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Complement opsonization promotes HSV-2 infection of human dendritic cells

Elisa Crisci¹, Rada Ellegård¹, Sofia Nyström¹, Elin Rondahl², Lena Serrander³, Tomas Bergström⁴, Christopher Sjöwall⁵, Kristina Eriksson⁶ and Marie Larsson¹#

¹Division of molecular virology, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden. ²Division of Infectious Diseases, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden. ³Division of Clinical Microbiology, Linköping University Hospital, Linköping, Sweden. ⁴Department of Infectious Disease, Institute of Biomedicine, University of Gothenburg, Gothenburg, Sweden. ⁵AIR Rheumatology, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden. ⁶Department of Rheumatology and Inflammation Research, University of Gothenburg, Gothenburg, Sweden.

# Corresponding author: Professor Marie Larsson, marie.larsson@liu.se

Running title: Role of complement in HSV-2 infection of DCs
Abstract

Herpes virus type 2 (HSV2) is one of the most common sexually transmitted infections globally with a very high prevalence in many countries. During HSV2 infection viral particles become coated with complement proteins and antibodies, both existent in the genital fluids, which could influence the activation of the immune responses. In genital mucosa, the primary target cells for HSV2 infection are epithelial cells, but resident immune cells such as dendritic cells (DCs) are also infected. The DCs are the activators of the ensuing immune responses directed against HSV2, and the aim of this study was to examine the effects opsonization of HSV2, either with complement alone or with complement and antibodies, had on the infection of immature DCs and their ability to mount inflammatory and antiviral responses. Complement opsonization of HSV2 enhanced both the direct infection of immature DCs and their production of new infectious viral particles. The enhanced infection required activation of the complement cascade and functional complement receptor 3. Furthermore, HSV2 infection of DCs required endocytosis of viral particles and their delivery into an acid endosomal compartment. The presence of complement in combination with HSV1 or HSV2 specific antibodies more or less abolished the HSV2 infection of DCs.

Our results clearly demonstrate the importance of studying HSV2 infection under conditions that ensue in vivo, i.e. when the virions are covered in complement fragments and complement fragments and antibodies, as this will shape the infection and the subsequent immune response and needs to be further elucidated.

Keywords: HSV2 infection, dendritic cells, complement, antibodies
During HSV2 infection viral particles should become coated with complement proteins and antibodies, both existent in the genital fluids, which could influence the activation of the immune responses. The dendritic cells are the activators of the immune responses directed against HSV2, and the aim of this study was to examine the effects of complement alone or complement and antibodies, on the HSV2 infection of dendritic cells and their ability to mount inflammatory and antiviral responses.

Our results demonstrate that the presence of antibodies and complement in the genital environment can influence HSV2 infection under in vitro conditions that reflect the in vivo situation. We believe that our findings are highly relevant for the understanding of HSV2 pathogenesis.
Introduction

Worldwide, herpes virus type 2 (HSV2) is one of the most common sexually transmitted infections with a high seroprevalence, over 50% in developing countries (1, 2). Many infected individuals are asymptomatic, and shedding of HSV2 in the genital tract can occur without any clinical symptoms (3). Notably, several studies indicate that preexisting genital herpes enhances the acquisition, transmission, and progression of human immunodeficiency virus (HIV-1) (1-4).

The innate immune response of the genital tract is the first line of defense against sexually transmitted viruses such as HSV2 (4). In the genital mucosa, epithelial cells are primary targets of HSV2 infection (1), but mucosa immune cells such as dendritic cells (DCs) can also become infected by HSV2 (5). The viral envelope of HSV2 contains an array of viral glycoproteins that are involved in the infection or the immune evasion (6, 7). The HSV2 glycoprotein C (gC) binds complement 3b (C3b) (7-11), which provides protection against complement-mediated virus neutralization, i.e. destruction (9, 12). HSV2 gC facilitates virus entry by attaching the viral particle to host cell-surface heparin sulfate and heparin (13) and the absence of gC sensitizes HSV2 to lysis through the classical complement pathway in epithelial cells (14).

It’s clear from in vivo studies in different mouse models that the complement pathway plays an important role in the HSV infection (15-17). Complement proteins are present in vaginal secretions (2) and seminal plasma (18, 19) and during a HSV2 infection the viral particles should be complement coated (9, 10), which might influence the infection and activation of the immune responses. Besides complement, the genital secretions contain antibodies which influence the mucosal immune response (20, 21). It is possible that preexisting HSV1 antibodies play a role in protecting individuals from acquiring HSV2 or in the clinical manifestations of the HSV2
infection (22-24). Individuals with HSV1 immunity tend to remain asymptomatic for HSV2 and
to have their first clinical manifestation of genital herpes only after experiencing an
immunosuppressive event (25).

Only few studies exist on the interaction between HSV2 and human DCs and they were
performed using monocyte derived DCs (MDDCs) (26, 27). HSV2 induces a productive viral
infection in MDDCs (5) and apoptosis in both infected and bystander cells (26). In DCs,
infectious HSV2 triggers the release of pro-inflammatory cytokines, most notably TNF-α, but
also IL-6 (26, 27) and antiviral factors such as IFN-β (27). Other effects exerted by HSV2 on
MDDCs include increased expression of aldehyde dehydrogenase member A1 (27), and impaired
antigen presentation (26).

