Langerhans cells and dendritic cells in innate defense against pathogens

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Chapter 11

General Discussion

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Part 1: Langerin and DC-SIGN: universal pathogen receptors?

In this thesis the function of LCs and DC-SIGN$^+$ DCs in innate defense against pathogens was investigated. Since LCs and DCs express distinct C-type lectins Langerin and DC-SIGN, respectively, the interaction of different pathogens with these C-type lectins was explored and resulted in the identification of novel ligands and pathogens. Thus far, HIV-1, *Candida albicans* and *Mycobacterium leprae* have been identified as ligands for Langerin (1-4). In this thesis it was shown that Langerin as well as DC-SIGN interact with HSV-1 and -2 (**chapter 4 and 8**). Furthermore, fungal species *Malassezia furfur, Saccharomyces cerevisiae* and a plethora of *Candida* species were identified as ligands for Langerin (**chapter 2**). These data suggest that Langerin, similar to DC-SIGN, is a pattern recognition receptor with a broad specificity for pathogens and this raises the question whether the immunological and virological outcomes are also similar.

1.1 Pathogen recognition

Langerin and DC-SIGN have overlapping carbohydrate specificities; they both interact with mannose and fucose structures (5;6). Many viruses and other micro-organisms express (host-cell derived) mannose structures on their surface, which facilitate recognition by Langerin and DC-SIGN (7-9). Therefore, based on the carbohydrate structure of ligands, one can make a prediction about the binding capacity to Langerin and DC-SIGN.

Langerin has high affinity for mannose, fucose and GlcNAc structures (6). However, Langerin does not interact with LeX and LeA, while it has strong affinity for LeY and LeB (**chapter 5**). LeY and LeB contain a terminal fucose structure, which is absent in LeX and LeA. These data suggest that Langerin is unable to bind internal fucose structures, but does interact with terminal fucose structures. In contrary, DC-SIGN interacts with all above mentioned Lewis structures (5). This is an apparent difference between the binding capacities of Langerin and DC-SIGN, and shows that Langerin has a distinct recognition pattern for fucose structures.

Fucose structures are often present on helminths, and may vary during the life-stage. As seen in table 1, several helminths interact with Langerin and/or DC-SIGN. However, distinct patterns are present: whereas Langerin recognizes *Haemoncus contortus* larvae, these are not recognized by DC-SIGN. On the other hand, DC-SIGN strongly interacts with cercariae from *Schistosoma mansoni*, while Langerin fails to interact with these cercariae. *S. mansoni* cercariae are the final larvae stage, which penetrates the skin of the human host. Upon entry, cercariae rapidly transform into a schistosomule, which is accompanied by profound changes in the antigenic structures and the schistosomule migrates throughout the tissues to the lung and liver (10). The finding that cercariae do not interact with Langerin suggests that this is a possible mechanism for the parasite to escape recognition by LCs. Together these data suggest that structures present on helminths and other pathogens determine the recognition by Langerin and DC-SIGN, which, dependent on the subsequent immune reaction, may provide an immune-evasive strategy.

Langerin interacts with the cell-wall of fungal pathogens, which contain mannose as well as β-glucan structures (**chapter 2**). Mannose structures present on fungal cell-walls are also ligands for DC-SIGN (5;6). Notably, Langerin interacts with both curdlan and laminarin, structures containing mainly β1-3 glucans. Thus far, dectin-1, present on DCs and expressed at low levels by LCs, has been the only C-type lectin identified to interact with β-glucans (11). Recently, α-glucans isolated from *M. tuberculosis* have been identified as a ligand for DC-SIGN (12). It remains to be determined whether Langerin interacts with α-glucans. In addition to the identification of Langerin as a receptor for β-glucans and fungi, these data show that Langerin acts as the main fungal receptor on LCs. Thus, these studies suggest that Langerin has a broad pathogen recognition pattern.

As listed in table 1, several pathogens fail to interact with Langerin and/or DC-SIGN. Together these data demonstrate the importance of carbohydrate structures that are present on pathogens in the recognition by Langerin and DC-SIGN. Extensive trimming of glycans present on a pathogen may provide an immune-evasive strategy in the interaction with Langerin and/or DC-SIGN. For instance, HBV is unable to interact with DC-SIGN: however, viral particles produced in the presence of an inhibitor that prevents trimming of complex mannose structures are able to interact with DC-SIGN (**chapter 9**).

Although Langerin and DC-SIGN recognize a large variety of pathogens and show overlapping recognition patterns, they have unique specificities that helps to diversify immune recognition. This is further supported by the differences in expression and immunological functions.

1.2 Host-pathogen interaction

Host-pathogen interaction can have a wide variety of outcomes. DCs are essential in shaping innate and adaptive immune responses to pathogens. In this section the interaction of pathogens with LCs and DCs and the contribution of C-type lectins DC-SIGN and Langerin are discussed (Figure 1).

1.2.1 DC-SIGN$^+$ DCs

C-type lectins on DCs play an important role in pathogen recognition for antigen-presentation. Antigens are taken up by C-type lectins such as DC-SIGN and targeted to the lysosome for (cross-)presentation onto MHC-I or -II (21). This requires an efficient maturation of DCs. However, several pathogens have evolved to subvert the antigen processing capacity and inhibit DC maturation. For example, HSV-1 actively down-regulates co-stimulatory molecules and MHC-I, which results in suppression of DC maturation and impaired antigen-presentation, while Measles Virus (MV) prevents CD8$^+$ T cell proliferation (22).

Another mechanism by which pathogens evade DC-mediated immunity is by interfering with antigen processing routing to not only prevent antigen presentation, but to also enhance pathogen survival and dissemination. HCV binding to DC-SIGN leads to targeting of HCV to non-lysosomal compartments, while antibodies and carbohydrates are normally directed through early endosomes to lysosomes (18;21). Binding to DC-SIGN also results in re-direction of the internalization route to

Table 1: Langerin and DC-SIGN binding

Ligand	Langerin	DC-SIGN
Carbohydrate structures	Mannose, terminal-fucose, GlcNAc (6)	Mannose, internal/ terminal fucose (5)
Lewis antigens	LeB, LeY	LeA, LeB, LeX, LeY (5)
α-glucans	nd	Yes (12)
β-glucans	Yes	No
Helminths		
Haemoncus contortus		
Adult	No	No
L3 larvae	Yes	No
Excretory/secretory proteins	Yes	Yes
Schistosoma mansoni		
Adult	Yes	Yes
Cercariae	No	Yes
Soluble egg antigen	Yes	Yes (13)
Bacteria		
E. coli	No	No
Borrelia burgdorferi	Yes	nd
M. tuberculosis (ManLam)	Yes	Yes (14)
S. aureus	No	nd
Virusses		
HIV-1 gp120 R5/ X4	Yes (1;2)	Yes (15)
HSV-1/ -2	Yes	Yes (16)
HBV	nd	No (17)
HCV	Yes	Yes (18)
MV	Yes	Yes (19)
Fungi		
Candida species	Yes	Yes (9)
Malassezia furfur	Yes	Yes
Cryptococcus neoformans	No	No
Cryptococcus gattii	No	No
Saccharomyces cerevisiae	Yes	Yes
Miscellaneous		
Salp15 (*Ixodes scapularis*)	Yes	Yes (20)

