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# Kinetics of *Candida albicans* and *Staphylococcus aureus* Biofilm Initiation on Herpes Simplex Virus (HSV-1 and HSV-2) Infected Cells

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## Abstract

This study examines the kinetics of *S. aureus* and *C. albicans* adherence as it relates to HSV replication and corresponding dynamic display of shared receptors. HeLa cells infected for various times with HSV-1 gL86 or HSV-2 333gJ-(MOI 50) were incubated with *S. aureus* ATCC 25923 or *C. albicans* yeast and CFU measured. Over time, *S. aureus* adherence to HSV-1 infected cells was relatively stable for 45 min then decreased to 0.8 of virus-free control, before cycling at 15-to-30 min intervals. In contrast, staphylococcal adherence to HSV-2 infected cells proceeded at a more gradual rate, increasing to control levels at ~105 min before decreasing to a nadir at 165 min. Yeast adherence to HSV-1 infected cells remained relatively unchanged for the first 75 min then increased 2-fold before returning to its original level. This pattern is repeated over the next 90 min. While a similar pattern with *C. albicans* and HSV-2 was measured, it occurred more rapidly. Our model shows that while the interaction of both HSV-1 and HSV-2 with *S. aureus* is both dynamic and inhibitory, *C. albicans* interaction with HSV-2 is more permissive than HSV-1. However, the interaction of both microbes with HSV-infected cells in this model system appears to be independent of α5B1, CD36 and HSP60 viral-regulated receptor expression. These findings indicate that microbiome interactions across taxonomic kingdoms are more complex than previously thought.

## Keywords

Polymicrobic, Microbiome, Biofilm, Adherence, Herpes Simplex Virus, *Staphylococcus aureus*, *Candida albicans*

## 1. Introduction

The initiation of colonization by the microbiome requires cellular adherence as an essential first step. Herpes simplex viruses (HSV-1 and HSV-2) cause permanent infections, and participate via asymptomatic shedding in not only person-to-person transmission but auto-infection in nearly all humans [1] [2]. During auto-infection, whether as a result of asymptomatic or symptomatic infection, the attachment, endocytosis and subsequent replication of the virus, causes differential receptor availability for other members of the microbiome [3] [4] [5]. Included amongst the high and low-affinity receptors that HSV-1 and HSV-2 can utilize for entry into cells are HSP60 (mitochondrial chaperonin) [6] [7], CD36 (platelet glycoprotein 4, fatty acid translocase (FAT)), and α5B1 (integrin and primary receptor for fibronectin) [6]. These receptors are shared by *S. aureus* and *C. albicans*, two pathogens that can persistently reside in the oro-nasopharynx with HSV. However, even though these co-shared receptors are present within the oro-nasopharynx there are distinct areas of colonization in hosts with natural teeth. One site shared by HSV-1/2 and *C. albicans* is the buccal and tongue mucosa [8]-[13]. In close proximity is the anterior nasal nares occupied by *S. aureus* [14]. This rather distinct separation in the colonization site is intriguing since *S. aureus* is isolated from oral specimens and adheres *in vitro* to buccal epithelial cells [15]. Despite this potential for HSV-candidal-staphylococcal colonization site overlap, *S. aureus* is only rarely isolated from normal buccal and tongue mucosa. This is interesting since *S. aureus* causes both gum and tissue infections, including chronic gingivitis and periodontal abscess [16] [17]. In contrast, when abiotic surfaces are present, as in the case of denture wearers, *S. aureus* forms a robust biofilm on the denture surface along with *C. albicans* [18]. This difference in epidemiology, and *in vivo* cell tropism may reflect differences in receptor turnover during viral entry. Initial studies examining the interaction between herpesvirus, *S. aureus* and *C. albicans* demonstrated that at the end of the time period encompassing the viral entry process (180 min), herpesvirus differentially regulates the interaction of *S. aureus* and *C. albicans* with HeLa 229 cells [12] [19]. This interaction was dependent on both herpes virus type (HSV-1 vs. HSV-2) and organism. HSV-1 and HSV-2 display differences in kinetics of cell binding and subsequent cell-cell-fusion [20] [21] [22]. A possible mechanism that would explain our observation is receptor changes during virus infection. To determine if differential expression by HSV of shared receptors is the mechanism through which this viral pathogen affects subsequent biofilm formation, we measured *S. aureus* and *Candida* adherence over time relative to the display of shared receptors.

## 2. Materials and Methods

### 2.1. Microbial Strains and Handling

HSV infection of HeLa cells is a well-defined viral replication system with early receptors and associated proteins defined. In addition, this system uses well

characterized genetically modified HSV-1&2 strains that, in HeLa cells, under-go the replicative cycle but HSV-1 progeny cannot infect other cells. Recombinant spread-deficient, entry and replication proficient strains from a single lot of either HSV-1 (KOS) gL86 or HSV-2 (KOS) 333gJ-encoding a beta-galactosidase reporter activity were used as previously described [12] [19]. HSV entry into cells was confirmed by o-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Research Products International) assays, as previously described [23].