The aim of this study was to examine the effects of opsonization of HSV2, i.e. with
complement alone or complement and HSV specific antibodies, exerted on the viral infection of
immature monocyte derived DCs and these cells’ ability to mount inflammatory and antiviral
responses in response to the viral exposure. Complement opsonized HSV2, both by human
serum and seminal plasma, gave enhanced infection of DCs and higher productive infection
compared to free, non-opsonized, HSV2. Furthermore, opsonization gave rise to significantly
higher gene expression of all inflammatory and antiviral factor tested but on the protein level
these differences between the free and complement opsonized HSV2 were not as clear as at gene
level. The presence of complement and HSV1 or HSV2 specific antibodies decreased infection
and inflammation and antiviral responses in the DCs. The HSV2 infection of DCs required
endocytosis and endosomal acidification as inhibition of these cellular events decreased the
infection. The enhanced infection induced by complement opsonized virions required functional
complement receptor 3 (CR3). This work clearly demonstrates the importance to study HSV2
infection under conditions that reflect the in vivo situation, i.e. virions covered in complement
fragments or complement fragment and antibodies need to be further explored.

### Materials and methods

#### Reagents

Dendritic cell (DC) culture medium RPMI-1640 (GIBCO, Sweden) was supplemented with
2mM glutamine, 20µg/ml gentamicin (GIBCO), 10mM HEPES (GIBCO), and 1% human
plasma. 100U/ml recombinant human GM-CSF (Genzyme) and 300U/ml recombinant human
IL-4 (R&D Systems, Minneapolis, MN, USA) were utilized for in vitro propagation of DCs.
Vero cells (ATCC, UK) were cultured in Dulbecco's Modified Eagle's medium (DMEM:
GIBCO) with 10% FCS, 2mM glutamine, 20µg/ml gentamicin and 10mM HEPES.

**Propagation of monocyte derived dendritic cells**

Whole blood was obtained from volunteers as well as from four individuals with a diagnosis of
systemic lupus erythematosus (SLE) based on the recent classification criteria (28) with
rs1143679 (R77H) mutation in CD11b (29) (Ethical Permits M173-07 and M75-08/2008) as
described previously (30). Peripheral blood mononuclear cells (PBMC) were separated by
density gradient centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway,
NJ, USA) and incubated on tissue culture dishes (BD, Europe) for 1h at 37°C to allow adherence
of the DC progenitors before the non-adherent cells were removed by washing with RPMI. The
progenitors were differentiated into immature monocyte derived DCs (hereafter referred to as
immature DCs) by adding 100U/ml GM-CSF and 300U/ml IL-4 at day 0, 2, and 4 of culture. On
day 5, the DCs were assessed for expression of CD14 and CD83 markers as quality control and then used for experiments.

Virus propagation and titration

HSV2 virus stock was prepared in African green monkey kidney cells (GMK) cultured in Eagles MEM supplemented with 10% FCS as previously described (31). The HSV2 strain used was strain 333. UV inactivation of HSV2 was accomplished by exposing the viruses to UV light for 0.5h.

Opsonization of HSV2

Complement opsonization of HSV2 was done by incubation of virions with an equal volume of human serum (HS) or seminal plasma (SP) (Ethical Permits M173-07 and M75-08/2008). Different types of HS were used for virus opsonization: HSV1 and HSV2 seronegative serum opsonized virus (C-HSV2), HSV1 seropositive serum opsonized virus (C1-HSV2) or HSV2 seropositive serum opsonized virus (C2-HSV2). The HS was tested for HSV antibodies using HerpeSelect® 1 ELISA IgG and HerpeSelect® 2 ELISA IgG kits (Focus Diagnostics, Cypress, CA, USA). We utilized nine HSV seronegative sera, eight HSV2+ sera, and eight HSV1+ sera for the experiments. For the experiments with seminal plasma, we used samples from four HSV1 and HSV2 seronegative donors. Free virus (F-HSV2) was treated with medium alone and mock (medium alone) was used as negative control. Additionally, DCs treated with serum or seminal plasma were used as negative control. All groups were incubated for 1h at 37°C and then directly used in the HSV2 infection experiments. Heat inactivation of the complements was done by incubation of human serum or seminal plasma at 56°C for 1h (HI-C).
HSV2 infection of Dendritic cell

Immature DCs (0.5 x10^6) were infected with mock, F-HSV2, C-HSV2, C1-HSV2, or C2-HSV2 with multiplicity of infection (MOI) of 1 to 3 for 2h at 37°C in RPMI alone or in 1% plasma from HSV seronegative donors. The different groups of DCs were then washed and cultured in 1% plasma from HSV seronegative donors for a total of 6h or 24h. The DCs were harvested, washed, and lysed with Bioline RLY lysis buffer (Bioline, UK) for RNA extraction or fixed with 4% paraformaldehyde (PFA) for 10min at 4°C for flow cytometry staining.

