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Vaccinia virus binds to the scavenger receptor MARCO on the surface of keratinocytes

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Abstract

Patients with altered skin immunity, such as individuals with atopic dermatitis (AD), can have a life-threatening disruption of the epidermis known as eczema vaccinatum (EV) after vaccinia virus (VV) infection of the skin. Here, we sought to better understand the mechanism(s) by which VV associates with keratinocytes. The class A scavenger receptor known as MARCO (macrophage receptor with collagenous structure) is expressed on human and mouse keratinocytes and found to be abundantly expressed in the skin of patients with AD. VV bound directly to MARCO, and overexpression of MARCO increased susceptibility to VV infection. Furthermore, ligands with affinity for MARCO, or excess soluble MARCO, competitively inhibited VV infection. These findings indicate that MARCO promotes VV infection and highlights potential new therapeutic strategies for prevention of VV infection in the skin.

Introduction

Vaccinia virus (VV) is a large DNA virus widely known for its use as a vaccine for the closely related variola virus, the causative agent of smallpox (Copeman and Banatvala, 1971; Fulginiti *et al.*, 2003a). Routine administration of this vaccine was stopped after smallpox was eradicated, however VV has been utilized for vaccination of selected high-risk individuals such as some individuals serving in the military (Fulginiti *et al.*, 2003a), and to study the disease mechanisms of smallpox. Vaccination is accomplished by inoculation in the skin by scarification of the epidermis. Inoculation typically results in a localized VV infection of keratinocytes at the inoculation site. This usually resolves following local innate immune defense and subsequent development of protective humoral and cell-mediated immunity. 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

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Conflict of Interest

The authors have no competing financials interests to disclose.

disseminated skin infection eczema vaccinatum (EV) (Copeman and Wallace, 1964; Fulginiti *et al.*, 2003b). Immunocompromised patients and children are also at risk of developing severe infections with VV (Lane and Millar, 1969; Redfield *et al.*, 1987; Sepkowitz, 2003). While these individuals are excluded from vaccination due to the associated risks, severe infections have also been known to occur in individuals coming into contact with others who have been recently inoculated with VV (Lane *et al.*, 1970; Vora *et al.*, 2008).

Although treatments for VV infection exist, none have been fully evaluated in a controlled setting (Bray, 2003). A better understanding of the factors controlling the pathogenesis of VV in the skin would reduce complications resulting from inoculation with live VV, and may also hint to alternative strategies for treatment of other viruses that share these pathogenic mechanisms. 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 using keratinocytes, the primary target of VV infection in the skin.

A number of cell surface molecules have been previously identified to mediate VV adsorption to the cell surface and promote infection. Some strains of VV bind initially to heparan sulfate (HS) or chondroitin sulfate (CS) glycosaminoglycans (GAGs), but others infect cells in a GAG-independent manner (Bengali *et al.*, 2009). Five viral proteins have been identified so far for their involvement in binding to the cell surface. A27 and H3 mediate binding to HS while D8 binds to CS (Chung *et al.*, 1998; Hsiao *et al.*, 1999; Lin *et al.*, 2000). GAG-independent binding is also possible with certain strains and certain cell types, and involves interactions between A26 and laminin on the cell surface, or between L1 and an undetermined receptor (Chiu *et al.*, 2007; Foo *et al.*, 2009). 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 (Bengali *et al.*, 2009). Studies focusing specifically on keratinocytes are required to determine which surface molecules are most critical for VV infection.

Cell surface and intracellular receptors are key elements of the innate immune system and are primarily thought to serve a protective role, as defects in innate immunity appear to be critical to pathogenesis of EV (Howell *et al.*, 2006a; Howell *et al.*, 2004; Howell *et al.*, 2007; Howell *et al.*, 2006b; Kim *et al.*, 2007). Additionally, numerous proteins expressed by VV function to interfere with host defense responses (reviewed in (Haga and Bowie, 2005)). 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 (Kristian *et al.*, 2003; Nizet *et al.*, 2001; Schauber *et al.*, 2007). Cells can detect VV infection through activation of cell-surface and intracellular pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs). Keratinocytes express TLRs 1–6, 9, and 10 (Köllisch *et al.*, 2005; Lebre *et al.*, 2006). A previous study implicated TLR2/6 in detection of VV on the cell surface, and melanoma differentiation-associated gene-5 (MDA-5) and the NOD-like receptor family, pyrin domain

containing 3 (NALP-3) inflammasome detecting VV inside the cell (Delaloye *et al.*, 2009). Additionally, VV DNA can stimulate innate immune responses in cells after recognition by TLR8 (Martinez *et al.*, 2010). Furthermore, Sphingosine-1-phosphate receptor 2 has recently been shown to recognize lipids derived from viral membranes and trigger the release of antimicrobial peptides (Wang *et al.*, 2012). 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 (DeWitte-Orr *et al.*, 2010a; Limmon *et al.*, 2008; Matsumoto *et al.*, 2003; Mukhopadhyay *et al.*, 2011; Saleh *et al.*, 2006; Yew *et al.*, 2010). TLR3 is hypothesized to enable recognition of a DNA virus such as VV since virtually all viruses produce dsRNA at some point during replication (Jacobs and Langland, 1996), which is then released into the extracellular space after lysis of infected cells (Majde *et al.*, 1998). Scavenger receptors have also been shown to bind to a variety of bacterial and viral products (Krieger *et al.*, 1993; Yew *et al.*, 2010). Therefore, it would be expected that scavenger receptors would function to alert keratinocytes to danger from VV infection and provide a protective role. However 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 herpes simplex virus type 1 (HSV-1) to bind to the surface of keratinocytes and increase infection (Macleod *et al.*, 2013). In this manuscript, we hypothesized that MARCO may also serve to enhance VV infection of keratinocytes. Our findings suggest MARCO can play a significant role in infection of keratinocytes, and that this interaction could have important implications for eczema vaccinatum.

