AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

VIRUS-CELL INTERACTIONS

Entry of Herpes Simplex Virus 1 into Epidermis and Dermal Fibroblasts Is Independent of the Scavenger Receptor MARCO

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ABSTRACT To enter host cells, herpes simplex virus 1 (HSV-1) initially attaches to cell surface glycosaminoglycans, followed by the requisite binding to one of several cellular receptors, leading to viral internalization. Although virus-receptor interactions have been studied in various cell lines, the contributions of individual receptors to uptake into target tissues such as mucosa, skin, and cornea are not well understood. We demonstrated that nectin-1 acts as a major receptor for HSV-1 entry into murine epidermis, while herpesvirus entry mediator (HVEM) can serve as an alternative receptor. Recently, the macrophage receptor with collagenous structure (MARCO) has been described to mediate adsorption of HSV-1 to epithelial cells. Here, we investigated the impact of MARCO on the entry process of HSV-1 into the two major cell types of skin, keratinocytes in the epidermis and fibroblasts in the underlying dermis. Using *ex vivo* infection of murine epidermis, we showed that HSV-1 entered basal keratinocytes of MARCO$^{-/-}$ epidermis as efficiently as those of control epidermis. In addition, entry into dermal fibroblasts was not impaired in the absence of MARCO. When we treated epidermis, primary keratinocytes, or fibroblasts with poly(I), a ligand for class A scavenger receptors, HSV-1 entry was strongly reduced. As we also observed reducing effects of poly(I) in the absence of both MARCO and scavenger receptor A1, we concluded that the inhibitory effects of poly(I) on HSV-1 infection are not directly linked to class A scavenger receptors. Overall, our results support that HSV-1 entry into skin cells is independent of MARCO.

IMPORTANCE During entry into its host cells, the human pathogen herpes simplex virus (HSV) interacts with various cellular receptors. Initially, receptor interaction can mediate cellular adsorption, followed by receptor binding that triggers viral internalization. The intriguing question is which receptors are responsible for the various steps during entry into the natural target tissues of HSV? Previously, we demonstrated the role of nectin-1 as a major receptor and that of HVEM as an alternative receptor for HSV-1 to invade murine epidermis. As MARCO has been described to promote infection in skin, we explored the predicted role of MARCO as a receptor that mediates adsorption to epithelial cells. Our infection studies of murine skin cells indicate that the absence of MARCO does not interfere with the efficiency of HSV-1 entry and that the inhibitory effect on viral adsorption by poly(I), a ligand of MARCO, is independent of MARCO.

KEYWORDS HSV-1, MARCO, SR-A1, SR-A6, dermal fibroblasts, entry receptor, epidermis, herpesviruses, keratinocytes, skin

During primary infection, herpes simplex virus (HSV) invades skin and mucocutaneous regions, which is followed by latent infection of sensory neurons. The extent of primary and recurrent infections in epidermal keratinocytes and dermal fibroblasts is

Received 22 March 2018 **Accepted** 10 May 2018

Accepted manuscript posted online 16 May 2018

Citation Thier K, Möckel M, Palitzsch K, Döhner K, Sodeik B, Knebel-Mörsdorf D. 2018. Entry of herpes simplex virus 1 into epidermis and dermal fibroblasts is independent of the scavenger receptor MARCO. J Virol 92:e00490-18. https://doi.org/10.1128/JVI.00490-18.

Editor Richard M. Longnecker, Northwestern University

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largely a function of the host's immune status. In immunocompromised patients and newborns, disseminated infections can occur, and a broader tissue tropism is observed. This implies that HSV has adapted a variety of entry pathways which are based on the accessibility of cellular receptors on various cell types.

Our focus is on the characterization of cellular receptors that mediate cellular entry of HSV-1 after invasion into natural target tissues such as skin or mucosa. Viral entry into individual cells involves the interaction of several viral glycoproteins with various cell surface receptors (1). Initially, HSV-1 can attach to the heparan sulfate (HS) side chains of cell surface proteoglycans via the two viral glycoproteins, glycoprotein C (gC) and glycoprotein B (gB). These interactions are not essential for the entry process, but their absence may reduce infection efficiency at least in cultured cells, while their impact on target tissues is mostly unclear (2, 3). Only after binding of the viral glycoprotein D (gD) to a receptor is fusion with cellular membranes induced in which the glycoproteins B (gB) and H/L (gH/gL) are involved in forming the core fusion machinery (4, 5). Important gD receptors are nectin-1, an intercellular adhesion protein, and herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor receptor superfamily (6, 7). The distinct roles of these two receptors during infection of the human host still need to be determined. Using an *ex vivo* infection model, we investigated the impact of nectin-1 and HVEM on HSV-1 entry into murine skin (8). When we compared the two major cell types of skin, keratinocytes in the epidermis and fibroblasts in the underlying dermis, we found that nectin-1 is less highly expressed on fibroblasts than on keratinocytes. In contrast, HVEM is present on nearly all fibroblasts but is expressed only on a few keratinocytes in the epidermis (9, 10). Interestingly, these expression levels show no direct correlation with the effectiveness as receptors. In both cell types, nectin-1 acts as major receptor, and HVEM can functionally replace it, but less efficiently in keratinocytes than in fibroblasts (9, 10).

Recently, the macrophage receptor with collagenous structure (MARCO) was described as a receptor that is exploited by HSV-1 to promote cell surface adsorption and infection in skin (11). MARCO (scavenger receptor A6 [SR-A6]) belongs to the class A scavenger receptors, one of eight classes of scavenger receptors comprising a group of pattern recognition receptors (12). Class A scavenger receptors are membrane-associated phagocytic receptors that are differentially expressed on immune cells (13). MARCO can bind various bacterial ligands and has been suggested to play an important role in host defense (14–16). The impact of MARCO as an HSV-1 receptor is based mainly on the observation that ligands of MARCO strongly inhibit HSV-1 adsorption in human keratinocytes, suggesting that MARCO plays a major role during the entry process (11). As we demonstrated that the absence of nectin-1 leads to a strong reduction of HSV-1 entry into murine epidermis and skin cells (9, 10), we here investigated the functional role of MARCO as an additional receptor in epidermal keratinocytes and dermal fibroblasts. Our results indicate that the absence of MARCO has no effect on the efficiency of infection, although poly(I), a ligand for class A scavenger receptors, reduced the number of infected cells. This reducing effect, however, was independent of the presence of MARCO.

