Chapter 7.2

Hepatitis C virus NS5A anchor peptide inhibits both HIV-1 and herpes simplex virus infection: The perfect microbicide?

Lot de Witte1, Michael D. Bobardt2, Guofeng Cheng3, Francis V. Chisari3, Teunis B.H. Geijtenbeek1, and Philippe A. Gallay2.

In the absence of an effective vaccine, there is an urgent need for safe and effective antiviral agents to prevent transmission of HIV-1. It was recently demonstrated that an amphipathic alpha-helical peptide (C5A) derived from the HCV NS5A membrane anchor domain has antiviral activity against HCV and HIV-1. In this study, we have investigated the potential of C5A to serve as a novel microbicide candidate that inhibits HIV-1 transmission. We have tested the microbicidal potential of C5A by examining the spectrum of HIV-1 isolates against which C5A is active, the mechanism of antiviral action of C5A against HIV-1, the modes of HIV-1 transmission that it can inhibit both in vitro and ex vivo, and the antiviral activity against HSV. We demonstrate that C5A efficiently inhibits both HIV-1 and HSV infection in vitro. Furthermore, C5A inhibits different mechanisms that are thought to mediate HIV-1 transmission. In conclusion, C5A is a promising candidate drug to treat and prevent HIV-1 infection as well as HSV infection and subsequent enhancement of HIV-1 transmission.

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**Introduction**

As discussed before in this thesis (Section 2, 6 and chapter 7.1), there is an urgent need for the development of a safe, effective topical agent (microbicide) to prevent the sexual transmission of human immunodeficiency virus type-1 (HIV-1) and stop the growing pandemic (Klasse et al., 2006; Moore et al., 2005; Dhawan et al., 2006). To date, detergents, pH modifiers, and polyanionic gels have been tested as microbicides (Klasse et al., 2008). Three efficacy trials have now failed (Klasse et al., 2008), and the results of others are pending. Thus, the identification of additional anti-HIV-1 transmission agents with varying mechanisms of action, and which may serve as safe and effective microbicides, is imperative.

There are some critical considerations in the development of topical microbicides against HIV-1 (Turpin et al., 2002): i) topical microbicides should exhibit potent activity against clade representatives and multi-drug-resistant viruses; ii) should be active at a low pH given the acidic environment of the genital epithelium; and iii) should be active for an acceptable length of time both prior to and after exposure.

In this thesis we have shown that different mechanisms might attribute to HIV-1 transmission, such as DC-mediated transmission, (activated) LC infection and subsequent transmission (Section 2, 5, 6). Moreover, T cell and macrophages in the lamina propria have been suggested as first target cells for HIV-1. The role of these mechanisms in vivo is unclear to date. Given the uncertain in vivo mode of HIV-1 transmission at present, microbicides should interfere with a wide range of transmission modes; and should mediate their inhibitory activity in the major in vivo HIV-1 targets including CD4+ T-lymphocytes, macrophages, DCs and LCs.

A broader antiviral specificity would be preferable since epidemiological studies have demonstrated that genital herpes simplex virus (HSV) infection is an important risk factor to both transmit and acquire HIV-1 (Freeman et al., 2006; McFarland et al., 1999; Royce et al. 1992).

We and others have shown that different protective factors are involved in HIV-1 transmission such as the integrity of the epithelial layer and Langerin-function (de Witte et al., 2007) and therefore a potential microbicide should exhibit no genital epithelial toxicity, keep the epithelial integrity and not interfere with Langerin function. Cheng et al. have recently shown that an amphipathic alpha-helical peptide (designated C5A) derived from the HCV NS5A membrane anchor domain has antiviral activity against HCV, other members of the Flaviviridae, and HIV-1 (Cheng et al., submitted). In this study, we have investigated the potential of C5A to serve as a novel microbicide candidate based on the considerations above. We have tested the microbicidal potential of C5A by examining the spectrum of HIV-1 isolates against which C5A is active, the mechanism of antiviral action of C5A against HIV, the modes of HIV-1 transmission that it can inhibit both in vitro and ex vivo, and the antiviral activity against HSV.

