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Exploitation of Skin Innate Immune Defense by Herpes Simplex Virus Type 1

and Vaccinia Virus

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Molecular Pathology

by

Daniel Travis MacLeod

Committee in Charge:

Professor Richard Gallo, Chair
Professor Victor Nizet
Professor Douglas Richman
Professor Robert Schooley
Professor Benjamin Yu

2012
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Chair

University of California, San Diego

2012
Dedication

I dedicate this dissertation to my family

for their unwavering support throughout my education.
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List of Abbreviations

ADAR1 – Adenosine deaminase, RNA-specific
AIM2 – Interferon-inducible protein absent in melanoma 2
AMP – Antimicrobial peptide
BMDM – Bone marrow-derived macrophages
Chx – Cyclohexamide
CNS – Central nervous system
COLEC12 – Collectin sub-family member 12
CS – Chondroitin sulfate
CSPG – Chondroitin sulfate proteoglycan
CQ – Chloroquine
CVA – Coxsackie A virus
CXCL16 – Chemokine (C-X-C motif) ligand 16
DC-SIGN – Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
Dxs – Dextran sulfate
EH – Eczema herpeticum
EGF – Epidermal growth factor
EV71 – Enterovirus 71
EV – Eczema vaccinatum
Fn – Fucoidan
GAG – Glycosaminoglycan
gB – Glycoprotein B

gC – Glycoprotein C


gD – Glycoprotein D


gH – Glycoprotein H


gL – Glycoprotein L

HAdV5 – Human adenovirus type 5

hBD – Human beta defensin

HCMV – Human cytomegalovirus

HCV – Hepatitis C virus

HIV – Human immunodeficiency virus

HS – Heparan sulfate

HSE – Herpes simplex encephalitis

HSPG – Heparan sulfate proteoglycan

HSV-1 – Herpes simplex virus type-1

HVEM – Herpesvirus entry mediator

IFI16 – Interferon gamma-inducible protein 16

IFN – Interferon

IL – Interleukin

IMV – Intracellular mature virus

IRAk2 – Interleukin-1 receptor-associated kinase 2

LAMP – Lysosomal-associated membrane protein 1

LDL – Low-density lipoprotein
LPS – Lipopolysaccharide
LTA – Lipoteichoic acid
MARCO – Macrophage receptor with collagenous structure
MDA-5 – Melanoma differentiation-associated gene-5
MDP – Muramyl dipeptide
MIP-3α – Macrophage inflammatory protein 3α
MSR1 – Macrophage scavenger receptor 1
MTA – Methyl-thioadenosine
MyD88 – Myeloid differentiation primary response gene (88)
NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells
NHEK – Normal human epidermal keratinocyte
NOD – Nucleotide-binding oligomerization domain-containing protein
NLRP3 – NOD-like receptor family, pyrin domain containing 3
OLR1 – Oxidized low density lipoprotein (lectin-like) receptor 1
PAMP – Pathogen-associated molecular pattern
PFU – Plaque forming unit
PGN – Peptidoglycan
PKR – Protein Kinase R
PLA – Proximity ligation assay
Poly(I) – Polyinosinic acid
Poly(I:C) – Polyinosinic:polycytidylic acid
Poly(C) – Polycytidylic acid
PS - Phosphatidylserine

PRR – Pattern recognition receptor

RIG-I – Retinoid acid inducible gene-I

RLR – RIG-I-like helicase receptors

SC – Stratum corneum

SCARB1 – Scavenger receptor class B, member 1

SCARB2 – Scavenger receptor class B, member 2

SCARA3 – Scavenger receptor class A, member 3

SCARA5 – Scavenger receptor class A, member 5

SCARF1 – Scavenger receptor class F, member 1

SCARF2 – Scavenger receptor class F, member 2

Sp1 – Specificity protein 1 transcription factor

SRCR – Scavenger receptor cysteine rich

STAB - Stabilin

STAT1 – Signal transducer and activator of transcription 1

TGFBR3 – Transforming growth factor beta receptor III

TNF – Tumor necrosis factor

TRAF6 – TNF receptor associated factor 6

TRIF – TIR-domain-containing adapter-inducing interferon-β

TLR – Toll-like receptor

USP7 – Ubiquitin-specific-processing protease 7

VIG – Vaccinia Immune Globulins
**VV** – Vaccinia virus

**ZBP1** – Z-DNA-binding protein 1
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written in the following pages, and I can honestly say I would not be the scientist and person I am today if I had not met her.
Vita

EDUCATION

2006-2012 Ph.D., Molecular Pathology
University of California, San Diego, La Jolla, CA, USA
Department of Medicine, Division of Dermatology
Advisor: Professor Richard Gallo

2001-2005 B.A., Double Major in Biochemistry & Psychology
Weinberg College of Arts and Sciences
Northwestern University, Evanston, IL, USA

WORK EXPERIENCE

09/2004-06/2005 Research Technician
Evanston Northwestern Healthcare, Research Institute
Supervisors: Dr. Mary-Jo LaDu, Dr. W. Blaine Stine

09/2003-04/2004 Laboratory Technician
Departments of Psychology, Neurobiology, Northwestern University
Supervisors: Dr. Aryeh Routtenberg, Dr. Jerome Rekart

ACADEMIC HONORS AND AWARDS

2012 San Diego Dermatology Research Day, Best Research Talk Award

2010 Med-Into-Grad Fellowship, Howard Hughes Medical Institute
Albert Kligman Travel Fellowship, *International Investigative Dermatology Conference*

**SCIENTIFIC AFFILIATIONS**

American Society for Microbiology
American Society for Virology

**RESEARCH**

**Original publications**

MacLeod, D.T., Nakatsuji, T., and Gallo, R.L. Vaccinia virus binds to scavenger receptors on the surface of keratinocytes. (In Preparation)

MacLeod, D.T., Nakatsuji, T., Yamasaki, K., Kobzik, L., and Gallo, R.L. HSV-1 Exploits the Innate Immune Scavenger Receptor MARCO to Enhance Epithelial Adsorption and Infection. (Submitted)


Book Chapters:

Scientific Talks


MacLeod, D.T., and Gallo, R.L. Keratinocyte cell surface heparan sulphate proteoglycans are required for uptake of double-stranded RNA prior to toll-like receptor 3 activation. 70th Annual Meeting of the Society for Investigative Dermatology, Atlanta, Georgia, USA, 2010.

MacLeod, D.T., and Gallo, R.L. Herpes simplex virus type 1 exploits scavenger receptors to infect human keratinocytes. Abstract for oral and poster presentation. 69th Annual Meeting of the Society for Investigative Dermatology, Montreal, Quebec, Canada, 2009.


Posters

MacLeod, D.T., and Gallo, R.L. Keratinocyte cell surface heparan sulphate Proteoglycans are required for uptake of double-stranded RNA prior to toll-like receptor 3 activation. 70th Annual Meeting of the Society for Investigative Dermatology, Atlanta, Georgia, USA, 2010.

MacLeod, D.T., and Gallo, R.L. Herpes simplex virus type 1 exploits scavenger receptors to infect human keratinocytes. 69th Annual Meeting of the Society for Investigative Dermatology, Montreal, Quebec, Canada, 2009.

ABSTRACT OF THE DISSERTATION

Exploitation of Skin Innate Immune Defense by Herpes Simplex Virus Type 1 and Vaccinia Virus

by

Daniel Travis MacLeod

Doctor of Philosophy in Molecular Pathology

University of California, San Diego, 2012

Professor Richard Gallo, Chair

Herpes Simplex Virus Type 1 (HSV-1) and Vaccinia Virus (VV) are two viruses that can infect the skin and occasionally cause severe disease, especially in patients with deficient cutaneous immunity. Chapter I of this dissertation discusses HSV-1 and VV infection and what is known about specific host-virus interactions that are critical to the pathogenesis of infection. One important determinant of pathogenesis is the presence of specialized innate immune receptors such as the scavenger receptor Macrophage Receptor with Collagenous Structure (MARCO), which facilitates the activation of Toll-Like Receptor (TLR)-3 by viral nucleic acids, promoting inflammatory responses to clear infections. We initially hypothesized that activation of TLR3 would inhibit HSV-1 and VV infection in keratinocytes. Testing this hypothesis led to the interesting finding that despite the role of MARCO in innate immune
responses, this receptor is usurped by HSV-1 to enhance infection of keratinocytes. We establish this is Chapter II by specifically targeting the interaction of HSV-1 and MARCO. Pharmacological inhibitors of MARCO prevented HSV-1 adsorption and infection of keratinocytes and reduced the size of lesions in a murine model of severe HSV-1 skin infection. Furthermore, HSV-1 co-localized with MARCO on the surface of keratinocytes, and a specific glycoprotein from the viral membrane, HSV-1 glycoprotein C (gC), bound directly to MARCO with high affinity. Importantly, increasing the expression of MARCO enhanced infection, while cells lacking MARCO had reduced susceptibility to infection.

We next hypothesized that MARCO could also serve as a receptor for VV infection of keratinocytes. This hypothesis is confirmed in Chapter III by demonstrating that pharmacological inhibitors of MARCO had potent antiviral activity, VV bound directly to MARCO, and MARCO expression also correlated with VV infection.

Taken together, this work identifies that an important component of the innate immune system can be exploited by HSV-1 and VV to enhance cutaneous virulence. This increases our knowledge of the basic events guiding early steps of infection in the skin, enhances our understanding of host-pathogen interactions occurring at this epithelial surface, and identifies MARCO as a new therapeutic target for the treatment of HSV-1 and VV skin infections.
Chapter I

Introduction:

Cutaneous HSV-1 and VV infection: What factors determine pathogenesis?
Summary

Human skin is constantly exposed to a variety of pathogens, including viruses such as HSV-1 and VV that can infect the skin and cause significant disease. The skin utilizes multiple strategies to prevent infection by such pathogens, and many of these strategies rely on the ability of the target cells to recognize foreign pathogens using pattern recognition receptors (PRRs) such as TLRs, and respond by the production of antiviral effector molecules. Conversely, HSV-1 and VV have evolved strategies of their own to rapidly gain access to target cells, avoid recognition, and impair host responses. The interplay of these factors ultimately plays a fundamental role in determining the pathogenicity of infection by HSV-1 or VV, and further characterization of these elements is an essential step toward controlling severe infections in the skin by HSV-1 and VV. It is also becoming increasingly clear that infection with both HSV-1 and VV differs in accordance with the cell type studied, highlighting the need for additional studies utilizing primary cells such as keratinocytes, which represent the vast majority of the cells in the skin and are a major target for these viruses during cutaneous infection.
**HSV-1 and VV are important skin pathogens**

Human skin is a dynamic environment that is constantly exposed to a range of organisms and potential pathogens including bacteria, viruses, fungi, parasites, and even insects (reviewed in (1)). The skin serves as a barrier to ensure that most of these microorganisms can be hosted or encountered without causing disease, although the frequency of these interactions does increase the likelihood of cutaneous infection. These infections are mostly limited to the skin, but some pathogens can occasionally spread systemically, increasing the severity of infection.

Pathogenicity of the many inhabitants of the skin is determined by both the virulence of the individual pathogen and the status of the skin surface on which it resides. Viruses are not typical residents of the skin, but exposure to these pathogens is frequent, presenting a formidable challenge for host defense systems (2). Cutaneous viral infections have a variable course of disease, often being cleared after a self-limiting infection, but some viruses may persist, causing long-term discomfort or life-threatening conditions, especially in individuals with compromised cutaneous immunity (2-6). Herpesviruses and poxviruses are particularly dermotropic, capable of infecting the skin and causing clinically relevant disease.

HSV is likely the most common virus infecting human skin (reviewed in (2, 7)). This large, enveloped DNA virus is divided into types 1 and 2 on the basis of genetic differences, although considerable homology exists in the
genomes of HSV-1 and HSV-2 (7). In immunocompetent hosts, HSV causes mucocutaneous infections, with HSV-1 more commonly localized to the oral mucosa and HSV-2 more commonly infecting the genital mucosa, although both types can infect either site (8). Additionally, herpetic whitlow is the name for a lesion caused by HSV on the finger or thumb, resulting from contact with either oral or genital lesions (8, 9). Infection with HSV can cause watery blisters at the site of infection that are typically confined to the upper layer of the skin, the epidermis, eventually progressing to form a scab that is characteristic of herpetic disease. During primary infection, the virus can also enter nerves innervating the site of infection and establish a latent phase of infection in neural ganglia, allowing the virus to avoid immune detection and clearance (reviewed in (7, 10)). Subsequent reactivation of the virus results in the virus traveling back down the nerves to the epithelial surface, causing the reoccurrence of lytic infection.

HSV can also occasionally cause life-threatening infections. Herpes simplex encephalitis (HSE) is an infection of the temporal lobes of the brain that can occur following reactivation of HSV (reviewed in (10)). HSE is fatal in the majority of untreated cases, and even with appropriate medical care, HSE still has a high mortality rate and a high incidence of serious neurological damage. Furthermore, individuals with altered skin immunity such as atopic dermatitis (AD) are predisposed to developing eczema herpeticum (EH), a disseminating HSV infection defined locally by an eruption of dome-shaped
vesicles on lesional skin, but is also associated with systemic symptoms including fever and malaise (reviewed in (6)). Significant mortality rates can accompany EH as a result of systemic viremia. Additionally, HSV infections in immunocompromised patients are often severe and prolonged, and carry an increased risk of morbidity and mortality (11). Moreover, in the oral mucosa, recurrence of HSV infection in immunocompromised patients is more frequent and aggressive (12). Finally, neonatal HSV infections are extremely serious and associated with high mortality (13). For these reasons, HSV-1 infection remains a serious public health issue and a highly relevant skin pathogen.

VV is a large, enveloped DNA virus best-known for its role as a protective vaccine against smallpox (reviewed in (14)). VV infects a variety of cells including keratinocytes and is widely studied as a prototypical member of the poxvirus family, preferred for the ease with which it can be cultured and this wide tissue tropism. Despite the astonishing success of VV as the first vaccine responsible for the eradication of a human disease, and the typically mild infection following exposure, vaccinia inoculation can have a number of potentially harmful effects, cautioning the widespread use of this vaccine (15). The eradication of smallpox in 1977 eliminated the need for widespread vaccination, although vaccination is still commonplace in the military (16, 17), highlighting the need to continue studying this virus and developing new ways of controlling severe infections. Complications of VV inoculation can include a wide range of local and systemic symptoms (15). Patients with AD are
especially susceptible to developing eczema vaccinatum (EV), a disseminating infection associated with a high mortality rate (18-20). Children and immunocompromised patients are also at increased risk of severe complications following contact with VV (21-23). For this reason, AD patients, children, and immunocompromised individuals are excluded from vaccination, however severe infections can even occur in individuals who come into contact with a person who was recently vaccinated (24, 25), emphasizing the need for a cautious approach to vaccination.

HSV-1 and VV are clearly important pathogens possessing the capacity to cause significant disease in the skin. However, whether contact with these viruses results in clinically relevant infection depends on a variety of important factors, outlined in greater detail below.

**Innate immune detection systems and skin antiviral defense**

Human skin employs numerous strategies to prevent infections by pathogens. The skin barrier has a number of inherent antimicrobial properties and the cells comprising this barrier perform multiple functions to directly inhibit the growth of microbes. Skin cells such as keratinocytes are able to detect pathogens using specialized receptors belonging to the innate immune system, enabling these cells to respond by producing antiviral mediators. Additionally, these cells secrete factors such as cytokines and chemokines responsible for the recruitment and activation of additional effector cells.
These functions all provide protection in the skin against pathogens such as HSV-1 and VV and are discussed in greater detail below.

**The barrier function of the skin**

The first level of protection against potential pathogens such as HSV-1 and VV relates directly to the structure and physical barrier function of the skin. As skin differentiates to form a stratified epithelial layer, the outermost keratinocytes become enucleate and exhibit properties indicating the initiation of programmed cell death (26). Although the effects of this process on viral infection and replication in the skin have not been specifically examined, the consequences of this process can be inferred, as both HSV-1 (27) and VV (28) rely on host cell nuclear function to complete their replication cycle. From these studies, it can be deduced that at some point in the differentiation process, keratinocytes become refractory to infection by HSV-1 and VV. This layer of outermost keratinocytes may in this way prevent access to basal keratinocytes and other skin cells that are more susceptible to infection. Additionally, the stratum corneum (SC) has an abundance of potentially antiviral lipids, peptides, and proteins deposited in the extracellular matrix which may act to inhibit infection by these viruses (29). In addition to these studies, the importance of an intact skin barrier in the prevention of infection by HSV-1 and VV is further underscored by the increased susceptibility to infection in
individuals with certain skin barrier defects such as AD where the physical and antimicrobial barrier function is impaired (3-5, 30-34).