Results

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

Patients with AD are susceptible to severe infections with VV (Copeman and Wallace, 1964; Howell *et al.*, 2006a; Howell *et al.*, 2004). We recently found that MARCO is expressed in keratinocytes and is a key receptor in cutaneous HSV-1 infection (Macleod *et al.*, 2013). Thus, given the susceptibility of AD patients to HSV-1 and VV, we decided to investigate whether the expression of MARCO in AD could have a role in the pathogenesis of these viral skin disorders. First, we tested the expression of MARCO in normal skin compared to lesional and non-lesional AD skin. MARCO was expressed evenly throughout the epidermis of normal skin and non-lesional AD skin (Figure 1a,b, and Supplementary Fig. S1). Strikingly, much greater staining for MARCO was seen in the epidermis of AD lesional skin. These data confirmed that the skin of AD has abundant MARCO on the surface of keratinocytes that could potentially interact with VV and play a role in the susceptibility of individuals with AD to VV infection. Stimulation of cultured normal human keratinocytes with cytokines elevated in AD (IL-4 and IL-13) did not significantly alter MARCO expression (Fig. 1c), suggesting that other factors contributing to AD were responsible for the increased MARCO expression.

Vaccinia virus binds directly to MARCO

We next sought to identify if MARCO is capable of interacting with VV. Keratinocytes express both MARCO and OLR1 scavenger receptors (Macleod *et al.*, 2013). To test for a direct interaction with VV, we employed a cell-free ELISA utilizing purified recombinant MARCO. Using this assay, we found that VV bound specifically to immobilized human and mouse MARCO protein (Fig. 2a,b), demonstrating that VV binds to MARCO in the absence of any other cell surface receptors. Importantly, this interaction could be disrupted by the addition of Poly(I) (Fig. 2c), a scavenger receptor ligand known to bind to MARCO (Chen *et al.*, 2006; Kodama *et al.*, 1990; Moriwaki *et al.*, 1998). VV did not bind to two additional scavenger receptors, OLR1 (Fig 2d) and MSR1 (Fig 2e), and an alternative enveloped virus, an HIV-1 pseudovirus (PsV), did not bind to MARCO (Fig. 2f).

Overexpression of MARCO increases susceptibility to VV infection

To evaluate the functional significance of the interaction of VV and MARCO we next infected wild-type (WT) and MARCO$^{-/-}$ mice with VV. Interestingly, both WT and MARCO$^{-/-}$ mice infected with VV had significant and comparable infiltration of inflammatory cells around the wound edge and throughout the dermis compared to uninfected control mice, and lesion sites appeared morphologically similar to each other (Figure 3a). Furthermore, at six days post-infection, there was no significant difference in the sizes of wounds in the skin of WT and MARCO$^{-/-}$ mice (Figure 3b), indicating that MARCO expression is not absolutely required for VV infection. However, although MARCO did not appear to be essential for infection in whole skin and the absence of MARCO did not alter the morphology of VV-induced lesions, it remained possible that the lack of MARCO could be compensated by additional receptors with similar characteristics. Thus, to further examine the relationship between the expression of MARCO and susceptibility to VV infection, we utilized a keratinocyte cell line that stably overexpresses human MARCO protein at levels approximately 3 fold higher than control transfected cells (Macleod *et al.*, 2013). VV infection of these cells resulted in significantly more viral plaques (Figure 3b) and viral DNA (Figure 3c) in the cells that overexpress MARCO compared to control cells. These experiments demonstrated that the interaction between MARCO and VV is functionally significant.

Scavenger receptor antagonists inhibit vaccinia virus infection of keratinocytes

A number of studies have previously demonstrated that there is overlap in the binding specificities and functions of class A scavenger receptors (DeWitte-Orr *et al.*, 2010b; Limmon *et al.*, 2008). Thus, as discussed above, the presence of additional receptors with similar specificity, such as SCARA3, COLEC12, or SCARF1, which are expressed by NHEK (Macleod *et al.*, 2013), may compensate for the absence of MARCO in MARCO$^{-/-}$ mice. In this instance, scavenger receptors ligands that exploit a similar binding specificity between MARCO and other such receptors would be able to competitively inhibit binding to VV. Polyinosinic acid (Poly(I)) and dextran sulfate (Dxs) are two such ligands that have been extensively characterized as ligands for class A scavenger receptors (Krieger *et al.*, 1993). Both of these compounds potently inhibited VV infection of keratinocytes in a dose-dependent manner (Figure 4a,b). Poly(I) provided levels of protection comparable to

Poly(I:C), which also binds to scavenger receptors (DeWitte-Orr *et al.*, 2010a; Limmon *et al.*, 2008; Mukhopadhyay *et al.*, 2011; Saleh *et al.*, 2006). However, unlike Poly(I:C), Poly(I) is not able to activate innate immune responses in keratinocytes (Macleod *et al.*, 2013). In contrast, the similar nucleic acid Poly(C), does not bind to scavenger receptors and had no effect on VV infection (Fig. 4a). Thus, these results showed a strong correlation between scavenger receptor binding and the capacity to inhibit infection. In light of the absence of an interaction of VV with OLR-1 and MSR1 (Fig. 2d,e), this revealed that while not all receptors that bind Poly(I) bind to VV, Poly(I) interferes with binding of VV to the cell surface.

Although the similarities in the capacity of Poly(I:C) and Poly(I) to prevent VV infection indicated that the inhibition could be attributed to a shared affinity for scavenger receptors rather than the ability of Poly(I:C) to activate keratinocyte innate immune responses, we designed experiments to confirm this. We first compared the ability of treatment of Poly(I:C) and other TLR ligands to protect against VV infection. Poly(I:C), significantly protected against VV infection of keratinocytes but treating with ligands that activate other TLRs (Lai and Gallo, 2008) did not (Fig. 4c). Notably, this inhibitory effect by Poly(I:C) as measured by viral plaque formation was dose-dependent and was comparable in both HaCat keratinocytes and normal human epidermal keratinocytes (NHEK) (Fig. 4d,e). This inhibitory effect was also seen when analyzing the amount of VV early gene mRNA at an early timepoint after infection (Fig. 4f), indicating that the inhibition was occurring at an early step in the infectious process.