**Results**

*C5A has antiviral activity against a broad range of immune deficiency viruses*

The range of action of a microbicidal candidate must be addressed early in the preclinical development pathway to provide evidence of its therapeutic utility against a broad and diverse sample of viruses. Thus we tested the antiviral activity of C5A against three distinct categories of viruses. The first category includes isolates representative of various HIV-1 subtypes with different co-receptor usage, either CCR5 (R5 viruses) or CXCR4 (X4 viruses). The second category includes drug-resistant HIV-1 isolates with developed resistance to RT, protease (PR) or fusion (T20) inhibitors. The third category of viruses that were tested were other retroviruses such as HIV-2 and SIV isolates. The antiviral activity of C5A was tested using CD4+ HeLa cells (called TZM cells), which produce beta-galactosidase upon HIV-1 infection (Wei et al., 2002). Viruses produced from human peripheral blood monocytic cells (PBMC) were added to TZM cells together with increasing concentrations of C5A (SWLRDIWDWICEVLSDFK)
for 4 hours, washed, and infection was measured 48 hours post-infection by beta-galactosidase activity. As negative control, we used a non-amphipathic peptide variant (SWRLDIWDICEVLSDFK), which was shown in the companion paper to have no antiviral activity against HCV (Cheng et al., submitted).

As shown in Table 1, C5A efficiently prevents infection of all HIV-1 and SIV isolates tested at submicromolar and low micromolar concentrations, including HIV-1 isolates derived from different clades, with various co-receptor usages, drug-resistant viruses, and even other lentiviruses (HIV-2 and SIV) (Table 1). This broad spectrum of antiviral activity of C5A among HIV-1 isolates is a precondition for an attractive microbicide candidate.

Table 1. C5A inhibits a broad spectrum of immunodeficiency viruses.
TZM cells (1x10^5) were exposed to different viruses (1 ng p24) in the presence of increasing amounts of wild-type SWLRDIWDICEVLSDFK C5A or its non-amphipathic variant (SWRLDIWDICEVLSDFK), which lacks antiviral activity. Viruses and peptides were washed away after 4 hours. Infection was determined 48 hours post-infection by measuring β-galactosidase activity. Results are representative of three independent experiments and are expressed in concentration (µM) of peptide required to inhibit 50% (IC50) or 90% (IC90) of virus infectivity.

<table>
<thead>
<tr>
<th>Name of HIV-1</th>
<th>C5A</th>
<th>Non-amphipathic C5A</th>
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<tr>
<td>Isolate</td>
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C5A prevents HIV-1 infection of the three in vivo targets: CD4+ T-lymphocytes, macrophages and DCs. An ideal microbicide candidate should prevent HIV-1 from infecting its cellular targets. Thus we tested the capacity of C5A to block HIV-1 infection in primary human CD4+ T-lymphocytes, macrophages and DCs. Purified CD4+ T-lymphocytes, macrophages or DC were exposed to HIV-1 (R5 NL4.3-BaL) (Chatterji et al., 2005) together with C5A or its non-amphipathic variant (5 µM). Twenty-four hours post-infection, cells were washed and viral replication monitored by measuring the amount of HIV-1 capsid present in the supernatant of the cell culture every three days. As shown in Fig 1a, C5A completely blocked HIV-1 infection of the three major in vivo targets of HIV: CD4+ T-lymphocytes, macrophages and DC. DCs capture HIV-1 and facilitate infection of the T-lymphocytes, resulting in a robust infection (Pope et al., 1994; cameron et al., 1992). C5A inhibits HIV-1 infection of DC-T-lymphocyte cultures (Fig. 1a), indicating that the peptide inhibits DC-mediated transmission. We obtained similar results using the primary HIV-1 R5 isolate JR-CSF (data not shown).