**TLRs and additional intracellular PRRs**

Beyond the overall structure of the skin organ and the antiviral capacities of this organ unit as discussed above, the individual cells comprising the skin contribute to antiviral defense by actively detecting and responding to pathogens. In order to detect potential pathogens, a variety of cells residing within or recruited to the epidermis, dermis, and underlying tissue, including keratinocytes, melanocytes, dendritic cells, macrophages, mast cells, neutrophils, NK cells, and T and B lymphocytes, express PRRs on the cell surface and in intracellular compartments (35-37). These PRRs include TLRs, Retinoid acid inducible gene -1 (RIG-I)-like helicase receptors (RLRs), additional intracellular dsRNA receptors such as protein kinase R (PKR), intracellular nucleotide sensors that act as components of the inflammasome such as Interferon-inducible protein absent in melanoma 2 (AIM2) and Nucleotide-binding oligomerization domain-containing protein (NOD)-like receptor family, pyrin domain containing 3 (NLRP3), and additional cytosolic DNA sensors including Interferon gamma-inducible protein 16 (IFI16) and Z-DNA-binding protein 1 (ZBP1) (reviewed in (35)). The ligands bound by these receptors are called pathogen-associated molecular patterns (PAMPs). Examples of PAMPs include bacterial, viral, and fungal surface proteins and
nucleic acids. Bearing PRRs thus allows cells to rapidly respond to PAMPs at the first stage of contact. Activation of PRRs after binding these PAMPs leads to the production of cytokines, chemokines, antimicrobial peptides, and other molecules involved in innate and adaptive immunity (38), enabling resident skin cells to limit infection and recruit additional effector cells to prevent spreading of the pathogens within the skin and to underlying tissues.

Many of these PRRs have the capacity to recognize and respond to PAMPs derived from viral pathogens, and thus it is not surprising that many have already been implicated in the recognition of HSV-1 and VV. TLR2 can recognize both HSV-1 and VV on the cell surface (39-45). The involvement of TLR2 also indicates that TLR1 and TLR6 may have roles in the detection of these viruses, as these two TLRs form heterodimers with TLR2, however many of the studies listed above did not specifically examine the requirement of TLR1 and TLR6 in HSV-1 and VV recognition. Regarding HSV-1, one study showed that both TLR1 and TLR6 were involved in TLR2 mediated responses using a transfected HEK293T cell line (45), while another study using murine peritoneal macrophages found that TLR6 was not required for TLR2-mediated HSV-1 recognition (39). For VV, Delaloya et al. directly demonstrated the involvement of TLR6, but not TLR1, in VV detection (42). In addition to TLR2, TLR4 has also been found to function on the cell surface to recognize a ligand derived from VV (46). Beyond these cell-surface TLRs, the intracellular TLRs TLR3 (47, 48) and TLR9 (45, 49-51) have both been shown to be involved in
both HSV-1 and VV recognition and responses, while TLR8 is also involved in recognition of VV nucleic acids (52). Collectively, these studies demonstrate that both cell-surface and intracellular TLRs can recognize components of HSV-1 and VV and initiate innate immune responses to infection.

In addition to these TLRs, a number of other PRRs are also important for innate recognition of HSV-1 and VV, particularly intracellular receptors involved in the detection of viral nucleic acids. For example, the RLRs RIG-I (53-56) and Melanoma differentiation-associated gene-5 (MDA-5) (42, 55-57) are both involved in the sensing of HSV-1 and VV nucleic acids. Initially, the RLR LGP2 was thought to be a negative regulator of responses to nucleic acid PAMPs (58), however this remains controversial, as recent studies have shown that this receptor enhances innate immune responses to VV (59). Another intracellular dsRNA receptor, PKR, has also been shown to play an important role in detection of both HSV-1 (60) and VV (61, 62). Intracellular DNA receptor systems also have a critical role in the detection of the DNA genomes of invading HSV-1 and VV virions. One such system is the inflammasome, which is comprised of protein complexes containing components that recognizes PAMPs such as viral dsDNA and promotes inflammatory responses and the maturation of Interleukin (IL)-1β and IL-18 (63). Both the AIM2-containing inflammasome (64) and NLRP3-containing inflammasome (42) have also been proposed to mediate innate responses to VV. HSV-1 also appears to activate the inflammasome, however it is unclear
which PRR is involved in this recognition (65). Additionally, viral DNA can be recognized by alternative cytosolic DNA receptors acting in an inflammasome-independent manner. IFI16 is a cytosolic innate immune sensor for both HSV-1 and VV dsDNA (66), while ZBP1 is an alternative cytosolic dsDNA receptor that mediates responses to HSV-1 (67).

**Scavenger receptors**

Scavenger receptors are a group of receptors classified together based on a shared ability to bind various forms of LDL including oxidized and/or acetylated LDL, and are thus best characterized for their roles in cardiovascular disease (reviewed in (68)). Scavenger receptors are grouped into 8 classes, A-H (Table 1). Scavenger receptors, primarily those belonging to class A and class B, have recently been demonstrated to participate in innate immunity as well, acting in a variety of ways to enable detection and clearance of pathogens. Multiple studies have demonstrated that class A scavenger receptors enable the detection of exogenous nucleic acids by mediating their uptake from the cell surface and presentation to other PRRs in intracellular compartments (Table 2). Scavenger receptors have also been shown to bind to additional PAMPs including components of bacterial cell walls such as lipoteichoic acid (LTA) and lipopolysaccharide (LPS), and these receptors are involved in the unopsonized phagocytosis of a variety of bacteria and yeast by macrophages (Table 2, and reviewed in (69-71)). It is clear from
the number of dsRNA and dsDNA receptors involved in HSV-1 and VV innate immune responses, as described above, that endosomal and intracellular nucleic acid detection are critical for the recognition of both viruses. Thus, scavenger receptors, particularly class A scavenger receptors, would be predicted to contribute to protection against infection by viruses such as HSV-1 and VV. Additionally, although class A scavenger receptors were initially thought to be restricted to macrophages, it has now been discovered that these receptors are expressed on a wide variety of cells, including dendritic cells (72), mast cells (73), and epithelial cells (74), expanding the importance of these receptors in the innate immune system. The expression and function of these receptors has not previously been evaluated in many resident skin cells including keratinocytes.

**Adaptive immune responses**

The early innate immune responses to viral PAMPs also drive subsequent adaptive immune responses to limit the spread of infection and promote clearance of the viruses (75, 76). Indeed, with HSV-1, it appears that initial interactions between the virus and TLRs help guide an adaptive response dominated by proinflammatory and T helper cell type-1 cytokines and cell-mediated immunity through recruitment and activation of macrophages, NK cells, and CD8+ T lymphocytes (77-79). VV is a prized example of adaptive immune activation following initial innate immune
responses because inoculation in the skin elicits a robust immune response resulting in long-lasting humoral and cell-mediated immunity (80). This extraordinary adaptive immune response is responsible for the success of VV as a vaccine protecting against smallpox infection, and has lead to extensive research examining the potential of recombinant VV to serve as an adjuvant for immunotherapy targeting important skin diseases including melanoma (81).

**Unresolved questions regarding cutaneous antiviral immunity**

Further experiments are needed to resolve important unanswered questions regarding innate immune recognition and cutaneous infection by HSV-1 and VV. Although keratinocytes express a number of innate immune receptors enabling rapid responses to cutaneous pathogens, and giving them the potential to recognize and respond to infection by HSV-1 and VV (Figure 1), it remains unknown which receptors are most essential for recognition of HSV-1 and VV infection of the skin and optimal initiation of innate immunity. Moreover, which of these early PRR recognition events are most critical for initiating and promoting subsequent adaptive immune responses? Importantly, these PRRs may not be required for protection against infection, but may still be capable of providing protecting against infection if activated by treatment with specific agonists prior to infection. Investigating PRR activation in the context of cutaneous viral infection will provide new information regarding how the skin immune system succeeds or fails in response to infection by HSV-1.
and VV, and may offer new therapeutic approaches to combating infections by these viruses.

**Factors that confer susceptibility to infection in the skin**

Whether a particular virus such as HSV-1 or VV can infect cells and cause disease in the skin depends on interactions between a variety of host and viral molecules in addition to the interactions described above pertaining to innate immune recognition. Early steps in infection require that the target cell expresses the proper repertoire of receptors and the additional cellular machinery needed by the virus to adhere to, gain entry, and replicate in the cells. Additionally, HSV-1 and VV counteract the early innate immune responses outlined in the previous section and thereby enhance their virulence by utilizing a variety of viral molecules specialized at evading host immune detection and responses. Furthermore, these epithelial host-pathogen interactions are also influenced by the cutaneous immune status of the individual, with certain skin conditions such as AD significantly increasing the likelihood and severity of infection by both HSV-1 and VV.

**Cellular receptors for HSV-1, and gaps in our current knowledge**

Many of the factors involved in the binding and entry of HSV-1 in mammalian cells have previously been determined, although it is becoming clear that infection is cell-type dependent, and a number of these generally
accepted steps are therefore of questionable relevance to understudied cell types such as keratinocytes. HSV-1 infection in general is believed to involve the following: the first step, initial absorption of HSV-1 to the cell surface, is due, at least in part, to an interaction between glycoprotein C (gC) in the viral membrane and cell surface heparan sulfate proteoglycans (HSPGs) (82-84). Glycoprotein B (gB) also contributes to HSPG adsorption, and this is particularly important in the absence of gC (85). More recently, Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) was identified as a dendritic cell receptor for both gC and gB, serving as a co-receptor with HSPGs during adsorption (86). Also, a number of cell types deficient in proteoglycan synthesis are still bound and infected by HSV-1 in a gB dependent manner, although a specific receptor has not been identified (87). Following attachment, HSV-1 virions enter some cells, including the commonly used Vero cell line, by direct fusion of the viral membrane with the plasma membrane (88). In other cell types, however, including HeLa cells and CHO cells, fusion is preceded by an endocytosis step (89). Cells lacking receptors required for fusion are still able to mediate uptake of HSV-1 by endocytosis, indicating that currently unidentified receptors participate in this infectious step (90). Whether occurring at the plasma membrane, or within an endosome, membrane fusion absolutely requires four viral glycoproteins, gB, glycoprotein D (gD), and glycoprotein H (gH) and glycoprotein L (gL) heterodimers (gHgL) (91-94). Two of these proteins, gD and gB interact with
distinct cell surface receptors during this process. Currently, three such receptors have been identified for gD: tumor necrosis factor (TNF) receptor superfamily member 14 (also referred to as herpesvirus entry mediator (HVEM)) (95), poliovirus receptor-related protein 1 (commonly referred to as Nectin-1) (96), and 3-O-sulfated heparan sulfate, a rare modified isoform of heparan sulfate (97, 98). Three receptors have been identified for gB, paired immunoglobulin-like type 2 receptor alpha (99), myelin-associated glycoprotein (100) and myosin-9 (also known as non-muscle myosin IIa) (101). After membrane fusion, HSV-1 viral cores are transported to the nucleus, and viral replication rapidly initiates.

The extent to which this vast body of research applies to primary keratinocytes is incomplete but has begun to be evaluated. Many assumptions can be made regarding receptor utilization during infection of keratinocytes by HSV-1 based on the expression of receptors on keratinocytes that have been shown to be involved in HSV-1 infection in alternative cell types (Table 2). However, further studies are needed to fully delineate similarities and differences in the mechanism of infection in keratinocytes compared to these other more commonly studied cells. As an example, mutant HSV-1 expressing gD at levels less than 1% of WT virus exhibit impaired entry into Vero and HeLa cells, but entry into HaCat keratinocytes or normal human epidermal keratinocytes (NHEK) was not altered (102). Furthermore, one study presented evidence that access to the Nectin-1 receptor favors entry of HSV-1
at the apical surface of polarized human epithelial cells (103), however another study suggests that HSV-1 entry into keratinocytes is primarily via basolateral membranes with no apparent correlation to Nectin-1 expression (104). Additionally, one study proposed that HSV-1 enters keratinocytes via a pH-dependent endocytic pathway(90), in agreement with some, but not all, commonly used cells types, as mentioned above, however a more recent study has demonstrated that HSV-1 can enter keratinocytes by both an endocytic pathway and by direct fusion with the plasma membrane (105). It is currently unknown how interactions of HSV-1 and its cell surface receptors result in alternative pathways of uptake and entry. These contrasting results emphasize the complexity of HSV-1 infection and underscore the need for further research into the cellular and viral requirements guiding infection of keratinocytes.

Incomplete understanding of the cellular receptors for VV

Infection by VV also differs not only depending on the cell type tested, but also on the strain of virus examined. Like HSV-1, the steps in infection include initial binding or adsorption to the cell surface, and membrane fusion, which can occur at the cell surface or following endocytosis (106, 107). Some strains of VV bind initially to HSPGs or chondroitin sulfate proteoglycans (CSPGs), but others infects cells in a Glycosaminoglycan (GAG)-independent manner (108). Five viral proteins have so far been identified for their
involvement in binding to the cell surface. A27 (109) and H3 (110) mediate binding to HSPGs, while D8 binds to CSPGs (111). GAG-independent binding is also possible with certain strains and certain cell types, and involves interactions between A26 and laminin on the cell surface (112), or between L1 and an undetermined receptor (113). The mechanisms of membrane fusion are complex, requiring at least 11 proteins on the surface of VV, and specific cellular proteins involved in this process have not yet been identified (108). Keratinocytes express some of the receptors mentioned above that are presumed to be involved in infection (Table 2), however, given the cell-type dependent characteristics and complexity of infection, further experiments are needed to determine the exact viral and cellular factors enabling VV infection of keratinocytes.

Immune evasion by HSV-1

Immune evasion is another critically important factor contributing to pathogenesis. The primary mechanism of immune evasion for HSV-1 is the latent infection of neurons innervating mucosal sites, where the virus persists in neural ganglions while avoiding the production of antigens that would label the cell as infected, thereby subverting recognition by cytotoxic lymphocytes. HSV-1 also carries a number of genes that contribute greatly to the ability of the virus to avoid immune detection and impede clearance by immune effectors. For example, it has been recently demonstrated that the Us3 protein
inhibits the upregulation of TLR3 in HSV-1 infected cells and may serve as a direct or indirect inhibitor of TLR3 function (114). In addition, the HSV-1 ICP0 protein responds to TLR activation by promoting the activity of Ubiquitin-specific-processing protease 7 (USP7), a host-cell nuclear protein which normally acts to attenuate TLR-mediated signaling events, a function that is exploited by HSV-1 to bring a premature halt to these immune responses (115). Furthermore, two HSV-1 proteins, Us11 and ICP34.5, act to counteract the effects of PKR, with Us11 directly interacting with this PRR and inhibiting its activity (116), and ICP34.5 acting on a major downstream target of PKR (117). Us11 also disrupts RLR-mediated recognition of the virus by interacting with and directly inhibiting RIG-I and MDA-5 (118). HSV-1 also expresses other proteins that interfere with multiple antiviral and pro-inflammatory pathways acting further downstream of these and other PRRs, largely targeting interferon (IFN) production and activity, as well as additional pathways important for controlling HSV-1 pathogenesis (reviewed in (119) and (120)).

**Immune evasion by VV**

VV has a large, extensively analyzed genome that has also been found to encode a number of protein that contribute to immune evasion by inhibiting pattern recognition receptors (PRRs), antiviral signaling pathways, and antiviral effectors. For example, the VV protein A52 prevents TLR-mediated
activation of pro-inflammatory nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways by binding directly to Interleukin-1 receptor-associated kinase 2 (IRAK2) and TNF receptor associated factor 6 (TRAF6), two key molecules in signaling pathways shared by the TLRs (121, 122), while the A46 protein also contributes to VV evasion of TLR-mediated signaling by mimicking a domain found in Myeloid differentiation primary response gene (88) (MyD88) and Toll/Interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF), two adapter molecules that associate with activated TLRs to allow downstream signal transduction (122, 123). Interestingly, a short peptide derived from A46 also possesses the capacity to specifically inhibit signaling via TLR4 by interacting with TLR4-specific adapter and bridging molecules (124). The VV E3 protein appears to have numerous antiviral functions. E3 prevents activation of the intracellular PRR PKR by both binding and sequestering intracellular dsRNA and by directly interacting with PKR (62, 125). E3 also inhibits the ZBP1 receptor by binding to intracellular dsDNA (126), inhibits the important antiviral mediator ISG15 by directly interacting with this protein (127), and prevents signaling via RIG-I/MDA-5 pathways by an unknown mechanism (128). Furthermore, numerous additional VV proteins have been discovered that target components acting downstream of these and other PRRs by inhibiting components of IFN and NF-κB signaling pathways, and by capturing and
inhibiting other important antiviral chemokines and cytokines such as TNF-\(\alpha\) and IL-1\(\beta\) (reviewed in (129)).