RESULTS

HSV-1 enters MARCO$^{-/-}$ cells efficiently. To address the functional role of MARCO for HSV-1 entry, we prepared epidermal sheets from tails of MARCO$^{-/-}$ mice (17) for *ex vivo* infection studies. After separation from the dermis, epidermal sheets were floated on virus suspension and infected cells were determined by visualizing the viral infected cell protein 0 (ICP0). ICP0 is expressed once the viral genome is released into the nucleus and localizes in nuclear foci, but it relocalizes to the cytoplasm later during infection (18, 19). Thus, the visualization of ICP0 expression serves as a marker for successful entry into individual cells. Previous results demonstrate that all basal keratinocytes express ICP0 at 3 h postinfection (p.i.) upon infection of murine epidermal sheets at 100 PFU/cell (9, 20). Here, we reduced the virus dose to 20 PFU/cell to visualize putative differences in the efficiency of infection. Immunostainings showed comparable

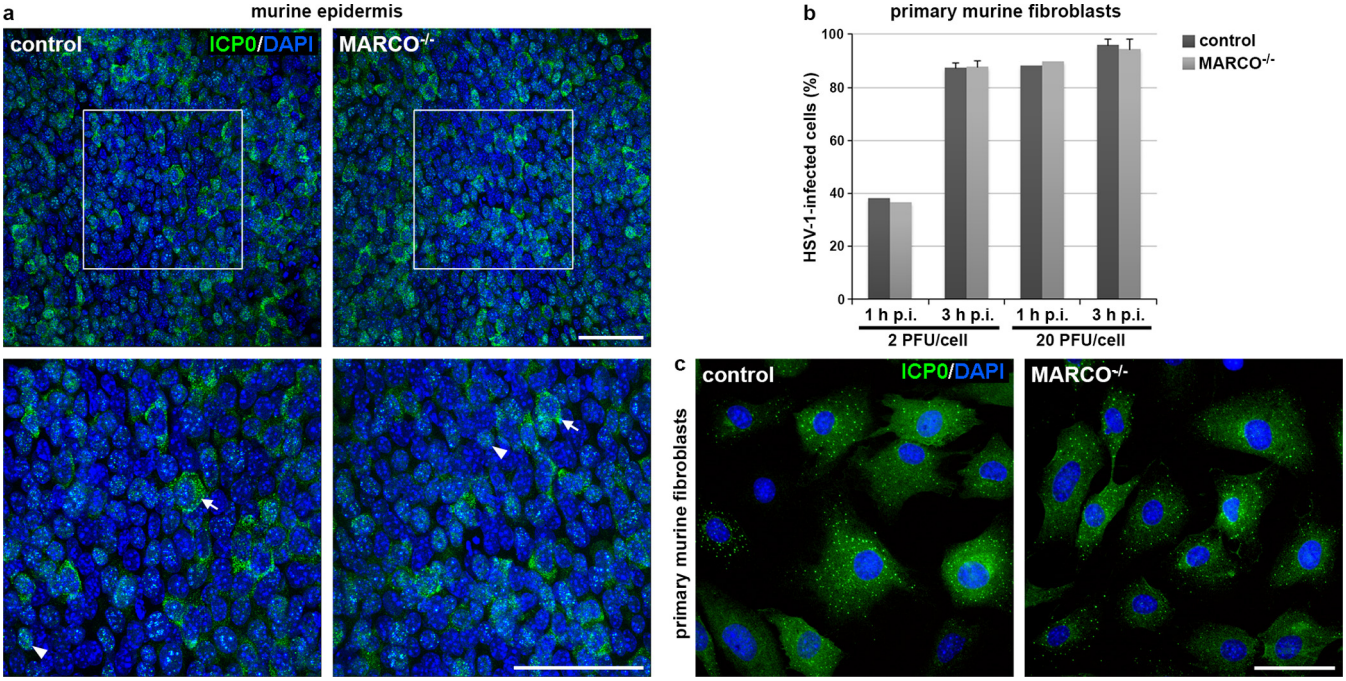

FIG 1 HSV-1 infection of epidermis and dermal fibroblasts from MARCO$^{-/-}$ mice. (a) Epidermal sheets from control and MARCO$^{-/-}$ skin were separated from the dermis by dispase II and infected with HSV-1 at 20 PFU/cell. Immunostainings of whole mounts show ICP0-expressing cells (green) in the basal keratinocyte layer at 3 h p.i. with DAPI (blue) as the nuclear counterstain. Examples of nuclear ICP0 (arrowhead) and cytoplasmic ICP0 (arrow) are indicated. Lower panels, two-fold magnifications of the boxed areas shown in the upper panels. Confocal projections and merged images show representative areas with 72% (control) and 78% (MARCO$^{-/-}$) infected keratinocytes, respectively. (b) Primary dermal fibroblasts from control or MARCO$^{-/-}$ skin were infected, and the number of ICP0-expressing cells was determined in two (1 h p.i.) or at least three (3 h p.i.) independent experiments. The results are shown as means + standard deviations. (c) Control or MARCO$^{-/-}$ primary fibroblasts were infected at 20 PFU/cell for 3 h. Confocal projections and merged images show ICP0-expressing (green) cells and DAPI (blue) as the nuclear counterstain in both control and MARCO$^{-/-}$ cells. Bars, 50 μm.

numbers of ICP0-expressing cells in the basal layer of epidermis from control and MARCO$^{-/-}$ mice (Fig. 1a). In addition, we isolated dermal fibroblasts from the skin of control and MARCO$^{-/-}$ mice and determined infection efficiency. To better visualize putative differences, we analyzed infected cells at 1 and 3 h p.i. Again, no difference in the number of ICP0-expressing cells was observed, even after infection with a low dose (2 PFU/cell) (Fig. 1b). In addition, the cytoplasmic localization of ICP0 indicated that there was even no delay in the onset of infection in MARCO$^{-/-}$ fibroblasts (Fig. 1c). Neither was delayed infection found in the MARCO$^{-/-}$ basal epidermal layer, as ICP0 was observed in nuclear foci and in the cytoplasm in both the presence and absence of MARCO (Fig. 1a). As MARCO$^{-/-}$ cells showed no decrease in the efficiency of HSV-1 entry, we assume that MARCO plays no direct role or has redundant functions for the internalization process into murine epidermal keratinocytes and dermal fibroblasts.

MARCO expression in murine and human skin cells. To address whether the missing role of MARCO as a receptor in murine skin cells correlates with its expression levels, we analyzed transcription by reverse transcription-PCR (RT-PCR). So far, MARCO is known to be constitutively expressed on specific subsets of macrophages and its expression can be induced upon inflammation at sites of bacterial infection (21). We detected rather weak MARCO signals for murine primary keratinocytes, while a strong signal was visible for bone marrow macrophages (Fig. 2a). To exclude effects on MARCO expression upon cultivation of keratinocytes, we also analyzed cells directly after dissociation of epidermal sheets. The signals, however, were as weak and variable as in primary cells (Fig. 2a). Signals for murine dermal fibroblasts were stronger than those for keratinocytes (Fig. 2a), and the level of MARCO transcripts did not change during early infection (data not shown). As expected, no signals were detected in fibroblasts of MARCO$^{-/-}$ skin (Fig. 2b). Our results show expression of MARCO in murine fibroblasts but only minor expression in murine epidermis, in comparison to bone marrow