Figure 1. C5A prevents HIV-1 infection of the three in vivo targets: CD4+ T-lymphocytes, macrophages and DCs. (a) CD4+ T-lymphocytes, macrophages, DCs (1x10^5 cells) or DCs and CD4+ T-lymphocytes were exposed to NL4.3 BaL (1 ng of p24) for 1 day, washed three times with medium, and cultured in a flat bottom 96-well plate. Wild-type C5A or its non-amphipathic variant (5 µM) were added together with virus to the cells or after three days (lower right panel). Supernatants were collected after different days and viral replication was monitored by p24 ELISA. Error bars represent standard errors of duplicates. These experiments are representative of three independent experiments using three different donors. (b) 293T-lymphocytes transfected with NL4.3 or NL4.3 BaL proviral DNA for 24 hours were treated with or without peptide (5 µM) for 1 hour at 37°C and washed to remove both previously released viruses and peptides. Twenty-four hours post-peptide treatment, infectivity of 293T-released de novo viruses was scored on TZM cells. Infection was measured 48 hours post-infection by beta-galactosidase activity. Error bars represent standard errors of duplicates. These experiments are representative of two independent experiments.
7.2: C5A inhibits both HIV-1 and HSV infection: The perfect microbicide?

Subsequently, we investigated whether C5A blocks both cell-free and cell-to-cell transmission during an established HIV-1 infection. Purified primary CD4+ T-lymphocytes were first exposed to HIV-1 (R5 NL4.3-BaL) in the absence of peptide for three days to establish infection. Three days post-infection, C5A or its non-amphipathic variant (5 µM) were added to infected cells. In this absence of treatment or in the presence of the non-amphipathic variant, HIV-1 replicates rapidly in T-lymphocytes with a peak of growth at day 12 (Fig. 1a). In sharp contrast, the addition of C5A at day 3 totally stopped HIV-1 replication at day 6 (Fig. 1a). These data demonstrate that C5A has the potential to interrupt an established HIV-1 infection in primary CD4+ T-lymphocytes. This finding is very important because it suggests that C5A not only prevents cell-free infection, but it also prevents cell-to-cell HIV-1 transmission. In addition, these data suggest that C5A can neutralize budding and released viruses. If C5A can also neutralize intracellular viruses that bud and/or assemble, one would anticipate that treating virus producer cells with C5A would result in the production and release of noninfectious particles. Next, we tested whether C5A inhibits production and release of non-infectious particles, by analyzing the production and infectivity of viruses released from cells transfected with HIV-1 and treated with or without C5A. Specifically, 293T-lymphocytes transfected with HIV-1 (X4 NL4.3 virus or R5 NL4.3-BaL virus) for 24 hours were treated with C5A or its non-amphipathic variant (5 µM). One hour post-treatment, cells were extensively washed to remove both previously produced and released viruses and peptides. Twenty-four hours post-peptide treatment, amounts and infectivity of de novo viruses released into the supernatant were analyzed as described above. Although C5A does not affect virus production (data not shown), it totally inactivated the infectivity of released viruses (Fig. 1b). This further suggests that C5A can neutralize cell-free particles as well as assembling and/or budding particles. Together these data demonstrate that C5A inhibits HIV-1 infection of target cells at the moment of initiation of infection and during established infection.

C5A prevents transmission of HIV-1 in vitro.

Subsequently, we investigated the potential of C5A to protect against HIV-1 transmission. Since vaginal epithelial lesions enhance HIV-1 transmission (Rustomjee et al., 1999), we investigated whether C5A is toxic for primary human genital epithelial cells (PGECs). PGECs were exposed twice daily to high concentrations (200 µM) of C5A for one week. No washes were performed in order to maintain a continuous exposure of cells to the anti-HIV-1 peptide. Cell viability was evaluated by methyl thiazol tetrazolium (MTT)-based colorimetric assessment. As a positive control for cell toxicity, cells were exposed to the detergent saponin. In contrast to 0.01% saponin, which starts killing cells in less than 4 days, C5A applied twice daily to cells at a concentration 10-100-fold greater than that which blocks HIV-1 infection, is not toxic to human cervical cells (Fig. 2a). We obtained similar results using Jurkat T-cells (data not shown). This result suggest that C5A will not interfere with the genital epithelial barrier.