**Increased susceptibility to infection in individuals with immune defects**

The presence of these numerous inhibitors of PRRs, their signaling pathways, and downstream effector molecules reinforces the importance of innate immune recognition in controlling the pathogenesis of HSV-1 and VV infection. Further evidence demonstrating the importance of innate immune detection in preventing cutaneous viral infection is derived from studies showing that defects in innate immune responses in the skin have been demonstrated to predispose individuals to developing more severe viral infections by HSV-1 and VV. As mentioned at the beginning of this chapter, patients with AD are susceptible to developing EH and EV, potentially lethal disseminated skin infections caused by HSV-1 and VV, respectively. The human cathelicidin LL-37 is an antimicrobial peptide (AMP) expressed by skin resident cells including keratinocytes, and TLR2 expression and activation is linked to the induction of cathelicidin (130). LL-37 has antiviral activity against both HSV-1 and VV (3, 4, 131), however AD is associated with deficient expression of this AMP (3, 4, 30). Additionally, Specificity protein 1 transcription factor (Sp1) is also expressed at deficient levels in AD skin, and at particularly low levels in patients with a history of EH (132). Expression of Sp1 is required for optimal protection against infection by HSV-1 and VV in
NHEK, limiting infection in part by enhancing the expression of the intracellular PRR PKR (132). AD skin also has deficient expression of S100A11, a protein that is also required for optimal innate immune responses to VV in keratinocytes (133). Furthermore, treatment of cells with IL-4 and IL-13, two cytokines associated with AD, inhibited the expression of human beta defensin (hBD) 3 and Macrophage inflammatory protein 3α (MIP-3α), two molecules with antiviral activity against VV that are also expressed at deficient levels in skin from patients with AD (32, 33, 134).

Additional innate immune defects have been linked to severe infection with HSV-1. Patients with genetic defects in TLR3 (47) and three additional genes involved in TLR3 function (135-137) have increased susceptibility to developing HSE, although these patients are apparently not more susceptible to infection in other organs, suggesting that TLR3 may have a redundant role in protection against infection in other tissues such as the skin.

Unresolved questions regarding susceptibility to infection in the skin

The large number of factors and cell-type-dependent variables contributing to susceptibility to infection complicates the study of HSV-1 and VV. The receptors identified to contribute to infection in alternative cell types have questionable relevance to infection of keratinocytes, thus further studies using keratinocytes are needed to confirm their involvement and identify the most important receptors. Additionally, the presence of a number of viral
inhibitors of PRR recognition and innate immune activation and function makes it difficult to analyze the role of these pathways during infection, as a lack of activation by either virus could be either due to the absence of specific viral PAMPs or could result from the expression of these viral inhibitors of innate immunity. Finally, the expression of viral receptors and specific PRRs may be influenced by skin conditions such as AD, and studies comparing AD skin and normal skin are needed to know if the receptors that confer susceptibility to infection are upregulated in AD skin or if activation of key PRRs is inhibited in AD skin compared to normal skin. Exploring these difficult questions is the key to the development of better therapeutics to control infection by HSV-1 and VV, especially in patients with conditions such as AD that have altered skin immune responses.

**Treatment of HSV-1 and VV infections in the skin**

A number of treatments are available for HSV-1 and VV infections. However, these drugs are not always successful at treating the most severe cases of infection, emphasizing the need for antivirals with greater therapeutic efficacy. It is important to note that many of these treatments work best in conjunction with innate and adaptive immune responses (138), and as such work best in patients with competent immune systems. In addition to traditional antivirals, recent attempts to prevent infection have also focused on immunomodulation and boosting host responses by activating some of the
PRRs and innate immune responses mentioned earlier in the chapter (139, 140), and also by preventing the interaction of these viruses with their cellular receptors and other molecules required for optimal infection.

**HSV-1 treatment**

The most effective drugs for treating HSV-1 are nucleoside analogues, which interfere with viral DNA replication (reviewed in (141)). These include acyclovir, valacyclovir, famciclovir, and penciclovir. Acyclovir was the first to be developed, and is still the most commonly prescribed anti-HSV drug. Typically, these treatments will be administered systemically, which has been shown to reduce symptoms and severity of infection (11). Topical treatment with acyclovir is also available, but the efficacy has not been well established (142). At best, these treatments reduce clinical symptoms of infection and decrease viral shedding, but do not eliminate the virus. Additionally, there are some instances where these antiviral treatments are insufficient to improve the outcome of infection or reoccurrence. In neonatal HSV-1 infections, administration of acyclovir appears to be effective if the disease is limited to the skin, eyes and mouth, but high morbidity and mortality rates are still observed in neonates with disseminated disease or central nervous system (CNS) involvement, even if they receive acyclovir treatment (143, 144). Also, a recent review of clinical studies including over 90 patients below the age of six presenting with primary herpetic gingivostomatitis concluded that there is only
very weak evidence that acyclovir is an effective treatment to reduce the number of oral or perioral lesions, or otherwise reduce symptoms associated with infection (145). Finally, 3.5-10% of HSV isolated from immunocompromised individuals, including those infected with HIV, are nucleoside-analogue resistant, further complicating the challenges involved with treating these patients (141, 146). Beyond these treatments, there is no vaccine available for HSV-1, and the development of an effective vaccine would be difficult due to the prevalence of the disease. Indeed, a vaccine currently undergoing clinical trials for prevention of HSV-2 has only been shown to be effective for patients who have never been exposed to HSV-1 (147). Therefore, the development of addition treatments serving as supplements to enhance the effectiveness of nucleoside analogues, and replace these drugs in cases of limited efficacy, will be critical for the improvement of antiviral therapy in the future.

Some studies have focused on the stimulation of innate immune responses as a way to protect against infection by HSV-1, although few have been conducted in the context of cutaneous infection. Using a mouse model of HSE, one study found a significant reduction in mortality by HSV-1 infection following intraperitoneal and intranasal administration of a TLR3 agonist, and moderate protection against infection by intranasal treatment of a TLR9 agonist (148), although the effects of pretreatment on cutaneous HSV-1 infection were not evaluated. Additionally, a number of TLR agonists have
been found to provide protection against HSV-2 genital infection, including TLR2/6 (149), TLR3 (150, 151), TLR4 (152), TLR7 (138, 153, 154), and TLR9 (155-157) agonists, however it is unclear if these results are translatable to HSV-1 infection of the skin. In addition to acting as standalone immunomodulators, many TLR agonists are also being examined for their ability to act as adjuvants to boost adaptive immune responses (158).

Disrupting the interaction of HSV-1 with vital host molecules such as receptors may be an additional manner of preventing infection by HSV-1. As described above, HSV-1 interacts with distinct receptors to mediate adsorption to the cell surface and entry into cells. Treatment with heparin can inhibit HSV-1 (159) presumably by preventing the adsorption of these viruses to HSPGs. Additionally, antibodies targeting HSV-1 gC can also prevent interaction of this protein with adsorption receptors and prevent infection (160, 161). Furthermore, inhibitors of HSV-1 entry in vitro also have therapeutic efficacy against infection in vivo (162), and antibodies targeting gD (161, 163) and gB (164, 165) prevent entry and infection. Moreover, in vitro studies have demonstrated that targeted downregulation of HSV-1 gE can interfere with the role of this protein in cell-to-cell spread and limit infection of keratinocytes (166).
**VV treatment**

For severe VV infections, limited treatment options include the administration of vaccinia intravenous immunoglobulins (VIGs), and administration of broad-spectrum antiviral drugs such as cidofovir and ribovirin (reviewed in (15, 167)). The biggest problem regarding these drugs is the lack of controlled studies determining their efficacy. All evidence for the antiviral effects of VIG treatment are derived from case reports describing physicians’ opinions of the improvement of VV-infected patients receiving VIG, not on placebo-controlled studies. Despite not having proven efficacy, administration of VIG is recommended by the Center For Disease Control and Prevention for treating complications following vaccination with VV (167). Additionally, while cidofovir has shown antiviral activity *in vitro* and *in vivo*, even in immunocompromised mice, the efficacy in humans has not been extensively characterized (168), and both cidofovir and ribovirin are not currently approved for treatment of VV infection.

Treatment with compounds that stimulate innate immune responses may also be useful in treating severe infections with VV. Agonists of TLR3 (169, 170), TLR7 (171), and TLR9 (172) have shown efficacy against VV infection. Furthermore, administration of the TLR3 agonist Polynosinic:polycytidylic acid (Poly(I:C)) worked best when given in combination with VIG and provided significantly better protection against infection than either treatment alone (170), demonstrating that
immunostimulatory compounds may function best in combination with the traditional antiviral agents described above.

VV infection can also be inhibited by blocking the interactions of specific viral glycoproteins with their cellular receptors. Indeed, it appears that the mechanisms of action of VIG treatment includes blocking receptor interactions, as some of the immunoglobulins comprising this treatment are neutralizing antibodies that target viral glycoproteins, in particular the H3 protein, which is involved in mediating interactions with cell surface HSPGs (173-176). Additional studies have shown that neutralizing antibodies recognizing A27 (174, 177-179), D8 (111, 179), L1 (177, 180), all viral glycoproteins involved in binding to cellular receptors, have been shown to prevent infection by VV to varying extents in vitro and in vivo. Interestingly, one study demonstrated that antibodies recognizing A27 were less potent in vivo compared to antibodies targeting L1, despite having similar neutralizing capacity in vitro (177), highlighting the need for thorough validation of in vitro inhibitory effects.

Moreover, combinations of antibodies appear to have more therapeutic efficacy in vivo compared to treatment with individual monoclonal antibodies, and specific antibodies targeting antigens such as L1, which are not highly represented in VIG, may even be able to provide better protection than VIG (181). Furthermore, pharmacological inhibitors may be therapeutically effective, because heparin treatment can also inhibit VV infection by blocking interactions of A27 and H3 with HSPGs (109, 110), CS can block interactions
of D8 and CSPGs (111), and soluble laminin can inhibit the interaction of A26 and laminin (112). Additional experiments are needed to determine whether blocking these interactions using immunoglobulins or pharmacological inhibitors will prevent the most important receptor interactions to provide therapeutic efficacy in humans.

**Conclusions**

HSV-1 and VV infect keratinocytes and can cause significant disease in the skin. Many factors determine the result of contact with either of these pathogens, and to a large extent, these factors are intrinsically linked together. The barrier function of the skin prevents penetration of these viruses to deeper levels of the skin containing cells that are more susceptible to infection. If this layer of protection is breach, resident skin cells can recognize HSV-1 and VV using receptors that belong to the innate immune system and are capable of recognizing viral proteins and nucleic acids. This field is complicated, however, by the variety of ways in which these viruses have evolved to utilize host molecules to mediate infection, and then subsequently deploy a number of strategies to inhibit recognition by PRRs.

Despite the wealth of knowledge regarding immune responses in the skin and pathogenicity of HSV-1 and VV, there are still critical questions that remain unanswered. Previous studies have identified a number of innate immune receptors that have the capacity to respond to viral PAMPs, however
which of these receptors are most critically important for detection and responses to HSV-1 and VV infection in the skin? Furthermore, of the host proteins that have been usurped to serve as receptors for HSV-1 and VV infection in other cell types, which of these are most relevant to infection of keratinocytes, and how does the interaction of these viruses with receptors promoting infection alter innate immune detection by PRRs? Finally, can greater knowledge of the factors contributing to pathogenesis of HSV-1 and VV infection of the skin lead to the development of targeted approaches to prevent infection, either by prevention of interactions with receptors promoting infection, or by stimulation of responses via PRRs important for defending against infection?

Chapter II begins with the hypothesis that stimulation of innate immune responses by activating TLR3 in keratinocytes will result in reduced infection by HSV-1. Thorough testing of this hypothesis lead to the finding that MARCO, a scavenger receptor that acts upstream of TLR3 to mediate the uptake of viral dsRNA, is surprisingly exploited to serve as a receptor for HSV-1 to promote infection of keratinocytes. In Chapter III, we test the hypothesis that MARCO is also a receptor for VV, and confirm MARCO is exploited by this virus as well. Thus, we identify MARCO as a new molecular target for skin infection by HSV-1 and VV, and discuss the consequences of these interactions for each of these viruses in Chapters II and III, respectively. Finally, in chapter IV, the long-term implications and advances made by these
findings in the fields of cutaneous innate immunity and virology are discussed, and new areas of research opened by these results are presented.
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Chapter II

HSV-1 exploits the innate immune scavenger receptor MARCO to enhance epithelial adsorption and infection
Abstract:

HSV-1 is an important epithelial pathogen and has the potential for significant morbidity in humans. Here we demonstrate that a cell surface scavenger receptor, MARCO, previously known to enhance antiviral defense by enabling nucleic acid recognition, is usurped by HSV-1 to mediate adsorption to epithelial cells. Ligands of MARCO dramatically inhibited HSV-1 adsorption and infection of human keratinocytes and protected mice against infection. HSV-1 virions and purified HSV-1 gC closely co-localized with MARCO at the cell surface, and gC bound directly to purified MARCO with high affinity. Increasing MARCO expression enhanced HSV-1 infection while cells from MARCO⁻/⁻ mice had reduced infection by HSV-1. These findings demonstrate that HSV-1 exploits a vital host defense element to enhance its capacity for disease. This observation provides a new therapeutic target and alters our understanding of the host-pathogen interactions determining pathogenicity of cutaneous HSV-1 infection.
Introduction

HSV-1 infections are a frequent occurrence with the potential to develop into life-threatening disease in immunocompromised individuals. Primary infection typically begins in the skin and mucocutaneous regions by targeting keratinocytes, the most abundant cell type in these tissues. If infection is not controlled, HSV-1 can spread to other target organs including the CNS. Despite the great advances that antiviral therapies have made in reducing HSV morbidity and mortality, it remains of considerable interest to understand in greater detail the elements contributing to HSV-1 virulence to enable a more targeted approach to HSV-1 therapy. Towards this end, the host elements that are utilized by the virus to promote infection, and conversely, elements of the mucocutaneous defense system that act to resist HSV-1 infection, require specific analysis in addition to the immune defense systems that act after systemic spread.

HSV-1 is successful in initiating disease by exploiting and repurposing host-derived molecules to enhance its virulence. The first step in this process is adsorption of the virus to the cell surface, and HSPGs are the cell surface molecules responsible for this process (82). Interestingly, HSV-1 and HSV-2 both bind to HSPGs yet recognize different structural features of heparan sulfate, thus showing that there is some degree of specificity to this interaction (182). gC is the major HSV-1 glycoprotein that binds to HSPGs (84, 183), but gB also contributes to HSPG adsorption, and this is particularly important in
the absence of gC (85). The HSPGs Syndecan-1 and Syndecan-4 are highly expressed by keratinocytes (184) and may serve as adsorption receptors for HSV-1, however studies have not addressed which specific receptors expressed by keratinocytes are most vital for adsorption. Membrane fusion events follow adsorption and additionally involve interactions of HSV-1 gD and gB with the cell surface. Multiple cell surface targets have been identified for viral entry including for gD: 3-O-sulfated heparan sulfate (97), TNF receptor superfamily member 14 (commonly known as HVEM) (96), or poliovirus receptor-related protein 1 (commonly known as Nectin-1) (95), and for gB: paired immunoglobulin-like type 2 receptor alpha (99), myelin-associated glycoprotein (100) or myosin-9 (also known as non-muscle myosin IIa) (101).

To mount an effective antiviral response cells must recognize the virus and initiate an appropriate immune response. Several types of intercellular receptors have been associated with the capacity to detect HSV infection (185). Class A scavenger receptors are an important element in this innate immune detection process as they bind to extracellular viral dsRNA at the cell surface to mediate dsRNA uptake and to enable this dsRNA to interact with TLR3 in the endosome (74, 186-189). Failures of this TLR3-dependent innate immune recognition pathway can have serious consequences. For example, patients with genetic defects in TLR3 (47), and other proteins involved in mediating the TLR3 dependent response to dsRNA (135-137), are predisposed to herpes simplex encephalitis, a severe, disseminated infection
of the CNS, and TLR3 deficiency renders astrocytes more susceptible to HSV infection and CNS disease (190). It is hypothesized that TLR3 is important for recognition of a DNA virus such as HSV-1 because virtually all viruses produce dsRNA during replication (191), and this dsRNA is released into extracellular space following lysis of infected cells (192). Thus, cells expressing TLR3 have the ability to defend against HSV-1 by using this receptor to respond to dsRNA, and this appears to be particularly crucial to innate antiviral defense in the CNS.