Viral binding and endocytosis

To evaluate the binding and uptake of virus particles, DCs were incubated for 2h at 37°C and at 4°C. Cells were collected, counted, and resuspended in distilled water and stored at -20°C. To assess the amount of DNA copies viral DNA was extracted with Qiagen, EZ1 Virus Mini 2.0 extraction Kit. A specific PCR was performed using Quantifast ROX Vial Kit (Qiagen, Sweden) or Takyon No ROX Probe Master mix dTTP (Eurogentec S.A. Belgium) with HSV2 gG forward primer: AGA TAT CCT CTT TAT CAT CAG CAC CA and HSV2 gG reverse primer: TTG TGC CAA GGC GA and the probe: CAG ACA AAC GAA CGC CG (33).

To determine whether the HSV2 infection of DC was dependent on acidification, we used the acidification inhibitors; NH₄Cl, (40mM, Sigma), and Bafilomycin A1 (BAF, 50nM; Sigma). Additionally, the requirement of endocytosis was assessed using cytochalasin D (CCD, 10µM; Sigma), clathrin mediated endocytosis was assessed using chlorpromazine (CP, 6.25µg/ml; Sigma), and protein transport was assessed with monensin (Mon, 4µl/ml; BD, Europe). All the agents were used 30min before the infection of the DCs.
Assessment of productive HSV2 infection of DCs

Supernatants from the HSV2 infection experiments were harvested at 6h and 24h and viral yields were quantified using a modified plaque assay method (35) on Vero cells. Briefly, DC supernatants were incubated in 2 or 10 fold dilution in 24-well plates with a confluent monolayer of Vero cells for 1h at 37°C. After the washing, the plates were coated with complete medium mixed with 2% agarose (1:1) and incubated for additional 3-4 days before assessing plaque forming units (PFU). Mock and UV-HSV2 were used as negative controls. All samples were tested in duplicates or triplicates.

Total RNA extraction, reverse transcription and qPCR

Total RNA from DCs exposed to mock, F-HSV2, C-HSV2, C1-HSV2, and C2-HSV2 was extracted using Isolate II RNA Mini Kit (Bioline, UK) and total cDNA was produced by SuperScript III Reverse Transcriptase First Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA, USA). Quantification of gene transcripts was performed using the SensiFAST SYBR® Hi-ROX Kit (Bioline, UK) using a 7900HT Fast Real Time PCR system with 7900 System SDS 2.3 Software (Applied Biosystems, Sweden). Primers targeting β-actin and GAPDH were used as housekeeping genes for reference as described by Vandesompele (36). Primers were purchased from CyberGene AB, Stockholm, Sweden ( Primer sequences; Supplementary Table 1). To compensate for variation between plates, values were normalized as described by Rieu (37), subtracting each value by the average of all values from the same experiment.

Assessment of inflammatory factors with ELISA
Levels of TNF-α, IL-6, IFN-α (Mabtech, Sweden), and IFN-β (VeriKine™ Kit, PBL Assay Science, USA) proteins were assessed in supernatants from mock, F-HSV2, C-HSV2, C1-HSV2, and C2-HSV2 24h infected DCs. In addition, these factors were also examined in supernatants from SP opsonized HSV2 infected DCs at 24h and for endocytosis studies at 6h. All the ELISAs were performed following the manufacturer’s instructions.

Flow cytometry

The quality of immature DCs were assessed by staining with anti-human CD83 and CD14 PE conjugated antibodies (BD, Europe). DCs were used if the purity was more than 95% and their expression of CD14 and CD83 were less than 10%. To evaluate the level of HSV2 infection in immature DCs, cells were permeabilized with PBS containing 0.2% saponin and 0.2% FCS and incubated with polyclonal Ab (pAb) against HSV2 (identifying all major glycoproteins in the viral envelope and at least one core protein; B0116, DAKO, Denmark) or monoclonal Ab (mAb) against HSV2 ICP8 (4E6) (Santa Cruz biotechnology, USA) followed by FITC or Alexa 488 conjugated secondary Ab (Abcam, Europe). Zombie Aqua™ Fixable Viability Kit (BioLegend, Europe) was used to discern viable/dead cells. Additionally, PE conjugated mAb against CD11b/Mac-1 (BD, Europe) was used to evaluate the presence of CD11b receptor on DCs. The samples were assessed by flow cytometry (FACS Canto, BD) and analyzed by FlowJo (Treestar, Ashland, OR, USA).

CD11b protein knockdown in dendritic cells by siRNA

Knockdown of the CD11b protein with siRNA in immature DCs was done by transfecting immature DCs at day 2 or 3 of culture with siRNA using the transfection reagent DF4
(Dharmacon) or HiPerFect (Qiagen) respectively. The transfection reagents were removed and 
the cells were used for experiments 2 days after transfection. The siRNA (SMART pool; 
Dharmacon) was specific for CD11b (Human ITGAM M-008008-01). Non targeting siRNA 
control pool (D-001206-13-05; Dharmacon) served as control. The transfection efficiency was 
determined by flow cytometry of cells transfected with siGLO RISC-Free Control siRNA (D-
001600-01; Dharmacon). Silencing of CD11b expression was verified by real-time PCR and 
flow cytometry.