To also test if protection by Poly(I:C) could be attributed to activation of innate immune responses, we treated cells with inhibitors of cellular activation. First, we used cycloheximide (Chx), a potent inhibitor of new protein synthesis. Treatment with Chx inhibited the ability of the cells to synthesize new proteins by 97% (Data not shown). However, VV early gene expression is, by definition, not altered by Chx treatment, thus allowing us to evaluate the ability of the virus to enter cells and begin viral RNA synthesis under conditions that block synthesis of new proteins (Amegadzie *et al.*, 1991). Poly(I:C) inhibited VV infection even under these conditions (Supplementary Fig. S2a), 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 chloroquine (CQ), a potent inhibitor of endosomal acidification. These conditions prevent the ability of Poly(I:C) to induce IL-6 and IFN-β by more than 80% (Macleod *et al.*, 2013). 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 (Supplementary Fig. S2b), further 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.

To complement these studies, we tested the ability of soluble recombinant MARCO to inhibit VV infection. Pretreatment with recombinant MARCO inhibited VV plaque formation in NHEK by 54% (Fig. 4g, $P < 0.001$), suggesting that the binding of MARCO to VV inhibits association of the virus with MARCO and related scavenger receptors on the cell surface, confirming the necessity of these molecules for optimal infection of skin cells.

Finally, to test the significance of blocking scavenger receptors to cutaneous VV infection, we topically applied a single dose of Poly(I) to the back skin of mice prior to infecting them with VV. This single dose of Poly(I) moderately reduced wound size at day 6 post-infection, while Poly(C) treated mice had wound sizes comparable to control PBS-treated mice (Fig. 4h).

These results demonstrated that multiple compounds that share the capacity to restrict access to cell-surface scavenger receptors are capable of inhibiting VV infection *in vitro* and *in vivo*, and further confirmed that the binding interactions observed between VV and MARCO are functionally relevant.

Discussion

We demonstrate in this manuscript that VV binds directly to MARCO, a scavenger receptor present on the surface of keratinocytes. Importantly, overexpression of MARCO increased susceptibility to VV infection, recombinant MARCO inhibited infection of keratinocytes, and ligands capable of blocking this interaction potently prevented infection of VV in keratinocytes and mouse skin. These results identify MARCO as a receptor that can be used by VV during infection in the skin. These observations also show a potential new therapeutic approach that may help limit VV infection in patients with AD who have abundant expression of MARCO in their thickened lesional epidermis.

A number of studies suggest that multiple receptors play complementary roles to enable VV binding, thus providing a complex and partially redundant system for the virus to infect a variety of cell types. One such class of molecules to which VV binds is cell surface heparan sulfate proteoglycans (HSPGs). MARCO was recently shown by us to act in conjunction HSPGs to mediate adsorption of HSV-1 to the cell surface (Macleod *et al.*, 2013). Based on our current results, we believe that a similar mechanism is functioning for VV. Furthermore, in addition to having multiple molecules to adhere to on the host cell, the strain of virus and the type of cell being infected also result in differences in the adsorption and entry of VV. Some strains of VV exhibit less dependence on HSPGs for adsorption compared to other strains (Bengali *et al.*, 2009; Carter *et al.*, 2005). To dissect the strain differences in viral binding it is therefore important to understand the molecules on the surface of the virus that are mediating adsorption. The viral preparation used in our experiments was primarily the intracellular mature virus (IMV) form of VV, which has at least seven proteins known or predicted to be expressed or associated with the cell surface (Duke-Cohan *et al.*, 2009). The interaction with MARCO was 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 glycosaminoglycans (GAGs) (Chung *et al.*, 1998; Hsiao *et al.*, 1999; Lin *et al.*, 2000). It is also possible that another viral glycoprotein, such as A26, which binds to laminin (Chiu *et al.*, 2007) is also 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 (Foo *et al.*, 2009). Further experiments will be needed to identify the specific viral glycoprotein capable of interacting with scavenger receptors, and to confirm whether the primary role of these receptors is solely in adsorption of the virus to

the cell surface, or whether they have an active role in uptake of the virus as well. VV is taken up by target cells via macropinocytosis, utilizing phosphatidylserine (PS) in the viral membrane to mimic apoptotic debris and trigger uptake by a cellular PS receptor (Laliberte and Moss, 2009; Mercer and Helenius, 2008; Mercer *et al.*, 2010). It is possible that scavenger receptors are contributing to this process as well, however further experiments will be needed to test this possibility.

Previously, it was expected that scavenger receptors serve a protective role due to their capacity to facilitate the detection of foreign pathogens. However, we have now demonstrated that MARCO increases the susceptibility to infection by binding to VV. This is consistent with a previously described interaction between HSV-1 and MARCO (Macleod *et al.*, 2013), and the binding of the closely related scavenger receptor MSR1 to Adenovirus 5 (Haisma *et al.*, 2009). This finding that an increasing number of diverse viruses exploit class A scavenger receptors is very interesting. It is also possible that these viruses use scavenger receptors as a way to escape recognition by the innate immune system. Indeed, it has been shown that although MSR1 and MARCO enhance uptake of extracellular Pathogen-associated molecular patterns (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 (Mukhopadhyay *et al.*, 2011). 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 (Scarselli *et al.*, 2002), Coxsackie A virus -7, -14, and -16 (Yamayoshi *et al.*, 2012) and Enterovirus 71 (Yamayoshi *et al.*, 2009).

The significant inhibition of VV by scavenger receptor ligands in human cells and mouse skin is important, as this shows that these ligands could potentially be used to block scavenger receptors and limit VV infection in human skin. Currently, treatment of severe cutaneous VV infection can involve administration of vaccinia immune globulin (VIG), however VIG is currently classified by the CDC as an investigational drug, and controlled studies determining the true efficacy of VIG and other potentially useful antiviral drugs such as Cidofovir and Riboviron are lacking (Bray, 2003). 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 either treatment alone (Worthington and Baron, 1973). 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. 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 as a treatment for severe infections such as EV that arise as complications from inoculation with VV.