nd: not determined

non-lysosomal compartments for protection and transmission of pathogens to target cells (23). This is beneficial to the pathogen since it would prevent efficient antigen presentation and subsequent immune stimulation, and in addition it could provide a mechanism by which pathogen transmission throughout the body occurs. It has been demonstrated that pathogens such as HIV-1 and *M. tuberculosis* survive within DCs (15;24;25) and that HIV-1 is subsequently transmitted to target cells, a mechanism known as trans-infection (15;24;25).Viruses exploit DC-SIGN binding to facilitate attachment and thereby enhance infection of the host. Several pathogens including HIV-1, MV, and HSV-1 exploit DC-SIGN binding for enhanced infection of DCs and transmission to target cells (15;16;19). Along these lines, the lack of binding to DC-SIGN can also provide a mechanism to escape from DC immune surveillance, such as demonstrated for HBV.

Interaction of pathogens with DCs often involves simultaneous activation of TLRs and C-type lectins and co-triggering of TLR and C-type lectin tailors immune responses (26). Synergistic C-type lectin and TLR activation results in altered cytokine production, which in turn can modulate T cell proliferation and T helper (Th) cell differentiation. Likewise, DC-SIGN and TLR-4-mediated tailoring of immune responses has been thoroughly investigated (27); stimulation of DC-SIGN alone fails to induce a cytokine response, while upon activation of TLR-4, DCs produce cytokines such as IL-10, IL-12 and IL-6. However, engagement of mannose structures with DC-SIGN together with TLR ligation enhances the production of IL-10 as well as pro-inflammatory cytokines IL-12 and IL-6 in DCs (28). Whereas TLR signaling is responsible for the recruitment of NF-κB to the nucleus, DC-SIGN triggering results in phosphorylation and subsequent acetylation of NF-κB subunit p65 via a signaling pathway including a signalosome containing LSP-1 and Raf-1. Together this results in prolonged and enhanced transcription of *IL10*, *IL12p35* and *IL6.* NF-κB modulation

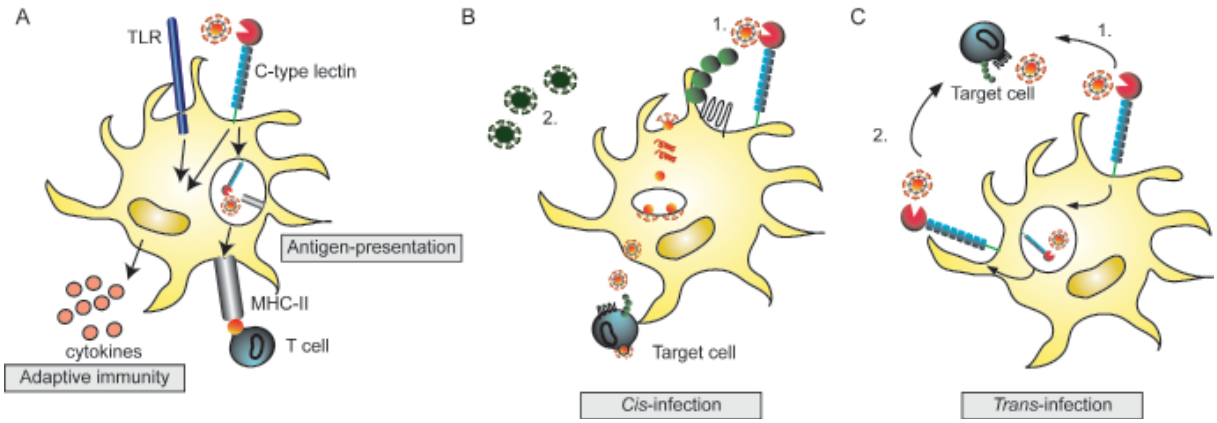


Figure 1. Immune evasion and modulation by pathogens. Pathogens evade and modulate host immune responses through several mechanisms. (A) Pathogens interfere with internalization, maturation and antigen presentation through receptors such as DC-SIGN. Both TLR and C-type lectin receptors interact with pathogens, which induces signaling that tailors the immune response. (B) Pathogen binding to DC-SIGN enhances attachment and subsequent DC infection (*cis*-infection). *De novo* produced particles are released and transmitted to target cells (1). Lack of interaction with DCs can lead to evasion from immune recognition (2). (C) Pathogen binding to DC-SIGN enhances infection of target cells independent of DC infection (*trans*-infection). This is mediated via cell-surface bound (1) or internalized (2) particles.

has been demonstrated for TLR-3, TLR-4 and TLR-5 stimulation in combination with DC-SIGN (27). In contrast, engagement of fucose structures together with TLR ligation results in enhanced IL-10, but decreased IL-6 and IL-12 production (28). DC-SIGN triggering by fucose structures results in dissociation of the signalosome containing Raf-1 from the cytoplasmic tail of DC-SIGN. The mechanism responsible for the fucose-specific cytokine profile remains unclear, although it is known that this process requires LSP-1, but not Raf-1, in the signalosome. Thus, DC-SIGN tailors TLR signaling by responding differently to mannose and fucose carbohydrate structures.

Helicobacter pylori is a human gastric pathogen that exists in two different phase variants, LeY^+ and LeY^- (29). Phase switching can be used by the pathogen as a mechanism to subvert the immune response by DCs. Indeed, LeY^+ bacteria increase anti-inflammatory cytokine IL-10 and suppress pro-inflammatory cytokines IL-12 and IL-6 production compared to LeY^- bacteria (28). This could account for the observed suppression of Th1 response during *H. pylori* infection (29). DC-SIGN signaling in response to LeY^+ variants can thus redirect the immune response towards a more balanced Th1/Th2 response, which is beneficial for the host compared to a dominant Th1 response, which has been associated with increased gastric inflammation (29;30). Thus, phase switching to a LeY^- variant would be more detrimental for the host and advantageous for the parasite compared to LeY^+ variants.