Surface epithelial cells are the initial target for viral entry and can activate a potent innate immune response through TLR3 (193-196). We initially hypothesized that NHEK could utilize TLR3 to defend against HSV-1 infection. However, testing this hypothesis led us to the unexpected discovery that instead of contributing to host defense, MARCO, a scavenger receptor with critical actions for TLR3 function, is exploited by HSV-1 to promote cell surface adsorption and infection in the skin.
Results

Scavenger receptor ligands inhibit HSV-1 infection of keratinocytes

Activation of TLR3 increases production of IFNs and other pro-inflammatory cytokines and antimicrobial peptides in skin and other tissues (197-199), and has been frequently linked with cutaneous immune and antiviral responses (193, 200-203). We first sought to determine whether activation of TLR3 could enable NHEK to directly defend themselves against HSV-1 by treating NHEK with Poly(I:C), a synthetic dsRNA (193-196). This treatment resulted in a significant dose-dependent inhibition of HSV-1 infection as assessed by measurement of viral plaque forming units (PFU) (Fig. 2a) and by quantifying levels of HSV-1 gD mRNA (Fig. 2b). However, interestingly, TLR3 activation leading to IFN production appeared to be dispensable for this protection against infection. Type-I IFNs have numerous antiviral functions (204, 205) and thus were initially examined as the potential effectors through which dsRNA conferred protection against HSV-1 following TLR3 activation. Inhibition of IFN-β production by methyl-thioadenosine (MTA) (206), a Signal Transducers and Activators of Transcription 1 (STAT1) inhibitor, did not alter the capacity of Poly(I:C) to reduce HSV-1 gD expression (Fig. 3a,b). These results indicated that although IFN-β has well-characterized antiviral effects, it was not responsible for protection against HSV-1 infection by Poly(I:C) under the conditions tested. We next utilized chloroquine (CQ) to more directly examine the effect of TLR3 inhibition on the ability of Poly(I:C) to protect
against HSV-1 infection, as CQ is known to be a potent inhibitor of endosomal acidification and TLR3 activation (188). As expected, pretreatment with CQ inhibited the capacity of Poly(I:C) to stimulate expression of IL-6 and IFN-β, thus confirming that TLR3 activation was inhibited (Fig. 3c). However, despite inhibition of these cytokine responses, CQ did not reduce the capacity of Poly(I:C) to protect against HSV-1 infection (Fig. 3d), further suggesting that cellular activation mediated by TLR3 was not required for the protective effects of Poly(I:C) under these conditions. Furthermore, treatment with Polyinosinic acid (Poly(I)), a ssRNA component of Poly(I:C) that does not activate TLR3, conferred protection against HSV-1 similar to that seen with Poly(I:C) (Fig. 4a). Poly(I) and Polycytidylic acid (Poly(C)) were not able to increase expression of IL-6, IL-8, IFN-β and hBD-2, four genes that were significantly increased by Poly(I:C) stimulation of NHEK (Fig. 4b). Since Poly(I) was capable of inhibiting HSV-1 infection, but Poly(C) was not, we considered an alternate explanation that could account for the antiviral activity of both Poly(I) and Poly(I:C).

Both Poly(I) and Poly(I:C) are ligands for class A scavenger receptors, a family of cell surface molecules that are required for dsRNA uptake prior to cell activation (74, 186, 187, 207). Poly(C), which did not protect against HSV-1, is not a ligand for class A scavenger receptors. Therefore, we next hypothesized that the capacity to inhibit infection was related to the ability to bind scavenger receptors. To test this hypothesis we examined additional scavenger receptors ligands: Fucoidan (Fn) and dextran sulfate (Dxs). These
were chosen since they also bind scavenger receptors (207) but are structurally distinct from polynucleotides such as Poly(I:C) or Poly(I). Dxs, Fn, and Poly(I) were all able to block Poly(I:C) stimulation of cytokine release (Fig. 5a,b), an observation consistent with their shared ability to block scavenger receptors. Chondroitin sulfate (CS) was used as a control for Dxs and Fn as it is another sulfated polysaccharide, but like Poly(C) it cannot bind scavenger receptors, and had no effect on the capacity of Poly(I:C) to function (Fig. 5a).

Diverse molecules that share the capacity to bind scavenger receptors (Poly(I), Dxs and Fn) all reduced HSV-1 plaque formation, but similar control molecules CS and Poly(C) did not (Fig. 6a). However, heparin also inhibited HSV-1 infection in NHEK (Fig. 6a). This effect was previously known and thought to occur by inhibiting interaction of HSV-1 with cell surface HSPGs (82). Therefore, we next examined if scavenger receptor ligands might act by interference with adsorption to HSPGs. To first test this, we compared the capacity of Poly(I) and heparin to inhibit infection by a mutant form of HSV-1 that has a truncated gC lacking amino acids 33-123, the C5/P domain (gCΔC5/P). The elimination of this domain dramatically reduces the affinity of gC for HSPG (183, 208). As expected, heparin treatment had a less potent inhibitory effect on the mutant virus compared to its capacity to inhibit WT virus (Fig. 6b). However, the ΔC5/P mutant maintained a similar relative capacity to WT virus to be blocked by Poly(I) (Fig. 6b). These results, together with our previous findings, led us to hypothesize that HSV-1 infection of keratinocytes
involves a previously unsuspected interaction with both scavenger receptors and HSPGs acting in an additive or synergistic manner to mediate HSV-1 adsorption to keratinocytes.

Scavenger receptor ligands inhibit adsorption of HSV-1 and purified gC to the cell surface

We next tested the action of scavenger receptors ligands in the initial adsorption of HSV-1 to cell surfaces. Poly(I) prevented HSV-1 adsorption to NHEK in a dose-dependent manner, while increasing doses of Poly(C) had no effect (Fig. 7a). Since adsorption of HSV-1 to the cell surface is primarily mediated by gC (84, 183), we next analyzed the effects of Poly(I) on the binding of this protein to the cell surface. The association of purified gC with keratinocytes was also inhibited by Poly(I), but not Poly(C) (Fig. 7b). Also, as predicted from prior work, heparin could potently block the association of gC with cells (Fig. 7b) by competition with HSPG (82). However, Poly(I) inhibited binding of gC by a different mechanism than heparin because Poly(I) could not displace gC from a heparin column (Fig. 7c), although abundant gC was bound to heparin and easily displaced by sodium chloride concentrations below 1.0 M (Fig. 7d), suggesting a relatively weak ionic interaction.
The scavenger receptor MARCO co-localizes with HSV-1 virions and purified gC on keratinocytes

We next sought to identify a specific scavenger receptor on keratinocytes that could associate with HSV-1. Analysis of NHEK determined that these cells express multiple scavenger receptors including MARCO and OLR1 (Fig. 8). Both of these receptors can bind Poly(I) (209-211), and thus we sought to determine if these receptors could co-localize with HSV-1 bound to the cell surface. To specifically measure co-localization with HSV-1, we employed a proximity ligation assay (PLA) that is designed to generate a fluorescent signal only when the antigens tested reside within less than approximately 40nm from one another. Assays were performed by maintaining cells at 4°C to inhibit viral entry, and comparisons were made between co-localization of HSV-1 with MARCO, syndecan-1, and OLR1. As expected, syndecan-1, an HSPG previously linked to HSV-1 infection (212, 213), generated a positive PLA signal in the presence of HSV-1 (Fig. 9c), confirming that this HSPG co-localized with HSV-1. MARCO also generated a strong positive PLA signal (Fig. 9b), indicating that MARCO and HSV-1 are also residing on the surface of the cells in close physical proximity. OLR1, although abundantly expressed, did not co-localize with HSV-1 (Fig. 9d). Similarly, purified gC also generated a strong PLA signal with both MARCO and syndecan-1, but not OLR1 (Fig. 10a-d), demonstrating that gC co-localized with both HSPGs and MARCO in the absence of other viral components.
MARCO directly binds to HSV-1 gC

The experiments described above implied that MARCO can interact directly with HSV-1 gC. To confirm and expand upon these results and to determine the affinity of this interaction, we next used purified MARCO to verify that gC can bind to this protein in the absence of other cellular receptors. gC bound at nM concentrations to MARCO, to saturation, and with association plots suggesting single order specific binding (Fig. 11a,b). This effect was specific to gC and MARCO, as another viral glycoprotein, gB, did not bind to MARCO (Fig. 11c), and gC did not bind to an alternative scavenger receptor, OLR1 (Fig. 11d). Notably, the interaction between MARCO and gC had a $K_D$ of $7.7 \times 10^{-10}$, a stronger affinity than that reported previously between gC and heparan sulfate or heparin ($K_D$ values of $1.3 \times 10^{-9}$ and $1 \times 10^{-7}$, respectively) (208).

Further evidence that HSV-1 gC adsorption to scavenger receptors is independent of the previously known adsorption to HSPG was seen by analysis of gC lacking the heparin binding (C5/P) domain (amino acids 33-123). This truncated form of gC binds heparin with approximately 42-fold lower affinity than the intact form of gC (208) but still bound to MARCO with relatively high affinity (Fig. 11e,f). The capacity of gC lacking the C5/P domain to bind to MARCO was consistent with our earlier finding that the infectivity of HSV-1 harboring this mutated gC was strongly inhibited by Poly(I), while
heparin had a less potent inhibitory effect compared to its capacity to inhibit infection by WT virus (Fig. 6b).

**HSV-1 infection correlates with MARCO expression in cells and is reduced in mouse skin treated with the scavenger receptor ligand Poly(I)**

To confirm the functional significance of the interaction of HSV-1 gC and MARCO, we first isolated and differentiated bone marrow-derived macrophages (BMDM) from WT mice and MARCO$^{-/-}$ mice and exposed the cells to HSV-1. Infected BMDM do not produce high titers of HSV-1 virions, but the virus enters the cells, produces viral RNA, and replicates its viral DNA genome, allowing quantification of viral infection (214). MARCO$^{-/-}$ cells infected with HSV-1 at an MOI of 5 had 45% less viral DNA 24 hours post-infection (Fig. 12a, $P=0.0051$). This difference between infection in WT and MARCO$^{-/-}$ cells was seen at the mRNA level and across a broad range of MOI (Fig. 12b, $P=0.0009$).

To complement these results, we next generated a keratinocyte cell line that overexpressed human MARCO. Keratinocytes overexpressing MARCO were approximately 3-fold more susceptible to infection than a control cell line (Fig. 12c), demonstrating that increasing the expression of MARCO resulted in increased infection by HSV-1.

Finally, to test the relevance of disruption of scavenger receptors using an *in vivo* system, we employed a model of severe cutaneous HSV-1 infection
to test the capacity of Poly(I) to prevent HSV-1-mediated disease. Mice were immunocompromised by prior systemic cyclophosphamide treatment (215), then infected with HSV-1 in the skin. Mice were treated locally with PBS, Poly(C) or Poly(I). Control mice treated with PBS or Poly(C) developed large necrotic skin lesions, with wound sizes averaging nearly 1cm$^2$ by five to six days post-infection, but Poly(I) treatment inhibited cutaneous lesion development by more than 50% (Fig. 12d).

Together, these findings indicate that the interaction of HSV-1 and MARCO is functionally significant in HSV-1 infection and is particularly relevant to cutaneous HSV-1 disease.
Discussion

We demonstrate using several independent lines of evidence that HSV-1 enhances adsorption and infection of keratinocytes by binding the scavenger receptor MARCO on the cell surface. First, the scavenger receptor ligand Poly(I) inhibited adsorption and infection of HSV-1 and inhibited gC binding to the cell surface. Second, proximity ligation unambiguously detected co-localization of HSV-1 gC and MARCO on cells bound with whole virions or purified gC. Third, MARCO directly interacted with gC in cell free systems. Finally, cells from MARCO-/− mice were less susceptible to infection by HSV-1, while overexpression of MARCO in keratinocytes increased susceptibility to infection. These findings enhance our understanding of the basic events guiding HSV-1 infection, as MARCO is a novel addition to the list of cellular factors capable of contributing to HSV-1 early infectious steps. Additionally, MARCO is an innate immune receptor, and the interaction of HSV-1 and MARCO may thus have important consequences regarding innate immune recognition of this virus. Finally, keratinocytes were used to establish the relationship between HSV-1 and MARCO, and the use of these primary cells highlights the clinical relevance and potential therapeutic significance of these findings.

Although previous studies have shown that HSV-1 infection can still occur in the absence of any cell surface HSPGs (216), it was entirely unsuspected that the virus can usurp a system used for innate viral recognition
and defense to promote adsorption. We confirmed the previously reported role of HSPGs in adsorption of HSV-1 (82) since heparin was able to suppress gC binding and HSV-1 infection of keratinocytes. Syndecan-1, an HSPG that is highly expressed on the surface of keratinocytes, co-localized with gC and is a likely candidate for the HSPG to which gC binds. In addition, we have now demonstrated that gC also co-localizes with MARCO on the cell surface, and gC bound directly to MARCO in the absence of other cellular or viral proteins. Notably, the staining pattern generated in the PLA assay differed between HSV-1 and gC, but this result was likely due to the fact that gC is more concentrated in the membrane of HSV-1, which could result in a higher density of PLA signals. Interestingly, the affinity of gC to MARCO was measurably higher than the previously known binding affinity of gC to heparan sulfate. Furthermore, gC bound to HSPGs and MARCO using distinct domains, because deletion of the C5/P (heparin binding) domain reduced gC binding to a much greater extent to heparin and heparan sulfate than to MARCO, and a mutant virus lacking this domain lost sensitivity to inhibition by heparin while remaining susceptible to inhibition by Poly(I). Thus, multiple lines of evidence show low affinity, high abundance (HSPGs) and high affinity, low abundance (MARCO) receptors are binding to gC and acting together to mediate adsorption and infection of keratinocytes.

Beyond promoting adsorption and infection, the interaction with MARCO may also benefit HSV-1 in another manner, by potentially enabling
HSV-1 to limit and evade innate immune responses. The binding of HSV-1 to MARCO may interfere with the normal function of MARCO to endocytose dsRNA, thereby limiting the ability of cells to respond to exogenous nucleic acids via intracellular innate immune receptors like TLR3. Additionally, a recent study has indeed shown that although MARCO increases intracellular TLR recognition of nucleic acids, the presence of these receptors actually has the opposite effect on the ability of cell-surface TLRs to detect pathogens by competing for binding to molecules recognized by these receptors (217). Thus, the interaction of HSV-1 with MARCO may both increase adsorption and infection, but also limit cell-surface recognition of viral components by alternative innate immune receptors such as TLR2, which is also involved in the detection of HSV-1 (39). Further studies will be needed to evaluate the full consequences that the interaction of HSV-1 with MARCO has regarding innate immune recognition.

From a clinical standpoint, the protection conferred against HSV-1 infection in vivo by scavenger receptor ligands validated the physiological relevance of our observations. Although the role of gC in viral attachment in vitro has been well established, the virulence mechanism of gC in vivo is more complex and also involves complement and antibody evasion, not only attachment (218). Although the efficacy of Poly(I) to inhibit HSV-1 infection in vivo was less than the effect seen in vitro, this decrease in effectiveness in mice could be due to a decreased dependence on gC, or it could possibly be
attributed to differences in the delivery or half-life of Poly(I) in vivo. Further studies will be needed to determine if Poly(I) can inhibit HSV-1 infection in human skin, and to optimize treatment conditions to specifically block the interaction of HSV-1 with scavenger receptors to limit disease.

Additional studies will be required to more fully define the family of scavenger receptors that may interact with HSV-1, as previous studies have shown that there are redundancies in the affinity and function of scavenger receptors in nucleic acid uptake (74, 187). In contrast to our findings with MARCO, Suzuki et al. previously reported that Macrophage scavenger receptor 1 (MSR1) knockout mice were more susceptible to HSV-1 infection compared to wild type mice (219). In this study, a different route of infection was used (intravenous), thus raising the important possibility that those findings may be specific to route of administration and cell target. Furthermore, since the subsequent recognition of viral nucleic acids by scavenger receptors is necessary for the later IFN response, it is possible that the observations with MSR1 deficient mice highlight the later immune defense role of this molecule rather than the early adsorption and infection process. Future studies will be needed to fully understand the outcome of the interaction of HSV-1 with scavenger receptors similar to MARCO in a variety of cell types, and whether these interactions are ultimately beneficial for the host or the virus in those contexts.
To our knowledge this is the first demonstration that HSV-1 can utilize a component of the danger recognition system to enhance attachment to the cell surface and cause disease. These studies concur with other examples of HSV-1 modulating innate and adaptive immune systems to enhance virulence, but conflict with the current common understanding of epithelial immune defense systems that predicts that these elements are beneficial to the host (130, 220), or that association with viral recognition systems leads to protection against infection (221, 222). Exploitation of the cell surface scavenger receptor system by HSV-1 further illuminates the complexity of the interactions between the host and pathogen at the epithelial surface.