Materials and Methods

Cells and Viruses

NHEK 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 (a gift from Rivkah Isseroff, University of California, Davis) were cultured in DMEM (Lonza, Basel, Switzerland), 10% FBS (Thermo Fisher Scientific, Waltham, MA), 2mM L-glutamine, Penicillin/Streptomycin (VWR, Radnor, PA). HaCat cells stably overexpressing MARCO and control HaCat cells were generated as previously described (Macleod *et al*., 2013). The Wyeth strain of VV (a kind gift from Michael Croft, La Jolla Institute for Allergy and Immunology, CA) vB5R-GFP VV, used for mouse experiments (a kind gift from Bernard Moss, National Institute of Allergy and Infectious Diseases, National Institutes of Health, MD), and HIV-1 JR-FL PsV (a kind gift from Pascal Poignard, International AIDS Vaccine Initiative, La Jolla, CA) were prepared and titered from crude extracts of BSC-1 or 293T infected cells, lysed and centrifuged to remove cellular debris, and stored at −80°C.

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%.

VV infection of murine skin

C57Bl/6 wild-type mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. $MARCO^{-/-}$ mice were a gift from Dr. Andrij Holian (Univerity of Montana, MT). 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. Mice were depilated, then infected one day later with 2×10^6 PFU vB5R-GFP VV by skin scarification, 25 scratches with a 25 gauge needle within a $1cm^2$ area on the back. Mice were photographed once daily, and lesion sizes were quantified with ImageJ. On day 6 post-infection, mice were sacrificed and skin from the wound site was biopsied and sent to the Moores Cancer Center Histology and Immunohistochemistry Core (La Jolla, CA) for fixation, sectioning, and staining with hematoxylin and eosin.

ELISAs

Proteins were bound to EIA/RIA plates at 2.5ug/ml in sterile PBS containing calcium and magnesium, pH 7.4 (Lonza, Basel, Switzerland), washed, blocked with PBS containing 3%BSA, then incubated with VV or HIV-1 PsV diluted in DMEM + 1% FBS. Unbound virus was removed by repeated wash steps with PBS. Bound virus was detected using a

rabbit polyclonal primary antibody recognizing MARCO (Abcam, Cambridge, MA) or a biotinylated human antibody recognizing HIV-1 (a kind gift from Pascal Poignard, International AIDS Vaccine Initiative, La Jolla, CA), an HRP-conjugated secondary antibody or streptavidin, respectively (Santa Cruz Biotechnology, Santa Cruz, CA), and TMB substrate reagent (BD Biosciences Pharmigen, San Diego, CA), and quantified using a microplate reader measuring absorbance at 450nm and subtracting absorbance at 570nm. Background binding (binding of the same concentrations of VV or HIV-1 PsV to plates coated with BSA) was subtracted.

Quantitative realtime PCR

RNA and DNA were isolated using Trizol (Life Technologies, Grand Island, NY), and RNA was reverse transcribed using iScript (Bio-Rad, Hercules, CA). Predesigned Taqman primers/probes (Life Technologies, Grand Island, NY) were used for the quantification of MARCO mRNA and custom Taqman Probes were used for VV DNA-dependent RNA polymerase (RPO35) and GAPDH mRNA and DNA quantification using Taqman Gene Expression Master Mix (Life Technologies, Grand Island, NY) with a 7300 Real-Time PCR System (Life Technologies, Grand Island, NY) using the primer sequences listed below. Fold changes of target genes normalized to host cell GAPDH levels relative to the control treatments were calculated using the $2^{(-\Delta\Delta Ct)}$ method, with untreated or vehicle treated samples normalized to 1 or 100% as indicated.

qPCR Primer/Probe sequences

GAPDH probe: 5′-CATCCATGACAACTTTGGTA-3′. VV RPO35 Probe: 5′-ATTGAATTCTCTTCCCGCGGATGCTG-3′. Primers were purchased from Sigma-Aldrich, St. Louis, MO: GAPDH: 5′-CCTAGCACCCCTGGCCAAG-3′, 5′-TGGTCATGAGTCCTTCCACG-3′; VV RPO35: 5′-GCCAATGAGGGTTCGAGTTC-3′, 5′-CAACATCCCGTCGTTCATCA-3′;

Reagents

Poly(I:C), Imiquimod, Peptidoglycan and CpG DNA were purchased from Invivogen, San Diego, CA. Malp-2 and Flagellin were purchased from Enzo Life Sciences, Farmingdale, NY. Bovine serum albumin (BSA), Lipopolysaccharide, Poly(I), Poly(C), Dextran Sulfate, and cyclophosphamide were purchased from Sigma-Aldrich, St. Louis, MO. Chloroquine phosphate was purchased from Spectrum, Gardena, CA. Recombinant human and mouse MARCO, and human OLR1, IL-4, and IL-13 were purchased from R&D Systems, Minneapolis, MN.

Stimulation with TLR ligands

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 and quantification of viral plaque forming units.

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 normal skin and 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 cold acetone, and stained for the presence of MARCO using a monoclonal antibody (a kind gift from Lester Kobzik, Harvard University, MA) and Keratin-14 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa-fluor 488- and 568-labeled secondary antibodies, respectively (Life Technologies, Grand Island, NY) and counterstained with DAPI. Images were captured using a BX41 microscope (Olympus, Center Valley, PA).

Statistical Analysis

Analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AD	atopic dermatitis
COLEC12	collectin sub-family member 12
CS	chondroitin sulfate
Dxs	Dextran sulfate
GAG	glycosaminoglycan
HS	heparan sulfate
HSPG	heparan sulfate proteoglycans
HSV-1	herpes simplex virus type 1
MARCO	macrophage receptor with collagenous structure
MSR1	macrophage scavenger receptor 1
NHEK	normal human epidermal keratinocytes

OLR1	oxidized low density lipoprotein (lectin-like) receptor 1
PAMP	pattern associated molecular pattern
Poly(C)	polycytidylic acid
Poly(I)	Polyinosinic acid
Poly(I,C)	polyinosinic:polycytidylic acid
PRR	pattern recognition receptor
SCARA3	scavenger receptor class A member 3
SCARF1	scavenger receptor class F, member 1
TLR	toll-like receptor
VIG	vaccinia immune globulins
VV	vaccinia virus

