Equine herpesvirus type 1 replication is delayed in CD172a+ monocyctic cells and controlled by histone deacetylases

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Equine herpesvirus type 1 (EHV-1) replicates in the epithelial cells of the upper respiratory tract and disseminates through the body via a cell-associated viraemia in monocyctic cells, despite the presence of neutralizing antibodies. However, the mechanism by which EHV-1 hijacks immune cells and uses them as ‘Trojan horses’ in order to disseminate inside its host is still unclear. Here, we hypothesize that EHV-1 delays its replication in monocyctic cells in order to avoid recognition by the immune system. We compared replication kinetics in vitro of EHV-1 in RK-13, a cell line fully susceptible to EHV-1 infection, and primary horse cells from the myeloid lineage (CD172a+). We found that EHV-1 replication was restricted to 4 % of CD172a+ cells compared with 100 % in RK-13 cells. In susceptible CD172a+ cells, the expression of immediate-early (IEP) and early (EICP22) proteins was delayed in the cell nuclei by 2–3 h post-infection (p.i.) compared with RK-13, and the formation of replicative compartments by 15 h p.i. Virus production in CD172a+ cells was significantly lower (from 10^1.7 to 10^3.1 TCID50 per 10^5 inoculated cells) than in RK-13 (from 10^5 to 10^5.7 TCID50 per 10^5 inoculated cells). Less than 0.02 % of inoculated CD172a+ cells produced and transmitted infectious virus to neighbouring cells. Pre-treatment of CD172a+ cells with inhibitors of histone deacetylase activity increased and accelerated viral protein expression at very early times of infection and induced productive infection in CD172a+ cells. Our results demonstrated that the restriction and delay of EHV-1 replication in CD172a+ cells are part of an immune evasive strategy and involve silencing of EHV-1 gene expression associated with histone deacetylases.

INTRODUCTION

Viruses are defined as obligatory intracellular parasites. They replicate inside the host cell using the host metabolic machinery. Following a primary infection, viruses can elicit a strong immune response. The host’s immune system employs a variety of strategies to eliminate the virus (Vossen et al., 2002). However, during co-evolution with their host, viruses have developed an impressive array of immune evasion mechanisms to escape their elimination by the host’s immune system (Alcami & Koszinowski, 2000; Favoreel et al., 2003). One strategy mastered by all herpesviruses is the capacity to establish life-long latency in immunocompetent hosts. During periods of reactivation, the virus may spread to contact individuals. During latency, the virus remains hidden from the immune system and can persist in its host (Efstathiou & Preston, 2005).

Equine herpesvirus type 1 (EHV-1), a member of the subfamily Alphaherpesvirinae, is a major pathogen of horses worldwide. The virus is responsible for respiratory disorders, abortion, neonatal foal death and neurological disorders (Allen & Bryans, 1986; Patel & Heldens, 2005; Dunowska, 2014). After primary replication in the epithelial cells of the upper respiratory tract, the virus can spread through the basement membrane to the connective tissues by the use of individual infected migrating monocyctic cells (Gryspeerdt et al., 2010; Vandekerckhove et al., 2010). The virus disseminates via a cell-associated viraemia in PBMCs to target organs such as the pregnant uterus or central nervous system. Here, the virus initiates a secondary replication that may lead in some cases to severe symptoms such as abortion and/or neurological disorders (Edington et al., 1991; Smith et al., 1996; Stierstorfer et al., 2002). Current vaccines do not provide full protection, as EHV-1 can cause a viraemia in the presence of virus-specific antibodies and CTL precursors (O’Neill et al., 1999; Kydd et al., 2006). This shows that recognition of EHV-1-infected carrier cells by the immune system is seriously hampered. Indeed, like other herpesviruses, EHV-1 appears to have developed sophisticated immune evasive strategies to counteract the immune response and to facilitate spread in an immune horse (van der Meulen et al., 2006).
Previous in vivo and ex vivo studies of the pathogenesis of EHV-1 in the equine upper respiratory tract identified CD172a+ monocytic cells as one of the main carrier cells of EHV-1. It was found that the expression of some late proteins in EHV-1-infected carrier monocytic cells is hampered at the early stages of infection (van der Meulen et al., 2000; Grispeerdt et al., 2012). These results suggested that infected leukocytes in the upper respiratory tract with restricted late viral protein expression could be the source of the cell-associated viraemia. An early block in the replication cycle of EHV-1 may protect infected leukocytes from efficient recognition by the immune system and hence allow these carrier cells to reach target organs. Interestingly, retroviruses such as human immunodeficiency virus (HIV) and maedi-visna virus, and other herpesviruses, such as pseudorabies virus and human cytomegalovirus (HCMV), are known to influence early response systems by blocking several steps of their replication cycle in their target cells in order to evade immune responses and promote viral pathogenesis (Rice et al., 1984; Nauwynck & Pensaert, 1994; Thornar, 2005; Bergamaschi & Pancino, 2010). However, until now, the mechanism of how EHV-1 uses CD172a+ cells as ‘Trojan horses’ to evade the immune system and disseminate within the host has not been described.

