to set their phosphorylation to background levels. Following starvation, THP1-DCs were incubated with the differentially opsonized HIV-1 particles. After 4h co-incubation, cells were lysed with RIPA Buffer (Sigma-Aldrich) containing protease and phosphates inhibitors and EDTA (Thermo-Scientific) for 20 min at 4ºC. The protein content was

determined by BCA (Thermo-Scientific). Lysates were separated using 10% SDS-PAGE gels, transferred to PVDF membranes and incubated with anti-human a-tubulin as loading control as well as anti-human phospho-IRF3(1:1000), p-TBK1 (1:1000), p-TAK1 (1:1000) and NF-κB (1:1000) (all from Cell Signaling Technology) and developed with the Lass 4000 Image Quant. For this, the peak values of the target protein were divided by the peak values of the loading control before doing a relative comparison. Quantification was performed using values from three to six different experiments.

Relative quantification by real-time RT-PCR.

THP1-DCs (WT & KOs) were infected with the differentially opsonized HIV-1 particles at different time-points from 1 h to 12 h at 37°C with a p24 concentration of 350 ng/mL for $0.5x10^6$ cells. Cells were lysed with RLT Buffer (Qiagen) and total RNA was purified according to the manufacturer's instructions. RNA was then quantified (NanoVue) and reverse transcribed into cDNA (iScript Reverse Transcription Supermix for RT-qPCR, BioRad). The cDNA was then used for multiplex qPCR (iQ Multiplex Powermix, BioRad) amplification, using PrimePCR™ Probes for IL-10, IL-6, IL-1B, IL12A, IL23A, TNFα and TGFβ (all from BioRad Laboratories). The RT-qPCR was run in the BioRad CFX96 Real Time PCR System. The cycling conditions were as follows: 3 min at 95°C, 44 cycles: 15 s at 95°C, 60 s at 60°C. For mRNA expression of IFNB1 real-time PCR using Sybergreen qPCR (Eva Green, BioRad) amplification and gene-specific primer/probe pairs (BioRad) were used. The cycling conditions were: 30 s at 95°C, 39 cycles: 5 s at 95°C, 10 s at 60°C with a melt curve 65°C to 95°C with an increment of 0.5°C for 5 s. A GAPDH (human) PCR using specific primer/probe pairs (BioRad) served as internal control to quantify the relative gene expression of target genes.

Data were analyzed with the BioRad CFX Manager Software (ΔΔCt method) and values were exported to GraphPad Prism.

Cytokine Analyses by ELISA

THP1-DCs were plated onto a 12 well tissue culture-plate at $0.5x10^6$ cells/well. Cells were infected with R5-tropic virus (R9Bal) at 12, 24 and 48 h. Supernatants were collected and inactivated with Igepal 5% (1:2). The amount of IL-1β was measured by ELISA (eBioscience).

Multicolor FACS analysis

Differentiation of THP1 into DCs exposed to cytokine cocktail (IL-4, GM-CSF, TNF-α) was analyzed by using anti-human CD11b-PE, CD11c-AlexaFluor488, CD18-APC, HLA-ABC-PerCP/Cy5.5, HLA-DR-APC-Cy7, DC-SIGN-PE, CD86-FITC, CD83-APC, CD1a-FITC, CD4-APC, CCR-

PerCP/Cy5.5 and CXCR4-PE as described [168] on a FACS Verse flow cytometer (BD Biosciences). Data were analyzed using FACS DIVA software (BD Biosciences).

(II) LCs

Ex vivo model and primary LC isolation

Human skin tissue was obtained from healthy donors undergoing corrective breast or abdominal surgery after informed consent in accordance with our institutional guidelines. Split-skin grafts of 0.3 mm in thickness were obtained using a dermatome (Zimmer Biomet, Indianapolis, IN, USA). After incubation with Dispase II (1 U/mL; Sigma Aldrich, Saint Louis, Missouri, USA), epidermal sheets were separated from the dermis, washed, cut in $1cm^2$ and cultured in Iscoves Modified Dulbecco's Medium (IMDM; Thermo Fischer Scientific) supplemented with 10% FCS, gentamycin (20 µg/mL; Centrafarm, Etten-Leur, The Netherlands), penicillin/streptomycin (10 U/mL and 10 µg/mL respectively; Invitrogen).