References

Amegadzie BY, Ahn BY, Moss B. Identification, sequence, and expression of the gene encoding a Mr 35,000 subunit of the vaccinia virus DNA-dependent RNA polymerase. J Biol Chem. 1991; 266:13712–8. [PubMed: 1856205]

Bengali Z, Townsley AC, Moss B. Vaccinia virus strain differences in cell attachment and entry. Virology. 2009; 389:132–40. [PubMed: 19428041]

Bray M. Pathogenesis and potential antiviral therapy of complications of smallpox vaccination. Antiviral research. 2003; 58:101–14. [PubMed: 12742570]

Carter GC, Law M, Hollinshead M, et al. Entry of the vaccinia virus intracellular mature virion and its interactions with glycosaminoglycans. J Gen Virol. 2005; 86:1279–90. [PubMed: 15831938]

Chen Y, Sankala M, Ojala JR, et al. A phage display screen and binding studies with acetylated low density lipoprotein provide evidence for the importance of the scavenger receptor cysteine-rich (SRCR) domain in the ligand-binding function of MARCO. J Biol Chem. 2006; 281:12767–75. [PubMed: 16524885]

Chiu WL, Lin CL, Yang MH, et al. Vaccinia virus 4c (A26L) protein on intracellular mature virus binds to the extracellular cellular matrix laminin. J Virol. 2007; 81:2149–57. [PubMed: 17166913]

Chung CS, Hsiao JC, Chang YS, et al. A27L protein mediates vaccinia virus interaction with cell surface heparan sulfate. J Virol. 1998; 72:1577–85. [PubMed: 9445060]

Copeman PW, Banatvala JE. The skin and vaccination against smallpox. Br J Dermatol. 1971; 84:169–73. [PubMed: 4926866]

Copeman PW, Wallace HJ. Eczema Vaccinatum. British medical journal. 1964; 2:906–8. [PubMed: 14185655]

Delaloye J, Roger T, Steiner-Tardivel Q-G, et al. Innate immune sensing of modified vaccinia virus Ankara (MVA) is mediated by TLR2-TLR6, MDA-5 and the NALP3 inflammasome. PLoS pathogens. 2009; 5:e1000480. [PubMed: 19543380]

DeWitte-Orr SJ, Collins SE, Bauer CM, et al. An accessory to the 'Trinity': SR-As are essential pathogen sensors of extracellular dsRNA, mediating entry and leading to subsequent type I IFN responses. PLoS Pathogens. 2010a; 6:e1000829. [PubMed: 20360967]

DeWitte-Orr SJ, Collins SE, Bauer CM, et al. An accessory to the 'Trinity': SR-As are essential pathogen sensors of extracellular dsRNA, mediating entry and leading to subsequent type I IFN responses. PLoS Pathog. 2010b; 6:e1000829. [PubMed: 20360967]

Duke-Cohan JS, Wollenick K, Witten EA, et al. The heterogeneity of human antibody responses to vaccinia virus revealed through use of focused protein arrays. Vaccine. 2009; 27:1154–65. [PubMed: 19146908]

Foo CH, Lou H, Whitbeck JC, et al. Vaccinia virus L1 binds to cell surfaces and blocks virus entry independently of glycosaminoglycans. Virology. 2009; 385:368–82. [PubMed: 19162289]

Fulginiti VA, Papier A, Lane JM, et al. Smallpox Vaccination: A Review, Part I. Background, Vaccination Technique, Normal Vaccination and Revaccination, and Expected Normal Reactions. Clin Infect Dis. 2003a; 37:241–50. [PubMed: 12856217]

Fulginiti VA, Papier A, Lane JM, et al. Smallpox Vaccination: A Review, Part II. Adverse Events. Clin Infect Dis. 2003b; 37:251–71. [PubMed: 12856218]

Haga IR, Bowie AG. Evasion of innate immunity by vaccinia virus. Parasitology. 2005; 130:S11–S25. [PubMed: 16281988]

Haisma HJ, Boesjes M, Beerens AM, et al. Scavenger receptor A: a new route for adenovirus 5. Molecular pharmaceutics. 2009; 6:366–74. [PubMed: 19227971]

Howell M, Gallo R, Boguniewicz M, et al. Cytokine milieu of atopic dermatitis skin subverts the innate immune response to vaccinia virus. Immunity. 2006a; 24:341–9. [PubMed: 16546102]

Howell M, Jones J, Kisich K, et al. Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum. J Immunol. 2004; 172:1763–70. [PubMed: 14734759]

Howell M, Streib J, Leung D. Antiviral activity of human beta-defensin 3 against vaccinia virus. J Allergy Clin Immunol. 2007; 119:1022–7. [PubMed: 17353034]

Howell MD, Boguniewicz M, Pastore S, et al. Mechanism of HBD-3 deficiency in atopic dermatitis. Clinical immunology. 2006b; 121:332–670. [PubMed: 17015038]

Hsiao JC, Chung CS, Chang W. Vaccinia virus envelope D8L protein binds to cell surface chondroitin sulfate and mediates the adsorption of intracellular mature virions to cells. J Virol. 1999; 73:8750–61. [PubMed: 10482629]

Jacobs BL, Langland JO. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology. 1996; 219:339–49. [PubMed: 8638399]

Kim B, Leung D, Streib J, et al. Macrophage inflammatory protein 3alpha deficiency in atopic dermatitis skin and role in innate immune response to vaccinia virus. J Allergy Clin Immunol. 2007; 119:457–520. [PubMed: 17141855]

Kodama T, Freeman M, Rohrer L, et al. Type I macrophage scavenger receptor contains alpha546 helical and collagen-like coiled coils. Nature. 1990; 343:531–5. [PubMed: 2300204]

Köllisch G, Kalali BN, Voelcker V, et al. Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes. Immunology. 2005; 114:531–41. [PubMed: 15804290]

Krieger M, Acton S, Ashkenas J, et al. Molecular flypaper, host defense, and atherosclerosis. Structure, binding properties, and functions of macrophage scavenger receptors. J Biol Chem. 1993; 268:4569–72. [PubMed: 8383115]