In order to understand the immune evasive strategies used by EHV-1, in vitro EHV-1 replication kinetics in equine nasal mucosal and blood CD172a+ monocytic cells, two major target cell types, were examined and compared with replication kinetics in rabbit kidney epithelial RK-13 cells, a cell line known to be fully susceptible to EHV-1 infection.

## RESULTS

### EHV-1 replication is delayed in CD172a+ cells compared with RK-13 cells

In a first experiment, equine nasal mucosal and blood CD172a+ cells were examined for their ability to support EHV-1 replication compared with RK-13 cells. To this end, the expression kinetics of the three major classes of protein were evaluated by indirect immunofluorescence staining: one immediate-early (IE) protein (IEP), one early protein (EICP22) and two late (gB and gC) proteins.

In EHV-1-inoculated RK-13 cells, IEP was detected in the nuclei of 3% of cells as early as 3 h post-infection (h.p.i.), the EICP22 protein in the nuclei of 5% of cells as early as 7 h p.i., and the late gB and gC glycoproteins in the cytoplasm of 2% of cells starting from 7 and 9 h p.i., respectively (Fig. 1a).

The number of RK-13 cells positive for all viral proteins increased over time. At 12 h p.i., 64% of the cells were found to be IEP positive, 37% were EICP22 positive, and 32 and 10% were gB and gC positive, respectively. At 24 h p.i., 100% of the cells were positive for all viral proteins.

In EHV-1-inoculated equine nasal mucosal and blood CD172a+ cells, IEP was first detected at 5 h p.i. The number of IEP-positive cells was significantly higher in blood CD172a+ cells than in nasal mucosal CD172a+ cells at 5 and 7 h p.i. (P<0.01) (Fig. 1b, c). From 5 to 7 h p.i., the percentage of IEP-positive cells increased from 0.3 to 0.9% and from 2.8 to 3.6% in nasal mucosal and blood CD172a+ cells, respectively. A maximum of 3.6 and 3.8% of IEP-positive cells was reached at 7 and 12 h p.i. in blood and nasal mucosal CD172a+ cells. EICP22 was first detected at 7 h p.i. in 0.2% of blood CD172a+ cells and at 9 h p.i. in 0.6% of equine nasal mucosal CD172a+ cells. Similarly to IEP expression, the percentage of EHV-1-inoculated CD172a+ cells expressing EICP22 did not increase after 12 h p.i. At 24 h p.i., the EICP22 protein was expressed in 2 and 1.3% of blood and nasal mucosal CD172a+ cells, respectively. The late gB protein was only detected in 0.3% of blood CD172a+ cells at 12 h p.i. and in 0.6 and 1.3% of blood and nasal mucosal CD172a+ cells at 24 h p.i. The late gC protein was only found to be expressed in 0.3% of nasal mucosal CD172a+ cells at 24 h p.i. The use of a higher m.o.i. (50) did not alter the number of IEP-positive cells (Fig. S1, available in the online Supplementary Material). In addition, EHV-1 replication was highly restricted in CD172a+ cells, independently of the abortigenic strains used (Fig. S2). Overall, these results demonstrated that the replication of EHV-1 in equine nasal mucosal and blood CD172a+ cells is delayed based on the kinetics of viral protein appearance in these cells.