LC-enriched epidermal single-cell suspensions were generated as described before [188, 286]. Briefly, epidermal sheets were incubating in PBS containing DNase I (20 units/mL; Roche Applied Science, Mannheim, Germany) and trypsin 0.05% (Beckton Dickinson, USA). Single-cell suspension was layered on Ficoll gradient (Axis-shield) and immature LCs were purified using CD1a microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). LCs were routinely 85% to 98% pure and expressed high levels of langerin and CD1a [214]. Mature LCs were generated as described before [188]. Briefly, epidermal sheets were cultured in IMDM (Thermo Fischer Scientific) supplemented with 10% FCS, gentamycin (20 µg/mL; Centrafarm, Etten-Leur, The Netherlands), penicillin/streptomycin (10 U/mL and 10 µg/mL respectively; Thermo Fisher Scientific) for three days and mature LCs were harvested. Isolated LCs were routinely 90% pure and expressed high levels of langerin and CD1a.

Multicolor FACS analysis

LCs expression markers were analyzed using CD11b-A_{488} (Sony Biotechnology), CD11c-PE (Biolegend), CD1a-APC (BD Pharmigen) and CD207-PE (Beckman Coulter) on FACSCanto II flow cytometer (BD Biosciences).

Primary LCs were infected with a multiplicity of infection of 0,2-0,4 and HIV infection was assessed by flow cytometry a day 5-7 after infection by intracellular p24 staining. Double staining with CD1a (LCs marker; HI149-APC; BD Pharmigen) and p24 (KC57-RD1-PE; Beckman Coulter) was used to discriminate the percentage of CD1a+p24+ infected LCs. LC infection and

transmission was assessed by FACSCanto II flow cytometer (BD Biosciences) and data analysis was carried out with FlowJo vX.0.7 software (TreeStar Inc, Ashland, OR, USA).

HIV-1 virus stock

The following virus stock was obtained through the NIH AIDS Reagent Program, division of AIDS, NIAID: HIV-1 JR-CSF Virus from Dr. Irvin Chen [287, 288]. HIV-1 virus stock was propagated on PHA-stimulated human PBMCs. Produced HIV-1 viruses were quantified by p24 ELISA (Perkin Elmer Life Sciences, Boston, MA, USA) and titrated using the indicator cells TYM-b1 (John C. Kappes, Xiaoyun Wu, Birmingham, Alabama, USA and Tranzyme Inc., the NIH AIDS Reagent Program, division of AIDS, NIAID) [289].

Opsonization of HIV-1

Incubation of HIV-1 with active normal human serum (NHS) or seminal fluid (SF) results in the covalent deposition of C3 fragments (C3b, iC3b, C3d, C3c) on the viral surface. We incubate purified R5-/X4 –tropic HIV-1 (concentration >1 μg p24/mL) for 1h at 37°C with NHS as a complement source (HIV-C) in a 1:10 dilution and with SF diluted in DBPS (1:1 ratio). Subsequent to opsonization, the virus was washed and pelleted by ultracentrifugation (14000 rpm for 90 minutes at 4°C), and resuspended in 500 μL of RPMI medium without supplements.

Virus Capture Assay (VCA)

The opsonization pattern was determined by using a virus-capture assay (VCA), as previously described [166] with antihuman C3c/C3d, IgG and mouse IgG antibodies as control for background binding. The coated VCA plates were incubated overnight with differentially opsonized viral preparations (1 ng of p24 per well) at 4°C and washed 4 times with RPMI medium to remove unbound virus. Bound virus was lysed (2% Igepal) and transferred to a pre-coated p24 ELISA [284] plate to confirm the opsonization pattern.