In this thesis we have shown two important pathways of HIV-1 transmission: infection of LCs and subsequent transmission (Section 5.6) and DC-SIGN+-DC-mediated transmission (Section 2). Since the attribution of these mechanisms to transmission in vivo is unknown, a potential microbicide candidate should interfere with both mechanisms. We used an ex vivo transmission model to mimic LC-mediated transmission of HIV-1 (Fig 2b). Epidermal sheets were infected with HIV-1 NL4.3-BaL-eGFP and directly incubated with C5A (10 µM). After 3 days, epidermal sheets were removed and T-lymphocytes were added for an additional 4 days. Migrated LCs (day 3) and samples of the co-cultures (day 5 and 7) were analyzed for infection by analyzing GFP expression by fluorescent microscopy and flow cytometry. C5A efficiently blocked LC infection by HIV-1 (Fig. 2c). Moreover C5A inhibited HIV-1 transmission to T-lymphocytes (Fig. 2d,e), and this was observed in different donors (Fig. 2f). Thus, C5A effectively prevents the transmission of HIV-1 by LCs ex vivo.
Figure 2 (Previous page). C5A inhibits HIV-1 transmission by LCs and DCs.

Primary genital epithelial cells (PGEC) were treated twice daily with 200 µM of C5A or 0.01% saponin for a week. No washes were performed in order to maintain a continuous exposure of cells to peptides. After overnight incubation, CellQuanti-MTT reagent was added and cell viability was quantified by OD 570 nm reading. (b-f) Epidermal sheets were infected with HIV-1 NL4.3-BaL-eGFP (100 ng p24) and directly incubated with 10 µM C5A or a DMSO control. After 3 days, epidermal sheets were removed and CCR5 + Jurkat cells (2x10^5) were added for an additional 4 days. Migrated DC/LC epidermal cells (day 3) and samples of the co-cultures (day 5 and 7) were analysed for GFP expression by flow cytometry. (c) HIV-1 infection of migrated LCs is depicted as percentage of total cells. Error bars represent standard errors of duplicates. (d) The co-cultures were analyzed for infection at day 7 by immunofluorescence microscopy (left panels GFP, right panels overlay of GFP and brightfield) and (e) flowcytometry. The percentage of GFP + cells is depicted. (f) The donor variability at day 5 is depicted. Error bars represent standard errors of duplicates. (g) DC (5x10^6) were exposed to HIV-1 NL4.3-eGFP (X4), NL4.3-BaL-eGFP (R5) or to NL4.3ΔEnv-eGFP pseudotyped with NL4.3 gp160 env together with 10 µM C5A or a DMSO control for 2 hours at 37°C. Cells were washed 3 times, activated CD4 + T-lymphocytes were added for 3 days and GFP expression was measured by flow cytometry. Error bars represent standard errors of duplicates. These results are representative of 3 independent experiments.

As described in Section 2, transmission of HIV-1 from DC to T-lymphocytes can occur irrespective of infection of the DC (trans-infection) and infected DCs can transfer de novo produced virus to T-lymphocytes (Geijtenbeek et al., 2000; Turville et al., 2004). We examined whether C5A can inhibit these two ways of HIV-1 transfer using a DC-T-lymphocyte co-culture system. Specifically, monocyte-derived DCs were exposed to HIV-1 NL4.3-eGFP (X4 virus), NL4.3-BaL-eGFP (R5 virus) or to the single-cycle NL4.3ΔEnv-eGFP (lacking HIV-1 gp160 envelope (Env) glycoprotein), but pseudotyped with NL4.3 gp160 env. DCs were infected together or not with C5A (10 µM) for 2 hours at 37°C. Cells were washed, T-lymphocytes were added for 3 days and infection was scored by GFP expression. Remarkably, C5A blocked transmission of replication competent HIV-1 but also single-cycle HIV-1 (Fig. 2g), indicating together with the results in figure 1a that C5A inhibits both mechanism of HIV-1 transmission from DCs to T-lymphocytes. This finding is critical because it suggests that C5A can inactivate infectious particles, which are transferred through the virological synapse without being exposed to the extracellular environment.