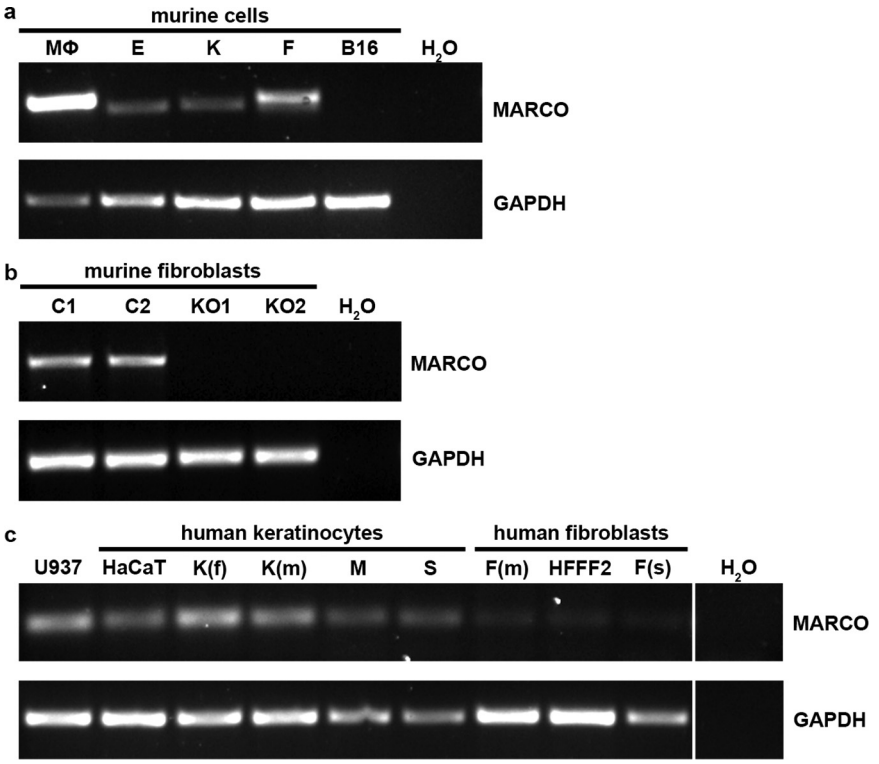

FIG 2 Expression of MARCO in murine and human skin cells. (a) RNA was isolated from bone marrow macrophages (Mϕ), murine epidermal sheets (E), primary murine epidermal keratinocytes (K), primary murine dermal fibroblasts (F), or B16-F1 (B16) cells. RT-PCR demonstrates MARCO transcripts (220 bp) and, as a control, GAPDH transcripts (240 bp). (b) The presence and absence of MARCO transcripts (602 bp) are shown in primary fibroblasts from two control (C1 and C2) and two MARCO$^{-/-}$ (KO1 and KO2) mice, respectively. GAPDH transcripts (240 bp) serve as a control. (c) MARCO transcripts (73 bp) were analyzed in U937 monocytes (U937), HaCaT cells (HaCaT), primary keratinocytes from human foreskin [K(f)], primary keratinocytes from human oral mucosa [K(m)], epithelial sheets from human oral mucosa (M), epidermal sheets from human skin (S), primary fibroblasts from human oral mucosa [F(m)], human fetal foreskin fibroblasts (HFFF2), or primary fibroblasts from human skin [F(s)]. GAPDH transcripts (240 bp) are shown as a control.

macrophages. Of note, in both murine epidermis and dermal fibroblasts, we detected no direct role of MARCO in HSV-1 entry, although expression levels of MARCO differed.

To address whether MARCO expression is comparable in murine and human skin, we analyzed human skin cells. MARCO was detected in the human keratinocyte cell line HaCaT, in primary keratinocytes, and in keratinocytes isolated either from mucosal epithelia or epidermal sheets, with some variations in intensity (Fig. 2c). As a positive control, MARCO expression was detected in the human monocyte cell line U937 (Fig. 2c). In contrast, rather weak signals were seen for human primary fibroblasts and for the cell line HFFF2 (Fig. 2c). Overall, expression of MARCO appears to be higher in human than in murine keratinocytes, while the opposite applies for dermal fibroblasts.

We next investigated whether human MARCO can facilitate viral entry by transiently overexpressing MARCO in the murine melanoma cell line B16-F1, which is one of the few cell lines that is nonpermissive for HSV-1 (data not shown) and which does not express MARCO (Fig. 2a). When we overexpressed the human cellular receptor HVEM in B16-F1 cells prior to HSV-1 infection, ICP0 expression was detected only in HVEM-positive cells (Fig. 3). This is in line with our findings that HVEM can act as alternative receptor in the absence of the major receptor nectin-1 in murine epidermis and in dermal fibroblasts (9, 10). In contrast, MARCO-overexpressing B16-F1 cells were not infected, indicating that MARCO cannot serve as a receptor that mediates viral internalization (Fig. 3).

Entry of HSV-1 into skin cells is inhibited by poly(I) in the presence and absence of MARCO. Previous studies described the inhibitory effect of the synthetic

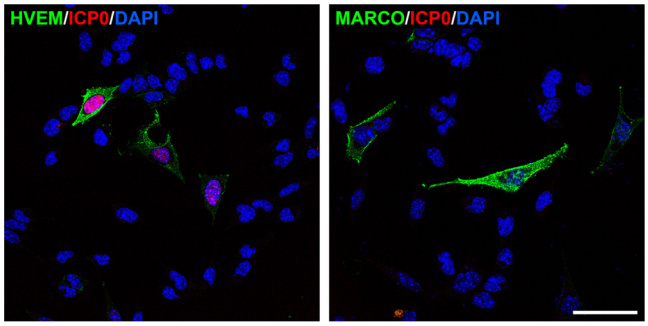

FIG 3 HSV-1 infection of B16-F1 cells transiently expressing HVEM or MARCO. The nonpermissive B16-F1 cells were transfected with HVEM- or MARCO-expressing plasmids and infected at 20 PFU/cell at 21 h posttransfection. At 3 h p.i., immunostainings show no ICP0 expression (red) in MARCO-expressing (green) cells, while HVEM-expressing (green) cells express ICP0 (red); DAPI (blue) is shown as the nuclear counterstain. Bar, 50 μm.

single-stranded RNA poly(I) on HSV-1 infection in human keratinocytes, while poly(C) was found to have no effect (11). Since poly(I) but not poly(C) can serve as a ligand for scavenger receptors, MacLeod et al. (11) concluded that poly(I) prevents adsorption of HSV-1 particles in keratinocytes by interfering with HSV-1 binding to the scavenger receptor MARCO.

When we treated skin cells with poly(I), we also found a reduction of infected cells with increasing concentrations of poly(I). After pretreatment of cells with poly(I) followed by infection with HSV-1 in the presence of the single-stranded RNA (ssRNA) component, we observed a block of viral entry into HaCaT cells, while 73% of the cells expressed ICP0 at 2 h p.i. after poly(C) pretreatment (Fig. 4a). A strong reduction in ICP0-expressing cells in the presence of poly(I) was found in primary human keratinocytes and in primary human fibroblasts, while treatment with poly(C) had no inhibitory effect (Fig. 4a).

To assess whether the inhibitory effect of poly(I) on HSV-1 entry depends on the presence of MARCO as described previously (11), we infected epidermal sheets prepared from control and MARCO$^{-/-}$ mice in the presence of poly(I). As a control, ICP0-expressing cells were also visualized in poly(C)-treated epidermis, where nearly all basal keratinocytes were infected at 3 h p.i. (Fig. 4b). In contrast, poly(I) treatment resulted in a strongly reduced number of infected cells, surprisingly in both control and MARCO$^{-/-}$ epidermis (Fig. 4b). We then analyzed the inhibitory effect of poly(I) in dermal fibroblasts in the presence and absence of MARCO expression. With increasing concentrations of poly(I), the number of infected primary fibroblasts decreased in both MARCO-expressing and MARCO-deficient cells, whereas poly(C) had no effect (Fig. 4c). The inhibitory effect of poly(I) was less prominent in murine fibroblasts than in primary human fibroblasts (Fig. 4a and c). Taken together, the effect of poly(I) on HSV-1 entry was independent of the presence of MARCO in murine skin cells. In human fibroblasts, a rather weak expression of MARCO correlated with a strong inhibitory effect of poly(I). A comparable effect was observed in human keratinocytes, where MARCO was much more strongly expressed than in fibroblasts. This finding suggests that the action of poly(I) in human skin cells is also unrelated to the presence of MARCO and most likely depends on the binding to other receptors.