Additional viruses are also known to interact with class A scavenger receptors. For example, MSR1 has been shown to be essential to the sensing of Human cytomegalovirus (HCMV) by endosomal TLRs (189), indicating a protective role for MSR1 in this context. In contrast, Human Adenovirus Type 5 (HAdV5) (223) has been recently shown to use MSR1 to facilitate infection. Additionally, it has been recently demonstrated that the presence of MARCO increases susceptibility to influenza, although MARCO does not enhance viral uptake and instead appears to suppress a beneficial early inflammatory response in the lungs (224). Combined with our current study, these results emphasize the nuanced relationship existing between class A scavenger receptors and viruses as a result of the role of these receptors in innate immunity and the ability of certain viruses to utilize them to enhance infection.
Furthermore, given the ability of additional viruses to bind to class A scavenger receptors, other viruses that infect the skin could also potentially exploit MARCO to mediate infection. In Chapter II, we test the hypothesis that MARCO is also exploited by VV, a virus that can also infect keratinocytes and cause significant cutaneous disease.
Methods

Cells, animals and viruses: NHEK (Life Technologies, Grand Island, NY) were cultured in Epilife media containing Epilife Defined Growth Supplement (Life Technologies, Grand Island, NY), 0.06mM calcium chloride, and 100 I.U. Penicillin and 100µg Streptomycin per ml (VWR, Radnor, PA). BSC-1 cells (ATCC, Manassas, VA) and HaCat keratinocytes were cultured in DMEM (Lonza, Basel, Switzerland), 10% FBS (Thermo Fisher Scientific, Waltham, MA), 2mM L-glutamine and 100 I.U. Penicillin and 100µg Streptomycin per ml (VWR, Radnor, PA). HaCat cells stably overexpressing MARCO and control HaCat cells were generated by transfecting HaCats with a pcDNA3 control plasmid or pcDNA3-MARCO, using the Nucleofector kit V and a Nucleofector device (Lonza, Basel, Switzerland) according to manufacturer’s instructions. Cells were then cultured for 3 weeks in media containing 500µg/ml G418 (Biopioneer, San Diego, CA) to select for transfected cells prior to use in experiments. These cells were continuously cultured in 500µg/ml G418 during experiments. C57Bl/6 mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. All animal studies were in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and were approved by UCSD IACUC (UCSD Animal Welfare Assurance # A3033-1). All procedures were performed under isofluorane anesthesia, and all efforts were made to minimize pain, discomfort, and suffering. Bone marrow from femurs of wildtype (WT) (C57Bl/6) and MARCO⁻/⁻ mice (225) was differentiated into bone
marrow derived macrophages (BMDM) in RPMI 1640 medium (Life Technologies, Grand Island, NY) with 10% FBS (Thermo Fisher Scientific, Waltham, MA), 2mM L-glutamine, 100 I.U. Penicillin and 100µg Streptomycin per ml (VWR, Radnor, PA), 0.05 mM 2-ME (Sigma-Aldrich, St. Louis, MO), and 5 ng/ml M-CSF (R&D Systems, Minneapolis, MN). The K26GFP strain of HSV-1, was a generous gift from Dr. Prashant Desai (Johns Hopkins University, MD) (Fig. 12a,b). The NS strain of HSV-1 (used for all plaque assays, Fig. 2a, 6a,b, and Fig. 12c), and the gCΔC5/P mutant (Fig. 6b) were a generous gift from Dr. Harvey Friedman (University of Pennsylvania, PA). The Schooler strain of HSV-1 was a generous gift from Dr. William Fenical (Scripps Institute of Oceanography, CA) (All other experiments).

**Plasmids:** A plasmid with a pcDNA3 backbone, expressing full-length human MARCO under the control of a CMV promoter, and also expressing a Neomycin resistance gene (a kind gift from Sanjunkta Ghosh, Harvard University, Boston MA) was used for transfection experiments. pcDNA3.1(-) (Life Technologies, Grand Island, NY) was used as a negative control plasmid.

**Reagents and Primary Antibodies:** Poly(I:C) was purchased from Invivogen, San Diego, CA. Poly(I), Poly(C), Dextran Sulfate, Fucoidan, Chondroitan Sulfate, cyclophosphamide, and methylthioadenosine was purchased from Sigma-Aldrich, St. Louis, MO. Chloroquine phosphate was purchased from
Spectrum, Gardena, CA. Recombinant OLR1 was purchased from R&D Systems, Minneapolis, MN. Purified HSV-1 gC (gC1(457t)), gB (gB1(730)), and gC(Δ33-123t) (lacking the ΔC5/P domain) were generous gifts from Dr. Roselyn Eisenberg and Dr. Gary Cohen (University of Pennsylvania, PA). A cell line for the production of recombinant MARCO was kindly provided by Dr. Andrij Holian (University of Montana, MT). MARCO protein was produced and purified as described (226). Primary antibodies recognizing HSV-1 gC (mouse monoclonal), HSV-1 gB (mouse monoclonal), MARCO (rabbit polyclonal), OLR1 (rabbit polyclonal) and all IgG controls were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. A monoclonal primary antibody recognizing HSV-1 gD was purchased from Abcam, Cambridge, MA. A primary antibody recognizing Syndecan-1 was previously characterized (184).

Fluorescence Microscopy: Cells were fixed with paraformaldehyde and blocked with 3% bovine serum albumin (Sigma-Aldrich, St. Louis, MO). Prolong DAPI anti-fade and fluorescent Alexa Fluor conjugated secondary antibodies were purchased from Life Technologies, Grand Island, NY. Images were captured using a BX41 microscope (Olympus, Center Valley, PA) or an Axio Observer microscope (Carl Zeiss Microscopy, Thornwood, NY).

Plaque Assay: Keratinocytes were infected with HSV-1 (NS strain) for two hours, then washed and incubated in fresh media for 48 hours. Cells were
fixed and plaque formation was visualized by staining cells with the anti-gD antibody, and an IR-Dye conjugated secondary antibody (LI-COR Biosciences, Lincoln, NE) and imaging stained plaques using an Odyssey Imager (LI-COR Biosciences, Lincoln, NE).

**Quantitative real-time PCR:** RNA and DNA were isolated using Trizol (Life Technologies, Grand Island, NY). RNA was reverse transcribed using iScript (Bio-Rad, Hercules, CA). Predesigned Taqman probes and primers were used to quantify IL-6, IL-8, hBD2 and IFN-β mRNA using Taqman Gene Expression Master Mix and a 7300 Real Time PCR System (Life Technologies, Grand Island, NY) according to manufacturer’s instructions. Custom Taqman Probes (Life Technologies, Grand Island, NY) were used for HSV-1 glycoprotein D and GAPDH RNA and DNA quantification. Fold change normalized to host cell GAPDH levels relative to the control was calculated using the $2^{(-ΔΔCt)}$ method.

**Quantitative real-time PCR Primer/Probe sequences:**

HSV-1 gD probe: FAM-CCATACCGACCACACCAGGAACC-MGB;

GAPDH probe: VIC-CATCCATGACAACTTTGG TA-MGB.

Primers were purchased from Sigma-Aldrich, St. Louis, MO:

HSV-1: 5’-CGGCCGTGTGACACTATCG-3’,
5’-CTCGTAAAATGGCCCCTCC-3’;

GAPDH: 5’-CCTAGCACCCTGGCCAAG-3’,
5'-TGGTCATGAGTCTCTCCACG-3';

RT-PCR: All genes were amplified from NHEK cDNA for 35 cycles and run on a 3% agarose gel with 0.05% ethidium bromide. Bands were visualized with a UVIDI gel imaging system (Major Science, Saratoga, CA). Primers were purchased from Sigma-Aldrich, St. Louis, MO:

MSR1: 5'-TGCAGCTAACTACCTTTGTTTCC-3',
5'-TGCAAACTCAAGCAATGTGGT-3';

MARCO: 5'-CAAGAGGGAAATGGGTGTA-3',
5'-AGGACTTTGGACCACCAGCAG-3';

SCARA3: 5'-TGGAGGGAATTCAGAAGCTG-3',
5'-TCCTGGGAGATTTGTCTGCT-3';

COLEC12: 5'-TGGCTATGTCACGAATCTGC-3',
5'-CTGGGTCAGGATTCTCTGCT-3';

SCARA5: 5'-GGAGAGAGGTACCCCAGGATT-3',
5'-GCTTCGGTCACCTTTGAAC-3';

SCARF1: 5'-CAGACACAGGCAGCTGTGAG-3',
5'-CAGTGAGGGCACTGCTGTT-3';

OLR1: 5'-TCCTTTGAGCCACAACATTAT-3',
5'-TTTCCGCATAAACAGCTCCT-3'.

ELISA: IL-6 concentration was determined using an OptEIA ELISA Set (BD Biosciences Pharmigen, San Diego, CA).
HSV-1 Immunofluorescence Adsorption Assay: HSV-1 was incubated with cells at 4°C for 2 hours, then cells were washed to remove unbound virus, fixed with paraformaldehyde, and stained using an HSV-1 gC antibody, and an Alexa-Fluor labeled secondary antibody (Life Technologies, Grand Island, NY). Images were taken by fluorescent microscopy as described above, and HSV-1 particles were quantified using ImageJ. The specificity of this assay was validated by control experiments using serial diluted HSV-1.

On-cell Western gC cell binding assay: gC was incubated with cells and the indicated compounds together at 4°C. Cells were washed before fixation. Bound gC was detected with the anti-gC antibody and an IR-Dye conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE), and an Odyssey imager (LI-COR Biosciences, Lincoln, NE). A standard curve was generated using serial dilutions of known concentrations of gC bound to cells to quantify the amount of bound gC.

ELISA binding assay: Capture protein was bound to EIA/RIA plates (Thermo Fisher Scientific, Waltham, MA) in PBS lacking calcium and magnesium, pH 7.4 (Life Technologies, Grand Island, NY), blocked with PBS containing 3%BSA (Sigma-Aldrich, St. Louis, MO), then incubated with viral glycoproteins diluted in PBS. Cells were washed with PBS containing 0.05% Tween-20. Bound glycoproteins were detected using primary antibodies, HRP-conjugated
secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and TMB substrate reagent (BD Biosciences Pharmigen, San Diego, CA). Standard curves for quantification of bound viral glycoproteins were generated using serial dilutions of known concentrations of the proteins and were used to quantify the bound proteins.

**HPLC:** A HiTrap Heparin HP Column and AKTA purifier HPLC system (GE Healthcare, Piscataway, NJ) were used for analysis. Briefly, 100µg of purified gC protein was loaded on to the heparin column and the column was washed to remove any unbound gC. Freshly prepared Poly(I) and NaCl solutions were used to generate the indicated concentration gradients used for elution. 1ml fractions were collected during the elution, and the amount of gC present in these fractions was quantified by dot blot using the anti-gC antibody, an IR-Dye conjugated secondary antibodies (LI-COR Biosciences, Lincoln, NE), and an Odyssey imager (LI-COR Biosciences, Lincoln, NE) with known concentrations of purified gC protein used to generate a standard curve for quantification.

**HSV-1 infection of immunocompromised mice:** Mice were injected intraperitoneally with 300mg/kg cyclophosphamide. One day later, mice were depilated. Two days after cyclophosphamide injection, subcutaneous (s.c.) injections with PBS, Poly(I) or Poly(C) were administered. One hour later, mice
were infected with HSV-1 on the treatment area by scarification with a 25G needle. S.c. injections of PBS, Poly(I) and Poly(C) continued twice per day. Mice were photographed once daily, and lesion sizes were quantified using ImageJ.

**Proximity Ligation Assay:** PLA was performed according to manufacturer instructions (O-link Bioscience, Uppsala, Sweden). Briefly, NHEK seeded into chamberslides (Thermo Fisher Scientific, Waltham, MA) were incubated with HSV-1 and gC for 2 hours at 4°C to allowing binding, but not internalization. Unbound virus or protein was removed by washing with cold PBS before cells were fixed with PFA at 4°C. Blocking buffer (O-link Bioscience, Uppsala, Sweden) was used to prevent non-specific antibody binding, and cells were incubated with two primary antibodies, one recognizing gC and the other recognizing the indicated proteins, MARCO, Syndecan-1, and OLR1. Secondary antibodies conjugated with oligonucleotides (O-link Bioscience, Uppsala, Sweden) were added, and hybridization, ligation, amplification, and detection steps were performed according to manufacturer’s instructions to generate an amplified fluorescent signal in areas where the antigens recognized by the two primary antibodies reside within less than approximately 40nm from each other. This fluorescent PLA signal was evaluated using fluorescence microscopy as described above.
**Statistical Analysis:** Analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com.
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Chapter III

VV binds to scavenger receptors on the surface of keratinocytes
Abstract:

VV is an important skin pathogen that can infect keratinocytes, the primary cell type in the skin, and can cause life-threatening infections such as EV in patients with altered skin immunity, especially individuals with AD. Scavenger receptors are important innate immune receptors, however they can also be exploited to serve as receptors for viruses. Here, we demonstrate that ligands with affinity for class A scavenger receptors potently inhibited VV infection of human keratinocytes. Additionally, VV bound directly to the class A scavenger receptor MARCO, but not to OLR1, an alternative scavenger receptor, and overexpression of MARCO in keratinocytes significantly increased susceptibility to VV infection. Furthermore, we found that MARCO is abundantly expressed in the skin of patients with AD. These findings indicate that VV utilizes MARCO as a receptor for infection of keratinocytes, and highlight MARCO as a potential new therapeutic target for VV infection in the skin.
Introduction

VV is a large DNA virus widely known for its use as a vaccine for smallpox. Routine administration of this vaccine was stopped after smallpox was eradicated, however the vaccine is still utilized for selected high-risk individuals such as some individuals serving in the military, based on fears that smallpox could be used as a weapon in biological warfare (17). Vaccination is accomplished by inoculation of VV in the skin. VV infects a broad variety of cells, but keratinocytes are the major target cell in the skin, and inoculation typically results in a localized infection at the inoculation site, which typically resolves and leaves the host with protective immunity against the closely related variola virus, the causative agent of smallpox (17, 227). However, individuals with altered skin immunity, such as those with AD are at increased risk of developing severe reactions to inoculation with VV, such as the disseminated skin infection EV (19, 20). Immunocompromised patients and children are also at risk of developing severe infections with VV (21-23). While these individuals are excluded from vaccination due to the associated risks, severe infections have even been known to occur in individuals coming into contact with others who have been recently inoculated with VV (24, 25).

Although treatments for VV infection exist, none have been fully evaluated in a controlled setting (15), and EV still presents a serious health risk. Improved techniques to limit the spread of VV in the skin and reduce complications resulting from inoculation with VV could arise from developing a
better understanding of the factors controlling the pathogenesis of VV in the skin. Some of the most important cellular factors that determine the outcome of contact with VV include receptors that are utilized by the virus to bind to and infect target cells, and alternatively, innate immune receptors that act to detect the virus and initiate immune responses. While some previous studies have been dedicated to examining these issues, few have done so in using keratinocytes, the primary target of VV infection in the skin.

A number of receptors have been previously identified as receptors targeted by VV to mediate adsorption to the cell surface and infection. Some strains of vaccinia bind initially to heparan sulfate (HS) or CS GAGs, but others infect cells in a GAG-independent manner (108). Five viral proteins have so far been identified for their involvement in binding to the cell surface. A27 and H3 mediate binding to HS while D8 binds to CS (109-111). GAG-independent binding is also possible with certain strains and certain cell types, and involves interactions between vaccinia A26 and laminin on the cell surface, or between vaccinia L1 and an undetermined receptor (112, 113). The mechanisms of membrane fusion are complex, requiring at least 11 proteins on the surface of VV, and specific cellular proteins involved in this process have not yet been identified (108). Studies focusing specifically on keratinocytes are required to determine which receptors are most critical for VV infection.
Conversely, receptors belonging to the innate immune system are primarily thought to serve a protective role, as defects in innate immunity appear to be critical to pathogenesis of EV (3, 5, 32, 33, 134). Additionally, numerous proteins expressed by VV function to interfere with host defense responses (reviewed in (228)). Keratinocytes are fully competent innate immune cells, expressing a number of receptors capable of detecting infection by pathogens such as VV, and can respond with the production of a number of antimicrobial mediators (130, 220, 229). Cells can detect VV infection through activation of cell-surface and intracellular pattern recognition receptors such as TLRs. Keratinocytes have been shown to express TLRs 1-6, 9, and 10 (194, 230). A previous study implicated TLR2/6 in detection of VV on the cell surface, and MDA-5 and the NLRP3-containing inflammasome detecting VV inside the cell (42). Additionally, VV DNA can stimulate innate immune responses in cells after recognition by TLR8 (52). Furthermore, Sphingosine-1-phosphate receptor 2 has recently been shown to recognize lipids derived from viral membranes and trigger the release of antimicrobial peptides (231). Further experiments are needed to know which of these innate immune receptors are most critical for keratinocyte responses to VV infection.

Class A scavenger receptors have important roles in innate immune defense as they bind to extracellular viral dsRNA and mediate uptake and presentation of dsRNA to TLR3 in the endosome (74, 186-189, 217). TLR3 is hypothesized to enable recognition of a DNA virus such as VV since virtually
all viruses produce dsRNA during replication (191), which is subsequently released into the extracellular space upon lysis of infected cells (192). Scavenger receptors have also been shown to bind to a variety of bacterial and viral products (189, 207), however the role of scavenger receptors in VV infection has not previously been evaluated.