Statistical analysis

The statistical program GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) was used for 
analysis of all data. Repeated measures ANOVA followed by a Bonferroni post-test or a two-
tailed paired t-test were used to test for statistical significance. Results were considered 
statistically significant if p<0.05. All experiments were performed a minimum of four times 
using cells derived from different blood donors or different cell line passages. When 
experimental values were normalized, the mean of free virus or mock were set to 1. qPCR results 
were normalized for variation between plates as previously described (37). In brief, each value 
was subtracted by the average of all values and then the obtained values were divided by the 
average of mock or free virus depending on the different experiments.

Results

Complement opsonization of HSV2 increased infection of immature dendritic cells whereas 
the presence of neutralizing HSV1 or HSV2 abs abolished the infection
HSV2 has the ability to infect immature monocyte derived DCs (26, 27) and give rise to a low level of productive infection (26). HSV2 expresses gC on the viral envelope and this is a glycoprotein which neutralizes complement factor C3 (7-11). Here we have assessed how complement opsonization affected the HSV2 infection of immature monocyte derived DCs (hereafter referred to as immature DCs) by infecting them with mock, free HSV2 (F-HSV2), HSV2 opsonized with complement using serum negative for HSV1 and HSV2 antibodies (C-HSV2), serum from HSV1 seropositive (C1-HSV2), or HSV2 seropositive (C2-HSV2). The mRNA expression levels of HSV2 thymidine kinase (TK), an enzyme involved in viral nucleotide biosynthesis and DNA metabolism, and glycoprotein D (gD), a receptor part of the core fusion machinery and important in viral entry, were assessed by qPCR at 6h and 24h post infection (Figure 1A-B). C-HSV2 induced significantly higher mRNA expression of HSV2 TK and gD at both 6h and 24h in DCs compared to free virus (Figure 1A-B). The presence of HSV1 or HSV2 specific antibodies in the serum used for opsonization almost abolished the infection as seen by the decreased mRNA expression levels of TK and gD compared to the free virus and C-HSV2 at both time points (Figure 1A-B). The gene expression pattern of HSV2 protein ICP0 was similar to TK and gD (Supplementary figure 1A). The mRNA profiles matched the profiles of HSV2 proteins expressed at 24h as assessed by flow cytometry, with 2-fold higher levels of HSV2+ cells in the C-HSV2 infected group compared to free HSV2 (Figure 1C-D). A similar protein expression pattern was obtained for HSV2 infected cell protein 8 (ICP8) (Supplementary figure 1B). To verify the results with a source of complement that exists at the site of infection, we assessed the effect opsonization with seminal plasma (SP) exerted on the HSV2 infection of DCs. The SP opsonized HSV2 (SP-HSV2) induced a higher infection of DCs compared to free virus at both 6h and 24h (Figure 1E) at gene transcripts level. This indicated
that even if less complement was present in the seminal plasma (32) the complement opsonized
capability to infect DC than free virus.

HSV2 infection is known to induce apoptosis in DCs (5) and we could confirm this effects in
our system with higher levels of dead and apoptotic cells for F-HSV2 and C-HSV2 compared to
C1-HSV2, C2-HSV2, and mock cells (Supplementary figure 1C and data not shown). As an
infection control, we assessed the effects of UV inactivated HSV2 (UV) and found that this
inactivation abolished the infection (Supplementary figure 2), which is consistent with previous
findings (26).

**Complement opsonization of HSV2 enhanced the productive infection of dendritic cells**

To assess the levels of productive HSV2 infection in DCs we measured the amount of released
infectious virions in the supernatants at 24h post infection by plaque forming assay. After 24h,
immature DCs infected with C-HSV2 had significantly higher production and release of viral
particles compared to free virus, C1-HSV2, and C2-HSV2 (Figure 1F). A similar pattern was
obtained when HSV2 was opsonized with seminal plasma (Figure 1G).

**Complement opsonization of HSV2 increased inflammatory and antiviral transcripts in
dendritic cells.**

Seeing that complement opsonized HSV2 enhanced the infection we assessed the effects the
opsonized virions had on the ability of DCs to take the action against the infection by producing
antiviral and inflammatory factors. We have recently demonstrated enhanced infection and
suppressed antiviral and inflammatory responses in DCs exposed to complement opsonized HIV-
1 (30). The mRNA expression levels of antiviral factors IFN-β, IFN-α, and MX1 and
inflammatory factors TNF-α, IL-6, and IL-1β were assessed for free and opsonized HSV2. The mRNA levels of IFN-β and MX1 induced by C-HSV2 were significantly higher compared F-HSV2 at 24h (**Figure 2A**), but no significant difference was observed for IFN-α levels between F-HSV2 and C-HSV2 (**Figure 2A**). The presence of HSV1 or HSV2 specific antibodies decreased IFN-β expression at 24h (**Figure 2A**). Surprisingly, there were similar protein levels of the antiviral factors, IFN-β and IFN-α, produced by DCs after F-HSV2 and C-HSV2 infection (**Figure 2B**), whereas the protein levels were significantly lower for C1-HSV2 and C2-HSV2 compared to F-HSV2 (**Figure 2B**). C-HSV2 significantly increased the mRNA levels of the inflammatory factors TNF-α, IL-6 and IL1-β in DCs at 24h compared to F-HSV2 (**Figure 2C**).