LC infection and transmission assay using ex vivo model

For infection, human epidermal sheets were exposed to differently opsonized (using NHS or seminal fluid as complement source) HIV-$1_{JR\text{-}CSF}$ (100μL/sheet, TCID50 of 38 x 10^3, determined in TZM-b1 cells), HIV-$1_{92BR030}$ and HIV-1_{Yu2B} (100μl/sheet, 500 ng p24/mL) for 3 days. For transmission, epidermal sheets were infected for 48 hours. After 48 hours, the epidermal sheets were removed and emigrated LCs were harvested and extensively washed to remove unbound virus. For transmission assay emigrated LCs were co-culture with U87 for 3 days at 37°C. For

monitoring HIV-1 infection in the emigrated LCs and LCs-mediated transmission of HIV-1 to U87 were determined by intracellular p24 staining.

Capture of HIV-1

Migrated LCs ($1x10^5$ cells/well in 100µl) were exposed to 25 ng p24/ml of R5 tropic non-opsonized (HIV) or complement-opsonized (HIV-C) HIV-1. After 6h incubation at either 4°C for binding or 37°C for internalization, cells were washed 4 times to remove unbound virus. Cell pellets were lysed and viral amount was assessed by p24 ELISA (Perkin Elmer Life Sciences) according to manufacturer instructions.

Cell treatment

Cells were infected with HIV and HIV-C as explained above. Preincubation with inhibitors or blocking antibodies was done for 2h: 10µmol/L AZT (NIH AIDS Research and Reference Reagent Program), 1µmol/L IDV (NIH AIDS Research and Reference Reagent Program), 8µg/mL α-CD11b/c (Biolegend), 20µg/mL 10E2 and 0,5mg/mL CR-isotype (Invitrogen).

U87 culture

Human astroglioma U87 cells expressing human CD4 [290] and CCR5 ($CD4^+$ $CCR5^+$U87) were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIH. $CD4^+$ $CCR5^+$U87 cell line were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) containing 10% fetal bovine serum (FBS) (Hyclone, Perbio, Erembodegem, Belgium), 0.01 M HEPES buffer (Gibco BRL), and 0.2 mg/ml geneticin (G-418 sulfate) (Gibco BRL). The cell cultures were maintained at 37°C in a humidified CO2-controlled atmosphere and subcultivations were done every 2 to 3 days by digestion of the monolayers with trypsin/EDTA (Gibco BRL).

Relative quantification by real-time RT-PCR.

IFNB and ISG mRNA levels was isolated using mRNA capture kit (Roche) and cDNA was synthesized with reverse transcriptase kit (Promega). PCR amplification was performed in the presence of SYBR Green in an ABI 7500 Fast PCR detection system (Applied Biosystems). Specific primers were designed using Primer Express 2.0 (Applied Biosystems). The sequences are as follows: IFNB, forward, ACAGACTTACAGGTTACCTCCGAAAC, reverse, CATCTGCTGGTTGAAGAATGCTT; MxA forward, TTCAGCACCTGATGGCCTATC, MxA reverse, GTACGTCTGGAGCATGAAGAACTG; APOBEC3G forward, TTGAGCCTTGGAATAATCTGCC and

reverse, TCGAGTGTCTGAGAATCTCCCC. Expression of target genes was normalized to GAPDH ($Nt = 2^{Ct(GAPDH)-Ct(target)}$).

HIV-1 integration Alu-PCR assay

Integrated HIV-1 levels were determined by a two-step Alu-LTR PCR. Total cell DNA was isolated at 4-5 days after iкection with peqGOLD DNA Mini Kit (Quiagen). In the first round of PCR, the DNA sequence between HIV-1 LTR and the nearest Alu repeat was amplified. The second round was nested quantitative real-time PCR of the first-round PCR products using primers to the aforementioned marker region in combination with another HIV-1-specific primer (R/U5) by real-time quantitative PCR. HIV-1 integration was normalized relative to GADPH DNA levels.