In addition to mannose and fucose induced DC-SIGN signaling, another mechanism is observed for Salp15. Salp15 is a salivary protein isolated from *Ixodes scapularis* (deer tick), the primary vector for *Borrelia burgdorferi* transmission. In **chapter 10**, Salp15 and whole tick saliva inhibited T cell proliferation upon TLR stimulation in DCs. Salp15 interaction with DC-SIGN on DCs synergistically with TLR-2 or TLR-4 triggering leads to suppression of pro-inflammatory cytokines IL-12, IL-6 and TNF-α. This suppressed cytokine production is dependent on intracellular signaling molecules Raf-1 and MEK. The decreased IL-6 and TNF-α production is caused by enhanced mRNA degradation, while IL-12 production is hampered due to impaired nucleosome remodelling at the *IL12-p35* promoter. This mechanism appears to be distinct from that of mannose or fucose structures and is not dependent on NF-kB phosphorylation or acetylation. However, the exact mechanism remains to be elucidated. Salp15 has been shown to bind to CD4, thereby inhibiting T cell receptor ligation-induced signals, resulting in reduced IL-2 production and impaired $CD4^+$ T cell activation and proliferation (31;32). Salp15 may alter the localization of intracellular signaling molecules involved in DC-SIGN signaling. In addition, other receptors, such as CD4 might affect the localization of Raf-1 or other downstream effector molecules to DC-SIGN. Together, the immunosuppressed environment created by tick saliva including Salp15 might facilitate *B. burgdorferi*'s escape from DCs and decreases activation of T cells. Subsequently, the immunosuppressed environment is advantageous for the dissemination of *B. burgdorferi* through the body. Thus, immune responses mediated by DC-SIGN are tailored through regulation of signaling pathways after triggering of different receptors or by ligands with different carbohydrate structures.

1.2.2 Langerin$^+$ LCs

As described previously, the interactions of pathogens with DC-SIGN have been thoroughly investigated. However, less is known about the interaction of Langerin and LCs with micro-organisms. In this thesis several pathogens have been identified as ligands for Langerin, including HSV-1 and -2, mycobacteria, helminths, and fungi, such as *Candida* species, *Saccharomyces cerevisiae* and *Malassezia furfur* (Table 1). In this section the interaction of LCs with different pathogens is discussed.

Opposite to DC-SIGN, the interaction of HIV-1 with Langerin does not result in enhanced infection, but rather results in degradation of HIV-1 by targeting HIV-1 to Birbeck granules (1). Unpublished observations suggest that a similar mechanism plays a role in interaction of Langerin with MV. MV binding to Langerin decreases infection of LCs (L. de Witte, unpublished observations). It is likely that HSV-1 and -2 interactions with Langerin also results in targeting to the Birbeck granules. Electron microscopy analysis could provide better insight. An important difference between HSV and HIV-1 is that HSV is highly infectious for LCs, surrounding keratinocytes and epithelial cells. In addition, LCs express other surface molecules with high affinity for HSV, such as heparan sulfates, which facilitate attachment of HSV to LCs (33;34). Therefore, it is plausible that under physiological circumstances Langerin on LCs might be less efficiently to prevent HSV infection of LCs.

HIV-1 and MV investigations suggest that Langerin binding results in targeting of these viruses to Birbeck granules for degradation. It remains to be determined whether this holds true for all Langerin-binding viruses and whether other micro-organisms can also be targeted to the Birbeck granules. Electron microscopic analysis of the interaction of *C. albicans* with Langerin demonstrates uptake of *C. albicans* by LCs via Langerin, although no internalization in Birbeck granules is observed (**chapter 2**). Indeed, the large size of the particles raises questions about the possibility of uptake of *C. albicans* into Birbeck granules.

LCs are also thought to play a role in antigen presentation. The classical paradigm has been that LCs have similar antigen presenting capacities as their DC-SIGN$^+$ DC counterparts. However, little evidence is available to date to support this. There are conflicting data about the role of murine LCs in inducing immune responses and careful interpretation is needed. The results of different studies are dependent on the stimuli and model used, especially since the recent identification of murine Langerin$^+$ CD103$^+$ dermal DCs that seem to be involved in certain processes previously attributed to LCs (35).

Current data do not support a prominent role for LCs in the induction of immune responses: LCs appear to be dispensable for immune activation such as contact hypersensitivity (36-38), graft-versus-host disease (39) and viral immunity (40;41). Together, this has lead to the hypothesis that LCs may have immune-regulatory properties that serve to constrain and contain adaptive immune responses in the skin. However, the majority of these studies have been performed in mice and under different conditions that might affect the outcome. Indeed, LC function in these mouse models might be dependent on the nature of the antigen, the type of pathogen, and the immunization route (42).

Murine studies have shown that LCs present antigens on MHC-I and MHC-II (43), however, LCs fail to cross-present viral antigens. In a murine HSV model, LCs do not present HSV antigens to $CD8^+$ T cells in lymph nodes. Instead it is thought that LCs transfer their antigens or apoptotic LC fragments to another DC subset (40). Whether this antigen transfer takes place in the epidermis, dermis or in the lymph nodes remains unclear (40;44). In a human MV-specific antigen presentation model, it is observed that LCs efficiently present antigens to $CD4^+$ T cells and that antigen presentation onto MHC-I occurs after viral infection of LCs. However, LCs fail to cross-present antigens to $CD8^+$ T cells (L. de Witte, unpublished observation). In contrast, LCs are able to transfer antigens to DC-$SIGN^+$ DCs, which subsequently cross-present antigens to $CD8^+$ T cells, which is independent of viral infection (de Jong, unpublished observation). These data support the hypothesis that LCs transfer viral antigens to DCs for further processing and presentation. Thus, LCs efficiently present viral antigens onto MHC-II and onto MHC-I upon viral infection. To overcome the lack of ability to cross-present antigens, LCs transfer viral antigens to DCs for cross-presentation. It remains to be determined whether this is specific for viral antigens.

Very little is known about the role of Langerin in antigen presentation. Targeting of Langerin with monoclonal antibodies results in delivery to non-lysosomal compartments but not to MHC-II molecules (45). In addition, Langerin-mediated antigen uptake results in delivery to Birbeck granules and may be involved in CD1a dependent antigen presentation (4). Nevertheless, in mice it has been demonstrated that LCs present OVA-labelled anti-Langerin antibodies both to $CD4^+$ and $CD8^+$ T cells (43). It remains to be determined whether pathogens captured by Langerin are similarly processed and presented to both $CD4^+$ and $CD8^+$ T cells, and whether this occurs in human LCs. Preliminary data suggest at least a partial contribution of Langerin in MV uptake for MHC-II presentation by human LCs (L. de Witte, unpublished observation).

The characteristics of LCs to deliver antibodies to Birbeck granules, which are thought to be involved in the endosomal recycling pathway, is very distinct from DC-SIGN and not completely understood. Langerin has a characteristic proline rich sequence in its cytoplasmic tail (46), while DC-SIGN bears a cytoplasmic tail containing internalization motifs with a di-leucine motif and a tri-acidic clusters (23). The differences in cytoplasmic tails can lead to recruitment of distinct adaptor proteins for each lectin, which are involved in internalization, intracellular sorting and even cytokine responses. It is currently unknown whether Langerin signaling affects adaptive immune responses and how the internalization pathway by Langerin is regulated at the molecular level.