In addition, the mRNA expression of the chemokines CCL3 and CXCL8 had similar pattern as the inflammatory factors at 24h post infection (**Supplementary Figure 3**). The secretion of IL-6 and TNF-α in DC supernatants were assessed at 24h post infection. Complement opsonized virus and F-HSV2 induced similar levels of these inflammatory factors (**Figure 2D**). The reason for this discrepancy between mRNA and protein expression levels could be due to a more suppressive cellular function in C-HSV2 infected DCs compared to free virus and/or activation of a different regulation of protein transcription by microRNAs in C-HSV2 compared to free HSV2 infected DCs. The TNF-α levels induced by C-HSV2 were much higher than the levels for C1-HSV2 and C2-HSV2 (**Figure 2D**). Moreover, similar to serum, HSV2 opsonized with seminal plasma induced higher TNF-α and IFN-β mRNA levels in DCs compared to free HSV2 (**Figure 2E**). At protein level seminal plasma opsonized virus tend to increase the secretion of TNF-α and IFN-β compared to free virus, but the data were not statistically significant, and exhibited a big variation between donors (**Figure 2F**).
Complement opsonization did not alter the amount of HSV2 particles binding to and taken up by dendritic cells.

To examine whether the higher level of infection induced by C-HSV2 was due to altered binding and internalization mechanisms, the levels of bound and internalized F-HSV2, C-HSV2, C1-HSV2, C2-HSV2 and heat inactivated C-HSV2 (HI-C) in DCs were assessed measuring the viral DNA copies/ml after 2h at 4°C (data not shown) or 37°C (Figure 3A). The levels of bound and internalized virions were similar for all virus groups with a tendency of higher viral uptake for C1-HSV2 and C2-HSV2 (Figure 3A).

Inhibition of endocytosis and endosomal acidification impeded complement opsonized HSV2 infection of dendritic cells.

HSV infection of epithelial cell lines has previously been shown to in part require an acid endosomal compartment and endocytosis (39, 40), therefore we examined the effects that inhibitors of acidification NH₄Cl, and Bafilomycin A1 (BAF), inhibitors of endocytosis Cytochalasin D (CCD) and chlorpromazine (CP), and monensin (Mon) a carboxylic ionophore exerted on the HSV2 infection of DCs. Drugs were used at concentrations previously shown to block viral uptake and endosomal acidification in DCs or other cell types (34, 41-43) without affecting the viral infectivity. Neutralization of endosomal acidification with NH₄Cl decreased the F-HSV2 and C-HSV2 infection of DCs, with almost 20-fold decreased C-HSV2 infection at 6h post infection (Figure 3B). Moreover, mRNA expression level of the antiviral response, as measured by IFN-β, gave the same profile as the infection with significantly decreased C-HSV2 levels (Figure 3B). The inhibition of DC endocytosis with CCD more or less abolished the F-
HSV2 and C-HSV2 infection at 6 hours (Figure 3C) and the same effects were seen for the antiviral response (Figure 3C).

F-HSV2 infectivity and antiviral response were also inhibited by monensin, chlorpromazine, and bafilomycin (Figure 3D), further confirming the involvement of endosomal acidification and clathrin mediated endocytosis in the HSV2 infection process of DCs. Our finding regarding the effect of bafilomycin on HSV2 infection correlated with a previous finding (43). C-HSV2’s infectivity was also inhibited by monensin and chlorpromazine but only to a significant level by monensin, whereas bafilomycin had no effect (Figure 3E). The inhibitors decreased also the antiviral response induced by C-HSV2 (Figure 3E). The treatment with these inhibitors had no direct effect on the viral infectivity, i.e. the ability of HSV2 to infect, or on the DCs’ baseline expression of antiviral and inflammatory factors (data not shown).

The elevated HSV2 infection in dendritic cells induced by complement opsonized virions required functional complement activation

The activation of the complement cascade is inhibited by heat inactivation of the serum and we confirmed the involvement of complement in the enhanced infection seen for C-HSV2 by using heat inactivated serum. Opsonization of HSV2 with heat inactivated serum gave the same binding and internalization level as F-HSV2 and C-HSV2 (data not shown). Heat inactivation of complement opsonized virus significantly decreased the mRNA expression of HSV2 TK and HSV2 gD compared to C-HSV2 and the levels were similar to free virus at 6h (Figure 4A). The same pattern was seen for the productive infection (Figure 4A) and for HSV2 protein expression at 24h (Figure 4B). The inactivation had no significant effects on C1-HSV2 and C2-HSV2 infection levels, nor on TK and gD mRNA or HSV2 protein levels (Figure 4B). In the case of
antiviral and inflammatory factors, the pattern was similar and HSV2 opsonized with heat inactivated serum restored IFN-β and TNF-α to the levels of free virus at 24h (Figure 4C).