We previously identified that keratinocytes express the class A scavenger receptor MARCO, and found that despite the role of this receptor in the innate immune system, MARCO is exploited by HSV-1 to bind to the surface of keratinocytes (Chapter II). In this chapter, we hypothesize that MARCO is also a receptor for VV infection of keratinocytes. Interestingly, similar to HSV-1, we find that VV binds specifically to the scavenger receptor MARCO, and that blocking the interaction of VV with cell-surface scavenger receptors prevents infection by VV. Furthermore, keratinocytes overexpressing this receptor are more susceptible to infection by VV, demonstrating that VV binding to MARCO plays a significant role in infection of keratinocytes, and that disruption of this interaction could have therapeutic implications. This may be particularly relevant for AD patients, as we also demonstrate that AD skin has abundant MARCO expressed throughout the thickened lesional epidermis.
**Results**

dsRNA protects keratinocytes against VV infection, but the protective effect does not depend on cellular activation

We had found in Chapter II that Poly(I:C) protected keratinocytes from HSV-1 infection in a manner that surprisingly involved competition for cell surface scavenger receptors, and not activation of cellular innate immune defenses through TLR3, the receptor for Poly(I:C). We hypothesized that Poly(I:C) treatment could also protect against VV in a similar manner. Treatment with Poly(I:C) protected against VV infection, while treating keratinocytes with ligands that bind to other TLRs (38) did not result in significant protection against infection (Fig. 13a). Furthermore, the inhibitory effect of Poly(I:C) was dose-dependent, as increasing concentrations of Poly(I:C) corresponded to increased inhibition of viral plaque formation in HaCat keratinocytes and NHEK (Fig. 13b,c). This inhibitory effect was also seen when analyzing the amount of VV early gene mRNA at an early timepoint after infection (Fig. 13d), indicating that the inhibition was occurring at an early step in the infectious process.

To test the hypothesis that protection by Poly(I:C) was also not due to activation of innate immune responses by TLR3, we utilized inhibitors of cellular activation. First, we used cyclohexamide (Chx), a potent inhibitor of new protein synthesis. Treatment with Chx inhibited the ability of the cells to synthesize new proteins by 97%, as demonstrated by the lack of expression of
VV intermediate genes, which require translation of early gene products in order to be produced (Fig. 14). Alternatively, VV early gene expression is, by definition, not altered by Chx treatment, allowing us to evaluate the ability of the virus to enter cells and begin viral RNA synthesis (232). Interestingly, Poly(I:C) inhibited VV infection even in cells that were not able to synthesize new proteins (Fig. 15a), indicating that the protective effect of Poly(I:C) did not involve the synthesis of new antiviral effector proteins.

Next, we tested the effect of treatment with CQ, a potent inhibitor of endosomal acidification, using conditions that prevented the ability of Poly(I:C) to activate TLR3 in keratinocytes and induce IL-6 and IFN-β by more than 80% (Fig. 3). Poly(I:C) was able to protect against VV infection in keratinocytes treated with CQ at levels comparable to the protection seen in untreated cells (Fig. 15b), demonstrating that cellular activation by Poly(I:C) was not required for the protective effect of Poly(I:C). These results with both Chx and CQ also confirmed that the protective activity of Poly(I:C) occurred at a very early timepoint during infection.

Scavenger receptor ligands inhibit vaccinia virus infection of keratinocytes

As we found with HSV-1, the protective effect of Poly(I:C) appeared not to be linked to its ability to stimulate innate immune responses in keratinocytes (Chapter II). We hypothesized that the ability of Poly(I:C) to protect against VV infection in keratinocytes was due to the capacity of this dsRNA to bind to
scavenger receptors (74, 186, 187, 217), and thus predicted that other ligands also capable of binding and competing for access to scavenger receptors would be able to inhibit infection by VV. Poly(I) and Dxs have been extensively characterized as ligands for class A scavenger receptors (207). As anticipated, both of these compounds also inhibited VV infection of keratinocytes in a dose-dependent manner (Figure 16). Poly(I) provided levels of protection comparable to Poly(I:C), despite not being able to activate innate immune responses in keratinocytes (Chapter II), while Poly(C), which does not bind to scavenger receptors, had no effect on VV infection (Fig. 16a). These results demonstrated that a variety of compounds that share the capacity to bind scavenger receptors are capable of inhibiting VV infection.

**Vaccinia virus binds directly to the scavenger receptor MARCO**

We next sought to identify a specific scavenger receptor capable of interacting with VV. We previously found that both MARCO and OLR1, two scavenger receptors previously shown to bind Poly(I), are expressed at high levels on keratinocytes (Chapter II). To test for a direct interaction between these scavenger receptors and VV, we employed a cell-free direct binding assay utilizing purified recombinant MARCO and OLR1. Using this assay, we found that VV bound specifically to MARCO protein, but not OLR1 protein (Fig. 17), demonstrating that VV binds specifically to MARCO in the absence of any other cell surface receptors. Importantly, this interaction could be disrupted by
the addition of the scavenger receptor ligand Poly(I) (Fig. 18). These results show that VV binds specifically to a scavenger receptor that is highly expressed by keratinocytes, and that this interaction can be inhibited by ligands capable of binding to scavenger receptors and preventing infection of keratinocytes by VV.

**MARCO is expressed in the skin of patients with atopic dermatitis**

Patients with AD are susceptible to developing severe infections with VV known as eczema vaccinatum (3, 5, 19). We found that VV binds directly to MARCO, and VV infection could be inhibited by scavenger receptor ligands such as Poly(I). Thus, we next analyzed non-lesional and lesional skin from patients with atopic dermatitis to compare the expression of MARCO to investigate whether the expression of this protein could potentially have a role in the pathogenesis and treatment of EV. In both lesional and non-lesional AD skin, MARCO was highly expressed throughout the epidermis (Figure 19). The epidermis of the lesional skin was much thicker than the epidermis of non-lesional skin, as expected, resulting in a much larger population of MARCO-expressing target cells in lesional skin, implicating this receptor in the pathogenesis of infection and highlighting the therapeutic potential of scavenger receptor ligands to block access to the abundant MARCO present in AD skin.
**Overexpression of MARCO increases susceptibility to VV infection**

To evaluate the function significance the interaction of VV and MARCO and to establish a relationship between the expression of MARCO and susceptibility to infection, we next created a keratinocyte cell line that stably overexpressed human MARCO protein. Infection of these cells and control cells with equal amounts of VV resulted in significantly more viral plaques in the cells that overexpress MARCO (Figure 20). These results demonstrate that increased expression of MARCO enhances the susceptibility of keratinocytes to VV infection and further confirm a significant role for this receptor in cutaneous VV infection.
Discussion

We demonstrate in this Chapter that VV is capable of binding directly to the scavenger receptor MARCO, which is present on the surface of keratinocytes. Importantly, ligands capable of blocking this interaction potently prevented infection of VV in keratinocytes, and overexpression of MARCO increased susceptibility to VV infection. These results identify MARCO as a novel receptor that is targeted by VV during infection in the skin, providing a potential new therapeutic approach which may be particularly important for patients with AD, who have abundant expression of MARCO in their thickened lesional epidermis.

Our results suggest that MARCO is serving as a cell surface receptor for VV. We previously found that MARCO functions in conjunction with HSPGs to mediate adsorption of HSV-1 to the cell surface, and based on our results, we believe that a similar mechanism could be functioning for VV. However, clear differences can be seen in the adsorption and entry of VV based on the strain of virus used and the type of cell being infected, with some strains, including the Wyeth strain used in this study, exhibiting less dependence on HSPGs for adsorption compared to other strains (106, 108). This raises the possibility that MARCO has a greater involvement in the adsorption of VV than HSV-1 to keratinocytes. Further experiments will be needed to fully quantify the extent of the contribution of MARCO to VV binding, although our studies demonstrating inhibition by the scavenger receptor ligand Poly(I) indicate a
major role for this receptor. The viral preparation used in the experiments is primarily the intracellular mature virus (IMV) form of VV, which means that the interaction with MARCO is possibly mediated by the viral glycoproteins H3, A27, D8, all of which are present on the surface of IMV and have been shown to have roles in the adsorption of VV by interactions with GAGs (109-111). It is also possible that another viral glycoprotein, such as A26, which has been shown to bind to laminin (112) is involved in binding to MARCO. The L1 protein is another interesting possibility, as this viral protein has been shown to bind to cell surfaces and blocks infection of VV in a GAG-independent manner by binding to an unidentified cellular receptor (113). Further experiments will be needed to identify the specific viral glycoprotein capable of interacting with this receptor, and to confirm whether the primary role of MARCO is solely in adsorption of the virus to the cell surface, or whether MARCO could have an active role in uptake of the virus as well. VV has been shown to be taken up by target cells via macropinocytosis, utilizing phosphatidylserine (PS) in the viral membrane to mimick apoptotic debris and trigger uptake by a cellular PS receptor (233-235). It is possible that MARCO is contributing to this process as well, however further experiments would be needed to test this possibility.

On the surface, it would be expected that scavenger receptors serve primarily a protective role in the detection of foreign pathogens based on their important functions in innate immunity, as described in Chapter I. In this Chapter, however, we have demonstrated that MARCO increases the
susceptibility to infection by serving as a receptor for VV. Additionally, we have previously discovered a similar interaction between HSV-1 and MARCO (Chapter II), while others have shown that the closely related scavenger receptor MSR1 is a receptor for HAdV5 (223). The finding that the capacity of MARCO and other similar class A scavenger receptors to serve as viral receptors is exploited by an increasing number of diverse viruses is very interesting. It is possible that exploitation of these receptors may benefit these viruses as a way to escape recognition by alternative innate immune receptors on the cell surface. Indeed, it has been shown that although MSR1 and MARCO enhance uptake of extracellular PAMPs for recognition by intracellular PRRs, the presence of these receptors actually inhibits recognition of extracellular PAMPs by cell surface PRRs, limiting the subsequent activation of cell surface innate immune defenses (217). Additionally, although class B scavenger receptors differ from the class A scavenger receptors in terms of structure and function, a number of these receptors have also been identified as receptors for viruses including Hepatitis C virus (HCV) (236), Coxsackie A virus (CVA) -7, -14, -16 (237) and Enterovirus 71 (EV71) (238).

The dramatic inhibition of VV by scavenger receptor ligands in human cells under a variety of conditions is significant, as this shows that these ligands could be used to block scavenger receptors and prevent VV infection in the skin. Currently, treatment of severe cutaneous vaccinia infection can
involves treatments such as the administration of VIG, however VIG is currently classified by the CDC as an investigational drug, and controlled studies determining the true efficacy of VIG and other seemingly useful antiviral drugs such as Cidofovir and Riboviron are lacking (15). Although these treatments appear to reduce the severity of infection, EV is still a life-threatening infection that could benefit from additional therapeutics. Furthermore, it is likely that a combination of approaches to target multiple aspects of the pathophysiology of VV infection would be the most effective therapy. For example, it has been shown that combined therapy of Poly(I:C) and VIG is more effective to treat severe VV infection in mice than than either treatment alone (170). Thus, developing optimal treatments may require a combination of strategies to block viral binding, entry, and replication, in addition to utilizing VIG to neutralize viral particles. We have shown in this Chapter that the skin of AD patients, who are susceptible complications related to VV inoculation, has abundant MARCO, and that keratinocytes expressing high levels of MARCO have increased VV infection. Blocking access to MARCO may thus be an important component of future approaches to antiviral therapy. Further studies will be needed to optimize treatment conditions and fully evaluate the efficacy of scavenger receptor blockade by Poly(I) and related compounds, as standalone therapies and in combination with additional antiviral drugs, as a treatment for severe infections such as EV that arise as complications from inoculation with VV.
Methods

Cells and Viruses: Normal Human Epidermal Keratinocytes (Invitrogen) were cultured in Epilife media containing Epilife Defined Growth Supplement (Invitrogen), 0.06mM calcium chloride, and 100 I.U. Penicillin and 100µg Streptomycin per ml (VWR). BSC-1 cells (ATCC) and HaCat keratinocytes were cultured in DMEM (Lonza), 10% FBS (Thermo Scientific), 2mM L-glutamine, Penicillin/Streptomycin (VWR). HaCat cells stably overexpressing MARCO and control HaCat cells were generated by transfecting of HaCats with a pcDNA3 control plasmid or pcDNA3-MARCO, using the Nucleofector kit V and a Nucleofector device (Lonza) according to manufacturer’s instructions. Cells were then cultured for 3 weeks in media containing 500µg/ml G418 (Invitrogen) to select for transfected cells prior to use in experiments. These cells were continuously cultured in 500µg/ml G418 during experiments. The Wyeth strain of VV (a kind gift from Michael Croft, La Jolla Institute for Allergy and Immunology, CA) was prepared and titered from crude extracts of BSC-1 infected cells, lysed and centrifuged to remove cellular debris, and stored at -80°C.

Plasmids: A plasmid with a pcDNA3 backbone, expressing full-length human MARCO under the control of a CMV promoter, and also expressing a Neomycin resistance gene (a kind gift from Sanjunkta Ghosh, Harvard
University, Boston MA) was used for transfection experiments. pcDNA3.1(-) (Invitrogen) was used as a negative control plasmid.

**Plaque assays:** Cells were infected with VV for 1 hour, then washed and incubated in fresh media for 24 hours. Plaque formation was visualized by staining cells with crystal violet. MOIs were typically selected to give approximately 100-500 plaques per well in a 6-well plate in each experiment. All plaques counts were compared to the controls displayed for each individual experiments, with plaque counts in the control wells normalized to 100%.

**Protein binding assays:** Capture protein was bound to EIA/RIA plates in PBS lacking calcium and magnesium, pH 7.4, blocked with PBS containing 3%BSA, then incubated with VV diluted in DMEM + 1% FBS. Plates were washed with PBS. Bound VV was detected using a primary antibody (Abcam), an HRP-conjugated secondary antibody (Santa Cruz Biotechnology), and TMB substrate reagent (BD Biosciences), and quantified using a microplate reader measuring absorbance at 450nm, and subtracting absorbance at 570nm.

**Quantitative realtime PCR:** RNA was isolated using Trizol (Invitrogen), and was reverse transcribed using iScript (Bio-Rad). Custom Taqman Probes (ABI) were used for VV DNA-dependent RNA polymerase (RPO35) and GAPDH mRNA quantification using Taqman Gene Expression Master Mix (ABI), and VV G8R gene expression was analyzed using SYBR Green PCR
Master Mix (ABI), with a 7300 Real-Time PCR System (ABI) using the primer sequences listed below. Fold change of VV RPO35 normalized to host cell GAPDH levels relative to the control was calculated using the $2^{(\Delta\Delta Ct)}$ method, with untreated or vehicle treated samples normalized to 100%.

**qPCR Primer/Probe sequences:**

GAPDH probe: 5'-CATCCATGACAACCTTTGGTA-3'.

VV RPO35 Probe: 5'-ATTGAATTCTCTTCCGGATGCTG-3'.

Primers were purchased from Sigma-Aldrich:

GAPDH: 5'-CCTAGCACCCCTGGCCAAG-3', 5'-TGGTCATGAGTCCTTCCACG-3';

VV RPO35: 5'-GCCAATGAGGGTTGAGGTTGAGGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAGGTTGAg

**Reagents:** Poly(I:C) (Invivogen). Poly(I), Poly(C), Dextran Sulfate, cyclophosphamide, (Sigma-Aldrich). Chloroquine phosphate (Spectrum). Recombinant MARCO and OLR1 (R&D systems).

**AD Skin Immunofluorescence staining:** All studies were approved by the Human Research Protection program at the University of California, San Diego, and were conducted in adherence to the Declaration of Helsinki Principles. Written, informed consent was obtained from all donors for all
procedures. Skin biopsy samples were obtained from lesional and non-lesional skin of AD patients, frozen in OCT, sectioned using a cryotome, and stored on microscope slides at -80°C until use. Briefly, cells were fixed with ice-cold methanol, and stained for the presence of MARCO using a monoclonal antibody (a kind gift from Lester Kobzik, Harvard University, MA) and an Alexa-fluor 488-labeled secondary antibody (Invitrogen).

**Statistical Analysis:** Analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com.
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Chapter IV

Conclusions and perspectives
Summary

We hypothesized that activation of keratinocyte innate immune responses through TLR3 would protect against HSV-1 infection and as a consequence have identified MARCO, a component of the TLR3 pathway, as a receptor for both HSV-1 and VV adsorption and infection of keratinocytes. The characterization of MARCO as a previously unknown element influencing infection by HSV-1 and VV is an important contribution to the fields of innate immunity and cutaneous viral infection. We have shown that interrupting the interaction of HSV-1 and VV with MARCO can prevent infection of primary human keratinocytes, a major target of infection, and that this therapeutic effect may be relevant for cases of severe cutaneous infection by these viruses. Furthermore, these results identify for the first time that two diverse viruses, HSV-1 and VV can exploit the same element of the host innate recognition system to enhance their capacity for disease, advancing the concept that multiple viruses may take advantage of recognition by scavenger receptors to enhance their virulence, exploiting the role of these receptors in the innate immune system.

Further studies will allow us to characterize the interaction of MARCO with HSV-1 and VV in greater detail, to identify the ligand on VV capable of binding to MARCO, to determine the domain of MARCO required for these interactions, and to conclusively examine whether this interaction mediates only adsorption to the cell surface, or whether MARCO also influences either
the uptake or entry of these viruses into cells. Specific studies are also required to understand whether these findings can be extrapolated to cell types other than keratinocytes. Additionally, future work will expand on our studies to more fully evaluate the therapeutic potential scavenger receptor antagonism, and to identify additional interactions between viruses and scavenger receptors and evaluate the consequences of these host-pathogen interactions.