*C. albicans* and *S. aureus* ATCC 25923 were maintained and cultured as previously described [12] [19]. Both organisms (−80˚C) were subcultured (37˚C; 18 hr) for use onto either Sabouraud Dextrose agar or mannitol salts medium, respectively. *C. albicans* yeast and *S. aureus* were prepared in Hanks Balanced Salts Solution (HBSS; $10^5$ CFU/ml final concentration; 37˚C; Corning) immediately prior to use.

## 2.2. Polymicrobic Biofilm Initiation

The number of HeLa cell-associated *S. aureus* and *C. albicans* was determined as an indicator of biofilm initiation, as previously described [12] [19]. Briefly, HeLa 229 cells (Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and L-glutamine, without sodium pyruvate; Mediatech; 10% heat-inactivated fetal bovine serum (FBS; VWR); gentamicin, 50 μg/ml; VWR) were grown overnight in 96-well plates ($4 \times 10^4$ cells/well; 85% final confluence) at 37˚C, 5% $CO_2$. HSV-1 or HSV-2 at a multiplicity of infection (MOI) of 50 was added to washed cells (Opti-MEM with HEPES, sodium bicarbonate and L-glutamine; Gibco) then incubated for various times. After viral infection, the cells were washed once with PBS then HBSS before incubation with *C. albicans* or *S. aureus* (5:1 target to cell ratio; n = 24). After incubation (30 min; 37˚C; 5% $CO_2$), cells were washed to remove unbound microbes (PBSx3) and lysed (RIPA, Life Technologies, 1:50 dilution; filter sterilized). The lysate (50 μl) was spread and plated onto mannitol salts and/or Fungisel agar to select for *S. aureus* and *C. albicans*, respectively. Controls consisted of HSV-uninfected HeLa cells handled as described for virus-infected HeLa cells. For each experiment, there was a separate control plate to confirm the viral MOI. In addition, maintenance of cell viability (trypan blue dye exclusion) was performed over the course of infection. Buffer controls showed no change in either staphylococcal or candidal CFU/ml during the 30 min incubation period.

## 2.3. Expression of HSV, *C. albicans* and *S. aureus* Co-Receptors

HSV-1 and HSV-2 effect on expression over time of receptors shared with *C. albicans* and *S. aureus*, *i.e.* HSP60 (mitochondrial chaperonin) [6] [7], CD36 (platelet glycoprotein 4, fatty acid translocase (FAT)), and α5B1 (integrin and primary receptor for fibronectin) [6] was determined by immunofluorescent microscopy imaging studies. Cells were infected with either HSV-1 or HSV-2, then washed

free of non-attached virus (PBSx3) and fixed (methanol). Cells were stained with FITC-conjugated Herpes Simplex Virus Type 1 + 2 gD antibody (OriGene), and 4',6-diamidino-2-phenylindole (DAPI; Life Technologies) to stain for nucleus. Cellular receptor staining was performed using mouse anti-HSP60, -CD36, or -α5β1 primary antibody. Secondary antibody used was Alexa Fluor 594 goat anti-mouse (Abcam). Fluorescence of cellular receptors was measured using ImageJ, and fluorescence was normalized to uninfected, virus-free control for each time point and sample (n = 40 total cells; 4 fields over 2 cover slips). In the absence of HSV, no receptor turnover was detected over the course of the assay.

### 2.4. Statistical Analysis

Each adherence time course (n = 24) was repeated once. Each imaging study was conducted twice. CFU were evaluated by analysis of variance (ANOVA; GraphPad InStat 3.10 for Windows, GraphPad Software Inc.). Mean values were considered significantly different at $p < 0.05$. T-test between groups was used to determine differences between fluorescence intensity as measured using ImageJ analysis.

## 3. Results

### 3.1. HSV-1 Regulation of *Candida* and *S. aureus* Adherence

To control colonization of pathogens on mucosal surfaces it is important to define the polymicrobic interactions that can occur. To accomplish this, an examination of factors regulating adherence, the initial step in biofilm formation, are required. Over the time period tested which represents the entry stage of HSV, *S. aureus* adherence (n = 24) was reduced as compared to HSV-1 free controls (**Figure 1(a)** and **Figure 1(c)**). The level of HSV-1 inhibition of *S. aureus* adherence reached the level of significant adherence reduction around 120 - 135 min ($p < 0.05$) post-HSV-1 infection. After this point, adherence cycled over the next 30 min returning to nearly control levels, before exhibiting significant reduction at 180 min. In contrast, yeast adherence (n = 24) to HSV-1 infected cells was relatively unchanged for the first 75 min as compared to uninfected controls (**Figure 1(b)** and **Figure 1(d)**). However, levels significantly ($p < 0.05$) increased (2-fold) at 90 min before decreasing to its original level by 105 min, with the pattern repeated at 180 min. During this time period, there was no decrease in cell viability that would account for changes in microbial adherence.