To characterize which step during HSV-1 entry was affected by poly(I), we performed infection studies in HaCaT cells and determined ICP0-expressing cells at 2 h p.i. When cells were preincubated with poly(I) but infected in the absence of poly(I), no effect on infection efficiency was observed (Fig. 4d). This suggests that the inhibitory poly(I) effect was based neither on the irreversible damage of the cell surface integrity nor on the induction of signaling pathways conferring cellular defense mechanisms. In contrast, when poly(I) was only added with the virus inoculum, viral entry was blocked, whereas poly(C) did not influence infection efficiency (Fig. 4e). As a control, we

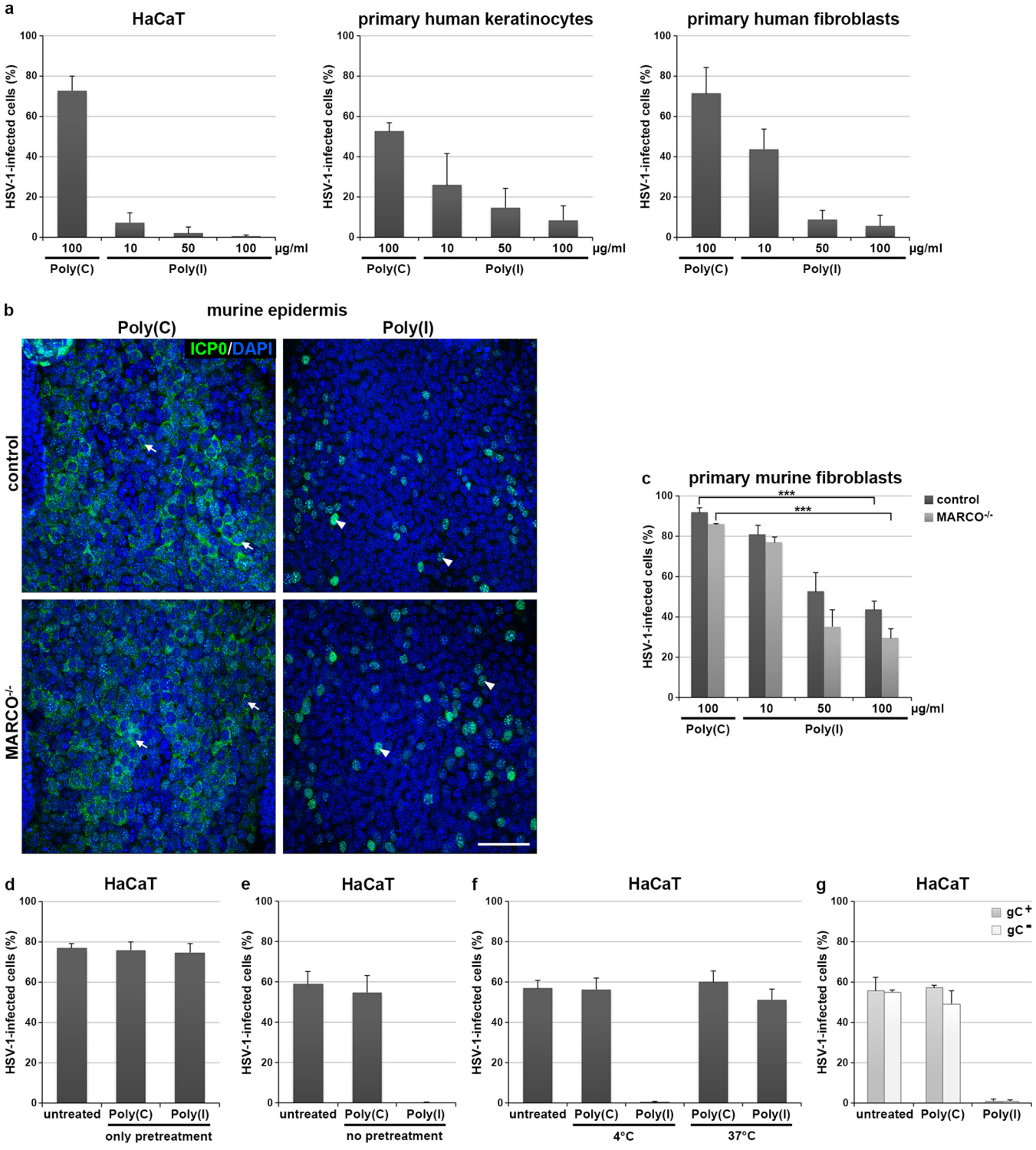

FIG 4 Inhibitory effect of poly(I) on HSV-1 infection. (a) HaCaT cells, primary human keratinocytes, and primary human fibroblasts were treated with poly(I) or poly(C) for 20 min at 37°C, followed by infection at 5 PFU/cell in the presence of poly(I) or poly(C). ICP0-expressing cells were determined in HaCaT cells and primary keratinocytes at 2 h p.i. and in primary fibroblasts at 3 h p.i., which revealed an inhibitory effect of poly(I) but not of poly(C). (b) Epidermal sheets from control and MARCO$^{-/-}$ mice were treated with 500 μg/ml of poly(I) or poly(C) for 20 min at 37°C, followed by infection at 20 PFU/cell in the presence of poly(I) or poly(C). Immunostainings of whole mounts demonstrate comparable inhibitory effects of poly(I) in both control and MARCO$^{-/-}$ epidermis, while poly(C) has no effect. Confocal projections and merged images show nuclear (arrowhead) and cytoplasmic ICP0 (arrow). Bar, 50 μm. (c) Primary fibroblasts from control and MARCO$^{-/-}$ mice were treated with poly(I) or poly(C) for 20 min at 37°C, followed by infection at 5 PFU/cell in the presence of poly(I) or poly(C) for 3 h. The inhibitory effect of poly(I) is observed in both control and MARCO$^{-/-}$ cells. ***, $P < 0.001$, unpaired t test. (d) HaCaT cells were treated with 100 μg/ml of poly(I) or poly(C) for 20 min at 37°C, followed by infection at 5 PFU/cell in the absence of poly(I) or poly(C) for 2 h. (e) HaCaT cells were infected at 5 PFU/cell for 2 h at 37°C in the presence of 100 μg/ml of poly(I) or poly(C). (f) HaCaT cells were incubated with HSV-1 at 5 PFU/cell for 1 h at 4°C in the presence

(Continued on next page)

pretreated the virus inoculum with increasing concentrations of poly(I). As cells were efficiently infected, we conclude that the poly(I) pretreatment causes no irreversible damage of the viral surface (data not shown). To address whether the adsorption or the internalization step during viral entry was affected by poly(I), we incubated the cells with virus and poly(I) at 4°C for 1 h to allow adsorption only to the cell surface. After removal of the supernatant, cells were incubated for 2 h at 37°C in the absence of poly(I). For the reverse experiment, incubation with virus at 4°C was performed in the absence of poly(I), followed by incubation at 37°C in the presence of poly(I). Interestingly, entry was blocked only after poly(I) incubation at 4°C, supporting that poly(I) interferes with viral adsorption (Fig. 4f). Once viruses were bound to the cell surface, poly(I) had no effect (Fig. 4f). As a control, poly(C) affected neither adsorption nor internalization (Fig. 4f).