1.3 Localization

LCs have a restricted localization within the human body: they line the epidermis and mucosal tissues, which are constantly exposed to the environment. The localization suggests that LCs play a regulatory role while DCs in the sub-epithelium and dermis, located below this delicate layer of LCs, play an important role in defense against pathogens. Based on the strategic location and compartmentalization of LCs and DCs it is tempting to speculate that high responsiveness of DCs to pathogens is necessary when the primary defense barrier is damaged and a pathogen has invaded the body. On the other hand, LCs are constantly exposed to micro-organisms in our environment and should therefore respond selectively. Several observations have been reported to support this hypothesis.

In general, LCs show a delayed migration from the epidermis to the lymph nodes compared to dermal DCs (38). These data have led to the hypothesis that LCs do not play a pivotal role in early antigen presentation events during infection. However, the unpublished observations demonstrating that LCs are capable of transferring viral antigens for cross-presentation to DCs, suggest that LCs do play a role in innate defense against viral pathogens. Therefore, antigen transfer may take place in the dermis. Further research is needed to clarify the functions of these cells.

Differences in C-type lectin and TLR expression contribute to the divergent function of LCs and DCs. Langerin and DC-SIGN have distinct functions in HIV-1 infection: DC-SIGN enhances infection and transmission, whereas Langerin captures HIV-1 for degradation. Under non-inflammatory conditions LCs form a first line of defense against HIV-1 infection. In contrast, HIV-1 subverts highly migratory DCs for transmission throughout the host. The interaction of LCs and DCs with HIV-1 is discussed in more detail in part 2 of this discussion. It remains to be determined whether this also holds true for other viruses and other classes of micro-organisms.

The repertoire of TLRs expressed by LCs is distinct from that expressed by DCs. Whereas DCs express TLRs that recognize bacteria, fungi and viruses, LCs lack expression of TLR-4, which recognizes gram-negative bacteria (47;48). In general, bacterial vaginosis is mainly caused by gram-negative bacteria. In addition, the responsiveness of LCs to bacterial TLR ligands is weak in terms of cytokine production and in general LCs produce low amounts of cytokines. LCs produce a repertoire of cytokines including IL-6, IL-8 and TNF-α but not IL-12 and type I interferons (47). Nevertheless, TLR-2 triggering of LCs induces IL-10 production, which suggests a specific response to commensal gram-positive bacteria (47). LCs do not respond to lactobacilli present in the healthy vaginal flora. This gram-positive bacterium is abundantly present, but does not usually cause an overt inflammatory response.

In contrast, LCs induce strong responses after viral TLR-3 triggering (49). Thus, LCs are more likely to respond to viral micro-organisms, while no or low responses are observed upon encounter with (commensal) bacteria. Together these data suggest that LCs selectively respond to micro-organisms, which depends on the nature of the microbe.

For defense against fungal pathogens, DCs express high levels of dectin-1. A main characteristic of dectin-1 is that TLR triggering is not required to induce cytokine responses (11), although TLR-2 ligation has a collaborative effect on yeast-induced activating signals (50). Triggering of dectin-1 on DCs by β-glucans results in IL-12p70, IL-1β, IL-6 and IL-23 production to generate both Th1 and Th17 immunity, which are important for the clearance of invading fungal pathogens (51). LCs express low amounts of dectin-1, but can recognize fungal β-glucans as well as mannan via Langerin (**chapter 2**). Currently, it remains to be determined what the immunological outcome of fungal interaction with LCs via Langerin is. Based on the similarities between Langerin and DC-SIGN and the observation that Langerin ligand mannan does not induce LC maturation or cytokine production, it is likely that Langerin needs TLR triggering to modulate immune responses. Monocyte-derived LCs strongly respond to ligands for TLR-2, which is involved in fungal recognition and this induces IL-10 secretion (52). Thus, hypothetically, synergistic triggering of TLRs and Langerin on LCs could result in a tailored immune response against fungi, similar to the response observed by DC-SIGN and dectin-1. However, how Langerin discriminates between pathogenic and opportunistic fungi, remains to be determined. Possibly Langerin signaling is altered based on different carbohydrate structures present on different fungi or on different fungal stages such as hyphae versus yeast form. Carbohydrate-specific signaling is also a possibility as shown for DC-SIGN (28).

LCs and DCs represent two distinct DC subsets. LCs are more quiescent under steady state conditions while DCs exhibit a more activated and mature phenotype (53). This appears to be an intrinsic difference although the micro-environment and location can further influence this. As sentinels lining the largest and most exposed interface with the environment, which is colonized with many commensal bacteria and fungi, there is more emphasis on the immune regulatory role and innate defense against viruses for LCs. Noteworthy, other immune cells and receptors also contribute to the induction of a proper immune response and therefore results should always be placed in the correct context.

Part 2: Langerin$^+$ LCs and DC-SIGN$^+$ DCs in HIV-1 acquisition

HIV-1 transmission can occur through the gastro-intestinal mucosa, via mother-to-child transmission, blood or plasma transfusion, shared-needle use and sharp-incidences, but the majority of HIV-1 transmission occurs via heterosexual transmission. At these anatomical sites LCs and DCs are present and in this section their role in HIV-1 infection and transmission is discussed.

2.1 Langerin$^+$ LCs versus DC-SIGN$^+$ DCs

The role of DCs in transmission has been extensively studied using monocyte-derived DCs. DCs facilitate HIV-1 transmission through several mechanisms *in vitro*, which are both infection-mediated and independent of DC infection (54). Productive infection of DCs leads to *de novo* production of virions, which subsequently infect target cells. In addition, independent of DC infection, cell surface attachment of HIV-1 to DCs results in the formation of an infectious synapse between DCs and T cells through which HIV-1 is transferred to T cells. In both mechanisms, capture of HIV-1 can be enhanced by attachment of HIV-1 via cell surface receptors DC-SIGN and Syndecan-3 (23;55). Moreover, HIV-1 transmission might also take place through an exocytic pathway that involves HIV-1 associated exosomes: endocytosed HIV-1 is incorporated into multivesicular bodies, which are rich in tetraspanins and subsequently HIV-1 is transferred to T cells by exosomes (56). Furthermore, HIV-1 transmission can occur when HIV-1 is captured by DCs and internalized via the endolysosomal pathway and subsequently directed towards the DCs-T cell synapse (54). It is likely that these mechanisms exist next to each other and involve several receptors, such as DC-SIGN and Syndecan-3 (23;55). Whether DC-SIGN$^+$ DCs play a role in *in vivo* HIV-1 transmission remains to be elucidated. Rectal mucosa is rich in DC-SIGN$^+$ DCs (57) where direct HIV-1 interaction with DC-SIGN$^+$ DCs takes place and it is suggested that in the absence of abrasions in the female genital tract, HIV-1 can reach DC-SIGN$^+$ DCs by transcytosis through or between epithelial cells (58). For a long time, HIV-1 transmission by LCs and DCs was thought to be mediated by similar mechanisms. However, the function of Langerin is distinct from DC-SIGN and this suggests different outcomes after HIV-1 encountering.