### 3.2. HSV-2 Regulation of *Candida* and *S. aureus* Adherence

Staphylococcal adherence to HSV-2 infected cells was also inhibited (**Figure 2(a)** and **Figure 2(c)**). The pattern of inhibition of *S. aureus* adherence by HSV-2 was immediate with a significant reduction ($p < 0.05$) by 15 min with adherence levels cycling to control levels between 75 to 150 min. The adherence level at 30 min was also similar to that of the staphylococcal control before decreasing to 0.65 of virus-free control at 165 to 180 min. The level of *C. albicans*

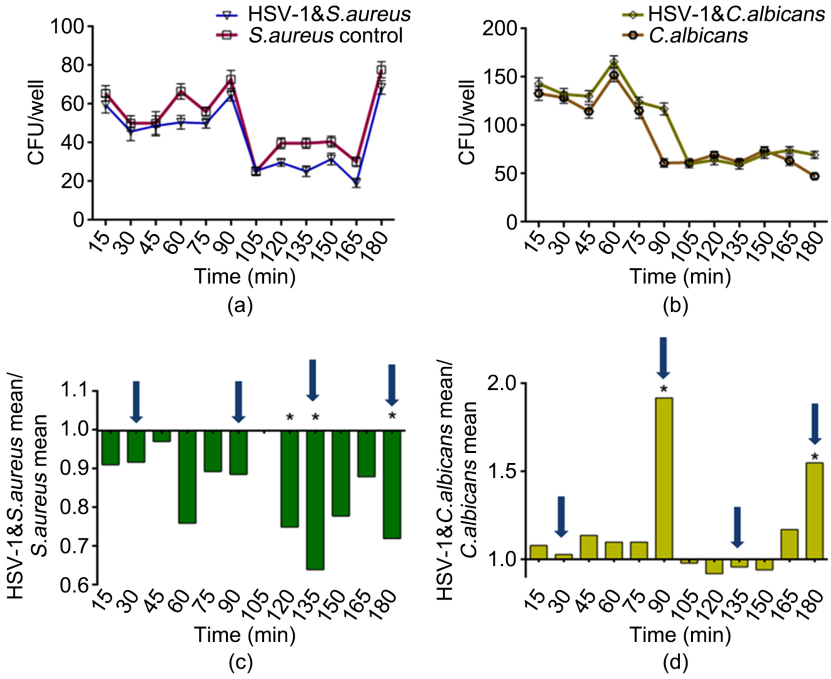


**Figure 1.** Kinetics of *Staphylococcus aureus* and *Candida albicans* adherence to HSV-1 infected HeLa 229 cells. HeLa 229 cells were infected with HSV-1 (KOS) gL-86 and co-incubated with (a) *S. aureus* or (b) *C. albicans*, for various time points. The adherence level of co-HSV-1 infection was normalized to (c) *S. aureus* or (d) *C. albicans* only time-matched control. Each adherence time course (n = 24) was repeated once. * indicates significantly different from virus-free time matched control ($p < 0.05$) as determined by ANOVA. Arrows indicate time points used for immunofluorescent staining.

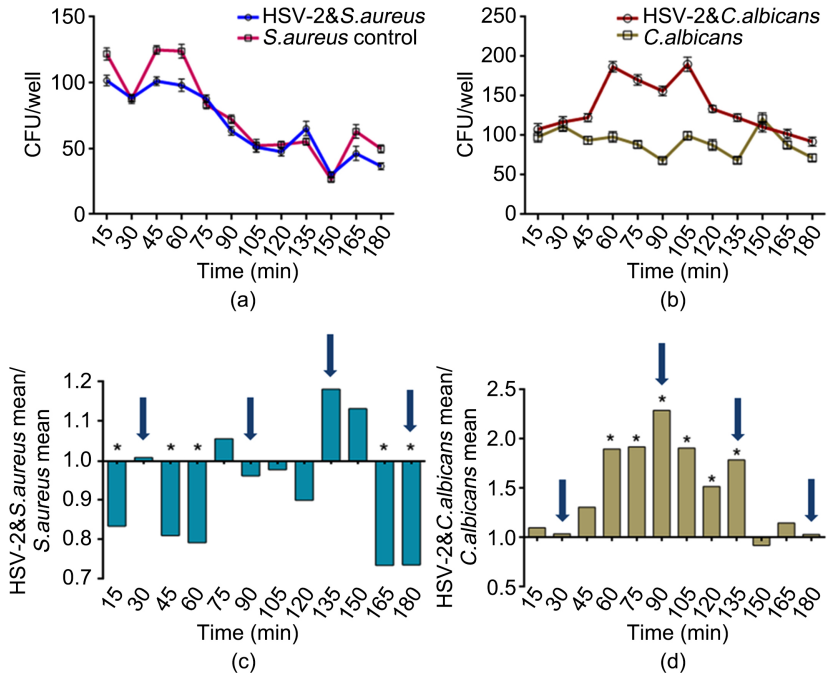


**Figure 2.** Kinetics of *Staphylococcus aureus* and *Candida albicans* adherence to HSV-2 infected HeLa 229 cells. HeLa 229 cells were infected with HSV-2 (KOS) 333gJ- and co-incubated with (a) *S. aureus* or (b) *C. albicans*, for various time points. The adherence level of co-HSV-2 infection was normalized to (c) *S. aureus* or (d) *C. albicans* only time-matched control. Each adherence time course (n = 24) was repeated once. * indicates significantly different from virus-free time matched control ($p < 0.05$) as determined by ANOVA. Arrows indicate time points used for immunofluorescent staining.

adherence to HSV-2 infected HeLa cells was significantly enhanced ($p < 0.05$) at 60 to 135 min before returning to HSV-2 free control levels (**Figure 2(d)**).