### Spatio-temporal distribution of EHV-1 viral proteins in CD172a+ compared with RK-13 cells

To determine whether the delay of EHV-1 replication in CD172a+ cells was correlated with a change in the distribution of EHV-1 viral proteins within the cell, we analysed the spatio-temporal distribution dynamics of IEP, EICP22, gB and gC in EHV-1-inoculated CD172a+ and RK-13 cells by immunofluorescence staining. The co-localization of IEP and EICP22 was also examined by means of immunofluorescence double staining.

In EHV-1-inoculated RK-13 cells, a weak and diffuse IEP signal was first detected at 1 h p.i. with a few foci of intranuclear staining, with intensity increasing by 3 and 5 h p.i. (Fig. 2). At 7 h p.i., IEP co-localized in one or two small globular structures, whilst IEP was also found distributed in speckles in the rest of the cell nuclei (see arrows). The localization of IEP in globular structures coincided with the appearance of EICP22 in the nuclei of cells. Both IEP and EICP22 co-localized in nuclear globular structures resembling the pre-replicative sites at 7 h p.i. However, IEP showed a change in nuclear distribution as infection progressed. The IEP signal progressively dispersed in the cytoplasm, and the size of the nuclear globular compartments increased and varied from 0–2 to 2–5 μm between 7 and 9 h p.i. At 12 h p.i., the IEP signal was found associated with massive globular compartments (5–8 μm), resembling the replicative compartments, which occupied most of the nuclear space, whilst IEP was found dispersed in the...
cytoplasm of infected RK-13 cells. EICP22 did not co-localize with IEP in the nucleus of cells but rather formed a shell around IEP-associated compartments. IEP expression progressed from individual IEP foci to large compartments, each consisting of several IEP foci. To confirm whether the formation of large IEP-associated compartments in the nuclei of EHV-1-inoculated RK-13 cells was dependent on viral DNA replication, we compared the nuclear distribution of IEP in EHV-1-inoculated cells in the presence or absence of phosphonoacetate (PAA), a specific inhibitor of the viral DNA polymerase. As expected, in PAA-treated cells, only small-punctuated IEP-associated nuclear structures were observed at the later stage of infection, whilst no large IEP-associated compartments were detected (data not shown). These results confirmed that IEP associates within distinct structures, called pre-replicative sites (7 h p.i.), that mature into replicative compartments (at 9 and 12 h p.i.) as infection progresses, and which constitute sites for viral DNA synthesis. However, in both cases, this correlated with the appearance of EICP22, which co-localized with IEP in the nuclei of both CD172a+ cell types. At 12 h p.i., a strong IEP staining was observed in the nucleus and to a lesser extent in the cytoplasm of both cell types, whilst no IEP-associated replicative compartments were detected. EICP22 was found to be expressed similarly to its expression at 9 h p.i. Interestingly, the expression of IEP within replicative compartments and surrounded by EICP22 was only detected at 24 h p.i. in the nucleus of both CD172a+ cell types (Figs 3 and 4, arrows). Finally, the cytoplasmic localization of gB and gC late proteins remained unchanged in nasal mucosal and blood CD172a+ cells compared with RK-13 cells (data not shown). These results showed that the delay of EHV-1 replication in CD172a+ cells compared with RK-13 cells is

presence of a cytopathic effect, which did not allow any further examination of the viral protein expression pattern. In both EHV-1-inoculated nasal mucosal and blood CD172a+ cells, a weak IEP signal was first detected in some parts of the nucleus and intensity increased from 5 to 7 h p.i. (Figs 3 and 4). At 9 h p.i., the IEP signal was diffuse throughout the nucleus and cytoplasm of blood CD172a+ cells, whilst in nasal mucosal CD172a+ cells, the IEP signal was still found exclusively restricted to some nuclear areas. However, in both cases, this correlated with the appearance of EICP22, which co-localized with IEP in the nucleus of both CD172a+ cell types. At 12 h p.i., a strong IEP staining was observed in the nucleus and to a lesser extent in the cytoplasm of both cell types, whilst no IEP-associated replicative compartments were detected. EICP22 was found to be expressed similarly to its expression at 9 h p.i. Interestingly, the expression of IEP within replicative compartments and surrounded by EICP22 was only detected at 24 h p.i. in the nucleus of both CD172a+ cell types (Figs 3 and 4, arrows). Finally, the cytoplasmic localization of gB and gC late proteins remained unchanged in nasal mucosal and blood CD172a+ cells compared with RK-13 cells (data not shown). These results showed that the delay of EHV-1 replication in CD172a+ cells compared with RK-13 cells is

Fig. 1. Expression kinetics of EHV-1 proteins in RK-13 cells (a), nasal mucosal (b) and blood CD172a+ (c) cells. Immunofluorescence stainings of immediate-early (IEP), early (EICP22) and late (gB and gC) proteins were performed in triplicate and data are represented as means ± SD.
correlated with a change in the spatio-temporal distribution dynamics of EHV-1 viral proteins within the cell.