In vivo and *ex vivo* data support a role for LC infection in HIV-1 transmission (59-61). Both the infection of LCs and the ability of LCs to transmit HIV-1 to T cells have been demonstrated to occur *ex vivo* and in primate models (59-63). However, high viral loads are needed to induce LC infection. In addition, sub-epithelial DCs are more efficient at transmitting HIV-1 to T cells compared to their epidermal counterparts (59). Therefore, there appears to be a barrier that needs to be overcome to infect LCs (1;64;65).

As discussed previously, Langerin on LCs captures HIV-1 for targeting of the virus to Birbeck granules for degradation, thereby protecting LCs against HIV-1 infection (1;2). Since LCs line the squamous stratified epithelium, they form a first line of defense against HIV-1 infection. The protective function of LCs may account for the relatively low infectiousness of HIV-1 and the need for high inoculum in order to achieve infection of LCs. However, Langerin function is abrogated by the use of high

viral loads, by saturating Langerin using antibodies or by inhibiting Langerin function by carbohydrates. This suggests that LCs have a dual role in HIV-1 transmission, depending on the activation state and the function of Langerin (65;66). This is highlighted by the effect of micro-organisms causing sexually transmitted infections (STIs) in HIV-1 acquisition, which is discussed in the next section.

2.2 Sexually transmitted infections

Epidemiological studies have demonstrated that the presence of STIs enhances the risk of acquiring HIV-1 (67-70). Since LCs are the first antigen presenting cells to encounter invading pathogens, they could play a role in HIV-1 acquisition. Several micro-organisms interact with Langerin, which might result in competition with HIV-1 for Langerin binding and subsequently enhances HIV-1 infection of LCs via CD4/CCR5. HSV-2 is the most common viral STI and is a ligand for Langerin (**chapter 4**). These data suggest that in the presence of HSV-2, HIV-1 has to compete with HSV-2 for Langerin binding. Saturation of Langerin by HSV-2 could subsequently lead to infection of LCs by HIV-1 via CD4/CCR5. Similarly, *C. albicans*, which causes vaginal candidiasis, also interacts with Langerin. Therefore, these data suggest that *C. albicans* increases the risk of HIV-1 infection through a similar mechanism. HSV-2 has an opposite effect on DC-SIGN-mediated transmission. HSV-2 binding to DC-SIGN and DC-SIGN down-regulation upon DC maturation by HSV-2 decreases HIV-1 transmission (de Jong, unpublished observation). This suggests that Langerin and DC-SIGN play opposing roles in the susceptibility to HIV-1 and that competition of micro-organisms with HIV-1 for Langerin might facilitate HIV-1 infection.

Micro-organisms not only interact with LCs via Langerin, but also directly activate LCs through TLRs. LCs residing the mucosa have an immature phenotype, which include high expression of Langerin. Interactions with pathogens induce maturation and subsequently down-regulation of Langerin from the cell surface. It has been demonstrated that HSV-2 and TLR-3 ligand Poly(I:C) induce maturation of LCs including a down-regulation of Langerin (**chapter 4**). Notably, both TLR-3-stimulated LCs and HSV-2 infected LCs capture less HIV-1 gp120 via Langerin, suggesting that these cells are more susceptible to HIV-1 infection and transmission. HSV-2 not only decreases Langerin function but also competes with HIV-1 for Langerin binding. In addition, both HSV-2- and TLR-3-mediated LC maturation might also induce a variety of phenotypical and transcriptional changes and subsequently affect HIV-1 infection, replication and transmission. TLR-2 ligation via either heat-killed *L. monocytogenes* or peptidoglycan (PGN) increases surface expression of CD4 in monocyte-derived LCs. In addition, post-entry restriction factor APOBEG3G is reduced after TLR-2 stimulation with heat-killed *L. monocytogenes* or Pam3CSK4, suggesting that Gram-positive bacteria enhance productive HIV-1 infection in LCs by decreasing APOBEC3G (71). Thus, Langerin function and the protective barrier of LCs can be breached by different mechanisms, including competition for Langerin and the induction of LC activation by micro-organisms.

TLR ligands also affect transmission of HIV-1 independent of infection. In **chapter 3** we have demonstrated that activation of primary human LCs through TLR-1/2

ligand Pam3CSK4 enhances HIV-1 transmission to target cells via increased HIV-1 capture. Pam3CSK4-mediated LC activation completely circumvents the protective effect of Langerin by increasing HIV-1 capture and subsequent transmission. The observed transmission is mainly mediated by cell-surface bound viral particles, since transmission is abrogated in the presence of neutralizing anti-HIV-1 antibodies (65). However, specific intracellular compartments in close contact with the cell-surface might also be involved, since mature DCs transmit HIV-1 via specialized intracellular compartments that are fully accessible for HIV-1 envelope specific inhibitors (72). Together, these data indicate that LC activation by Pam3CSK4, induces or activates receptors that have a high affinity for HIV-1 compared to Langerin, since even at low viral concentrations HIV-1 transmission is enhanced in the presence of Pam3CSK4. However, the molecular mechanism involved remains to be determined. Activation of CD34-derived LC-like cells with LPS and TNF-α increases HIV-1 transmission independent of infection by internalization of HIV-1 in CD1a-containing multivesicular bodies (66). These data suggest that in LCs HIV-1 is protected from degradation in intracellular compartments and efficiently transmitted upon interaction with target cells through trans-infection, similar to mechanisms observed by DC-SIGN$^+$ DCs.

STIs induce the production of pro-inflammatory cytokines, which can affect the susceptibility of LCs to HIV-1 transmission (65). As demonstrated in **chapter 3**, both *C. albicans* and *Neisseria gonorrhoea* induce production of TNF-α in skin and vaginal tissue (65). TNF-α induces HIV-1 replication of LCs *ex vivo* thereby increasing HIV-1 transmission to CD4$^+$ T cells (65). TNF-α has been shown to enhance HIV-1 replication through activation of the transcription factor NF-κB that increases HIV-1 transcription (73;74). In agreement with these data, TNF-α enhances HIV-1 replication in LCs, which increases HIV-1 transmission to T cells due to enhanced *de novo* HIV-1 production by LCs (65). However, it is important to note that although TNF-α enhances replication, it does not increase HIV-1 entry into cells. Therefore, the observed TNFα-mediated increased transmission is dependent on Langerin function and is only detected when Langerin is inhibited or saturated by high viral concentrations (65). TNF-α is induced in genital mucosal fluids from women with bacterial vaginosis (75). These data strongly suggest that STIs induce TNFα, which enhances HIV-1 replication in LCs, and HIV-1 transmission to T cells (65). It has been demonstrated that TNF-α exerts a similar effect on DC-SIGN$^+$ DCs (76). Cytokines induced by STIs can also regulate the expression and function of HIV-1 co-receptor CXCR4. On DCs, CXCR4 expression is increased by IL-4 and TGFβ, and inhibited by IFN-α,-β and -γ, while CCR5 expression on DCs is not susceptible to cytokines (77). In addition to invasive STIs, sexual intercourse and (non-ulcerating) STIs can induce (micro) abrasions in the epithelium. This can induce inflammatory cytokine production, such as TNF-α, in the epithelium as a reaction to trauma, thereby forming a risk factor for HIV-1 acquisition.