### 3.3. Kinetics of HSV-1/2, *S. aureus* and *C. albicans* Co-Receptor Expression

Herpes viral infection of HeLa cells causes a dynamic expression of receptors shared by HSV-1/2, *S. aureus* and *C. albicans*, with the kinetics of receptor expression different between the viruses (**Figure 3(a)**, **Figure 3(b)**, **Figures S1-S6**). Based on the CFU adherence studies (**Figure 1**) HSV-1 had minimal effect on either *S. aureus* or *Candida* adherence. Since the receptors chosen were detectable despite the depression of *S. aureus* adherence, receptor display was measured for those time periods which best reflected changes in enhanced adherence for *C. albicans*. Over time, HSV-1 regulated expression of α5β1, CD36 significantly ($p < 0.05$) increased. HSP60 expression significantly ($p < 0.05$) increased by 30 min to maximally measured levels where it remained over the course of the assay. However, the pattern of receptor expression measured does not correlate with the pattern of *S. aureus* or *C. albicans* adherence to HSV-1 infected cells. The inverse correlation between staphylococcal adherence and receptor expression likely indicates internalization of required receptor upon viral endocytosis. HSV-2 associated with HeLa cells was higher at the same MOI (50) than HSV-1 (**Figure S4-S6** and **Figure S1-S3**, respectively). This difference in viral entry pattern parallels that of α5β1 and CD36 receptor expression (~4-fold increase and ~4 - 6-fold increase, respectively). However, HSV-2 regulation of receptor expression like that of HSV-1 does not appear to directly correlate with either *S. aureus* or *C. albicans* adherence.

## 4. Discussion

The co-habitation of HSV with other taxonomic members of the microbiome, *i.e.* bacteria and fungi, occurs commonly on the buccal and tongue surfaces [8] [9] [10] [11]. The communal interaction between these microbes is also of importance in disease states such as atopic dermatitis (eczema herpeticum) where there is an association between HSV infection causing increased staphylococcal skin disease as well as geographically distal candida infections [24] [25] [26]. On these surfaces, the intermittent viral shedding, either during asymptomatic or symptomatic infections, likely causes an alteration in the display of available receptors to which other members of the resident microbiome biofilm would attach. This differential display of receptors could be due to either viral occupation of specific receptors during their attachment process, or conversely, as a result of exposure or internalization of receptors during virus endocytosis [27] [28]. Alterations in expression of receptors shared between HSV, *S. aureus* or *C. albicans* could have a significant impact on infectious processes.

HSV utilizes a variety of receptors in the initial stage of its entry process including α5β1, CD36, HSP60 and fibronectin, which are receptors HSV-1/2