Themal conditions for R/U5 and full-length HIV-DNA consisted of 10 min at 95°C and 50 cycles of 95°C for 15 s and 60°C for 30a, and melt curve analysis for specification of PCR products. The Alu-HIV-1 PCR cycling conditions included a denaturation step (98°C for 10 min), followed by 22 cycles of denaturation (98°C for 30s), annealing (60°C for 30s), and extension (70°C for 10min), together with a Melt curve part. Data were analyzed with the BioRad CFX Manager Software (ΔΔCt method) and values were exported to GraphPad Prism.

Viral Fusion Assay

LCs were plated into 96-well plates ($1x10^5$ cells/well in 100 µl) and infected with 250 ng p24/ml of non-opsonized or opsonized R9Bal/β-lam and VSV/ β-lam. After 5h incubation, cells were washed and loaded for 1h with CCF2-AM substrate solution according to the manufacturer's instructions (LiveBLAzer™ FRET-B/G Loading Kit with CCF2-AM, LifeTechnologies). Cells were washed again and developed for 16h in CO_2-independent medium (Gibco) containing 10 % FCS and 2.5 mM probenicid. Cleavage of CCF2 was analyzed by flow cytometry after fixation of DCs in 4% paraformaldehyde.

Microscopy

To visualize the intracellular HIV-1 localization by HC/HT screening analyses, LCs (~100.000/well in 100 µl) were seeded in CellCarrier Ultra plates (Perkin Elmer) and exposed to R9Bal/mCherry or –GFP and YU2B/mCherry or –GFP (350 ng p24/mL) for 3h. LCs were fixed with 4% paraformaldehyde, stained using Hoechst 33342 (Cell Signalling Technologies), permeabilized (Permeabilization Wash Buffer, Biolegend) and labelled for CD207 (Beckman Coulter), CD1a (BD Pharmigen), CD11b (ThermoFisher) and C3b (ImmunoTools).

Statistical analyses

Differences were analyzed by the GraphPad prism software using the unpaired student's t-test (2-tailed) or one-way ANOVA with Bonferroni post-test for multiple comparisons depending on the analyses.

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10. Abbreviations

Ab	Antibody
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
AP	Alternative complement pathway
APCs	Antigen-presenting cells
APOBEC3G	Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G
C1Inh	C1 Inhibitor
C4bp	C4 binding protein
CA	Capsid
cART	Combined antiretroviral therapy
CCL	CC-Chemokine Ligand
CCR	CC- Chemokine receptor
CoML	Complement-mediated lysis
CP	Classical complement pathway
CR(s)	Complement receptor(s)
CTLs	Cytotoxic T cells
CXCR	CXC chemokine receptor
DC(s)	Dendritic cell(s)
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
dGTP	Deoxyguanosine triphosphate
DHN-melanin	Dihydroxynaphthalene-melanin
dNTPs	Deoxynucleotide triphosphate
dsDNA	Double-stranded DNA
Env	Envelope proteins
ER	Endoplasmatic reticulum

ESCRT	Endosomal sorting complex required for transport
fB	Factor B
fD	Factor D
fDCs	Follicular dendritic cells
fH	Factor H
FHL-1	Factor H-like protein 1
fI	Factor I
Gag	Group-specific antigen
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAART	Highly active antiretroviral therapy
HIV-1	Human immunodeficiency virus
HIV	Human immunodeficiency virus non opsonized
HIV-C	Complement opsonized HIV
HIV-SF	Semen fluid opsonized HIV
iDCs	Immature dendritic cells
iLCs	Immature langerhans cells
IN	Integrase
ITAM	Immunoreceptor tyrosin-based activation motif
kDa	Kilodalton
KO	Knock-out
LCs	Langerhans cells
LP	Lectin complement pathway
LTNPs	Long-term non-progressors
LTRs	Long terminal repeats
MA	Matrix
MAC	Membrane attack complex
MBL	Mannose-binding lectin