Overall, HIV-1 infection and transmission by DC-SIGN$^+$ DCs and Langerin$^+$ LCs can be modulated at different levels. A fundamental difference exists between Langerin and DC-SIGN in HIV-1 degradation and transmission under non-inflammatory conditions. In contrast to DC-SIGN, Langerin has not been shown

to enhance HIV-1 infection. The protective barrier formed by LCs is breached by several mechanisms, which are both Langerin-dependent and -independent (Table 2). Most of the mechanisms have been explored *ex vivo*, often with epidermal LCs or LC-like cells and monocyte-derived DCs, and further studies are necessary to investigate the involvement of these mechanisms *in vivo*.

Table 2. A role for LCs in HIV-1 acquisition

Mechanism	Result	Example
Functional Langerin	HIV-1 degradation via Langerin on LCs → no HIV-1 infection	Non-inflammatory conditions
Langerin saturation Langerin inhibition	Infection of LCs → *de novo* HIV-1 production	High viral load
Langerin competition	Infection of LCs → *de novo* HIV-1 production	Fungi or HSV-2-mediated competition for Langerin
Decrease in Langerin expression, phenotypical/ transcriptional changes as a result of LC maturation	Infection of LCs → de novo HIV-1 production	Poly(I:C), HSV-2
Up-regulation of HIV-1 entry receptors, decrease in restriction factors	Enhanced HIV-1 entry, enhanced *de novo* HIV-1 production and release	TLR-2 ligation: CD4 and APOBEC3G
Enhanced HIV-1 binding to LCs (Langerin independent)	Trans-infection of target cells	TLR-1/-2 agonist Pam3CSK4
Inflammation-induced TNF-α production	Enhanced HIV-1 replication in LCs	*C. albicans*, *N. gonorrhoea*

2.3 Other players in the game?

There is a gap in our knowledge about the initial events during HIV-1 acquisition, including the mechanisms of how HIV-1 enters the body and what cells are the primary target cells in the mucosa (78). LCs lining the mucosal tissue account for 1-3% of the total cell population and therefore other cells can also influence HIV-1 acquisition. Mucosal LCs are surrounded by mainly epithelial cells and these genital epithelial cells express proteins that mediate attachment of HIV-1 (79). However, whether epithelial cells themselves are productively infected with HIV-1 remains controversial (80;81). Likewise, the region at the interface of the endocervix and ectocervix is rich in $CD4^+$ T cells. Therefore, these cells can provide a route of entry for HIV-1 (58). Furthermore, cell-conjugates of LCs and T cells provide a mechanism of HIV-1 transfer (59;82).

The lamina propria of ectocervix and the dermis contain not only DCs but also macrophages (83;84). These vaginal macrophages constitute approximately 10% of the total number of leukocytes in these tissues and support HIV-1 replication (85). In addition, other immune cells such as NK cells and pDCs play a role in the innate defense against HIV-1 (58). Together the cells in tissues approached by HIV-1 create a specific micro-environment by not only interacting directly with HIV-1 and other types of cells but also by producing an array of soluble factors such cytokines, defensins, interferons, and mucins, which can further affect HIV-1 acquisition by

different mechanisms. None of the available research models completely mimic the human HIV-1 exposed tissues, and it is therefore a future challenge to unravel the events occurring in this specific micro-environment.

2.4 Host-defense mechanisms

Several biological factors influence the susceptibility to acquire and transmit HIV-1. These include viral load, viral strain variants and different soluble factors (86;87). This section discusses the effect of different soluble factors in human milk, semen and body fluids, the effect of the composition of the female and male genital tract and the results of genetic alterations in HIV-1 acquisition and transmission.

Semen contains a variety of products that affect HIV-1 transmission. Spermatozoa themselves interact with heparan sulfates on DCs, thereby facilitating HIV-1 transmission (88). In addition, seminal fluid affects the genital tissue by neutralizing the acidic environment, which could prolong survival of HIV-1 in the female genital tract. Several soluble factors in semen have an effect on HIV-1 susceptibility; anti-leukoprotease Secretory Leukocyte Protease Inhibitor (SLPI) present in saliva and semen is able to protect $CD4^+$ T cells against HIV-1 infection (89), while Semen-derived Enhancers of Virus Infection (SEVI) captures HIV-1 virions and enhances attachment of HIV-1 to T cells and macrophages (87) and Mucin-6, from seminal plasma, enhances DC-mediated HIV-1 transmission by interacting with DC-SIGN (90). It remains to be determined what the effects of semen are on LC-mediated HIV-1 transmission.

In addition to semen, human milk components possess a high level of anti-HIV-1 activity; lactoferrin and bile salt-stimulated lipase inhibit HIV-1 infection (91;92). In **chapter 6** MUC-1, from human milk, has been demonstrated to interact with DC-SIGN thereby preventing DC-mediated HIV-1 transmission. Postnatal mother-to-child transmission is mediated via human milk, and in the gastro-intestinal tract $DC\text{-}SIGN^+$ DCs are present, while Langerhans cells are absent. Therefore, MUC-1 provides a protective factor in human milk against HIV-1 transmission. It is likely that different components of semen or breast milk have opposing effects on HIV-1 transmission, and therefore the net effect on HIV-1 infection is dependent on many variables. Increased understanding of the factors involved may provide insight in HIV-1 acquisition.

The composition of female genital tissue also affects the susceptibility to HIV-1 infection. In general, HIV-1 infection occurs through the endocervix as well as the multi-layered squamous epithelium of the vagina and ectocervix. Although the endocervix consists of a single-layer columnar epithelium, it is covered with mucus, which provides a protective barrier. LCs are expressed throughout the female genital areas, but the density of the LCs in the female tissue, such as the cervix, is low compared to male tissues, such as foreskin or glans penis (93). This may account for the higher male-female transmission than female-male transmission. In general, the female vagina is of low pH and commensal bacteria, such as *Lactobacillus*, have a microbicidal effect that prevent HIV-1 acquisition (94;95). When this milieu is altered, for example due to the colonization by pathogenic micro-organisms, an

environment can arise that promotes HIV-1 infection. In addition, the female genital tissue is under continuous influence of hormones. During the menstrual cycle there is a constant increase and decrease of the female epithelium; estrogen thickens while progesterone thins the epithelium (96). Although the density of the cells is not altered during menstrual cycle (95), the net number of LCs acting as a protective barrier will be lower when the epithelium is thinner and the epithelium will be more prone to damage. Furthermore, the expression of HIV-1 receptors varies during the menstrual cycle (97). In addition, during ovulation hydration and alkalization of the mucus occurs, which possibly decreases its barrier function (58).