The observation that poly(I) interferes with viral adsorption is in line with the findings of MacLeod et al. (11), who described that binding of the purified viral protein gC to MARCO was inhibited by poly(I). To assess the impact of gC for the inhibitory effect of poly(I), we performed infection studies with the HSV-1 mutant HSV1(17$^+$)Lox-ΔgC^{P62FS}, which does not express gC (data not shown). HSV-1 gC can bind to cell surface heparan sulfate as well as to the C3b fragment of the third component of complement, leading to downregulation of the complement cascade (22–24). In the absence of gC, HSV-1 still initiates infection, but binding to the cell surface is reduced (24). In the presence of poly(I), entry of HSV1(17$^+$)Lox-ΔgC^{P62FS} into HaCaT cells was blocked as efficiently as entry of the control virus strain HSV1(17$^+$)Lox-gC$^+$ with restored gC expression (Fig. 4g). Again, poly(C) had no effect on infection efficiency (Fig. 4g). Thus, we conclude that the inhibitory effect of poly(I) during viral adsorption is not mediated by blocking gC binding.

Overall, we demonstrate that poly(I) can block HSV-1 entry into both human and murine skin cells, although to different extents. This effect most likely relies on the interference of poly(I) with the viral attachment to the cell surface, which, in contrast to previous results (11), is gC independent and does not rely on the presence of MARCO.

The inhibitory effect of poly(I) on HSV-1 entry does not depend on the presence of the scavenger receptor A1. As we found no evidence that the inhibitory effect of poly(I) on HSV-1 entry depends on MARCO as a receptor, we investigated whether poly(I) acts as a ligand for scavenger receptor A1 (SR-A1), a structural relative of MARCO (25). To exclude compensatory functions of the two class A scavenger receptors, we infected skin cells prepared from MARCO$^{-/-}$/SR-A1$^{-/-}$ double-knockout (dKO) mice (26) with HSV-1 and initially determined infection efficiency. After *ex vivo* infection of epidermal sheets, we surprisingly observed fewer infected cells in the basal keratinocyte layer of MARCO$^{-/-}$/SR-A1$^{-/-}$ dKO epidermis than in the basal layer of control epidermis (Fig. 5a and b). While approximately 57% of the basal cells of control epidermis expressed ICP0 at 3 h p.i., ICP0 expression was detected in only 26% of the basal cells of dKO epidermis (Fig. 5b). In addition to the reduced number of infected cells, entry was also delayed, as nuclear ICP0 was detected in dKO cells, while ICP0 was already translocated to the cytoplasm in control epidermis (Fig. 5a). These results indicate that viral entry into MARCO$^{-/-}$/SR-A1$^{-/-}$ dKO epidermis is less efficient than in the epidermis of control mice.

To investigate whether infection efficiency is also reduced in fibroblasts isolated from MARCO$^{-/-}$/SR-A1$^{-/-}$ dKO skin, we infected dermal fibroblasts with HSV-1 at 2 and 20 PFU/cell. The comparison of infected control and dKO cells revealed no difference even when the number of infected cells was determined at 1 h p.i. or after infection with a low infection dose (Fig. 5c). This implies that HSV-1 entry is reduced

FIG 4 Legend (Continued)
of 100 μg/ml of poly(I) or poly(C), washed, and incubated for 2 h at 37°C. In addition, cells were incubated with HSV-1 at 5 PFU/cell for 1 h at 4°C, washed, and incubated for 2 h at 37°C with 100 μg/ml of poly(I) or poly(C). (g) HaCaT cells were infected with HSV1(17+)Lox-ΔgC^{P62FS} (gC$^-$) or HSV1(17+)Lox-gC$^+$ (gC$^+$) at 50 PFU/cell for 2 h at 37°C in the presence of 100 μg/ml of poly(I) or poly(C). The inhibitory effect of poly(I) was observed in the presence and absence of gC. (a, c, d, e, f, and g) The effects of poly(I) were shown in at least three independent experiments. The results are shown as means + standard deviations.