Kristian SA, Lauth X, Nizet V, et al. Alanylation of teichoic acids protects Staphylococcus aureus against Toll-like receptor 2-dependent host defense in a mouse tissue cage infection model. J Infect Dis. 2003; 188:414–23. [PubMed: 12870123]

Lai Y, Gallo RL. Toll-like receptors in skin infections and inflammatory diseases. Infectious Disorders - Drug Targets. 2008; 8:144–55. [PubMed: 18782031]

Laliberte JP, Moss B. Appraising the apoptotic mimicry model and the role of phospholipids for poxvirus entry. Proc Natl Acad Sci U S A. 2009; 106:17517–21. [PubMed: 19805093]

Lane JM, Millar JD. Routine Childhood Vaccination against Smallpox Reconsidered. N Engl J Med. 1969; 281:1220–4. [PubMed: 4186804]

Lane JM, Ruben FL, Abrutyn E, et al. Deaths Attributable to Smallpox Vaccination, 1959 to 1966, and 1968. JAMA. 1970; 212:441–4. [PubMed: 4392370]

Lebre MC, van der Aar AMG, van Baarsen L, et al. Human Keratinocytes Express Functional Toll-Like Receptor 3, 4, 5, and 9. J Invest Dermatol. 2006; 127:331–41. [PubMed: 17068485]

Limmon GV, Arredouani M, McCann KL, et al. Scavenger receptor class-A is a firs cell surface receptor for double-stranded RNA. FASEB J. 2008; 22:159–67. [PubMed: 17709607]

Lin CL, Chung CS, Heine HG, et al. Vaccinia virus envelope H3L protein binds to cell surface heparan sulfate and is important for intracellular mature virion morphogenesis and virus infection in vitro and in vivo. J Virol. 2000; 74:3353–65. [PubMed: 10708453]

Macleod D, Nakatsuji T, Yamasaki K, et al. HSV-1 exploits the innate immune scavenger receptor MARCO to enhance epithelial adsorption and infection. Nature communications. 2013; 4:1963.

Majde JA, Guha-Thakurta N, Chen Z, et al. Spontaneous release of stable viral double-stranded RNA into the extracellular medium by influenza virus-infected MDCK epithelial cells: implications for the viral acute phase response. Arch Virol. 1998; 143:2371–80. [PubMed: 9930193]

Martinez J, Huang X, Yang Y. Toll-like receptor 8-mediated activation of murine plasmacytoid dendritic cells by vaccinia viral DNA. Proc Natl Acad Sci U S A. 2010; 107:6442–7. [PubMed: 20308556]

Matsumoto M, Funami K, Tanabe M, et al. Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol. 2003; 171:3154–62. [PubMed: 12960343]

Mercer J, Helenius A. Vaccinia Virus Uses Macropinocytosis and Apoptotic Mimicry to Enter Host Cells. Science. 2008; 320:531–5. [PubMed: 18436786]

Mercer J, Knebel S, Schmidt FI, et al. Vaccinia virus strains use distinct forms of macropinocytosis for host-cell entry. Proc Natl Acad Sci U S A. 2010; 107:9346–51. [PubMed: 20439710]

Moriwaki H, Kume N, Sawamura T, et al. Ligand specificity of LOX-1, a novel endothelial receptor for oxidized low density lipoprotein. Arterioscler Thromb Vasc Biol. 1998; 18:1541–7. [PubMed: 9763524]

Mukhopadhyay S, Varin A, Chen Y, et al. SR-A/MARCO-mediated ligand delivery enhances intracellular TLR and NLR function, but ligand scavenging from cell surface limits TLR4 response to pathogens. Blood. 2011; 117:1319–47. [PubMed: 21098741]

Nizet V, Ohtake T, Lauth X, et al. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature. 2001; 414:454–7. [PubMed: 11719807]

Redfield RR, Wright DC, James WD, et al. Disseminated Vaccinia in a Military Recruit with Human Immunodeficiency Virus (HIV) Disease. N Engl J Med. 1987; 316:673–6. [PubMed: 3821799]

Saleh M-C, van Rij RP, Hekele A, et al. The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nat Cell Biol. 2006; 8:793–802. [PubMed: 16862146]

Scarselli E, Ansuini H, Cerino R, et al. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. 2002; 21:5017–25. [PubMed: 12356718]

Schauber J, Dorschner RA, Coda AB, et al. Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. J Clin Invest. 2007; 117:803–11. [PubMed: 17290304]

Sepkowitz KA. How Contagious Is Vaccinia? N Engl J Med. 2003; 348:439–46. [PubMed: 12496351]

Vora S, Damon I, Fulginiti V, et al. Severe Eczema Vaccinatum in a Household Contact of a Smallpox Vaccinee. Clin Infect Dis. 2008; 46:1555–61. [PubMed: 18419490]

Wang Z, Lai Y, Bernard JJ, et al. Skin mast cells protect mice against vaccinia virus by triggering mast cell receptor S1PR2 and releasing antimicrobial peptides. J Immunol. 2012; 188:345–57. [PubMed: 22140255]

Worthington M, Baron S. Effect of polyriboinosinic-polyribocytidylic acid and antibody on infection of immunosuppressed mice with vaccinia virus. J Infect Dis. 1973; 128:308–11. [PubMed: 4728690]

Yamayoshi S, Iizuka S, Yamashita T, et al. Human SCARB2-dependent Infection by Coxsackievirus A7, A14, A16 and Enterovirus 71. J Virol. 2012

Yamayoshi S, Yamashita Y, Li J, et al. Scavenger receptor B2 is a cellular receptor for enterovirus 71. Nat Med. 2009; 15:798–801. [PubMed: 19543282]

Yew KH, Carsten B, Harrison C. Scavenger receptor A1 is required for sensing HCMV by endosomal TLR-3/-9 in monocytic THP-1 cells. Mol Immunol. 2010; 47:883–93. [PubMed: 19914718]