The elevated infection induced by complement opsonized HSV2 in dendritic cells required functional complement receptor 3

Complement receptor 3 (CR3) is exploited by several pathogens for suppression of innate responses (44, 45). DC expression of CR3 is required for complement opsonized HIV’s augmentation of infection (30), consequently we examined if the enhanced HSV2 infection of DCs by complement opsonized HSV2 also was dependent on viral binding to CR3. Here we used DCs derived from individuals with SLE with mutated CR3 alpha-integrin CD11b (rs1143697: R77H), which gives decreased expression of CD11b and impairs the signaling through CR3 (30, 46, 47). The free HSV2 gave the same level of infection in DCs derived from individuals with SLE as in cell from healthy donors (Supplementary figure 4). The enhanced infection seen for C-HSV2 in DCs from healthy individuals was abolished when the DCs had dysfunctional CR3 (Figure 5A). Moreover, the enhanced gene expression of inflammatory and antiviral factors normally seen for C-HSV2 exposed DCs was not detected when DCs with mutated CR3 were used (Figure 5B). To confirm the findings from the SLE patient derived DCs with dysfunctional CR3, we knocked down CD11b expression with siRNA and assessed infection, inflammation and antiviral responses (Figure 5C-D). The knockdown of CD11b abolished the increased C-HSV2 infection, whereas this increase was still present in the control siRNA transfected cells (Figure 5C). Moreover, the higher gene expression of inflammatory and antiviral factors seen for C-HSV2 exposed DCs was not present in the CD11b knockdown DCs (Figure 5C).
Discussion

Langerhans cells (LCs) and DCs are one of the initial responder cells during the establishment of HSV2 infection in the mucosa and are involved in the induction of the HSV2 specific adaptive immunity via DC cross presentation (5, 48). At the site of infection, the HSV2 virions are exposed to soluble factors, such as complement and antibodies, which should influence the effects virions exert on their target cells as well as the local infection. We found that HSV2’s capacity to utilize the complement system, using serum or seminal plasma, enhanced the virus’ ability to directly infect DCs and that the enhanced infection required functional CR3. Presence of HSV1 or HSV2 specific antibodies during the opsonization more or less abolished the ability of HSV2 to infect the DCs. The HSV2 infection of DCs required endocytosis of virus particles and acidification of the endosomal compartment. Complement opsonized HSV2 induced higher mRNA expression but not statistically significant higher protein secretion of antiviral and inflammatory factors in the DCs compared to free HSV2.

HSV2 has the ability to infect several cell types such as epithelial cells, nerve cells and DCs located in the genital mucosa (1, 5) and in vitro studies have shown that immature monocyte derived DCs and LCs are highly susceptible to HSV2 infection (49-51). Our findings support that HSV2 infects DCs and that this gives rise to a low level of productive infection. The low level of productive infection is not surprising since previous studies have found HSV2 and HSV1 infection of DCs to be predominantly abortive (26, 52-54). This should be the explanation for the discrepancies we see with between the high levels of viral mRNA transcripts and the low levels of infectious HSV2 produced by infected DCs. One additional explanation could be the existence of cellular inhibitors of infection that influence events after the mRNA transcription, i.e. inhibiting the production of viral proteins and/or infective virions.
The first steps in the HSV infection of target cells, i.e. binding and uptake of viral particles, have been investigated and almost all studies focused on HSV1 and epithelial cell lines (34, 39, 40, 55). The mechanism for HSV1 and HSV2 uptake in epithelial cells is rapid endocytosis (34). The HSV1 endocytosis requires several of the viral envelope glycoproteins, i.e. gB, gD, and gH-gL (56, 57) and in keratinocytes HSV1 uptake is a cholesterol and dynamin mediated process. In the endosomal compartment HSV1 and HSV2 require the gD receptor to access the host cell cytosol (34) and in the case for HSV1 dynamin to be able to infect (58). The binding and uptake of HSV2 by DCs has not been examined previously, and we found that complement opsonization had no effect on the amount of HSV2 virions bound or internalized. The HSV2 infection for both free and complement opsonized virions was dependent on endocytosis and endosomal acidification. Interestingly, the infection with free virus involved clathrin dependent endocytosis, whereas complement opsonized virus seemed to utilize a clathrin independent mechanism for infection. In addition to suppressing infection, the neutralization of endosomal acidification decreased the antiviral responses in DCs. This indicates that the antiviral responses require a degradation of the viral particles and/or active infection of the DCs. TLR3 and TLR2 have been suggested to be as important PRRs in the immunological control of the HSV2 infection (59-62). Furthermore, sensors such as STING and DAI can also sense HSV2 (63). The exact PRRs involved in the activation of the antiviral and inflammatory responses in the human DCs in our system of HSV2 infection, remain to be determined. Our finding that HSV2 antiviral responses in DCs required endocytosis of the virus into an acidified endosomal compartment, suggests the involvement of endosomal and/or cytosolic PRRs.