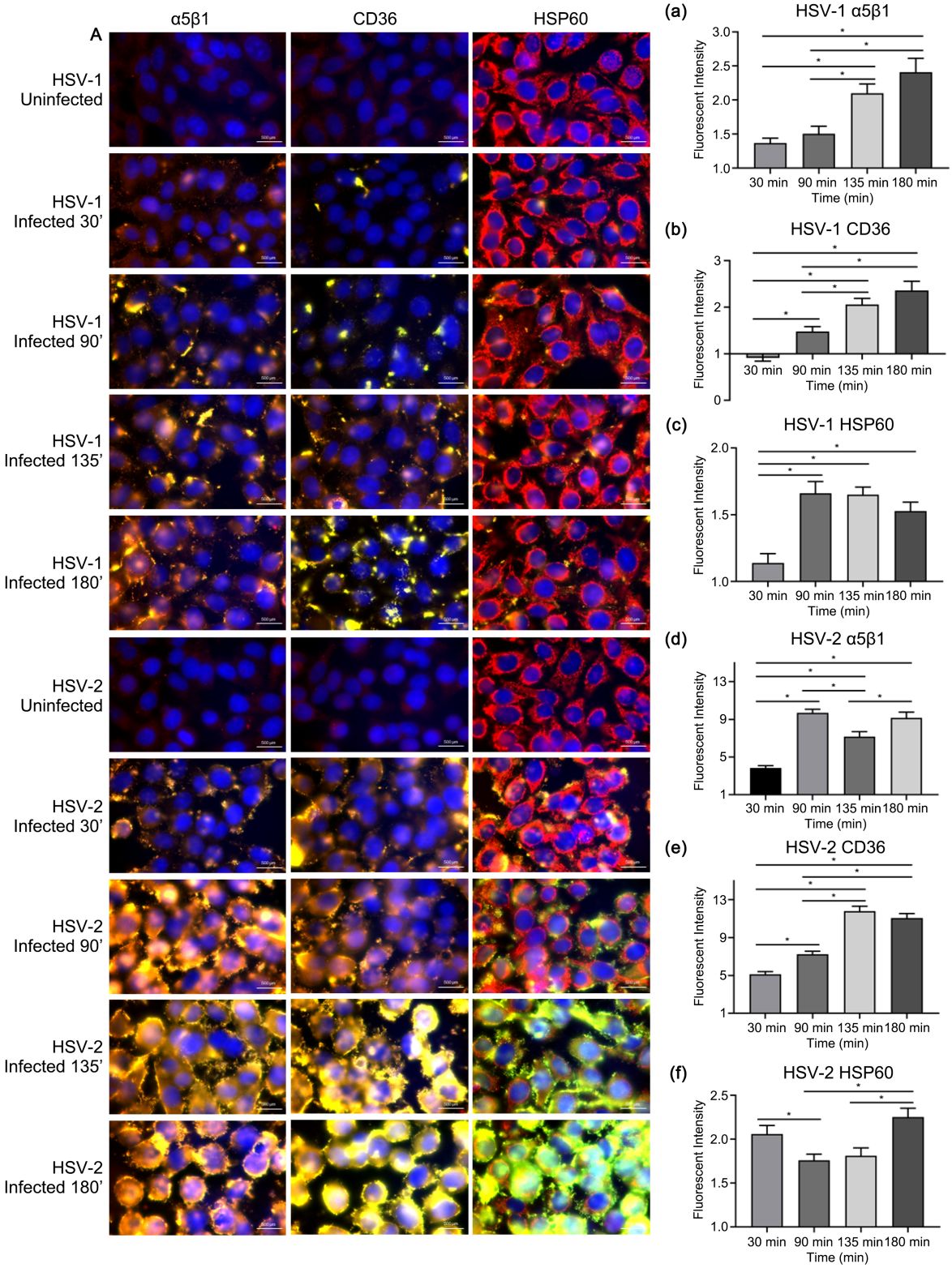


**Figure 3.** Kinetics of HSV-1/2 *Candida* and *S. aureus* shared cell receptor display (A) HeLa 229 cells infected with either HSV-1 or HSV-2 for various time points, followed by fixation and immunofluorescent staining of nucleus (DAPI), HSV-1 or HSV-2, and cellular receptors α5β1, CD36, and HSP60. DAPI, HSV-1/2 and cellular receptor merged images are shown. Scale bar (A) represents 500 μm. ((a)-(f)) α5β1, CD36, and HSP60 fluorescence was measured using ImageJ, and normalized to uninfected, virus-free control for each time point and sample (5 fields; n = 10/field). * indicates significant difference ($p < 0.05$).

shares with *S. aureus* and *C. albicans* [29]-[46]. A distinct advantage in the utilization of HeLa cells for the study of microbiome interactions is that these cells lack surface fibronectin expression, which *in situ* is also differentially lacking on the apical surface of mucosal epithelia [47]-[54]. Thus, initial interactions by the microbial mucosal biofilm with HSV-infected cells can be examined in the absence of this confounding extracellular matrix material. Defining the kinetics of virus-mediated receptor display, relative to the interaction between virally infected cells and putative members of the microbiome in cell association, i.e. adherence, is an important first step in characterization of the virus-regulated microbe membership. The findings for *S. aureus* and *C. albicans* adherence to HSV-1 or HSV-2 infected cells demonstrate that it is a dynamic virus-specific process. Interestingly, the dynamic display of virus-microbe shared receptors α5β1, CD36 and HSP60 does not correlate with adherence patterns for either *S. aureus* or *C. albicans* despite their shared receptors [47] [55] [56]. Thus, alternative receptors may be involved in regulating subsequent *S. aureus* or *C. albicans* adherence to HSV-infected cells. To date, most studies measuring microbial adherence to living substrate are done at a "point-in-time", as was the foundational study measuring HSV-staphylococcal-candidal interaction (180 min) [12]. The importance of measuring the kinetics of complex interkingdom interactions in biofilm formation as they pertain to a dynamic substrate, such as a living cell membrane, has been shown by the findings herein. This observation also illustrates the complexity in dissecting apart the interactions that take place between members of the microbiome, especially since the virion endocytic process could result in the masking or unmasking of alternative receptors.

## 5. Conclusion

Further studies are needed to clarify this complex relationship between microbiome members, particularly with regards to additional specific *S. aureus* or *C. albicans* receptors that could be hidden, or unmasked, as part of HSV's cell entry process [12]. These studies also further support the previous findings that HSV-*S. aureus* has an antagonistic relationship while that of HSV-*Candida* is a supportive interaction, relative to adherence. Characterization of how HSV regulates microbiome membership in HeLa cells may prove useful in identifying factors that can be applied to studies of candidal or staphylococcal colonization of HSV infected keratinocytes towards the elucidation of the pathophysiology of specific diseases such as eczema herpeticum.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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## Supplemental Data