mDCs	Mature dendritic cells
MDMs	Monocyte-derived Macrophages
MHC I	Major histocompatibility complex type I
MHC II	Major histocompatibility complex class II
miLCs	Migratory langerhans cells
moDCs	Monocyte-derived dendritic cells
mRNA	Messenger RNA
NC	Nucleocapsid
Nef	Negative Regulatory Factor
NHS	Normal Human Serum
NNRTIs	Non-nucleoside RTIs
NRTIs	Nucleoside analogue RTIs
PAMPs	Pathogen associated molecular patterns
PIC	Pre-integration complex
p.I	Post infection
Pol	DNA polymerase
Pro	Protease
PRRs	Pattern recognition receptors
pVL	Plasma viral load
RCAs	Regulators of complement activation
Rev	Regulator of expression of virion proteins
ROS	Reactive oxygen species
RT	Reverse transcriptase
RTIs	Reverse transcriptase inhibitors
SAMHD1	SAM domain- and HD domain- containing protein 1
SIV	Simian immunodeficiency virus
Tat	Trans-Activator of Transcription

TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-β	Tumor necrosis factor beta
VCA	Virus Capture Assay
Vif	Virion infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
VSV	Vesicular stomatitis virus non opsonized
VSV-C	Complement opsonized VSV
WT	Wild type
WT-THP1 DCs	Wild type differentiated THP1 cells to dendritic cells
CD11b KO-THP1 DCs	CD11b knock-out differentiated THP1 cell line to dendritic cells
CD11c KO-THP1 DCs	CD11c knock-out differentiated THP1 cell line to dendritic cells
CD18 KO-THP1 DCs	CD18 knock-out differentiated THP1 cell line to dendritic cells

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I would also like to thank Dr. Igor Theurl and Dr. Lukas Huber for being members of my PhD thesis committee and for our fruitful discussions (basic immunology, statistics, etc).

Next, I would like to thank all the members of AG Wilflingseder who have continuously maintained an amicable atmosphere, inside and outside the lab. Ranging from scientific methods to chilling at the Inn, learning how to ski and hike the biggest peak in Südtirol, I have learned a lot from all of them. I have been through highs and low lows, and it would have been hard for me to keep the spirit alive without being part of such wonderful team. We all use to look out for each other as part of a big family. Ulla, Marion, Manuela, Vicky, Paula, Parul, David, Karo and Greta – it’s hard to put in words …. THANK YOU again for everything. Ulla, Marion and Manuela, my HIV-1 team, who has taught me through long discussions about troubleshooting, new hypothesis and some dancing in the BSL3 at 12am. Special mention to Paula, our Spanish PostDoc, who arrived in my longest lowest moment but being very patient with my mood, she gained all my affection and respect — she never hesitate to continue helping and teaching me. I would like to thank Karo and her amazing pair of hands. As well to Greta, both were essential in the organization of everything in the labs and helped in discussing and performing experiments. To the new group members, Maureen and Eliott, thanks to helped me realize I can still learn showing you how to perform some protocols.

I am further deeply grateful to all the people in the PhD office, especially Samyr, my ski-partner who was always available for serious scientific discussions, beer after work, exhausted ski days and trips.

A special mention to Bjiliana, for always be the first person asking me " Wie geht es dir?" , for our deep conversations during the lunch time, about live, family, lazy people around … thanks for sharing with me part of your life. It was a pleasure meeting you.

I would especially like to thank Ana, Fraga and Valentina for their numerous scientific help, but also and even more so for their infectious positive attitude, good advices and tranquil words. These words have often calmed my nerves and anxiety helping me to grow as a person. Thanks for our often CCB-Lunch and coffee breaks, you made me part of our CCB-VIP group!