A number of earlier studies have suggested that a prior oral HSV1 infection can provide partial protection against genital HSV2 acquisition (22-24), whereas others have proposed that it
has no protective effect (64-66). At present, most evidence indicates that preexisting HSV1 antibodies do not inhibit the infection, rather they will make the HSV2 infection less pathogenic with milder symptoms (22-25). We found that the presence of HSV1 specific antibodies more or less abolished the ability of HSV2 to infect DCs and surprisingly they neutralized the infection with the same efficiency as HSV2 specific antibodies. The presence of HSV1 or HSV2 antibodies did not decrease the uptake of virus by DCs, rather slightly increased it, so the absence of infection was not due to inability of the virus to bind and be internalized but rather to neutralization of HSV2 infectivity. This raises questions such as how the adaptive immune response induced by a prior oral HSV1 infection, affects the genital mucosal responses during primary genital HSV2 infection, i.e. whether the existing HSV1 antibodies will neutralize the virus and render the virions less cytopathic and suppressive for the DCs functionality and this needs further elucidation.

HSV2 uses many strategies to establish persistent infection and inhibition of complement activation/cascade and antibody binding by gC and gE/gI proteins on the viral surface are part of these mechanisms (7, 67). gC on HSV2 (gC2) and HSV1 (gC1) both bind C3b (8-10) and HSV1 gC also interferes with the binding of C5 and properdin to C3b (12, 15, 68, 69). In fact, HSV1 gC1 contains a C5- and P-interacting domain that accelerates the decay of the alternative complement pathway C3 convertase and this domain is important in modulating complement activity, since HSV1 lacking this domain is more readily neutralized by complement alone, and is significantly less virulent than wild-type virus in vivo. Interestingly, this domain is absent in HSV2 gC2, suggesting that the mechanism by which HSV2 evades the complement cascade may be distinct from that of HSV1 (12, 14, 70).
In our study we found that complement fragments binding to the HSV2 enhanced the direct
and productive infection of DCs. The enhanced HSV2 infection of DCs induced when the virus
is complement opsonized has also been observed for HIV-1 by our group and by others (30, 71,
72). In the case for HIV-1 and the bacteria *Francisella tularensis*, the complement opsonization
suppresses the pathogen induced antiviral and inflammatory immune response by CR3 mediated
modulation of TLR signaling pathways (30, 44). Even if the enhanced infection achieved by
complement opsonized HSV2 was due to CR3 interaction, it did not suppress the secretion of
antiviral and inflammatory factors. The mechanisms behind these differences between different
pathogens could be diversity in receptors, such as type of pattern recognition receptors activated,
due to presence of different PAMPS, in combination with the CR3, giving rise to distinct
signaling and activation of the DCs.

The HSV2 infection of DCs in our hands induced an array of inflammatory and antiviral factors
with higher mRNA levels induced by the complement opsonized viruses compared to free virus,
whereas the protein expression levels were the same. The reason for these discrepancies between
mRNA and protein expression levels could be due to a more suppressive cellular function in C-
HSV2 infected DCs compared to free virus and/or activation of a different regulation of protein
transcription by microRNAs in C-HSV2 versus free HSV2 infected DCs.

In conclusion, the HSV2 exploitation of our innate defense, i.e. the complement system,
enhanced the virus ability to infect DCs. Presence of HSV antibodies clearly render the virus
virtually noninfectious and less toxic to the DCs and should function as a source of antigens for
activation of adaptive immune responses. We clearly demonstrate the importance to study HSV2
infection under conditions that reflect the in vivo situation, i.e. virions covered in complement
fragments or complement fragments and antibodies, as these factors will have a profound effect on the virus’ interaction with the host.
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References


Figure 1. Complement opsonization of HSV2 increased infection in immature DCs.

Immature DCs (10^6/ml) were exposed to mock or 3 MOI of free HSV2 (F), HSV2 complement opsonized with HSV1/2 seronegative serum (C), HSV2 opsonized with HSV1 (C1), HSV2 (C2) seropositive serum or HSV2 opsonized with seminal plasma (SP) from HSV1/2 seronegative donor for 6h or 24h. (A-B) mRNA expression levels at 6h and 24h for HSV2 TK and gD were assessed by qPCR. qPCR values were normalized with free virus mean values set to 1. (C-D) HSV2 infection was assessed by flow cytometry at 24h using a pAb against HSV2, and representative dot plots with percentage of HSV2 positive cells are shown (mean + SEM). Data are shown as box and whiskers of 4-6 independent experiments. (E) PFU assays were performed to assess productive HSV2 infection, i.e. production of infectious viral particles by DCs. Supernatants (24h) from DCs exposed to mock, F, C, C1 and C2 were tested and HSV2 PFU/ml calculated. A representative experiment (left) and normalized experiments (N=5) with PFU values of free virus values set to 1 (right). Data are shown as box and whisker plots of 5 independent experiments. (F) mRNA expression levels at 6h and 24h for HSV2 TK and gD were assessed by qPCR. qPCR values were normalized with free virus mean values set to 1. (G) PFU assays were performed to assess productive HSV2 infection in DCs. Supernatants (24h) from DCs exposed to F and SP were tested and HSV2 PFU/ml calculated. Normalized experiments (N=4) with PFU values of free virus values set to 1. *p<0.05. **p<0.005. ***p<0.0005.