**Kinetics of EHV-1 production in CD172a\(^+\) cells compared with RK-13 cells**

To determine the kinetics of viral production in blood and nasal mucosal CD172a\(^+\) cells compared with infected RK-13 cells, virus titrations of supernatants (extracellular fraction) and cells (intracellular fraction) were performed.

No significant increase in intracellular and extracellular virus titres was observed in any of the cell types before 9 and 12 h p.i., respectively. At 12 h p.i., virus titration of the intracellular fraction showed a 10-fold lower virus titre in blood and nasal mucosal CD172a\(^+\) cells compared with RK-13 cells \((P<0.01)\), but no significant difference in extracellular virus titre was observed between infected CD172a\(^+\) and RK-13 cells (Fig. 5). At 24 h p.i., virus titration of the extracellular fraction revealed a 100-fold and 1000-fold lower virus titre in blood \((10^{5.1} \text{ TCID}_{50} \text{ per } 10^5 \text{ inoculated cells}; \ P<0.01)\) and nasal CD172a\(^+\) cells \((10^{4.4} \text{ TCID}_{50} \text{ per } 10^5 \text{ inoculated cells}; \ P<0.001)\) compared with RK-13 \((10^{5.7} \text{ TCID}_{50} \text{ per } 10^5 \text{ inoculated cells})\). In addition, virus titration of the intracellular fraction showed a 1000-fold and 10 000-fold lower titre in blood \((10^2 \text{ TCID}_{50} \text{ per } 10^5 \text{ inoculated cells})\) and nasal CD172a\(^+\) cells \((10^{1.7} \text{ TCID}_{50} \text{ per } 10^5 \text{ inoculated cells})\).
cells) than in RK-13 (10⁵ TCID₅₀ per 10⁵ inoculated cells). No increase in extracellular and intracellular virus titres was observed in nasal mucosal CD172a⁺ cells over time post-inoculation. The virus remained infectious throughout the experiment, as confirmed by the inactivation curve.

Next, we evaluated and compared the percentage of cells that produced and transmitted infectious EHV-1 to neighbouring cells, between infected CD172a⁺ and RK-13 cells. Co-cultivation of inoculated CD172a⁺ cells and RK-13 cell monolayers revealed that less than 0.01 and 0.02% of nasal mucosal and blood CD172a⁺ cells, respectively, were productive. The number of EHV-1-producing CD172a⁺ cells was significantly less than the number of EHV-1-producing RK-13 cells (31%); *P*<0.0001.

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**Fig. 3.** Double immunofluorescence of EHV-1 IEP (green) and EICP22 (red) in nasal mucosal CD172a⁺ cells. Nuclei were counterstained with Hoechst (blue). Each image represents a single section through a cell. Arrows indicate the formation of IEP-associated replicative compartments. Bar, 10 μm.
Histone deacetylase (HDAC) inhibitors relieve the delay of EHV-1 protein expression in CD172a+ cells at very early times of infection

Next, we determined whether HDACs were involved in the temporary block of EHV-1 protein expression in CD172a+ cells at early times of infection. We tested whether treatment of EHV-1-inoculated CD172a+ cells with the deacetylase inhibitors trichostatin (TSA) and sodium butyrate (NaBut) increased IEP, EICP22 and gB protein expression in these cells at early times of infection. These experiments were performed using blood CD172a+ cells only, as the kinetics of EHV-1 replication was found to be similar in both nasal and blood CD172a+ cells.