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Furthermore, I would like to thank my friends, which I have met during my stay here. One can only dream to have the kind of friend I have been lucky to be associated with. Most of them I just named them, but there are more like Ezgi, Maria, Petro, Marcos …. .

Of course I cannot forget my friends back home, in particular, "Las Pakis". They are the kind of person always there for you, doesn't matter what, for laugh, cry, anger, anything. They are always available to do a WhatsApp call anytime, to include me in their full-stories-life even from the distance, to sacrifice their plans to be with me when I am in Sevilla.

Antonio you have been my anchor and force.

Lastly and most importantly, I would like to thank my family, my parents and grandparents for their love and unmitigated support, although it is very hard for them to have me so far all these years.

12. Curriculum Vitae

Marta Bermejo Jambrina, MSc

EDUCATION

2006-2012	Biology Degree - University of Seville (Spain)
2012-2013	Erasmus Student - TU-Braunschweig (Germany)
2012-2015	Biochemistry Degree – University of Seville (Spain)
2015- present	PhD Student at the Molecular Cell Biology and Oncology Program (MCBO)
	Division of Hygiene and Medical Microbiology, Innsbruck (Austria)

WORK EXPERIENCE

07/2007-08/2007	Natural History Museum (London) (Dr. Mark Carine)
	"Diversity patterns at regional level: the Azores diversity enigma"
06/2008-08/2008	Natural History Museum (London) (Dr. Mark Carine and Dr. Emma Sherlock)
	"Endemism and evolution of the Canary Island endemic flora"
07/2009	WWF ADENA (Canary Island)
2009-2010	University of Seville, Dept. of Plant Biology and Ecology (Prof. Dr. Mari Cruz Díaz Barradas)
04/2012	XLAB Course (Göttinger Experimentallabor für junge Leute – Georg August University of Göttingen & Max Planck Society)
04/2013-09/2013	Helmholtz Center for Infection Research (Braunschweig) (Dr. Esteban A. Hernández Vargas)
10/2013-12/2013	Georg August University of Göttingen. Dept. of Crop Sciences Section of Plant Nutrition and Crop Physiology (Prof. Dr. Klaus Dittert)
02/2014-09/2014	Julius-Maximilians-University of Würzburg. Institute of Molecular Infection Biology/Research Center for Infectious Diseases. "The minimal CRSPR/Cas9 Type II-C system in *C. jejuni*" (Prof. Dr. Cynthia Sharma)

2015 – present	Medical University of Innsbruck. Division of Hygiene and Medical Microbiology. "DC-iphering complement receptor mediated HIV-1 incorporation and effects on DC function in search for novel therapeutical targets" (Prof. Dr. Doris Wilflingseder)
10/2017-04/2018	University of Amsterdam. Academish Medisch Centrum-Experimental Immunology. "Mechanistic insights into complement-induced mechanisms of HIV-1 Langerhans Cells" (Prof. Dr. Doris Wilflingseder and Prof. Dr. T.B.H. Geijtenbeek)

PERSONAL COMPETENCES

Laboratory Experience:

- Basic research in immunology
- Cell culture
- Virus culture BL3
- Flow Cytometry
- Standard molecular and immunological techniques
- Basic microscopy
- Generation of KOs using CRSIPR/Cas9

Languages: English (B2+), German (C1) and Spanish (mother tongue)

IT knowledge: MS-Windows, MS-Office, Adobe Photoshop, Adobe Illustrator, Corel Draw, GraphPad Prism, Image J, FlowJo, Matlab.