Male circumcision has been demonstrated to decrease HIV-1 infection (98). The outer foreskin and the glans penis are protected by a keratinized epithelium, which act as a strong mechanical barrier, whereas the inner foreskin is poorly keratinized, which increases vulnerability to HIV-1 infection (58). In addition, in non-circumcised men the area between the foreskin and glans may provide a receptive environment for STIs. These STIs might affect the protective barrier against HIV-1 transmission by LCs localized in the foreskin, thereby greatly increasing HIV-1 susceptibility (65). Epidemiological studies have demonstrated that circumcision prevents the acquisition of STIs, such as HSV-2 and HPV (99).

Host genetic predispositions can further affect the susceptibility to HIV-1 infection and include variation in the functionality and expression of CCR5, Langerin and DC-SIGN. Individuals with a mutation in HIV-1 co-receptor CCR5, a homozygous 32 nt deletion, are protected from primary HIV-1 infection (100). These data support the observation that HIV-1 transmission occurs primarily by R5-tropic strains. Changes in the function or binding affinity of Langerin due to genetic alterations could also affect the susceptibility to HIV-1 infection. Several polymorphisms in the Langerin gene might affect the binding activity or stability (101). Clinical correlations between mutations in the carbohydrate recognition domain of Langerin and HIV-1 susceptibility have recently been identified and preliminary data suggest that protection of LCs against HIV-1 is affected by mutations in the carbohydrate recognition domain (M. van der Vlist, unpublished publications), which decrease HIV-1 binding. In contrast, the carbohydrate recognition domain of DC-SIGN contains no polymorphisms that affect amino acid sequence, suggesting that DC-SIGN has been maintained under a strong selective pressure that prevents accumulation of any amino acid changes (102). On the other hand, a low number of repeat units in the neck region of DC-SIGN is associated with a decreased risk of HIV-1 acquisition (103). This is likely to be due to the reduced stability or reduced expression of DC-SIGN (104). Finally, variation in responsiveness of human epidermal tissue to TLR ligands in combination with HIV-1 susceptibility exists (65). Hence, this suggests that genetic variation in CCR5, Langerin, DC-SIGN, and TLR expression play a role in the susceptibility to HIV-1 infection.

2.5 Microbicides in HIV-1 prevention: future prospectives

To date, no curative treatment or protective vaccine against HIV-1 is available, while the HIV-1 pandemic is still expanding world-wide. Therefore, alternative protective measures, such as the development of topically applied HIV-1 microbicides in the form of genital creams, are needed to put a hold to HIV-1 acquisition (105). These protective measures include microbicidal creams, and compounds targeting HIV-1 attachment, entry, reverse transcriptase and integration. LeX (**chapter 5**) and mermaid (**chapter 7**) exemplify the use of compounds to interfere with DC-SIGN-mediated HIV-1 transmission.

The studies described in this thesis investigate the role of LCs and DCs in the acquisition and transmission of HIV-1 through mucosal genital tissue. Acting as a delicate barrier against HIV-1 by capturing HIV-1 through Langerin, it is essential to maintain the LC barrier function, while it is favourable to inhibit DC-SIGN binding to HIV-1 to prevent HIV-1 infection and transmission. Microbicides or mucosal vaccines should prevent direct and indirect activation of LCs. LC activation can lead to HIV-1 acquisition by lowering the viral threshold needed to induce direct LC infection, by increasing HIV-1 replication and allowing trans-infection of target cells. Along the same line, the fact that LCs do not respond well to certain bacterial products could be an advantage. In addition, vaginal creams could possibly cause epithelial disruptions and inflammation at cellular levels (106), which in turn could actually enhance HIV-1 infection. Therefore, the safest mucosal microbicide would be one that is non-irritating, does not interfere with host-defense mechanisms and at the same time does not induce viral resistance. Hence, in the rational design of new topical microbicides it is important to carefully determine the effects at all levels. Although DC-SIGN targeting appears to be a favorable target to decrease HIV-1 transmission, DC-SIGN is most likely also involved in innate immune processes that are beneficial for humans. Therefore, blocking DC-SIGN could also have adverse effects. Appropriate models to investigate the interaction of microbicides with LCs or DCs are a prerequisite.

In **chapter 5**, it is demonstrated that LCs derived from a Mutz-3 cell-line (Mu-LCs) are a valuable tool to screen microbicides in their interaction with Langerin. Mu-LCs and DCs resemble their *in vivo* counterparts and Mu-DCs are functional in inducing anti-tumor T cell immunity (107;108). In addition, Mu-DCs are a valuable tool as an unlimited source of DCs in cytotoxic T lymphocyte priming and lymph-node homing capabilities (108;109). Although Mu-LCs are useful to screen potential microbicides in Langerin-mediated HIV-1 transmission, a drawback is that this cell-line exhibits a semi-mature phenotype and upon TLR stimulation produces a limited amount of cytokines (110). Other LC-like cells have been generated from $CD34^+$ cord blood (111) and blood monocytes (112). Like Mu-LCs these cells have a LC-like phenotype and express Langerin. However, it is important to keep in mind their limitations. These LC-like cells can express receptors that are not normally found on primary LCs, such as the C-type lectins DC-SIGN. Moreover, some of these LC-like cells have a different TLR expression profile compared to primary LCs, which might confound certain results obtained with these cells. Therefore, it is necessary to verify

obtained results with primary LCs. Since genital tissue is not readily available for research purposes, skin obtained from breast or abdominal reconstructive surgery are the most closely related models used as source of primary research material. Noteworthy, human skin tissue is composed of a keratinized layer, which is absent in the female genital tissues. Nevertheless, research using primary human material is scarce, but very valuable since LCs are maintained in their physiological environment.

Monocyte-derived DCs have provided a great model to study the function of DC-SIGN$^+$ CD1a$^+$ DCs in virology and immunology. However, questions remain whether these cells resemble merely one specific DCs subset *in vivo*. Identification of immune cells in the dermis and sup-epithelium has been hampered by the lack of distinct markers and it is thought that monocyte-derived DCs exhibited a phenotype that combines several dermal and sup-epithelial subsets *in vivo*.

In the identification of potential microbicides, microbicide transmission and sterilization assays may be very valuable (113). Furthermore, attempts are made to design skin equivalents in the laboratory by adding different cell(line)s into a matrix, which is a promising tool to investigate the interaction between pathogens and the different cell types (114). However, it will be a challenge to incorporate all primary cell subsets into these systems.

Although the development of mouse models for HIV-1 infection has made great improvements over the past few years (115-117), Rhesus macaque monkeys are still the most often used *in vivo* model to study HIV-1 transmission. Primate studies are laborious, relatively expensive and minimalized for ethical reasons. Therefore, hormone treatments and high viral loads are often used to facilitate HIV-1 infection. These conditions do not necessarily resemble HIV-1 transmission in humans which is rather inefficient, therefore making it difficult to translate research between models. In addition, interspecies differences should not be underestimated. Overall, each method has its limitations, which should be carefully weighed against the advantages.