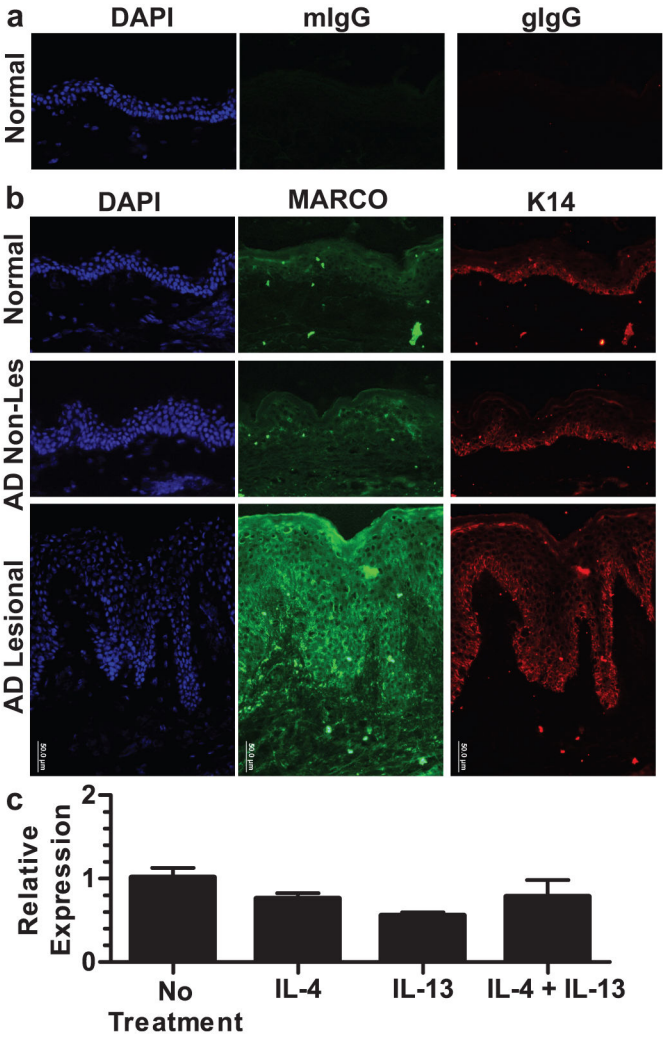

Figure 1. MARCO expression in atopic dermatitis

(**a,b**) Skin biopsy samples taken from a normal donor and an AD donor at non-lesional and lesional sites were analyzed for Keratin-14 (K14), which is predominantly expressed by basal keratinocytes, and human MARCO expression, and compared to goat IgG (gIgG) and mouse IgG (mIgG) controls. Scale bars = 50μm. Images in (**b**) are one set of representative images from an experiment with 2 normal skin donors and 2 AD donors (see Supplementary Fig. S1). (**c**) NHEK were treated with 50ng/ml IL-4 and/or IL-13 for 24 hours before RNA isolation and qPCR for MARCO expression. Error bars indicate SEM, n=3. Differences between treatment conditions were not significant, as determined by One-way ANOVA with Tukey post-tests.

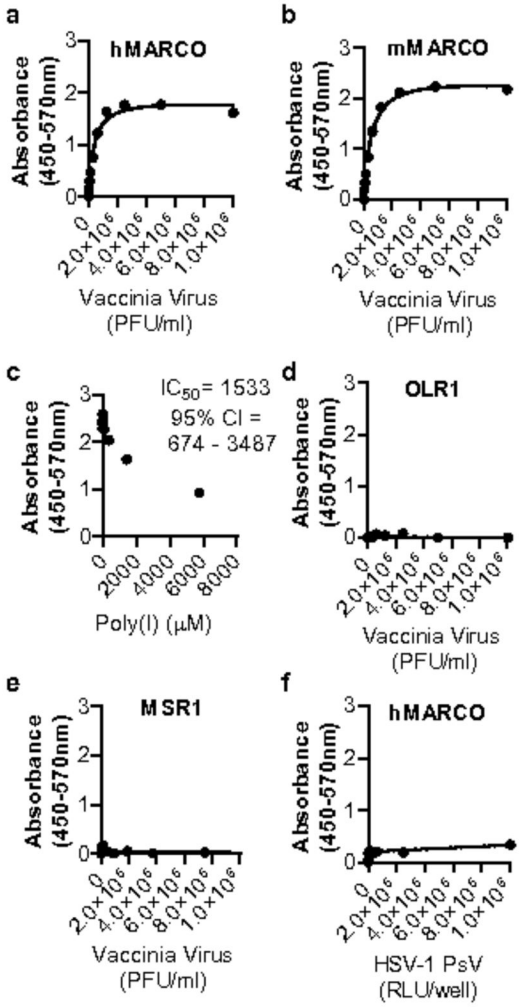

Figure 2. Vaccinia binds directly to human and mouse MARCO

Indicated concentrations of VV were added to plates coated with human MARCO (hMARCO) (**a**), mouse MARCO (mMARCO) (**b**), human OLR1 (**d**) and human MSR1 (**e**). Virus bound to the immobilized protein was quantified by ELISA. Error bars indicate SEM, n=3, with non-linear regressions plotted as solid lines. **c**, Plates coated with hMARCO were incubated with 6×10^6 PFU/ml VV in the presence of Poly(I). Bound virus was quantified by ELISA. Each individual data point is plotted. Data in (**c**) was transformed using a logarithmic x-axis, and the half-maximal inhibitory concentration (IC_{50}) value and the 95% confidence interval for this value were calculated using Graphpad Prism. Poly(I) molarity was calculated based on the molecular weight of each individual nucleotide in the polymer, with average molecules ranging in length up to 1000 nucleotides. **f**, indicated amounts of HIV-1 pseudovirus (PsV) were added to plates coated with hMARCO and bound virus quantified by ELISA. Error bars indicate SEM, n=2, with non-linear regression plotted as a solid line. **a–f**, data presented are from one representative experiment of two independent experiments.