We found that the number of IEP-, EICP22- and gB-positive blood CD172a+ cells was significantly higher in NaBut- and TSA-treated CD172a+ cells compared with

![Fig. 4. Double immunofluorescence of EHV-1 IEP (green) and EICP22 (red) proteins in blood CD172a+ cells. Nuclei were counterstained with Hoechst (blue). Each image represents a single section through a cell. Arrows indicate the formation of IEP-associated replicative compartments. Bar, 10 μm.](http://vir.sgmjournals.org)
untreated cells from 7–9 to 24 h p.i. ($P<0.05$) (Fig. 6). In NaBut-treated cells, IEP was detected in the nuclei of 0.6% of the cells as early as 1 h p.i., EICP22 protein in the nuclei of 1.6% of the cells as early as 5 h p.i. and gB in the cytoplasm of 2.3% of the cells starting from 7 h p.i. At 12 h p.i., 12% of the cells were IEP positive, 9% were EICP22 positive and 7.3% were gB positive. At 24 h p.i., 14% of the cells were positive for all viral proteins. Similar results were observed in TSA-treated cells. Titration of the supernatant of NaBut- and TSA-treated cells showed a significant rise in virus titre compared with non-treated cells at 12 and 24 h p.i., respectively ($P<0.05$) (Fig. S3). This result confirmed productive EHV-1 infection in these cells. TSA and NaBut cell treatment did not affect the kinetics of EHV-1 protein expression in EHV-1-inoculated RK-13 cells (data not shown).

EHV-1 proteins were expressed earlier in HDAC inhibitor-treated CD172a+ cells than in untreated cells and were comparable to those found in non-treated and EHV-1-infected RK-13 cells. At 1 h p.i., the IEP signal was bright and homogeneously distributed in the nucleus of NaBut-treated cells within few foci of intranuclear staining. At 7 h p.i., EICP22 was found to be expressed in the cell nuclei around newly formed IEP-associated compartments (Fig. 7). At 12 h p.i., cells harboured a massive nuclear IEP-associated replicative compartment, and gB was found to be expressed in the cytoplasm and at the plasma membrane of the cell (Fig. 8a). From 12 to 24 h p.i., intercellular contacts were observed between CD172a+ cells, which resulted in the formation of 'ring-shaped' cell clusters, consisting of at least two and up to 20 cells. A single cell containing a nuclear IEP-associated replicative compartment initiated intercellular contacts with neighbour cells via membrane extensions that were found to be IEP and gB positive (Fig. 8b). Recruited neighbouring cells progressively formed clusters that were characterized by cytoplasmic IEP expression as well as cell-surface and polarized cytoplasmic gB expression, whilst no IEP-associated replicative compartment was detected in these neighbouring cell nuclei (Fig. 8c).

**DISCUSSION**

In the present study, we demonstrated that approximately 4% of equine nasal mucosal and blood CD172a+ cells, two target cells of EHV-1, were susceptible to EHV-1 infection in vitro. This is in agreement with the study of van der Meulen et al. (2000), demonstrating that EHV-1 replication is highly restricted in equine PBMCs. As little is known about the regulation of monocytic cell tropism and susceptibility to EHV-1, we hypothesized that a low efficiency of binding of EHV-1 to CD172a+ cells may be responsible for the restriction of EHV-1 replication in these cells, suggesting the presence of specific cell-surface receptors with restricted expression patterns. Preliminary in vitro studies demonstrated significant differences in the binding efficiency of EHV-1 between CD172a+ and RK-13 cells in terms of percentage of infected cells and number of virus particles bound per cell. We found that EHV-1 does not bind efficiently to CD172a+ cells compared with RK-13 cells, and the low efficiency of EHV-1 binding to CD172a+ cells was not shown to be virus-dose dependent. This indicated that a block at the virus entry level was partially responsible for the restricted virus replication in CD172a+ cells.

As well as the restriction of EHV-1 replication in CD172a+ cells, we demonstrated significant differences in the kinetics of EHV-1 protein expression between susceptible CD172a+ cells and control RK-13 cells at very early times of infection. We found that IEP was detected later in the nuclei of blood and nasal CD172a+ (5 h p.i.) than in the nuclei of control RK-13 cells (3 h p.i.). Similarly, EICP22 and late (gB and gC) protein expression was delayed in both CD172a+ cell types. Moreover, we observed that
EICP22 and the late gB and gC proteins accumulated more slowly in blood CD172a^+ cells than IEP, suggesting a delay associated with a consecutive block of viral protein expression in CD172a^+ cells. In addition, a progression of stages could be defined for the nuclear association of IEP with EICP22 during EHV-1 infection, as shown for HSV-1 infection (Knipe et al., 1987; Taylor et al., 2003; Chang et al., 2011). In EHV-1-inoculated RK-13 cells, IEP showed a diffuse nuclear distribution and co-localized with EICP22 at early times of infection, whilst both proteins presented a distinct nuclear distribution at later times of infection. By the use of PAA, an inhibitor of viral DNA polymerase, we