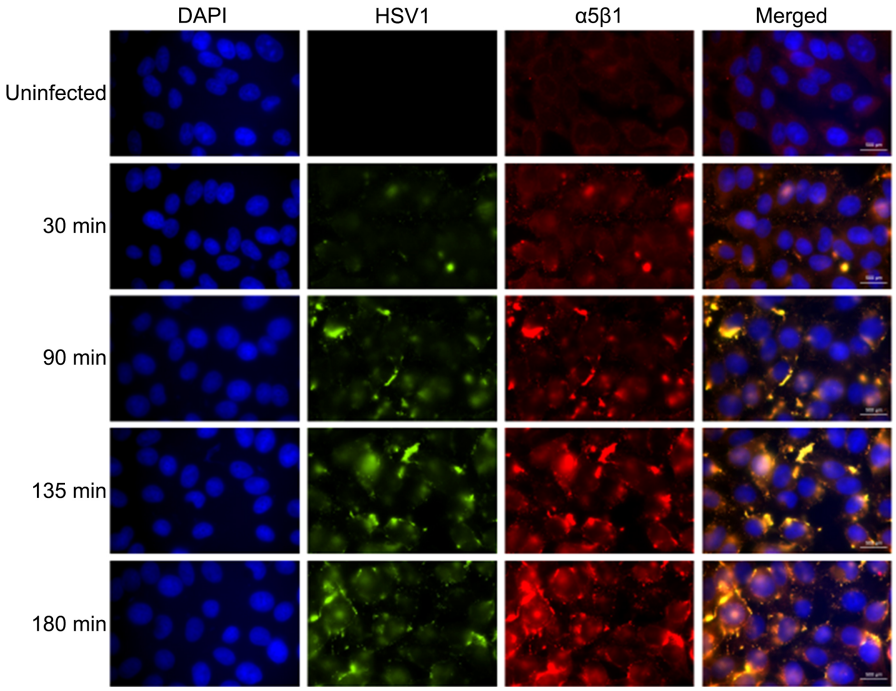


**Figure S1.** Kinetics of HSV-1 and α5β1 cellular receptor. HeLa 229 cells were infected with HSV-1 for 30, 90, 135 and 180 minutes, followed by fixation and staining of nucleus (DAPI), HSV-1, and cellular receptor α5β1 by immunofluorescent microscopy. Individual channel images are shown. α5β1 fluorescence was measured using ImageJ, and normalized to uninfected, virus-free control for each time point and sample (40 cells total/4 fields over 2 cover slips). Scale bar represents 500 μm.

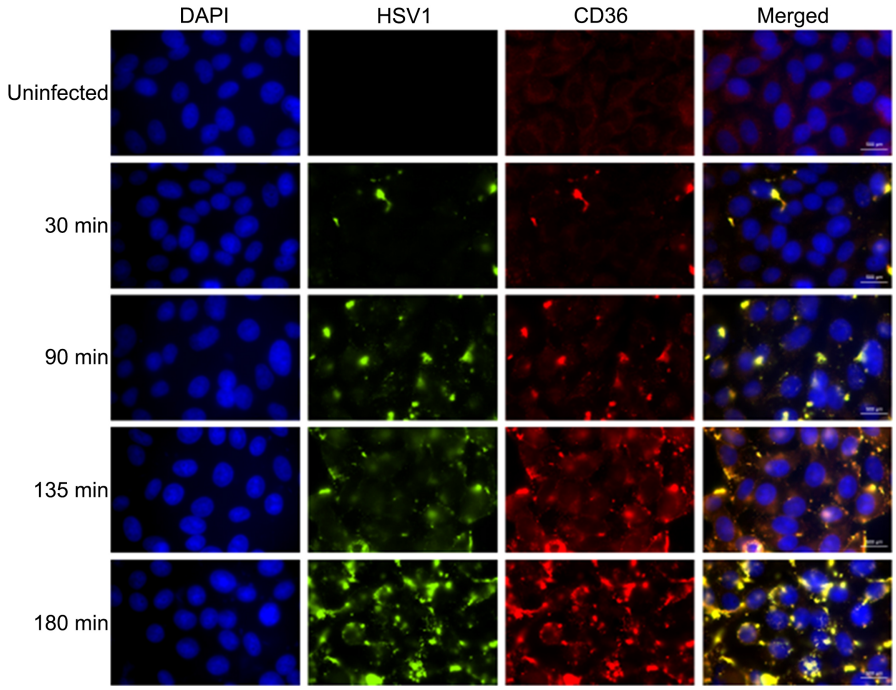


**Figure S2.** Kinetics of HSV-1 and CD36 cellular receptor. HeLa 229 cells were infected with HSV-1 for 30, 90, 135 and 180 minutes, followed by fixation and staining of nucleus (DAPI), HSV-1, and cellular receptor CD36 by immunofluorescent microscopy. Individual channel images are shown. CD36 fluorescence was measured using ImageJ, and normalized to uninfected, virus-free control for each time point and sample (40 cells total/4 fields over 2 cover slips). Scale bar represents 500 μm.