SEMINARS – WORKSHOPS AND CONFERENCES

- Course "Industrial Microbiology" from 25-29 June 2012 at UNIA (International Universtiy of Andalucía)(Spain)
- 3rd Mol Micro meeting Würzburg from 7-9 May 2014 at Julius Maximilians Universiy of Würzburg (Germany)
- CRISPR 2014 Berlin from 14-16 May 2014 (Germany) (Poster presentation)
- ÖGAI Meeting 2016 from 16-19 November, Innsbruck (Asutria) (Poster presentation)
- 2nd MidWinter Conference – Advances in Immunobiology" from 21-25 January, 2017. Seefeld in Tirol (Austria) (Poster presentation)
- 1st Meeting of Study Group Dendritic Cells (AKDC) in Mainz from 1st-3rd February 2017 (Germany)
- Life Science PhD Meeting from 18th – 19th April 2017, Innsbruck (Austria) (Poster presentation)

- Xlab-Alumni Symposium from 18th-22th August, Göttingen (Germany) (Invited Speaker)
- Netherlands Conference HIV Pathogenesis (NCHIV2017) on 21th November 2017, Amsterdam (The Netherlands)
- NVVI2017 Annual Meeting from 13th-14th December 2017, Noordwijkerhout (The Netherlands)
- Life Science PhD Meeting from 4th -6th April 2018, Innsbruck (Austria) (Poster presentation)
- 15th International Symposium on Dendritic Cells from 10th – 14th June 2018, Aachen (Germany) (Poster presentation)
- 5th European Congress of Immunology (ECI2018) from 2nd -5th September 2018, Amsterdam (The Netherlands) (Poster presentation)
- Keystone Symposia "HIV Vaccines X7" from 23th – 29th March 2019, Whistler-British Columbia (Canada) (Poster presentation)

AWARDS AND SCOLARSHIPS

January 2017	ÖGAI travel grant for MidWinter Conference (Seefeld, Austria)
January-April 2018	EFIS-IL Short-Term Fellowships
June 2018	Travel grant to attend the 15th International Symposium on Dendritic Cells (Aachen, Germany)
September 2018	Travel grant to attend the 5th European congress of Immunology-ECI2018 (Amsterdam, The Netherlands)
March 2019	ÖFG Travel grant for the Keystone Symposia "HIV Vaccines X7" (Whistler-Bristish Columbia, Canada) (waiting for the resolution)

PUBLICATIONS

- Carlos E. Torres-Cerna, Alma Y. Alanis, Ignacio Poblete-Castro, **Marta Bermejo Jambrina**, Esteban Abelardo Hernandez-Vargas; "A comparative study of differential evolution algorithms for parameter fitting procedures", 2016 IEEE Congress on Evolutionary Computation (CEC)
- **Bermejo-Jambrina, M.,** Eder, J., Helgers, L. C., Hertoghs, N., Nijmeijer, B. M., Stunnenberg, M., & Geijtenbeek, T. B. H. (2018). C-Type Lectin Receptors in Antiviral Immunity and Viral Escape. *Frontiers in Immunology*, *9*, 590. http://doi.org/10.3389/fimmu.2018.00590
- Steger, M*., **Bermejo-Jambrina, M***., Yordanov T., Wagener J., Brakhage A., Pittl, V., Huber, L., Haas H., Lass-Flör C., Posch, W., Wilflingseder, D. (2018). β-1,3-glucan-lacking Aspergillus fumigatus mediates an efficient antifungal immune response by activating complement and dendritic cells : Virulence

- **Bermejo-Jambrina, M**., Yordanov T., Huber, L., Posch, W., Wilflingseder, D. (2019). Characterization of Human Complement Receptors 3 and 4 as IFN type I inducers: A potential role upon HIV-1 infection. (in preparation)
- Nijmeijer, B. M.*, **Bermejo-Jambrina, M*.,** Wilflingseder, D., Geijtenbeek, T.B.H. (2019). HIV-1 hijacks the complement system to escape degradation and promote viral dissemination by human Langerhans cells (in preparation)
- Jáuregui P., Steger M., **Bermejo-Jambrina M**., Lass-Flörl C., Posch W., Wilflingseder D. Efficient antiviral host defense mechanisms are induced in DCs by complement-opsonized HIV-1 via MAVS signaling. (in preparation)