C5A inhibits herpes simplex virus infection in vitro and ex vivo.

Since genital herpes increases transmission of HIV-1 (Freeman et al., 2006; McFarland et al., 1999; Royce et al. 1992), an ideal microbicide would inhibit HIV-1 as well as HSV infection. We therefore investigated whether infection of HSV-1 and -2 could be inhibited by C5A. Vero cells were infected with HSV-1, HSV-1-GFP and HSV-2 in the presence of C5A. After 2 days the cells were analyzed by microscopy and flow cytometry. In DMSO-control conditions, the monolayer of cells was destroyed and the cells obtained a round morphology, indicative of HSV infection (Figure 3a). In contrast, the monolayers in the cultures treated with C5A were mostly unaffected, indicating that C5A prevents HSV infection (Figure 3a). Moreover, GFP expression was only observed in the untreated HSV-1 GFP-infected cultures but not in those treated with the C5A peptide (Figure 3a).

To measure the efficacy of the C5A treatment, vero cells were infected with different concentrations of HSV and either treated or not treated with C5A. Infection was determined by staining for cell surface expression of HSV gB and GFP. A decreased percentage of infected cells expressed HSV gB and GFP after treatment with the C5A peptide (Figure 3b). More specifically, infection of the cells with either HSV-1 or HSV-2 cells was more than 100 times less efficient in presence of the peptide (Figure 3b). The peptide lost its capacity to completely block HSV infection of vero cells at an MOI of 1 (Figure 3b). Together, these results indicate the C5A efficiently inhibits HSV infection in vitro. We then investigated the capacity to block HSV infection ex vivo. The primary target cells for HSV-1 are keratinocytes. We therefore infected epidermal sheets with different concentrations of HSV-1-GFP. At the moment of infection the sheets were treated with different concentrations of C5A. After 2 days, the sheets were analyzed by microscopy and flow cytometry. The peptide efficiently inhibited HSV-1 infection. At the
highest concentration of C5A, the peptide blocked HSV-1 GFP also at higher MOI (Figure 4a,b), indicating that a micromolar concentration of C5A is required to inhibit infection. At the lower concentrations, the level of inhibition was dependent on the inoculum of virus (Figure 4a,b). Thus, C5A inhibits HSV infection in vitro and ex vivo.

Discussion

In this study, we report that a non-toxic amphipathic alpha-helical peptide, designated C5A, has potent antiviral activity against HIV-1 and HSV. C5A was identified by screening a synthetic peptide library covering the HCV polypeptides for antiviral activity against HCV (Cheng et al., in press). C5A is derived from the membrane anchor domain of HCV NS5A and destabilizes HCV integrity and has membranolytic activity (Cheng et al., in press). C5A is thought to target the viral membrane and therefore displays a unique antiviral activity. Here we demonstrated that C5A exhibits a broad range of antiviral activity against HIV-1 primary isolates. C5A inhibits different mechanisms that are thought to be involved in HIV-1, including HIV-1 cell-to-cell transmission, LC- and DC-mediated transmission and HSV infection. Moreover, Bobardt et al., demonstrated that C5A is stable at low pH, prevents trans-epithelial transmigration of HIV-1 and the peptide is effective from 2 hours prior to infection to 2 hours after infection (Bobardt et al., submitted). C5A is thought to disrupt the integrity of HIV-1 particles by destabilizing the physical linkage between the mature conical capsid core and the viral envelope (Bobardt et al., submitted). It is therefore thought that C5A targets the lipid composition of HIV-1 membranes, and this would indicate that C5A will not select for viral escape variants and, if used in combination with agents that do select for resistance variants, it could prevent their spread. Thus, C5A meets different of the considerations of a novel candidate microbicide, and appears to represent the prototype of a new generation of antiviral
agents. protective barrier of the vaginal epithelium (Klasse et al., 2008). Moreover, LC activation and decreased Langerin function might also have attributed to enhanced HIV-1 transmission (Chapter 5,6). Similarly, efficacy trials of the cellulose sulfate polyanion Ushercell were stopped due to increased rate of infection (Klasse et al., 2008). We therefore stress the need to further investigate the effect of C5A on the genital tract, the influx of HIV-1 target cells as well as for LC activation and the protect function of Langerin. Treatment of C5A inhibited LC mediated transmission, suggesting that the antiviral capacity