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**Fig. 6.** Replication kinetics of EHV-1 in blood CD172a^+ cells following treatment with HDAC inhibitors TSA and NaBut. Experiments were performed in triplicate. Results are shown as means ± SD, and a two-way ANOVA test was performed to evaluate significant differences between HDAC inhibitor-treated and untreated cells. *P* < 0.05.

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**Fig. 7.** Double immunofluorescence staining of EHV-1 IEP (green) and EICP22 (red) proteins in EHV-1-inoculated and NaBut-treated blood CD172a^+ cells at 1 and 7 h p.i. Nuclei were counterstained with Hoechst (blue). Each image represents a single section through a cell. Bar, 10 μm.
confirmed that IEP-associated nuclear structures, so-called replication compartments, were essential for viral DNA replication (Quinlan et al., 1984). Interestingly, we observed that IEP localized into replication compartments more slowly in CD172a^+ cells (24 h p.i.) than in RK-13 cells (7 h p.i.). In addition, we found that viral production and the percentage of EHV-1 producing cells were significantly lower than in RK-13 cells. Taken together, we demonstrated that the replication of EHV-1 in CD172a^+ cells was delayed at a very early time of infection, starting from the nuclear expression of IEP, which successively slowed down the replication machinery in these cells, thus leading to a non-productive infection. We believe that the restriction/delay of replication in CD172a^+ cells is part of an immune evasive strategy employed by EHV-1. By slowing its replication down, EHV-1 allows better survival of CD172a^+ cells, whilst the virus uses them as ‘Trojan horses’ to disseminate undetected throughout the body. However, we believe that not all infected leukocytes will transfer the virus to endothelial cells, not every transfer will lead to effective spread to neighbouring endothelial cells and not every successful infection of endothelial cells will cause infection of surrounding tissues. Indeed, studies have demonstrated that EHV-1 viraemia is detected in pregnant mares, which do not abort, and only a minority of viraemic horses (10 %) develop neurological disorders during equine herpes myeloencephalopathy outbreaks (Goehring et al., 2006; Slater, 2007). In addition, we are also convinced that these outcomes of infections of leukocytes with neurovirulent and non-neurovirulent leukocytes are quite different, based on preliminary in vitro experiments we performed demonstrating strikingly different outcomes in EHV-1 replication between the two strains in target CD172a^+ cells.

Furthermore, we showed that the delay of EHV-1 protein expression in susceptible blood CD172a^+ cells could be relieved following treatment with TSA and NaBut, two HDAC inhibitors. HDACs are known to condense the chromatin structure by deacetylation of histones in order to repress cellular and/or viral transcription. According to this, our results suggested that HDACs play a role in the silencing of gene expression in EHV-1 target cells. This was confirmed by the expression of EICP22 (in the nucleus) and gB (in the cytoplasm) starting from 5 and 7 h p.i. in HDAC inhibitor-treated CD172a^+ cells, accompanied by the rapid formation of a nuclear IEP-associated replication compartment (at 7–9 h p.i.) and resulting in a productive EHV-1 infection in these cells. These results were consistent with recent work demonstrating the importance of protein acetylation and control of IE gene expression via HDACs during herpesvirus infections (Meier, 2001; Everett et al., 2009). In addition, we found that a higher number of CD172a^+ cells (12–14 %) were susceptible to EHV-1 infection following treatment with HDAC inhibitors compared with untreated cells (4 %). This suggests that the permissiveness for EHV-1 infection might be linked to