Figure 2. Complement opsonized HSV2 induced higher mRNA levels of inflammatory and antiviral factors in immature DCs compared to free virus.
DCs (10⁶/ml) were exposed to 3 MOI of free HSV2 (F), complement opsonized HSV2 (C), HSV2 opsonized with both complement and specific antibodies against HSV1 (C1), or HSV2 (C2) or mock treated for 24h. mRNA expression of antiviral factors IFN-β, IFN-α, and MX1 (A) and inflammatory factors TNF-α, IL-6, and IL-1β (C) was determined by qPCR. qPCR values were normalized with mock mean values set to 1. Protein levels of IFN-α and IFN-β (B) and IL-6 and TNF-α (D) were assessed in the supernatants from DCs by ELISA. Data are shown as box and whisker plots of 5-6 independent experiments. (E) DCs (10⁶/ml) were exposed to 3 MOI of free HSV2 (F), complement opsonized HSV2 (C), HSV2 opsonized with seminal plasma (SP) from HSV1/2 seronegative donor or mock treated for 24h. mRNA expression of inflammatory factor TNF-α and antiviral factor IFN-β were determined by qPCR. qPCR values were normalized with mock mean values set to 1. Data are shown as box and whisker plots of 5-6 independent experiments.* p<0.05. **p<0.005. ***p<0.0005.

Figure 3. HSV2 infection of DCs required endocytosis and endosomal acidification

DCs (10⁶/ml) were exposed to 3 MOI of free HSV2 (F), complement opsonized HSV2 (C), HSV2 opsonized with both complement and specific antibodies against HSV1 (C1) or HSV2 (C2), heat inactivated C-HSV2 (HI-C) or mock treated for 2h or 6h. (A) Levels of HSV2 viral DNA copies in immature DCs exposed to F, C, C1, C2 or HI-C for 2h were assessed by qPCR. (B-E) DCs were pre incubated for 30 min with 40mM Ammonium Chloride (NH₄Cl) (B), 10µM Cytochalasin D (CCD) (C), 50nM Bafilomycin A1 (BAF), 4µl/ml Monensin (Mon) and 6.25µg/ml Chlorpromazine (CP) (D-E) before HSV2 infection. (B-C) mRNA expression levels of HSV2 TK and IFN-β were determined by qPCR. Values have been normalized with free virus mean values set to 1 and data are shown as mean+SEM of 4-5 independent experiments. (D-E)
mRNA expression levels of HSV2 TK and IFN-β were determined by qPCR at 6h. Values have been normalized with free virus or C opsonized virus mean values set to 1. Protein levels of IFN-β were assessed in the supernatants from DCs by ELISA at 6h. Data are shown as mean+SEM of 4-5 independent experiments. * p<0.05. **p<0.005. ***p<0.0005.

Figure 4. Complement is required for the elevated HSV2 infection in DCs induced by complement opsonized virions

DCs (10^6/ml) were exposed to mock, 3 MOI of free HSV2 (F), HSV2 complement opsonized with HSV1/2 seronegative serum (C), HSV2 opsonized with HSV1 (C1) or HSV2 (C2) seropositive serum or heat inactivated sera (HI) for 6h and 24h. (A) mRNA expression levels for HSV2 TK and gD assessed by qPCR at 6h. PFU assays were performed to test productive infection at 24h and values are expressed as PFU/ml (representative experiment). (B) mRNA expression levels for HSV2 TK, gD were assessed by qPCR or by HSV2 protein expression determined by flow cytometry at 24h using a pAb against HSV2 to assess the percentage HSV2 positive cells. (C) mRNA expression levels for IFN-β and TNF-α assessed by qPCR at 24h. qPCR values were normalized with mock set to 1. Data are shown as mean + SEM of 4-5 independent experiments. * p<0.05. **p<0.005. ***p<0.0005.

Figure 5. Functional complement receptor 3 was required for the elevated HSV2 infection in DCs induced by complement opsonized virions

(A-B) DCs (10^6/ml) propagated from individuals with mutated nonfunctional CD11b, i.e. CR3, were exposed to mock or 3 MOI of free HSV2 (F) or HSV2 complement opsonized with HSV1/2
seronegative serum (C) for 24h. mRNA expression levels for HSV2 TK, gD, TNF-α, IFN-β, and IFN-α were assessed by qPCR at 24h. qPCR values were normalized with free virus mean values set to 1. Data are shown as mean + SEM of 3-5 independent experiments. (C-D) DCs (10^5/100µl) were transfected with CD11b siRNA (white) or control siRNA (black) at day 2 or 3 and used for experiments 2 days after transfection. (C) mRNA expression levels for HSV2 TK, TNF-α and IFN-β were assessed by qPCR at 24h. qPCR values were normalized with C-HSV2 control siRNA mean value set to 1. (D) CD11b expression was evaluated by qPCR (left) and flow cytometry (right). Flow cytometry representative histogram (grey histogram: untreated; dotted line: control siRNA; solid line: CD11b siRNA) and summary graph are shown. Data are shown as mean + SEM of 3-5 independent donor experiments. * p<0.05. **p<0.005. ***p<0.0005.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5