**Figure 4. C5A inhibits herpes simplex virus infection of epidermal sheets *ex vivo***

(a,b) Epidermal sheets were prepared from human skin and infected with different concentrations of HSV-1-GFP for 2 days. Different concentrations of C5A (10µM) or a DMSO control were added together with the viruses (a) The cultures were analyzed by fluorescence microscopy and representative pictures are depicted. (b) The epidermal cells were harvested and analysed for GFP expression by flow cytometry. Error bars represent standard errors of duplicates. These results are representative of 2 independent experiments.

To launch C5A as a candidate microbicide two additional features need to be addressed: safety and *in vivo* efficacy. C5A was not toxic at high levels for genital epithelial cells *in vitro*, indicating that C5A can be safely applied in vaginal formulation. However, the safety of C5A as a microbicide should be further investigated. Nonoxynol-9 (N9), a former candidate microbicide, although very potent against HIV-1 *in vitro*, increased the rate of new HIV-1 infections in a clinical trial, probably by recruiting HIV-1 target cells to the mucosa by damaging the naturally of C5A is stronger than acute activation of LCs. However, microbicides will be used on a long-term basis, and this could result in a chronic influx of HIV-1 target cells and LC dysfunction.
A lack of complete effectivity of the microbicide, for instance by incomplete patient compliance, may then enhance transmission instead of protect against transmission. The next requirement will be to test the antiviral activity of C5A in vivo. In our in vitro and ex vivo experiments, C5A was effective between 1-10 µM. Whereas other anti-HIV-1 peptides have an IC₅₀ between 10 and 100 nM (Kliger et al., 2001), and the anti-HSV drug acyclovir has an average IC₅₀ of 0.15 (HSV-1) and 1.62 (HSV-2) µM (Crumpacker et al., 1979). Since we propose to use C5A as a topical agent, the absorption in the mucosal fluids and tissues needs to be high enough to obtain concentrations of C5A in the vaginal fluid, throughout the epithelial and subepithelial layers. Interestingly, in our HIV-1 and HSV-1 ex vivo experiments using epidermal sheets the same concentrations of C5A were required to efficiently inhibit infection, suggesting that C5A does penetrate the tissue or works before the virus enters the tissue. These results should be confirmed in genital epithelial tissues. Small modifications of the peptide might enhance the biological activity and allow topical administration. Thus, in light of the failure of two recent microbicide trials (Klasse et al., 2008), it is a challenge to obtain evidence for the safety and efficacy C5A treatment. In conclusion, C5A inhibits different mechanisms that are thought to mediate HIV-1 transmission in vitro. Therefore, C5A is a promising candidate drug to prevent HIV-1 transmission as well as HSV infection. We focused on C5A as a candidate microbicide; however, C5A should also be further investigated as candidate antiviral drug for treatment of HIV-1 and recurrent and systemic herpes infection, especially with acyclovir-resistant HSV strains (Bacon et al., 2003).

Methods

Cells. Immature DCs, CD4⁺ T-lymphocytes and monocyte-derived macrophages were isolated and characterized as described previously (Saphire et al., 2001; de Witte et al., 2007). TZM-bl cells express CD4, CXCR4 and CCR5 and contain an integrated lacZ gene driven by the HIV-1 LTR (Wei et al., 2002). Primary genital epithelial cells (PGEC) were isolated as described previously (Bobardt et al., 2007).