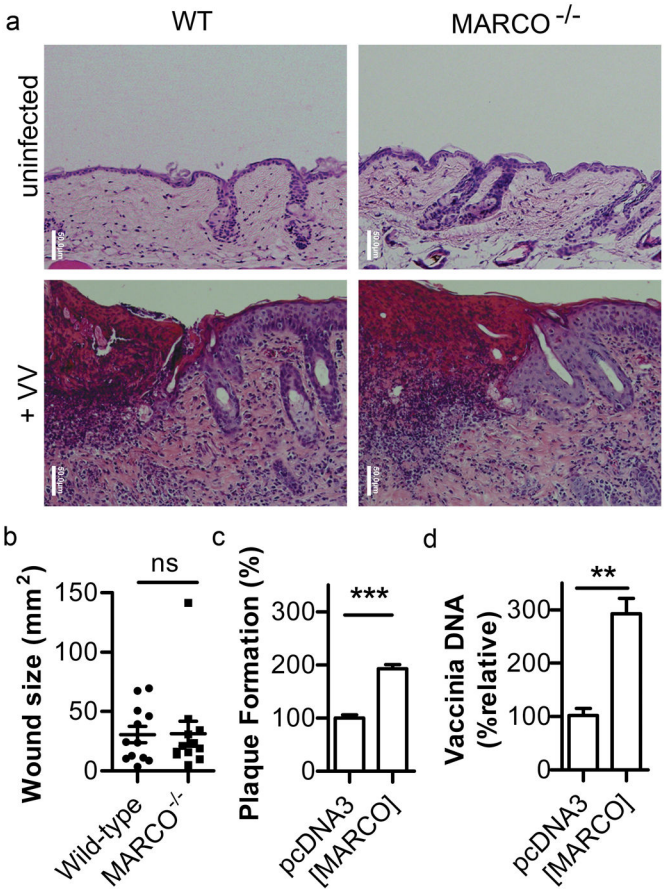

Figure 3. Overexpression of MARCO increases VV infection in keratinocytes

Age-matched female Wild-type (WT) and MARCO$^{-/-}$ mice were infected with VV by skin scarification, and six days after infection wounds were (**a**) biopsied for histology and (**b**) sizes were quantified. **a**, Representative images of the skin of uninfected WT and MARCO$^{-/-}$ mice and skin from the edges of the wounds of mice infected with VV are displayed. Scale bars = 50μm. **b**, Wound sizes were quantified with ImageJ, n=12. **c**, HaCat keratinocytes stably overexpressing MARCO (labelled [MARCO]), or control cells (pcDNA3) were infected with VV. Plaques were stained and quantified 24 hours after infection, n=3. **d**, HaCat keratinocytes stably overexpressing MARCO (labelled [MARCO]), or control cells (pcDNA3) were infected with 1000 PFU/ml VV for 24 hours before quantification of viral DNA, n=3. **b–d**, error bars indicate SEM, all comparisons were made using two-tailed Student's T-tests, ns = not significant, ** $P < 0.01$, *** $P < 0.001$. All data are from representative experiments repeated at least two times.

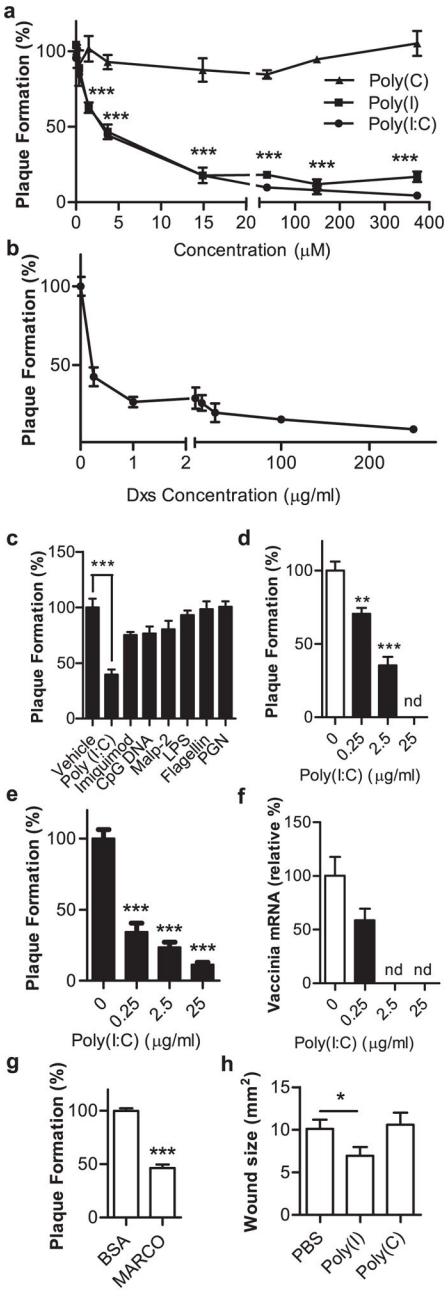

Figure 4. Scavenger receptor antagonists prevent vaccinia infection

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. **a**, 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 up to 1000 nucleotides in length. **a**, two-way ANOVA with bonferroni post-tests was used to compare Poly(I) and Poly(I:C) treatment to Poly(C) treatment at each concentration, *** $P < 0.001$. **c**, HaCat were pretreated with TLR ligands for 24 hours before infection with VV. HaCat (**d**) and NHEK (**e,f**) were infected with VV after Poly(I:C) pretreatment for 24 hours. **c–e**, plaques were quantified 24 hours

after infection. **f**, Viral mRNA was quantified by qPCR 8 hours after infection. **c–f**, statistical significance was determined using One-way ANOVA with Tukey post-tests, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, nd, not detectable. **g**, VV was pretreated for 1 hour at 37°C with 50μg/ml bovine serum albumin (BSA) or recombinant human MARCO before adding to NHEK to assess viral plaque formation. A two-tailed Student's T-test was used to determine statistical significance, *** $P < 0.001$. **a–g**, error bars indicate SEM, n=3. h, 50μl of PBS, or PBS containing 5mg/ml Poly(I) or Poly(C) were applied to depilated back skin of age-matched wild-type female mice for 15 minutes prior to infection with VV. Wound sizes were quantified at 6 days post-infection. Error bars indicate SEM, n=4, statistical significance determined using One-way ANOVA and a Bonferroni post-test, * $P < 0.05$.