**Further characterization of MARCO in HSV-1 and VV infection**

We propose a novel mechanism for HSV-1 and VV adsorption to keratinocytes as summarized in Figure 21. In Chapter II we demonstrated that MARCO is a receptor for HSV-1 infection. We further demonstrate that HSV-1 gC binds specifically to MARCO, enabling MARCO to act as a co-receptor for viral adsorption along with HSPGs. VV also binds to MARCO (Chapter III), but we have not yet identified a specific glycoprotein capable of mediating this interaction. If MARCO is also acting as a co-receptor with HSPGs for VV adsorption, it appears likely that either H3 (110) or A27 (109), glycoproteins that bind to HSPGs, would also bind to MARCO. VV proteins D8 (111), A26 (112), and L1 (113) could also be the primary glycoproteins binding to MARCO, as these glycoproteins have been shown to be involved in binding to the cell surface. A candidate-based approach could potentially be used to identify the specific VV glycoprotein capable of binding to MARCO, similar to
the work with HSV-1 described in Chapter II, where we identified that purified HSV-1 gC co-localizes with MARCO on the cell surface and directly binds to MARCO.

Identifying MARCO as a receptor for both HSV-1 and VV raises another important question regarding MARCO: which domain(s) of MARCO are required for binding to these viruses? MARCO forms a trimeric type II transmembrane protein with three extracellular domains: domain III, a spacer region residing proximal to the plasma membrane, domain IV, a collagenous triple helix, and domain V, the Scavenger receptor cysteine rich (SRCR) domain (239). Domain V appears to be the most likely domain mediating the interaction of MARCO with HSV-1 and VV, as this SRCR domain has been implicated in the binding of MARCO to other ligands including modified LDL and bacteria (211, 240-243) and thus appears to be the major ligand-binding domain. Furthermore, although MARCO is already a trimeric molecule, domain V is itself capable of forming dimers and oligomers, allowing for the clustering of multiple trimeric MARCO molecules, which is speculated to facilitate MARCO binding to large molecules such as bacteria and modified LDL (242), which are both larger than even the (relatively) large viruses HSV-1 and VV. We have demonstrated in Chapter II and III that HSV-1 and VV, respectively, bind directly to purified MARCO. Furthermore, gain of function analyses using keratinocytes revealed that increasing levels of MARCO expression increased infection by both viruses. Utilizing truncated forms of MARCO in either of these
assays will enable identification of the viral binding domain(s) in future studies, and will clarify whether oligomerization of trimeric MARCO molecules enhances viral binding.

MARCO may also participate in other aspects of HSV-1 and VV infection, such as endocytic uptake following adsorption. Infection by either virus clearly requires multiple interactions with distinct receptors, however as discussed in Chapter I, a large gap in understanding remains regarding the uptake process existing between adsorption to the cell surface and entry (fusion of cellular and viral membranes). The function of the intracellular domain of MARCO has not been well characterized, but it may be involved in mediating signaling events in response to ligand recognition (244, 245). MARCO is clearly involved in the phagocytosis of bacteria and other ligands in phagocytic cells, however it is unclear if it is MARCO or co-receptors that are generating the internalization signals (246, 247). Mutagenesis of the intracellular domain of MARCO overexpressed in keratinocytes will elucidate whether this domain is involved in HSV-1 and VV uptake and infection, or whether the sole role of MARCO is in adsorption of these viruses to the cell surface.

One final further consideration regarding the role of MARCO in HSV-1 and VV infection pertains to the generalizability of our findings in keratinocytes to additional cell types. The use of primary keratinocytes in Chapters II and III to establish the role of MARCO boosts the potential physiological significance
of our findings, as this represents the most thorough investigation of HSV-1 and VV receptor utilization in cells that are a major target of infection in humans. Expression of specific viral receptors is clearly an important determinant of tissue tropism (248, 249), and it is thus possible that expression of MARCO by cells in other tissues enhances the spread of HSV-1 and VV during systemic infection and plays a role in determining which tissues become infected. Initial characterization of MARCO suggested that this receptor is expressed only by a subset of macrophages (250), however in Chapter II we demonstrate that this receptor is expressed by keratinocytes, raising the possibility that it is also expressed by additional cell types in other tissues. Experiments analyzing the expression of MARCO in the liver and brain, for example, will help to elucidate whether MARCO expression is an important factor for HSV-1 targeting these tissues during severe systemic infections (215). However, care must also be taken when extrapolating from our findings using keratinocytes to alternative cell types, particularly macrophages, which are known to express high levels of MARCO (239). Contact of HSV-1 and VV with keratinocytes clearly results in infection of the target cell, replication of viral RNA and DNA, and production of a large number of infectious virions that are eventually capable of lysing the infected cell. In contrast, macrophages possess intrinsic resistance to HSV-1 infection, and can restrict replication of the virus at various stages of the infectious process (251). As noted in Chapter I, macrophages have an important role in
controlling the spread of HSV-1 infection. Thus, since contact of HSV-1 with macrophages does not typically result in infection, it will be interesting to determine the contribution of MARCO to macrophage-mediated antiviral defense. Interactions of VV with macrophages are not as well studied, and under certain circumstances may result in infection of these cells and production of new virions (252-254). Macrophage and keratinocyte interactions with HSV-1 and VV mediated by MARCO may thus have differing outcomes. Several studies indeed suggest that this may be true and emphasize the importance of examining cell-type specific interactions. As mentioned briefly in Chapter II and Chapter III, Haisma et al., demonstrated that overexpression of MSR1 on CHO epithelial cells results in increased infection by HAdV5, however MSR1 on macrophages enabled uptake and degradation of HAdV5 (223). Further experiments are needed to determine if interactions between MARCO and either HSV-1 or VV in macrophages are protective against infection, in contrast to our findings with keratinocytes. This could be accomplished by transferring WT bone-marrow into MARCO−/− mice, generating a model where MARCO is expressed only by cells of haematopoietic origin such as macrophages but not by keratinocytes, and subsequently infecting these mice with HSV-1 and VV. In either case, scavenger receptor blockade by treatment of Poly(I) protected against HSV-1 skin infection, indicating that blocking the interaction of scavenger receptors
and HSV-1, and possibly also VV, are ultimately protective in the context of skin infection.

**Therapeutic efficacy of scavenger receptor blockade**

In Chapters II and III we demonstrated that scavenger receptor ligands such as Poly(I) and Dxs have the capacity to block infection of keratinocytes by HSV-1 and VV, respectively. Given the ability of both of these viruses to cause severe infection in the skin of AD patients, it will be important to more extensively characterize the therapeutic efficacy of Poly(I) and Dxs. Specifically, future studies will determine whether treatment with these compounds can be optimized to achieve good standalone prevention of infection in the skin of AD patients, or if they can successfully used as supplements to standard therapeutics. However, Dxs may not have considerable therapeutic potential, as it has previously been evaluated for *in vivo* effects against HSV-1, and was not found to significantly protect against cutaneous infection (255). Also, despite the finding that this compound demonstrated significant *in vitro* antiviral activity against HIV (256), systemic treatment was seen to have significant toxicity and to actually increase the amount of HIV in patients (257).

Poly(I) may have more therapeutic potential than Dxs. As discussed above, MSR1 appears to mediate infection of cell expressing this receptor and uptake of HAdV5 by macrophages (223). Poly(I) treatment *in vivo* prevented
sequestration of HAdV5 in liver macrophages, indicating that systemic Poly(I) treatment is successful at blocking this interaction in mice (258), and also demonstrated that high-dose treatments of Poly(I) are not associated with toxicity (258-260). Assuming that this lack of toxicity translates to humans, the key to treatment of HSV-1 and VV skin infections will be delivery of Poly(I) to the infection site. Further experiments comparing the capacity of systemic and topical Poly(I) treatment to protect against infection are critical to fully evaluating the therapeutic potential of scavenger receptor blockage.

The inosine-containing RNAs Poly(I) and Poly(I:C) are synthetic RNAs and their physiological relevance was thought to be limited, due to the fact that inosine is not a standard nucleoside used in eukaryotic and prokaryotic RNA. However, recent studies have revealed that this is not strictly correct, as cells possess an enzyme, known as Adenosine deaminase, RNA-specific (ADAR1), which has the capacity to “edit” host cell RNAs, creating adenosine-to-inosine substitutions, thus increasing the level of inosine-containing RNAs inside the cells (reviewed in (261, 262)). Interestingly, ADAR1 is IFN inducible and has been linked to both proviral and antiviral responses (263). Furthermore, it has recently been described that ADAR1-edited inosine-containing RNA is taken up from the cell surface by scavenger receptors to activate cells via TLR3 and PKR (264). ADAR1 may thus serve critical roles in antiviral defense against HSV-1 and VV by generating inosine-containing RNAs that compete for access to scavenger receptors and also initiate innate immune responses.
ADAR1 is indeed highly expressed by NHEK (Fig. 22), and the VV protein E3L has been shown to antagonize RNA-editing by ADAR1 (265). However, further studies are required to determine the sufficiency or necessity of ADAR1 in the natural capacity of keratinocytes to protect against viral infection.

**Interactions of scavenger receptors with multiple viruses**

We have shown in this dissertation that MARCO is a receptor for two distinct DNA viruses, HSV-1 and VV. The idea of viruses sharing the same receptor is certainly not unprecedented (266, 267). Indeed, an increasing number of viruses have been shown to utilize scavenger receptors. As discussed in Chapter III, Class B scavenger receptors have been identified as receptors for HCV (236), CVA7, CVA14, and CVA16 (237) and EV71 (238). Additionally, as discussed above, the class A scavenger receptor MSR1 is a receptor for HAdV5 when overexpressed in CHO cells (223). Our current knowledge of the expanding number of viruses that can usurp scavenger receptors is summarized along with the roles of scavenger receptors in antiviral innate immunity in Figure 23. Interactions between scavenger receptors and viruses may represent a large and underappreciated aspect of host-pathogen interactions given the broad ligand binding specificities of scavenger receptors such as MARCO, the number of different scavenger receptors that exist, the multiple roles of scavenger receptors in innate immunity, and the potential for these scavenger receptors to be exploited as
receptors as described above. Further studies are needed to screen for additional virus-scavenger receptor interactions, and followup studies will be need to fully determine the consequences of these interactions for both the host and pathogen.

**Final Conclusions**

This dissertation began with the hypothesis that activation of TLR3 in keratinocytes would enable these cells to protect against HSV-1 and VV infection. Thorough investigation of this hypothesis led us to identify and characterize that the innate immune receptor MARCO, a scavenger receptor acting upstream of TLR3, is a novel receptor on keratinocytes for HSV-1. We additionally demonstrated that MARCO serves as a high-affinity receptor for the specific HSV-1 glycoprotein gC, revealing that HSV-1 adsorption to keratinocytes involves the interaction of gC with MARCO in addition to the previously known binding of gC to HSPGs, which was thought to be solely responsible for adsorption. We also hypothesized that MARCO could serve as a receptor for VV, a distinct virus that is also capable of infecting the skin. We confirmed this hypothesis, demonstrating that exploitation of scavenger receptors may represent a previously unappreciated mechanism of virulence for diverse viral pathogens. Taken together, this work demonstrates that MARCO represents a previously unknown therapeutic target for HSV-1 and VV skin infections, and that blocking the interaction of HSV-1 or VV with
MARCO may have important implications for patients predisposed to severe skin infections by these viruses.

Future experiments expanding our knowledge of the role of MARCO and other scavenger receptors in the pathogenesis of HSV-1, VV, and other viruses will enhance our knowledge in the fields of innate immunity and virology, and will provide valuable new information regarding viral recognition and exploitation of host defense systems. Future studies will allow us to better explain why certain interactions with scavenger receptors benefit the host, while others predispose to infection, information that can ultimately guide the prevention and treatment of viral infections in humans.
Appendix
Figure 1: Potential mechanisms of PRR recognition of HSV-1 and VV by keratinocytes. Keratinocytes express multiple PRRs including TLR1 (230, 268, 269), TLR2 (230, 268-270), TLR3 (230, 269), TLR4 (270), TLR6 (230), TLR9 (269), RIG-I (271), MDA-5 (271), PKR (272), AIM2 (273), IFI16 (274), NLRP-3 - (275), that have been demonstrated to have a role in the detection of HSV-1 and VV proteins and nucleic acids in alternative cell types.
Figure 2: Poly(I:C) inhibits HSV-1 infection. a, NHEK were treated with Poly(I:C) at the indicated concentrations for 20 minutes before infection with HSV-1 at an MOI of 0.0005. PFU were quantified 48 hours after infection. b, NHEK were treated with Poly(I:C) for 20 minutes prior to infection with HSV-1 at an MOI of 0.005 for 6 hours. HSV-1 gD mRNA was quantified by qPCR. All data are means ±s.e.m., n=3 from representative experiments repeated at least two times. One-way ANOVA with Tukey post-tests were used for statistical analysis, * P<0.05; ** P<0.01; *** P<0.001. P values were derived from comparisons to untreated samples.
Figure 3: dsRNA protects against HSV-1 infection in the absence of cellular activation.  

a, b, HaCat keratinocytes were pretreated with 0.1mM methylthioadenosine (MTA) for 2 hours before treatment with 12.5µg/ml Poly(I:C) and HSV-1 at an MOI of 0.005 for 7 hours. Expression of IFN-β (a) and HSV-1 (b) mRNA were evaluated by qPCR. c, d, HaCat cells were incubated with 10µg/ml chloroquine (CQ) for 1 hour before stimulation with 50µg/ml Poly(I:C) for 2.5 hours (c) or a combination of Poly(I:C) and HSV-1 at an MOI of 0.5 for 7 hours (d). Gene expression was analyzed by qPCR. a-d, error bars indicate s.e.m., n=3. One-way ANOVA with Tukey post-tests were used for statistical analysis, * P<0.05; ** P<0.01; *** P<0.001. c, P values were derived from comparisons to Poly(I:C) treated samples. All data are from representative experiments repeated at least three times.
Figure 4: Poly(I) protects against HSV-1 infection but does not activate keratinocytes. c, HaCats were treated with 100µg/ml Poly(I:C), or 50µg/ml of Poly(I) or Poly(C) 20 minutes before the addition of HSV-1 at an MOI of 0.01. Cells were incubated for 5.5 hours before quantification of HSV-1 mRNA by qPCR. d, NHEK were treated with 10µg/ml Poly(I), Poly(C) and Poly(I:C) for 3 hours before qPCR analysis of gene expression. All data are means ±s.e.m., n=3 from representative experiments repeated at least two times. One-way ANOVA with Tukey post-tests were used for statistical analysis, *** P<0.001. b, P values were derived from comparisons to vehicle treated samples.
Figure 5: dsRNA-mediated gene induction is inhibited by scavenger receptor ligands. 

a, NHEK were treated with Dxs, Fn, and CS at the indicated concentrations and simultaneously stimulated with 1 µg/ml Poly(I:C) for 6 hours. IL-6 was quantified by ELISA. Two-way ANOVA with bonferroni post-tests was used for comparisons to CS treated cells at each dose. Error bars indicate s.e.m., n=3, *** P<0.001. 

b, HaCat keratinocytes were treated with 5 µg/ml Poly(I) for 10 minutes prior to stimulation with 10 µg/ml Poly(I:C) for 6 hours. Secreted IL-6 was quantified by ELISA. One-way ANOVA with Tukey post-test was used to compare treatments as indicated. Error bars indicate s.e.m., n=3, *** P<0.001. All data are from representative experiments repeated at least three times.
Figure 6: Diverse scavenger receptor ligands protect against HSV-1 infection using a different mechanism than heparin. 

a, NHEK were treated with 10µg/ml of the indicated compounds for 20 min prior to the addition of HSV-1 at an MOI of 0.0005. PFU were quantified 48 hours after infection. 