Viruses. 293T-lymphocytes were transfected with proviral plasmid (9 µg) and VSV-G envelope plasmid (1 µg). At day 2, VSV-G pseudotyped viruses were harvested and used to acutely infect Jurkat T-cells. Two days post-infection, viruses were harvested and p24 content was measured by ELISA (Perkin Elmer Life Sciences). The following proviral constructs were used, wild-type pNL4.3 (X4), pNL4.3-BaL (R5) in which wild-type NL4.3 envelope was switched for the R5 BaL envelope, the pNL4.3-ΔEnv, which lacks gp160, the pNL4.3-eGFP (X4) and the pNL4.3-BaL-eGFP (R5), which encode the GFP gene instead of the Nef gene (Chatterji et al., 2005). Primary HIV-1, HIV-1 drug-resistant, HIV-2 and SIV viruses were obtained through the NIH AIDS Research and Reference Reagent Program and amplified in PHA/IL-2 stimulated PBMC. The HSV-1 virus strain Syn17⁺, the recombinant HSV-1 VP16-GFP strain number V44 (LaBossière et al, 2004), and HSV-2 strain 333 were grown and titrated on vero cells.

HSV-1/-2 infection. Vero cells were seeded into a 24 well plate (Greiner Bio-One, Frickenhausen Germany). Cells were infected with different concentrations of HSV and the C5A peptide (10µM) or the DMSO control was added immediately. 48 hours post-infection cell morphology and GFP expression were measured using a Leica DMIL fluorescence microscope (Leica Microsystems Wetzlar, Germany) and pictures were taken using a Leica DFC 320 camera (Leico Microsystems). Cells were harvested and stained with antibodies against HSV gB (T111; Novus biologicals, Littleton, CO, USA). The cells were washed and fixed with 4% PFA/PBS and analyzed by flow cytometry. Epidermal sheets were prepared as previously described (de Witte et al., 2007). The sheets were infected with different concentrations of HSV-1 GFP by pipetting the virus underneath the sheets into the medium. Different concentrations of C5A or the appropriate volume of the DMSO control solution was added in a total volume of 500 µL. After 2 hours, 1.5 ml complete IMDM was added and the sheets were cultured for 2 days. The sheets were analyzed by fluorescence microscopy or by measuring GFP expression by flow cytometry.

Infections. TZM cells (1x10⁵ cells/ml) were exposed to HIV-1 (1 ng of p24) for 4 hours in the presence or absence of C5A, washed, and infection was measured 48 hours post-infection by beta-galactosidase activity. Without washing, TZM cytotoxicity is observed at >100 µM of C5A (data not shown). DCs, CD4⁺ T-lymphocytes or
macrophages (1 x 10^5 cells) were exposed to virus (1 ng of p24) for 1 day, washed three times with medium, and cultured in a flat bottom 96-well plate (Greiner Bio-One). Supernatants were collected after different days and viral replication was monitored by p24 ELISA.

**Peptide.** As reported (Cheng et al., in press), C5A composed of D-amino acids exhibits prolonged anti-HIV-1 activities in serum than C5A composed of L-amino acids (data not shown). Interestingly, C5A composed of L-amino acids is not degraded in serum (i.e. after 4 hours at 37°C), but instead is tightly bound (interaction maintained in an SDS-gel) to unknown seric molecules (data not shown). This tight association may result in the attenuated anti-HIV-1 activity of the L-amino acids peptide in serum overtime. Thus we exclusively synthesized peptides composed of D-amino acids for all experiments conducted in this study. Peptides were dissolved in DMSO and subsequently diluted in RPMI or DMEM in the absence of serum.

**Ex vivo infection and transmission.** Human epidermal sheets were prepared as described previously (de Witte et al., 2007) and infected with HIV-1 NL4.3-BaL-eGFP (100 ng p24) by pipetting the virus underneath the sheets into the medium. Either 10 µM of C5A or the appropriate volume of the DMSO control solution was added in a total volume of 500 µL. After 2 h, 1.5 mL complete IMDM was added and the sheets were cultured for 3 days. The epidermal sheets were removed and CCR5+ Jurkat cells (2x10^5) were added for an additional 4 days. Migrated epidermal cells (day 3) and samples of the co-cultures (day 5 and 7) were harvested, fixed in 4% paraformaldehyde/PBS and analysed for GFP expression using flow cytometry. To determine the phenotype of the infected migrated cells, the cells were double-stained with the LC marker Langerin. All infected cells were Langerin positive (data not shown). We did not observe any changes after addition of C5A concerning LC migration, LC maturation and LC viability as determined by flow cytometry (data not shown).