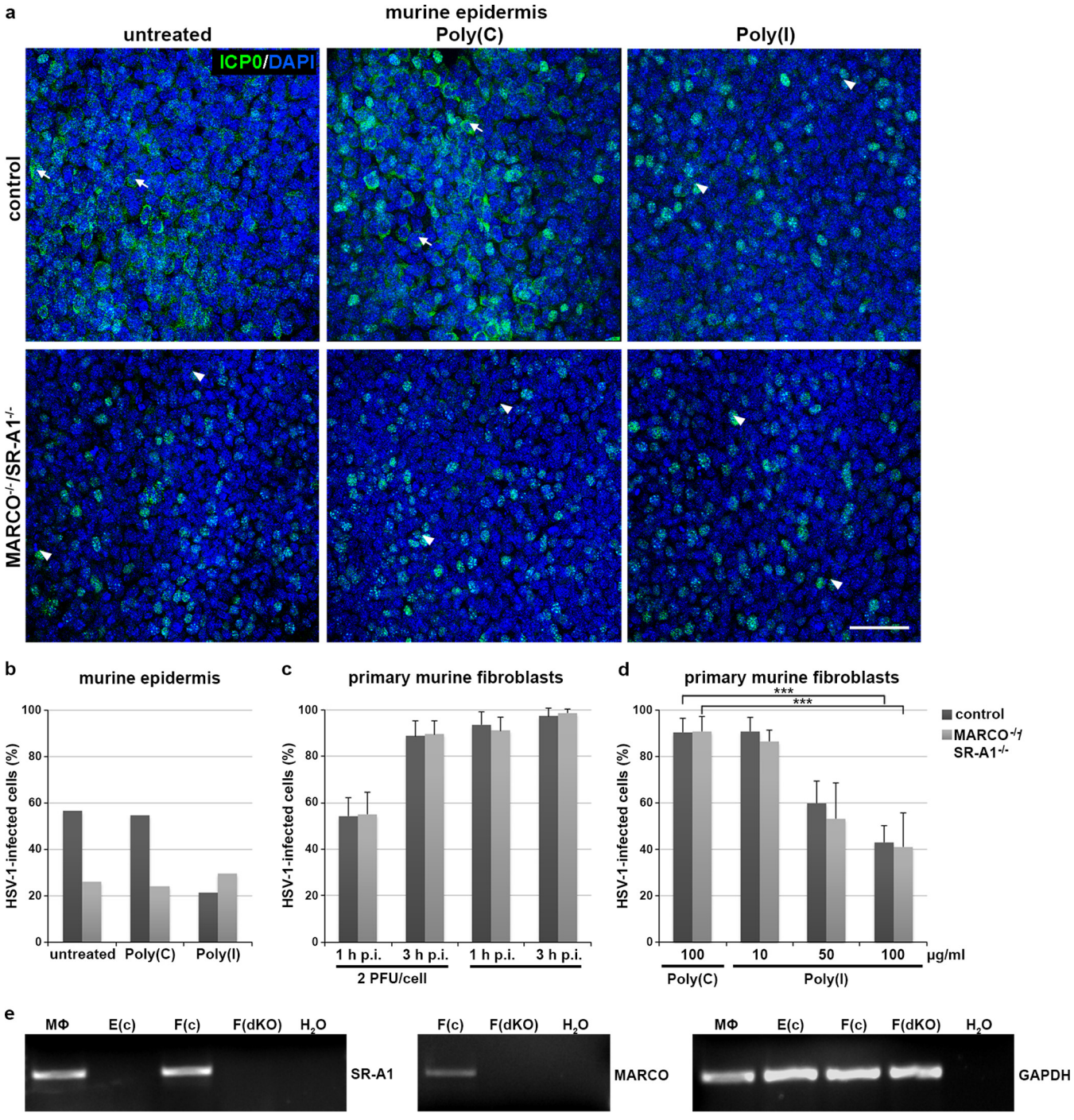

FIG 5 Inhibitory effect of poly(I) on HSV-1 infection in epidermis and dermal fibroblasts from MARCO$^{-/-}$/SR-A1$^{-/-}$ mice. (a) Epidermal sheets from control and MARCO$^{-/-}$/SR-A1$^{-/-}$ mice were treated with 500 μg/ml of poly(I) or poly(C) for 20 min at 37°C, followed by infection at 20 PFU/cell for 3 h in the presence of poly(I) or poly(C). For comparison, untreated epidermis is shown. Confocal projections and merged images show immunostainings of whole mounts. Bar, 50 μm. (b) Quantification of infected epidermis from two mice shows the inhibitory effect of poly(I) only in control epidermis, not in MARCO$^{-/-}$/SR-A1$^{-/-}$ epidermis. The number of ICP0-expressing cells was already reduced in untreated MARCO$^{-/-}$/SR-A1$^{-/-}$ epidermis compared to control epidermis. (c) Primary fibroblasts from control and MARCO$^{-/-}$/SR-A1$^{-/-}$ mice were infected, and ICP0-expressing cells were determined in at least three independent experiments. The results are shown as means + standard deviations. (d) Primary fibroblasts from control and MARCO$^{-/-}$/SR-A1$^{-/-}$ mice were treated with poly(I) or poly(C) for 20 min at 37°C, followed by infection at 5 PFU/cell in the presence of poly(I) or poly(C). ICP0-expressing cells were determined in at least three independent experiments. The results are shown as means + standard deviations. (e) RNAs were isolated from bone marrow macrophages (Mϕ), epidermis of control mice [E(c)], or primary dermal fibroblasts from control [F(c)] or MARCO$^{-/-}$/SR-A1$^{-/-}$ [F(dKO)] mice. RT-PCR indicates the presence or absence of SR-A1 (317 bp) and MARCO transcripts (602 bp). As a control, GAPDH transcripts (240 bp) are shown.

only in dKO keratinocytes, while entry into dKO fibroblasts is unaffected. As we observed no difference in infection efficiency in keratinocytes and fibroblasts of MARCO$^{-/-}$ skin, our results suggest a role of SR-A1 during viral entry into epidermis. To address whether the putative role of SR-A1 is related to a stronger expression in epidermis than in fibroblasts, we analyzed SR-A1 expression by RT-PCR. Surprisingly, no expression of SR-A1 was detected in control epidermis, while signals were detected in primary dermal fibroblasts and macrophages (Fig. 5e). As a control, we confirmed the absence of MARCO and SR-A1 expression in dKO fibroblasts (Fig. 5e) and the comparable expression of nectin-1 and HVEM in control and dKO cells (data not shown). Our observations suggest that SR-A1 plays no direct role during HSV-1 entry into epidermis.

To investigate whether poly(I) still affects the reduced viral entry into MARCO$^{-/-}$/SR-A1$^{-/-}$ dKO epidermis, we treated epidermal sheets with poly(I) or poly(C). No inhibitory effect was observed for poly(I) in dKO epidermis in comparison to poly(C)-treated or untreated tissue (Fig. 5a and b). This implies that epidermis prepared from MARCO$^{-/-}$/SR-A1$^{-/-}$ dKO skin is resistant to the poly(I)-induced reduction of HSV-1 entry observed in control and MARCO$^{-/-}$ epidermis. As expected, we detected an inhibitory effect of poly(I) in control epidermis, where SR-A1 expression was not detected, while the number of infected cells after treatment with poly(C) was comparable to that of untreated control epidermis (Fig. 5a and b). These results led us to speculate that the epidermis of MARCO$^{-/-}$/SR-A1$^{-/-}$ dKO mice exhibits changes on the cell surface that abrogate the inhibitory effects of poly(I) on viral adsorption.

We then addressed the putative effect of poly(I) in MARCO$^{-/-}$/SR-A1$^{-/-}$ dKO fibroblasts, where the absence of SR-A1 in addition to MARCO did not influence infection efficiency. Interestingly, the infection efficiency declined with increasing amounts of poly(I) in both control and dKO fibroblasts (Fig. 5d). This finding further supports that the inhibitory effect of poly(I) is independent of SR-A1, as SR-A1 is present in control but not in dKO fibroblasts.

Overall, we conclude that MARCO and SR-A1 are not direct targets for the inhibitory effect of poly(I) on HSV-1 entry into epidermis and dermal fibroblasts, suggesting that poly(I) blocks an essential interaction of HSV-1 with the cellular surface, which is still unknown.

DISCUSSION

Various receptors on the cell surface can interact with multiple HSV-1 envelope glycoproteins, which finally enable the virus to penetrate into the cytosol. The challenge is to identify those interactions that play a functional role in target tissues. Recently, MacLeod et al. (11) described the unexpected finding that the scavenger receptor MARCO is involved in adsorption and infection of HSV-1 in keratinocytes rather than in contributing to host defense mechanisms. So far, MARCO is best characterized in macrophages, where it is involved in sensing and clearing bacterial pathogens (13). Less is known about the expression of MARCO in cells other than macrophages and its potential role in antiviral defense. When we investigated the role of MARCO during HSV-1 entry into murine epidermis, the site of primary and recurrent infection in skin, we found successful viral entry into keratinocytes of epidermal sheets prepared from MARCO$^{-/-}$ mice which was as efficient as the entry into wild-type (wt) epidermis. This result is in line with the detection of only low expression of MARCO in murine epidermis, suggesting no direct role of MARCO in this tissue. As expression of MARCO was stronger in murine dermal fibroblasts, we performed infection studies in primary fibroblasts. Our results demonstrate that successful viral entry was as efficient in MARCO-deficient fibroblasts as in the presence of the receptor. This indicates that the differential expression of MARCO in epidermis and dermal fibroblasts has no functional consequences for HSV-1 entry, which further supports its independence of MARCO. Interestingly, the robust expression level of MARCO in murine fibroblasts was not observed in primary human fibroblasts from different sources, suggesting that MARCO expression might differ between murine and human skin cells. To address whether MARCO can in principle serve as a receptor, we overexpressed human MARCO in cells

nonpermissive for HSV-1. MARCO expression did not render those cells susceptible, indicating that MARCO is unlikely to serve as a receptor for internalization of HSV-1 in human skin. However, the viral interaction with MARCO might influence additional steps that are distinct from viral entry but can contribute to later HSV-1 infection steps in target tissues. Based on recent observations, MARCO might play a role during the development of pathogenesis, as HSV-1 infection of MARCO$^{-/-}$ mice via scarification led to skin lesions that were larger in wt mice than in MARCO$^{-/-}$ mice (11). To reveal the relevance of MARCO during HSV-1 infection of skin *in vivo*, it is important to know on which cells, in addition to macrophages, MARCO is expressed to further explore the functional consequences of viral interactions with MARCO.