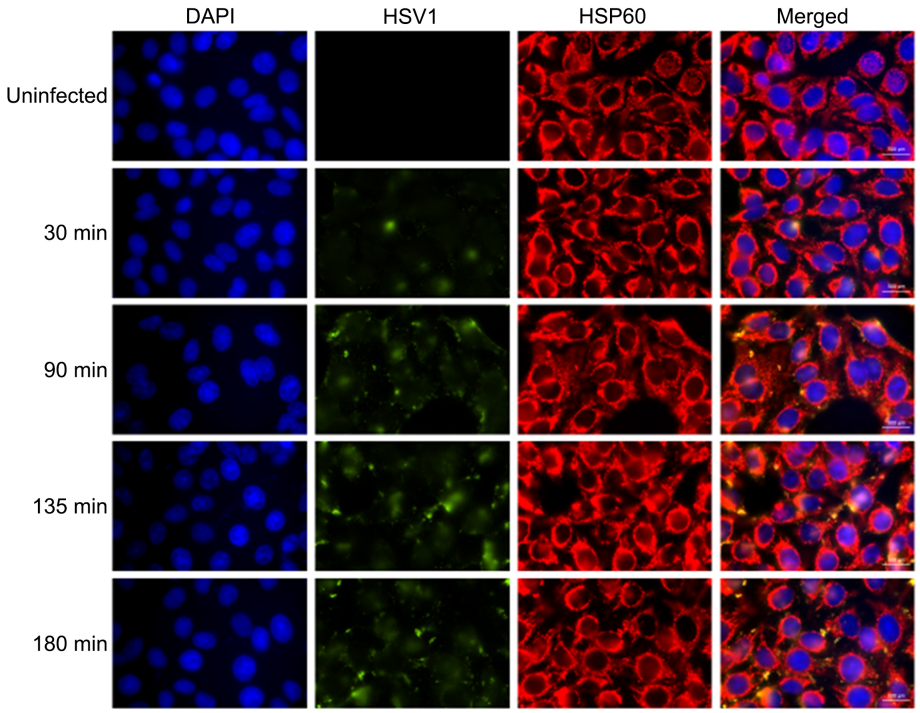


**Figure S3.** Kinetics of HSV-1 and HSP60 cellular receptor. HeLa 229 cells were infected with HSV-1 for 30, 90, 135 and 180 minutes, followed by fixation and staining of nucleus (DAPI), HSV-1, and cellular receptor HSP60 by immunofluorescent microscopy. Individual channel images are shown. HSP60 fluorescence was measured using ImageJ, and normalized to uninfected, virus-free control for each time point and sample (40 cells total/4 fields over 2 cover slips). Scale bar represents 500 μm.

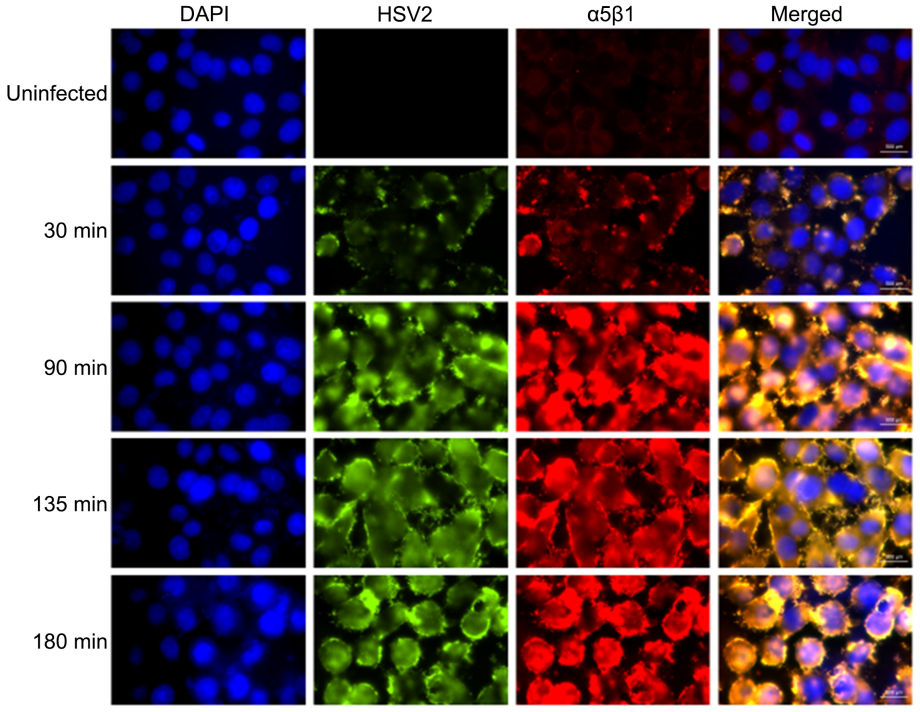


**Figure S4.** Kinetics of HSV-2 and α5β1 cellular receptor. HeLa 229 cells were infected with HSV-1 for 30, 90, 135 and 180 minutes, followed by fixation and staining of nucleus (DAPI), HSV-2 and cellular receptor α5β1 by immunofluorescent microscopy. Individual channel images are shown. α5β1 fluorescence was measured using ImageJ, and normalized to uninfected, virus-free control for each time point and sample (40 cells total/4 fields over 2 cover slips). Scale bar represents 500 μm.