b, NHEK were treated with 10µg/ml Poly(I) or Heparin 20 min before infection with WT HSV-1 at an MOI of 0.0005 or an equivalent amount of HSV-1 gCΔC5/P viral particles. PFU were quantified 48 hours after infection. PFU are displayed as relative % compared to untreated cells for each virus. All data are means ±s.e.m., n=3 from representative experiments repeated at least two times. One-way ANOVA with Tukey post-tests were used for statistical analysis, ** P<0.01; *** P<0.001. P values were derived from comparisons to vehicle treated samples.
Figure 7: Scavenger receptor ligands inhibit adsorption of HSV-1 and purified gC to cells but do not block gC binding to Heparin. a, HaCat keratinocytes were incubated with HSV-1 at an MOI of 100 at 4°C with the indicated concentrations of Poly(I) and Poly(C). Bound HSV-1 was visualized by fluorescence microscopy. Five images of bound HSV-1 per concentration were captured and the number of HSV-1 particles quantified using ImageJ. Each image contained 15-20 cells. Error bars indicate s.e.m. Two-way ANOVA with bonferroni post-tests was used to compare the effect of Poly(I) to Poly(C). n=5, *** P<0.001. b, purified gC was incubated with cells in the presence of 100µg/ml heparin, Poly(I), or Poly(C) at 4°C. Unbound gC was removed by multiple wash steps, then cells were fixed and bound gC was detected and quantified using an on-cell western assay. One-way ANOVA with Tukey post-tests were used for statistical analysis with comparisons made to vehicle treated cells, n=2, ** P<0.01. ND, not detectable, NS, not significant. c,d, 100µg of purified gC was bound to a Heparin column, and eluted with a linear gradient (broken line) of Poly(I) (c), or NaCl (d). Eluted gC was quantified from 1ml fractions by dot blot and plotted as circles representing each individual fraction connected by a solid line. All data are from representative experiments repeated at least two times.
Figure 8: MARCO and OLR1 are expressed by keratinocytes. a, cDNA from NHEK was amplified by PCR using primers designed to amplify regions of approximately 100 basepairs of the following scavenger receptors: 1) MSR1, 2) MARCO, 3) SCARA3, 4) COLEC12, 5) SCARA5, 6) SCARF1, 7) OLR1. b, NHEK were fixed and stained with a control rabbit IgG or rabbit polyclonal primary antibodies to human MARCO and human OLR1, and an Alexa Fluor 488 secondary antibody, and counterstained with DAPI. Scale bar = 50µM.
Figure 9: MARCO co-localizes with HSV-1 on the keratinocyte cell surface. NHEK were incubated alone (a) or in the presence of HSV-1 (b-d) for 2 hours at 4°C to allow binding of the virus to the cell surface. Unbound HSV-1 was removed by multiple wash steps before cells were fixed and incubated with both a mouse monoclonal antibody targeting HSV-1 gC, and rabbit polyclonal antibodies targeting MARCO (a,b), syndecan-1 (c), or OLR1 (d). Physical proximity of MARCO, syndecan-1 and OLR1 to HSV-1 was determined using a fluorescence-based proximity ligation assay that produces a red fluorescent signal only when the antigens recognized by the antibodies utilized in the assay reside within less than 40 nm of each other. One representative frame with similar cell numbers is displayed for each condition. a-d, scale bar = 200 µm. All data are from representative experiments repeated at least two times.
Figure 10: MARCO co-localizes with HSV-1 gC on the keratinocyte cell surface. NHEK were incubated alone (a) or in the presence of purified HSV-1 gC (b-d) for 2 hours at 4°C to allow binding of the virus or glycoprotein to the cell surface. Unbound gC was removed by multiple wash steps before cells were fixed and incubated with both a mouse monoclonal antibody targeting HSV-1 gC, and rabbit polyclonal antibodies targeting MARCO (a,b), syndecan-1 (c), or OLR1 (d). Physical proximity of MARCO, syndecan-1 and OLR1 to HSV-1 gC was determined using a fluorescence-based proximity ligation assay that produces a red fluorescent signal only when the antigens recognized by the antibodies utilized in the assay reside within less than 40nm of each other. One representative frame with similar cell numbers is displayed for each condition. a-d, scale bar = 100µm. All data are from representative experiments repeated at least two times.
Figure 11: MARCO binds directly to HSV-1 gC. Plate-bound purified MARCO protein was incubated with increasing concentrations of purified gC (a), gB (c) or gCΔC5/P (gC with a deletion of the heparin binding domain, amino acids 33-123) (e). gC, gB, and gCΔC5/P remaining bound after washing was detected and quantified by ELISA. $K_D$ and $B_{max}$ were determined using nonlinear regression with background subtracted using Graphpad Prism. In (b) and (f), data in (a) and (e), respectively, were transformed to create double-reciprocal plots to show linear binding kinetics. d, plate-bound recombinant OLR1 was incubated with purified gC. gC remaining bound after washing was detected and quantified by ELISA. a-f, all individual replicate values plotted with offset overlapping points. All data are from representative experiments repeated at least two times.
Figure 12: HSV-1 infection correlates with MARCO expression in cells and is reduced in mouse skin treated with the scavenger receptor ligand Poly(I). a, BMDM were infected with HSV-1 (MOI = 5), and viral DNA was quantified 24 hours after infection. Data are from one representative experiment of two independent experiments. \( n=6 \), error bars indicate s.e.m., evaluated by student’s t-test, two-tailed. b, BMDM were infected with HSV-1 at increasing MOI, and viral RNA was quantified six hours after infection, \( n=3 \). Mean ± s.e.m., effect of genotype analyzed by two-way ANOVA. Data are from one representative experiment of two independent experiments. c, HaCat cells selected after transfection with a control plasmid (pcDNA3) or a plasmid enabling high expression of human MARCO (pcDNA3-MARCO) were infected with HSV-1 at an MOI of 0.0005. PFU were quantified 48 hours after infection and compared using a two-tailed T-test. \( n=3 \). Error bars indicate the s.e.m. d, 16 week old female mice were immunocompromised with cyclophosphamide prior to infection with \( 10^5 \) PFU HSV-1. Mice were injected locally with PBS, or 125µg Poly(C) or Poly(I) twice daily. Photographs were taken once daily. Lesions were quantified using ImageJ. Error bars indicate the s.e.m. Two-way ANOVA with bonferroni post-tests was used to compare wound sizes in Poly(I) and Poly(C) treated mice to PBS treated mice, \( n=4 \), ** \( P<0.01 \); *** \( P<0.001 \).
Figure 13: The synthetic dsRNA Poly(I:C) protects keratinocytes against VV infection. a, HaCat keratinocytes were pretreated for 24 hours with 10µg/ml Poly(I:C), 5µg/ml Imiquimod, 5µg/ml CpG DNA, 100ng/ml Malp-2, 1ng/ml Lipopolysaccharide (LPS), 100ng/ml Flagellin, 1µg/ml Peptidoglycan (PGN), before infection with VV. Plaques were quantified 24 hours after infection. b, HaCat keratinocytes were infected with VV after pretreatment with the indicated concentrations of Poly(I:C) for 24 hours. Plaques were quantified 24 hours after infection. c, NHEKs were infected with VV after pretreatment with the indicated concentrations of Poly(I:C) for 24 hours. Plaques were quantified 24 hours after infection. d, NHEK were infected with 240 PFU/ml VV after pretreatment with the indicated concentrations of Poly(I:C) for 24 hours. Viral mRNA was quantified 8 hours after infection. a-d, error bars indicate s.e.m., n=3, ** P < 0.01, *** P < 0.001; nd, not detectable.
Figure 14: Cyclohexamide inhibits VV G8R mRNA synthesis. HaCat keratinocytes were pretreated with Chx for 30 minutes prior to infection with VV for 4.5 hours. mRNA levels of VV G8R, an intermediate gene, were quantified by qPCR. mRNA levels in vehicle treated, VV infected cells were set to 100%. Error bars indicate SEM. nd, not detectable. n=3, *** $P < 0.001$. 
Figure 15: dsRNA protects against VV infection in the absence of cellular activation. a, HaCat keratinocytes were pretreated with 10µg/ml cyclohexamide for 30 minutes prior to infection with 2.4x10^3 PFU/ml VV in the presence or absence of 25µg/ml Poly(I:C), as indicated, for 3 hours. Viral mRNA was quantified by qPCR. b, NHEK were pretreated with 10µg/ml chloroquine (CQ) for 1 hour before the addition of 5x10^3 PFU/ml VV in the presence or absence of 50µg/ml Poly(I:C), as indicated. Viral mRNA was quantified by qPCR 2.5 hours after infection. Error bars indicate s.e.m., n=3, * P < 0.05, *** P < 0.001; ns, not significant.
**Figure 16: Additional ligands with affinity for scavenger receptors prevent VV infection.**

HaCat keratinocytes were infected with 100 PFU/well VV in the presence of the indicated concentrations of (a) Poly(I:C), Poly(I), and Poly(C), or (b) dextran sulfate (Dxs). Plaques were quantified 24 hours after infection. Poly(I:C)/Poly(I)/Poly(C) molarities were calculated and normalized based on the molecular weight of each individual nucleotide in the polymer, with average molecules ranging from 200-1000 nucleotides in length. Error bars indicate s.e.m., n=3, *** P < 0.001.
Figure 17: VV binds directly to MARCO. MARCO (a) and OLR1 (b) were coated on plates at 2.5ug/ml. Increasing concentrations of VV was added to the wells and allowed to bind to the proteins. Unbound virus was removed by repeated wash steps, and virus remaining bound to the immobilized protein was quantified using an HRP-linked secondary antibody and a chromogenic substrate solution, and detected by measuring absorbance (Abs) at 450nm, with Abs 570 subtracted. Each individual replicate is plotted using staggered overlapping points, with non-linear regressions plotted as solid lines.
Figure 18: VV binding to MARCO is inhibited by Poly(I). a, Plates coated with 2.5ug/ml MARCO were incubated with 6x10⁶ PFU/ml vaccinia virus in the presence of increasing concentrations of Poly(I). Unbound virus was removed by multiple wash steps, and bound virus was quantified using an HRP-linked secondary antibody and a chromogenic substrate solution, and detected by measuring absorbance (Abs) at 450nm, with Abs 570 subtracted. Each individual data point is plotted. b, data in (a) was transformed using a logarithmic x-axis, and the half-maximal inhibitory concentration (IC₅₀) value was calculated using Graphpad Prism. The 95% confidence interval (CI) for this IC₅₀ value is also displayed. Poly(I) molarity was calculated based on the molecular weight of each individual nucleotide in the polymer, with average molecules ranging from 200-1000 nucleotides in length.
Figure 19: MARCO expression in skin from patients with atopic dermatitis. Skin biopsy samples taken from an AD donor at non-lesional (a) and lesional (b) sites from were analyzed by immunofluorescence staining. MARCO expression was analyzed using an antibody recognizing human MARCO, and compared to IgG controls. Scale bars = 100µm.
Figure 20: Overexpression of MARCO increases VV infection in keratinocytes. HaCat keratinocytes stably overexpressing MARCO (pcDNA3-MARCO), or control cells (pcDNA3) were infected with VV. Plaques were stained and quantified 24 hours after infection. Error bars indicate s.e.m. n=3.
Figure 21: Model of the novel role of MARCO in HSV-1 and VV adsorption to keratinocytes.
Figure 22: ADAR1 expression in NHEK. cDNA from NHEK was amplified with a custom probe/primer set for GAPDH as described in the Methods sections of Chapters II and III, and a predesigned primer/probe set for ADAR1 (Life Technologies, Grand Island, NY) in triplicate. The amplification plot of each individual replicate is displayed.
Figure 23: Summary of the known roles of scavenger receptors in antiviral defense and as receptors for infection. Numerous scavenger receptors have important roles in mediating antiviral defense. MSR1 recognizes HCMV and HAdV5 (on macrophages) (223), and nucleic acids PAMPs from viruses including CpG DNA, dsRNA, and ssRNA (74, 187, 217, 244, 264, 276). MARCO also binds to these three nucleic acid PAMPs (217, 244), and SCARB1 also binds to CpG DNA (277). Conversely, some viruses exploit recognition by these receptors. HAdV5 uses MSR1 as a receptor on certain cell types (223), HCV uses SCARB1 as a receptor (236), SCARB2 is a receptor for CVA7, CVA14, CVA16 (237), and EV71 (238), and we have now shown in this dissertation that both HSV-1 and VV use MARCO as a receptor.
Table 1: Mammalian scavenger receptors organized by class. (reviewed in (68, 278)). Seven classes of scavenger receptors are expressed by mammalian cells, distinguished from each other by a number of key structural features. Class A scavenger receptors include Macrophage scavenger receptor 1 (MSR1), Macrophage receptor with collagenous structure (MARCO), Scavenger receptor class A, member 3 (SCARA3), Collectin sub-family member 12 (COLEC12), and Scavenger receptor class A, member 5 (SCARA5). Class B scavenger receptors include Scavenger receptor class B, member 1 (SCARB1), Scavenger receptor class B, member 2 (SCARB2), and CD36. Class D scavenger receptors include CD68 and Lysosomal-associated membrane protein (LAMP) -1, -2, and -3. Class E includes only a single member, Oxidized low density lipoprotein (lectin-like) receptor 1 (OLR1). Class F scavenger receptors are named Scavenger receptor class F, member 1 (SCARF1) and Scavenger receptor class F, member 2 (SCARF2). Class G includes a single scavenger receptor, Chemokine (C-X-C motif) ligand 16 (CXCL16). Class H is comprised of two scavenger receptors, Stabilin (STAB) -1 and -2.

<table>
<thead>
<tr>
<th>Class</th>
<th>Receptors</th>
<th>Structural Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>MSR1, MARCO, SCARA3, COLEC12, SCARA5</td>
<td>Type II transmembrane glycoproteins that can form homotrimers</td>
</tr>
<tr>
<td>B</td>
<td>SCARB1, SCARB2, CD36</td>
<td>Type III glycoproteins with two transmembrane domains connected by a large extracellular loop</td>
</tr>
<tr>
<td>D</td>
<td>CD68, Lamp-1, Lamp-2, Lamp-3</td>
<td>Type I transmembrane glycoproteins, endosome/lysosome-associated</td>
</tr>
<tr>
<td>E</td>
<td>OLR1</td>
<td>Type II transmembrane glycoprotein, extracellular region contains C-type lectin-like domain</td>
</tr>
<tr>
<td>F</td>
<td>SCARF1, SCARF2</td>
<td>Type I transmembrane proteins containing multiple epidermal growth factor (EGF)-like domains in the extracellular region, and a long cytoplasmic tail</td>
</tr>
<tr>
<td>G</td>
<td>CXCL16</td>
<td>Type I transmembrane protein with an extracellular chemokine domain joined to a mucin-like region</td>
</tr>
<tr>
<td>H</td>
<td>STAB1, STAB2</td>
<td>Type I transmembrane proteins with extremely large extracellular domains mostly consisting of multiple EGF-like domains</td>
</tr>
</tbody>
</table>
Table 2: Scavenger receptors contribute to innate immunity by binding to various PAMPs.

<table>
<thead>
<tr>
<th>Function</th>
<th>PAMPs</th>
<th>Scavenger receptors involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uptake of RNA from the cell surface, presentation to intracellular PRRs including TLR3 and RLRs</td>
<td>dsRNA ssRNA</td>
<td>MSR1 (74, 187, 217, 264), MARCO (217), SCARA3 (187), COLEC12 (187), SCARA5 (187)</td>
</tr>
<tr>
<td>Binding DNA at the cell surface, mediating TLR9-independent responses and modulation of TLR9-dependent responses</td>
<td>CpG DNA</td>
<td>MSR1 (244, 276), MARCO (244), SCARB1 (277), CXCL16 (279)</td>
</tr>
<tr>
<td>Uptake of bacterial cell wall components, presentation to NOD2</td>
<td>MDP</td>
<td>MSR1, MARCO (217)</td>
</tr>
<tr>
<td>Binding to Gram-positive and Gram-negative bacteria, non-opsonic phagocytosis</td>
<td>LPS, LTA, Malp-2, and/or Bacteria surface proteins</td>
<td>MSR1 (280-283), MARCO (226, 250, 284), COLEC12 (285), SCARA5 (286), CD36 (287-290), SCARB1 (290-292), SCARB2 (290, 292), OLR1 (293, 294), SCARF1 (294), CXCL16 (295), STAB1 (296), STAB2 (296)</td>
</tr>
<tr>
<td>Non-opsonic phagocytosis of yeast</td>
<td>Zymosan</td>
<td>MSR1 (297), MARCO (297), COLEC12 (285, 298)</td>
</tr>
<tr>
<td>Binding to fungal pathogens, modulation of immune responses</td>
<td>Beta-glucans</td>
<td>CD36 (299), SCARF1 (299)</td>
</tr>
</tbody>
</table>
Table 3: Potential receptors for HSV-1 and VV expressed by keratinocytes.

<table>
<thead>
<tr>
<th>Infectious Step</th>
<th>Virus</th>
<th>Receptors expressed by keratinocytes</th>
<th>Viral glycoprotein mediating interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorption</td>
<td>HSV-1 &amp; VV</td>
<td>HSPGs: <strong>Syndecan-1</strong> (184, 300, 301), <strong>Syndecan-2</strong> (301), <strong>Syndecan-4</strong> (184, 301), <strong>CD44 – HS</strong> (301, 302), <strong>Transforming growth factor beta receptor III (TGFBR3, also known as betaglycan)</strong> (303), <strong>Glypican-1</strong> (301, 304)</td>
<td>HSV-1 gC &amp; gB VV A27 &amp; H3</td>
</tr>
<tr>
<td>Adsorption</td>
<td>VV</td>
<td>CSPGs: <strong>CSPG4</strong> (305), <strong>CD44 – CS</strong> (306), <strong>TGFBR3</strong> (303)</td>
<td>VV D8</td>
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<tr>
<td>Entry</td>
<td>HSV-1</td>
<td><strong>HVE M</strong> (96), <strong>Nectin-1</strong> (96, 104)</td>
<td>HSV-1 gD</td>
</tr>
<tr>
<td>Entry</td>
<td>HSV-1</td>
<td><strong>Myosin-9</strong> (307)</td>
<td>HSV-1 gB</td>
</tr>
<tr>
<td>Entry</td>
<td>VV</td>
<td>?</td>
<td></td>
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