Poly(I) but not poly(C) can inhibit adsorption of HSV-1 in keratinocytes and protect mice against infection (11). Poly(I) is a ligand for scavenger receptors (SR) that also stimulates intracellular toll-like receptor 3 to release inflammatory cytokines, while poly(C) is a cognate nonligand (27). Here we show that poly(I) can efficiently inhibit entry into primary human keratinocytes and fibroblasts and confirm the inhibitory effect of poly(I) on adsorption of HSV-1 in human keratinocytes, as recently described (11). These inhibitory effects were less pronounced in murine skin cells and, surprisingly, occurred in the presence and absence of MARCO. As previous studies predicted that poly(I) prevents interaction of viral gC with MARCO (11), we infected human keratinocytes with HSV-1 mutants lacking gC expression. Viral entry was equally efficient in the absence and presence of gC in poly(C)-treated cells but was blocked in the presence of poly(I) irrespective of gC expression. Hence, the inhibitory effect of poly(I) appears unrelated to the interaction of gC with cell surface structures. Although soluble gC can interact with MARCO, as previously shown (11), this interaction might not mimic those between virions and cell surface molecules. Alternatively, poly(I) might interfere with the interaction of viral gB with cell surface structures. gB, which is required for viral entry, is involved in viral attachment by interacting with heparan sulfate proteoglycans (HSPGs), and, more importantly, functions as a fusion protein (1).

To exclude that the inhibitory effect of poly(I) is based on an interaction with further scavenger receptors, we chose mice that are deficient in MARCO and the structural relative SR-A1 for infection studies. Surprisingly, entry into the epidermis of dKO mice was less efficient than into wt epidermis, although wt epidermis showed no SR-A1 expression. In contrast, SR-A1 expression was detected in murine dermal fibroblasts, where viral entry was unaffected by the absence of MARCO and SR-A1. Together with our results obtained with MARCO$^{-/-}$ mice, this shows that HSV-1 entry into dermal fibroblasts is not influenced by the absence of both MARCO and SR-A1. The epidermis of dKO mice, however, exhibits a phenotype that can delay viral entry and results from a deficiency of SR-A1 in skin. Furthermore, this phenotype abrogates the inhibitory effect of poly(I), suggesting cell surface alterations other than the absence of MARCO and SR-A1 in dKO epidermis that affect a target of both poly(I) and HSV-1 entry. The conclusion that MARCO and SR-A1 are not direct targets of the inhibitory effect of poly(I) is further supported by our finding that poly(I) interferes with viral entry into dKO fibroblasts. Hence, a deficiency of SR-A1 in addition to MARCO in murine skin leads to modifications, whose identification might help to understand further determinants of HSV-1 entry into epidermis. In this context, the impact of viral attachment to cell surfaces in tissue comes to the fore. The question will be to which extent receptor interactions leading to viral internalization are preceded by adsorption of virions in epidermis. As HSV-1 can exploit the ubiquitously expressed HSPGs as an initial receptor, we investigated the effect of heparin on viral entry into murine epidermal sheets. Heparin was shown to block virus adsorption by interfering with viral interactions of HSPGs (28). Our initial results demonstrated that heparin can efficiently inhibit entry into epidermis (data not shown), suggesting that HSPGs might contribute to the efficiency of viral entry in tissue. This is in line with previous infection studies in human gingival samples suggesting that HSPGs participate in the viral binding to the basement membrane connecting the epithelium with the lamina propria (29). The experi-

mental challenge will be to demonstrate the impact of adsorption in skin or mucosa in the absence of HSPGs.

In conclusion, we found that the efficiency of HSV-1 entry into epidermal keratinocytes and dermal fibroblasts is not influenced by the presence or absence of MARCO. Intriguingly, the scavenger receptor ligand poly(I) can very efficiently reduce viral entry, presumably by interfering with viral adsorption, but MARCO and SR-A1 do not represent direct targets. The finding that the inhibitory effect of poly(I) is independent of MARCO is, in turn, logical given the observation that the efficiency of viral entry does not depend on MARCO. Of note, if the inhibitory effect of poly(I) is indeed based on interference with viral adsorption, attachment of HSV-1 to the corresponding target plays a major role during entry into skin.

MATERIALS AND METHODS

Mice, preparation of murine epidermis, and isolation of primary murine fibroblasts, primary human fibroblasts, and primary human keratinocytes. Murine skin samples were taken from the tails of five control (C57BL/6) mice and five adult (6- to 7-week-old) MARCO$^{-/-}$ mice (17) (tails were kindly provided by Siamon Gordon). In addition, murine skin was taken from the tails of six control (C57BL/6) mice and six adult (5-week-old) MARCO$^{-/-}$/SR-A1$^{-/-}$ dKO mice (26) (tails were kindly provided by Subhankar Mukhopadhyay). Epidermal sheets were prepared by the removal of the dermis by dispase II treatment and incubated in Dulbecco's modified Eagle's medium (DMEM, high glucose, GlutaMAX supplement [Life Technologies]), as described previously (30). Primary fibroblasts were isolated from murine dermis of control, MARCO$^{-/-}$, or MARCO$^{-/-}$/SR-A1$^{-/-}$ dKO adult mice or from lamina propria of human oral mucosa after 3 h of treatment at 37°C in serum-free DMEM-high glucose-GlutaMAX (Life Technologies) containing 400 U of collagenase I (Worthington)/ml as described previously (10). Primary fibroblasts were cultured in DMEM-high glucose-GlutaMAX (Life Technologies) containing 10% fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Experiments were performed in primary murine and human fibroblasts at 2 to 4 passages after isolation.

Primary human keratinocytes from foreskin (31) were cultured in the presence of mitomycin C-treated 3T3 fibroblasts (strain J2) as feeder cells at 37°C and maintained in DMEM-high glucose-GlutaMAX (Life Technologies)–Ham's F-12 (Gibco) (3.5:1.1) containing 10% FCS, penicillin (400 IU/ml), streptomycin (50 μg/ml), adenine (1.8×10^{-4} M), glutamine (300 μg/ml), hydrocortisone (0.5 μg/ml), epidermal growth factor (EGF) (10 ng/ml), cholera enterotoxin (10^{-10} M), insulin (5 μg/ml), and ascorbic acid (0.05 mg/ml).

Cells, viruses, and plasmids. HaCaT cells (32) and B16-F1 mouse melanoma cells (33), which express neither nectin-1 nor HVEM (data not shown), were maintained in DMEM-high glucose-GlutaMAX (Life Technologies) containing 10% FCS, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Infection studies were performed with purified preparations of HSV-1 wild-type strain 17 as described previously (31).