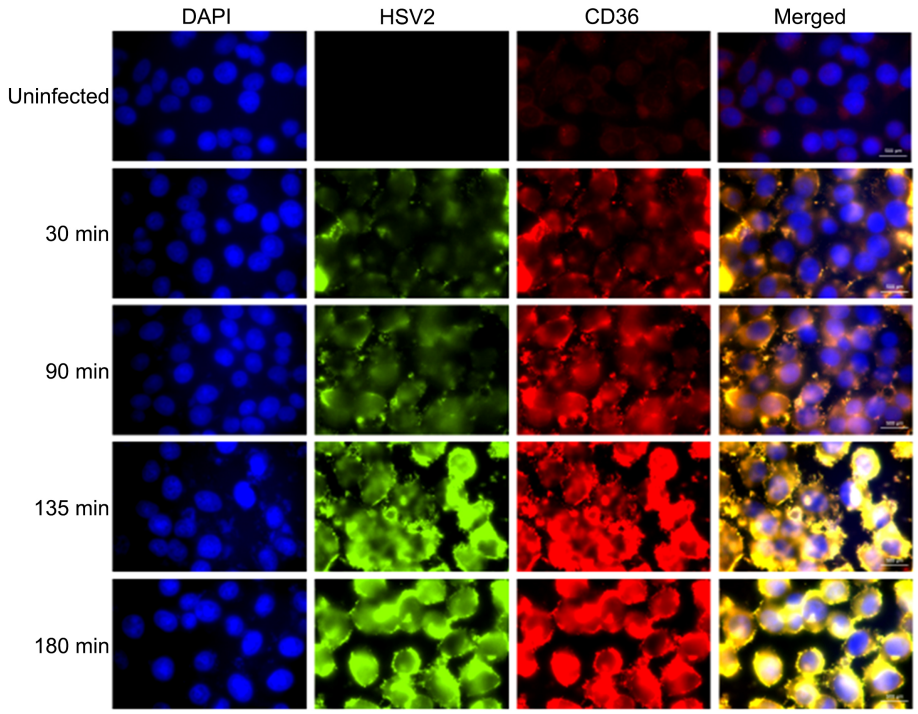


**Figure S5.** Kinetics of HSV-2 and CD36 cellular receptor. HeLa 229 cells were infected with HSV-1 for 30, 90, 135 and 180 minutes, followed by fixation and staining of nucleus (DAPI), HSV-2, and cellular receptor CD36 by immunofluorescent microscopy. Individual channel images are shown. CD36 fluorescence was measured using ImageJ, and normalized to uninfected, virus-free control for each time point and sample (40 cells total/4 fields over 2 cover slips). Scale bar represents 500 μm.

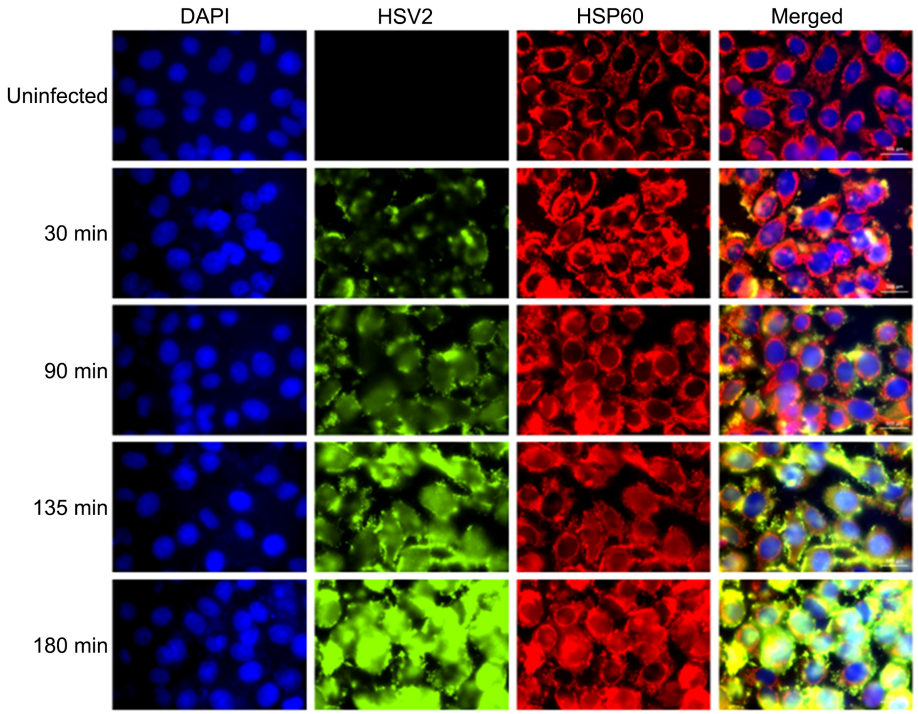


**Figure S6.** Kinetics of HSV-2 and HSP60 cellular receptor. HeLa 229 cells were infected with HSV-1 for 30, 90, 135 and 180 minutes, followed by fixation and staining of nucleus (DAPI), HSV-2, and cellular receptor HSP60 by immunofluorescent microscopy. Individual channel images are shown. HSP60 fluorescence was measured using ImageJ, and normalized to uninfected, virus-free control for each time point and sample (40 cells total/4 fields over 2 cover slips). Scale bar represents 500 μm.