The development of innovative strategies against HIV-1 infection is essential in the fight against HIV-1. Promising compounds are currently tested in phase III trials (118). However, it is still a long way for the wide-spread distribution of functional microbicides, especially to those who have limited access to healthcare. Therefore, prevention and early treatment of STIs, education, advocacy for safe sex and condom-use, and male circumcision are currently the main protective measures against HIV-1 and other STIs. Knowledge attained from all levels of research can contribute the understanding of HIV-1 infection, transmission and pathology and ideally will lead to the development of a preventive HIV-1 vaccine.

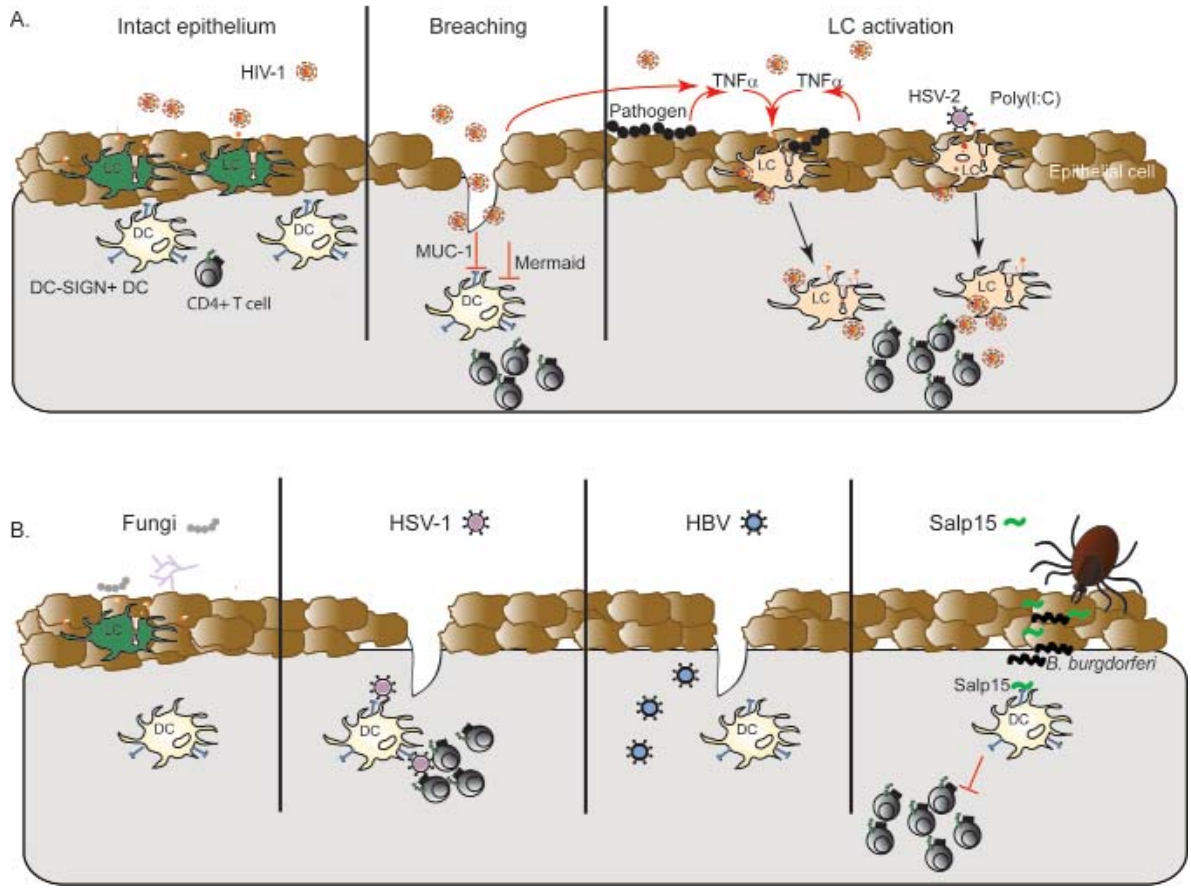
Figure 2. Thesis outline: LC and DC interaction with pathogens. (A) Interaction with HIV-1. In intact epithelium, LCs form a protective barrier against HIV-1 infection. Several conditions might affect the protective function of LCs and induce HIV-1 transmission. Pro-inflammatory cytokines or pathogenic components induced by STIs and inflammation activate LCs, which upon HIV-1 exposure, lead to enhanced HIV-1 replication in LCs (TNF-α) or increased capture and transmission of HIV-1 (Pam3CSK4) (chapter 3). Viral STI HSV-2 competes with HIV-1 for Langerin binding and induces down-regulation of Langerin expression. Viral TLR-3 agonist Poly(I:C) induces strong maturation of LCs, resulting in Langerin down-regulation in addition to phenotypical/transcriptional changes. Both HSV-2 and Poly(I:C) enhance HIV-1 infection of LCs and subsequent *de novo* HIV-1 production (chapter 4).

Concluding remarks

LCs were discovered over 200 years ago by Paul Langerhans, while only 10 years ago Langerin and DC-SIGN have been identified. Whereas DC-SIGN research has taken a big leap since the development of monocyte-derived DCs and the function has been widely explored, Langerin research has lagged behind and thus far Langerin has mainly been used as a marker to identify Langerhans cells.

The studies in this thesis describe the role of Langerin$^+$ LCs and DC-SIGN$^+$ DCs in the innate defense against pathogens, with a focus on HIV-1. This thesis has elaborated on the effect of viral, bacterial and fungal sexually transmitted infections on LC function and subsequent susceptibility to HIV-1 infection and has identified potential compounds to interfere with DC-SIGN-mediated HIV-1 transmission.

Together this thesis provides knowledge and insight in the pathogen recognition of Langerin and DC-SIGN and can contribute to new therapeutic strategies in microbial infections and the development of effective microbicides and mucosal vaccines.



Breaches in the epithelium, induced by inflammation, STIs or sexual intercourse, allow HIV-1 to interact directly with DC-SIGN$^+$ DCs in the sub-epithelium, resulting in efficient HIV-1 transmission. MUC-1 and mermaid (chapter 6 and 7) prevent binding of HIV-1 to DC-SIGN, thereby preventing HIV-1 transmission. Inflammation induces an influx of T cells that are susceptible to direct infected by HIV-1. LCs derived from Mutz-3 cell line provide a valuable tool to screen potential microbicides (not depicted, chapter 5). (B) Langerin is a receptor for fungal species *Candida* species, *Malassezia furfur* and *Saccharomyces cerevisiae* (chapter 1). DCs mediate HSV infection and transmission through DC-SIGN (chapter 8). HBV does not interact with DCs (chapter 9), and Salp15 binding to DC-SIGN prevents T cell activation needed against *B. burgdorferi* infection (chapter 10). This figure is modified from *de Jong,* Journal of Internal Medicine, 2009.

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