In addition, HaCaT cells were infected with HSV1(17$^+$)Lox-ΔgC^{P62FS}, which contains a single nucleotide deletion in UL44 leading to a premature stop codon and thus preventing expression of gC (data not shown). HSV1(17$^+$)Lox-ΔgC^{P62FS} was generated from pHSV1(17$^+$)blueLox (34) by repairing the thymidine kinase gene, into which the bacterial artificial chromosome (BAC) sequences had been initially inserted, and removing the β-galactosidase gene. The gC-reconstituted virus HSV1(17$^+$)Lox-gC$^+$ was generated by repairing the UL44 mutation using the primers 5′-ACA TCG GGG TCC CCC GGG TCA GCC GCC AGC CCG GAG GTC ACC CCC ACA TCG ACC CCA AAC CTA GGG ATA ACA GGG TAA TCG ATT T-3′and 5′-GTT TTG TGT GAC ATT GTT GGG GTT TGG GGT CGA TGT GGG GGT GAC CTC CGG GCT GGC GGC TGC CAG TGT TAC ACC CAA TTA ACC-3′ and the plasmid pEP-Kan-S2 (35). The HSV-1 mutant strains were propagated and titrated as described previously (36).

Virus inoculum was added to epidermal sheets or to cells at 37°C, defining time point 0. For infection of epidermal sheets, the calculation of the virus dose was based on the estimated cell number in the basal layer (1×10^4 cells/mm^2). Primary human foreskin keratinocytes were seeded without 3T3 fibroblasts prior to infection.

The open reading frame (ORF) of full-length human MARCO was cloned into a pcDNA4/TO/myc-His A vector (Thermo Fisher). The expression vector pSC386 encoding human HVEM (37) was used as a control.

Ethics statement. The preparation of human fibroblasts was carried out in strict accordance with the recommendations of the local ethics commission. The study was approved by the Ethics Commission, Medical Faculty, University of Cologne (approval no. 10-205 and no. 08-144).

RNA preparation and RT-PCR. RNA was isolated from primary murine bone marrow macrophages, murine epidermis, primary murine epidermal keratinocytes, primary murine dermal fibroblasts, U937 human monocytes (38), HaCaT cells, primary human oral mucosa keratinocytes, human oral mucosa epithelium, human epidermis, primary human oral mucosa fibroblasts, HFFF2 human fibroblasts (ECACC no. 86031405), or primary human skin fibroblasts by use of TRIzol (Life Technologies) or Nucleozol reagents (Macherey-Nagel). cDNA was synthesized using SuperScript II reverse transcriptase (Life Technologies); PCR was performed with *Taq* DNA polymerase (Life Technologies), and after testing at least three primer pairs for each cDNA, the indicated primer pairs were used as follows: (i) to detect transcription in general, murine MARCO primers (forward, 5′-ACTCCAGAGGGAGAGCACTT-3′; and reverse, 5′-GGTGGAACCCAAAGACACCT-3′) generate a 220-bp fragment spanning exons 16 and 17; (ii) to

detect transcription in control and MARCO$^{-/-}$ fibroblasts, which carry the β-galactosidase and *neo* resistance genes leading to the partial loss of exon 2 and the deletion of exon 3, murine MARCO primers (forward, 5′-CTCAGCTGGGTCCATACCAG-3′; and reverse, 5′-TCACCCTTGGCACCTGAAAG-3′) generate a 602-bp fragment spanning exons 3 to 11; (iii) to detect transcription in human skin cells, human MARCO primers (forward, 5′-CTACAGTGGTACCTGGGGGA-3′; and reverse, 5′-CATGCGGCAGAAGACAATGG-3′) generate a 73-bp fragment in exon 16; (iv) murine SR-A1 primers (forward, 5′-AAAATGGCCCCTCCCTT CAG-3′; and reverse, 5′-ATTCTGGAAGCGTTCCGTGT-3′) generate a 317-bp fragment spanning exons 3 and 4 (in MARCO$^{-/-}$/SR-A1$^{-/-}$ dKO mice, exon 4 of SR-A1 is deleted); (v) GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers (forward, 5′-TGATGACATCAAGAAGGTGGTGAAG-3′; and reverse, 5′-TCCTTGGAGGCCATGTGGGCCAT-3′) generate a 240-bp fragment.

Immunocytochemistry and antibodies. For whole mounts (18, 39), murine epidermal sheets were fixed with 3.4% formaldehyde for 2 h, stained overnight with mouse anti-ICP0 (11060; 1:60) (40), and visualized with the corresponding secondary antibody and DAPI (4′,6-diamidino-2-phenylindole) as described previously (30).

Infected human and murine cells were fixed with 2% formaldehyde for 10 min, permeabilized with 0.5% NP-40 for 10 min, and stained for 60 min with mouse anti-ICP0 (11060; 1:60) (40), followed by incubation with the corresponding secondary antibody and DAPI, all at room temperature.

Transfected B16-F1 cells were fixed with 2% formaldehyde for 10 min, stained for 90 min with rabbit anti-myc (1:200) (Santa Cruz) to detect MARCO expression or with rabbit anti-HVEM (R140; 1:1,000) (41), followed by incubation with the corresponding secondary antibodies. Subsequently, cells were permeabilized with 0.5% NP-40 for 10 min, stained for 60 min with mouse anti-ICP0 (11060; 1:60) (40), and visualized with the corresponding secondary antibody to detect infected cells. Cells were counterstained with DAPI.

Microscopy was performed using a Leica DM IRB/E microscope linked to a Leica TCS-SP/5 confocal unit or a Zeiss Axiophot. Images were assembled using Photoshop (version CS2; Adobe).

Poly(I)/poly(C) treatment. Murine epidermal sheets or cells were treated with poly(I) (Sigma) or poly(C) (Sigma) for 20 min at 37°C, followed by infection with HSV-1 at 5 PFU/cell in the continued presence of the respective components. Alternatively, HaCaT cells were treated for 1 h with poly(C) or poly(I), followed by HSV-1 infection (5 PFU/cell) in the absence of the ligands, or were infected in the presence of poly(C) or poly(I) without pretreatment. In addition, HaCaT cells were incubated for 1 h with HSV-1 (5 PFU/cell) on ice in the presence of poly(C) or poly(I) to allow viral attachment, followed by three washing steps with ice-cold DMEM-high glucose-GlutaMAX (Life Technologies) and incubation for 2 h at 37°C. Furthermore, HaCaT cells were incubated for 1 h with HSV-1 (5 PFU/cell) on ice in the absence of poly(C) or poly(I), washed three times with ice-cold DMEM-high glucose-GlutaMAX (Life Technologies), and then treated with poly(C) or poly(I) for 2 h at 37°C.

Transient expression. B16-F1 cells were transfected with a plasmid expressing *myc*-tagged human MARCO or a plasmid expressing human HVEM (1 μg per 4×10^4 cells) using X-tremeGENE HP transfection reagent (Roche). At 21 h posttransfection, cells were infected for 3 h with HSV-1 at 20 PFU/cell.

ACKNOWLEDGMENTS

We are grateful to Siamon Gordon and Subhankar Mukhopadhyay for providing tails of MARCO$^{-/-}$ and MARCO$^{-/-}$/SR-A1$^{-/-}$ mice, respectively. We thank Roger Everett for the antibodies against ICP0, Anika Steffen for B16-F1 cells, Stefan Höning for U937 monocytes, and Claude Krummenacher, Roselyn Eisenberg, and Gary Cohen for plasmid pSC386 encoding human HVEM. Additional thanks go to Juha Ojala for the expression plasmid of human MARCO and for very helpful comments. We also thank Mats Paulsson for his suggestions to improve the manuscript.

This research was supported by the German Research Foundation (KN536/16-3) and the Köln Fortune Program/Faculty of Medicine, University of Cologne.

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