**DC transmission assay.** DC were plated at 5x10^4 cells/well in 96-well v-bottom plate (Greiner Bio-One). Cells were incubated with HIV-1 NL4.3-eGFP (X4), NL4.3-BaL-eGFP (R5) or the single round NL4.3ΔEnv-eGFP-pseudotyped with NL4.3 gp160 (X4) (25 ng p24) for 2 hours at 37°C. Cells were washed 3 times with warm medium and PHA/IL-2-activated CD4+ T-lymphocytes (2x10^5) were added. Cells were cultured in a flat bottom 96-well plate (Greiner Bio-One), harvested after 3 days, fixed in 4% paraformaldehyde/PBS and GFP expression was measured by flow cytometry.

**Cytotoxicity.** PGEC were plated in clear bottom 96-well plates (Greiner Bio-One). Cells were serial diluted from 55,000 cells to 25 cells in 100 µL complete DMEM. Fifteen µL of the CellQuanti-MTTTM reagent (Gentaur Belgium) was added and cells were incubated for 4hoursat 37°C. Then 100 µL of the solubilization solution was added and the plate was shaken for 1hoursat room temperature. The OD 570 nm was measured on a Molecular Devices SpectraMax 384 Plus reader. A linear relationship was observed between OD 570 nm and the cell number. The detection limit was estimated to be 950 cells from the blank control. To determine the cytotoxicity of peptides on PGEC, 55,000 cells were plated per 80 µL well in clear bottom 96-well plates (Greiner Bio-One). Cells were treated twice daily with 200 µM of C5A or 0.01% saponin for a week. No washes were performed in order to maintain a continuous exposure of cells to peptides.

**References**


Chapter 7.3

Concluding remarks Section 7

*Dendritic cells: to target or not to target? That is the question...*

At present the role of different subsets of DCs at the site of HIV-1 entry *in vivo* is not fully elucidated. DC-SIGN⁺ DCs are thought to mediate HIV-1 transmission, whereas LCs protect against transmission. Therefore, care is required with candidate microbicides that target DC subsets. The distinct outcomes of these DC subsets upon HIV-1 encounter are attributed to the HIV-1 receptors, Langerin, DC-SIGN and syndecan-3. The *trans*-receptors syndecan-3 and DC-SIGN are attractive microbicide targets. Importantly, the carbohydrate specificities of DC-SIGN and the protective receptor Langerin partly overlap. This indicates that interference of the interaction between HIV-1 and DC-SIGN needs to be done at the site of the receptor and with specific compounds, to preserve the function of Langerin. Candidates are a blocking antibody against DC-SIGN or Lewis X containing structures, which specifically block DC-SIGN but not Langerin (Chapter 5.2). Recombinant DC-SIGN and high mannose containing ligands have been proposed to block interaction of DC-SIGN with HIV-1 (Table 7.1.2). However, these compounds interfere with Langerin function, and thus need to be revisited. Importantly, activation of LCs and DCs enhances HIV-1 transmission (Section 6). Thus, care should be directed to evaluate the effect of DC-specific compounds for activation. To avoid activation of the different DC subsets, it might be worthwhile to directly target the virus, such as with the compound C5A. C5A is a promising microbicide candidate, inhibiting HIV-1 transmission at different levels: epithelial transcytosis, DC-mediated transmission and direct infection of target cells, including LC infection. To take this candidate into microbicide trials, the next steps will be to manipulate the molecule to higher biological activities and carefully test the compound for inflammation, activation and efficiency to inhibit HIV-1 transmission in an *in vivo* model.