![Fig. 8. Double immunofluorescence staining of IEP (green) and gB (red) proteins expressed in EHV-1-inoculated and NaBut-treated blood CD172a^+ cells at 12 h p.i. Nuclei were counterstained with Hoechst (blue). (a) Formation of an IEP-associated replicative compartment in the nucleus and cytoplasmic expression of gB in CD172a^+ treated cells. (b) Intercellular contact between an infected cell and a non-infected cell. (c) ‘Ring-shaped’ CD172a^+ cell clusters of EHV-1 infection. Bar, 10 μm.](image-url)
EHV-1 replication is delayed in CD172a+ monocylic cells

driven by cellular adhesion molecules or by indirect contact via paracrine signalling, which induces cell migration to the developing focus of infection, as occurs in HIV infection (Boomker et al., 2005; Nikolic et al., 2011). Both contacts may result in the formation of cell clusters, which trigger cell-to-cell fusion events. These ‘microfusion’ events are likely to be responsible for infectious virus transfer and cytoplasmic transfer of viral material from an infected cell to uninfected surrounding cells, as described in the transmission of HCMV infection in vitro (Gerna et al., 2000; Digel et al., 2006). The adhesion molecules involved in cell-to-cell interactions as well as the exact composition of the cytoplasmic material transferred from an infected to uninfected CD172a+ cells remain to be investigated. However, the results of direct cell-to-cell transmission of EHV-1 between CD172a+ cells, which undergo complete virus replication under HDAC inhibition, constitute relevant findings in light of the pathogenesis of EHV-1 in vivo and in vitro.

In conclusion, we have demonstrated that the replication of EHV-1 is restricted and delayed at the level of viral gene transcription in nasal and blood CD172a+ cells compared with RK-13 cells. These data substantiate the hypothesis that EHV-1 hijacks CD172a+ cells and silences its replication within the cells, acting like a ‘Trojan horse’, in order to survive in the blood circulation and reach target organs and to evade immune surveillance. Silencing of EHV-1 gene expression in CD172a+ cells was shown to be tightly regulated by histone deacetylation events. A better understanding of the molecular details of chromatin regulation could give new insights into the pathogenesis of EHV-1, both in vivo and in vitro, and provide future therapeutic strategies.

METHODS

Viruses. The Belgian EHV-1 non-neurovirulent strain 97P70, first isolated in 1997 from the lungs of an aborted fetus, was used in this study (van der Meulen et al., 2000). Virus stocks used for inoculation were passaged five times in equine embryonic lung cells (EEL) and one time in RK-13 cells.

Cells.

Isolation of equine blood CD172a+ cells. Healthy horses between 8 and 10 years old were used as blood donors. The horses were seronegative for EHV-1. The collection of blood was approved by the ethical committee of Ghent University (EC2013/17). Blood was collected by jugular venipuncture on heparin (15U ml−1) (Leo) and diluted in an equal volume of Dulbecco’s PBS (DPBS) without calcium or magnesium (Gibco). PBMCs were isolated by density-gradient centrifugation on Ficoll-Paque (1.077 g ml−1) (GE Healthcare, Life Sciences) at 800 g for 30 min at 18°C. The interphase cells, containing the PBMCs, were collected and washed three times with DPBS. The cells were resuspended in leucocyte medium based on RPMI (Gibco) supplemented with 5 % FCS (Grainer), 1 % penicillin, 1 % streptomycin and 0.5 % gentamicin (Gibco). Afterwards, cells were seeded on 24-well plates (Nunc A/S) at a concentration of 105 cells ml−1 and cultivated at 37 °C with 5 % CO2. After 12 h, non-adhering lymphocytes were removed by washing the cells three times with RPMI. The adherent cells consisted of >90 %

Finally, we observed the formation of clusters of CD172a+ cells composed of one cell containing a nuclear IEP-associate replication compartment and surrounded by cells presenting cytoplasmic IEP and gB expression at a later stage of infection in HDAC-treated cells (12–24 h p.i.). This result indicated that EHV-1 can efficiently spread by cell-to-cell contact in productively infected CD172a+ cells and transfer cytoplasmic viral proteins to uninfected neighbouring cells. This finding is partially in agreement with previous studies by van der Meulen et al. (2000), who demonstrated the formation of intercellular contacts between EHV-1-inoculated PBMCs within clusters after mitogen stimulation, thus facilitating transmission of virus between cells. As little is known about the mechanism underlying efficient EHV-1 cell-to-cell transmission in CD172a+ cells, we hypothesize that, after productive viral infection in individual CD172a+ cells, EHV-1 recruits neighbouring cells either by direct contact(s) via induction of membrane extensions (synapses)

repression of the IE promoter, as described for HCMV infection (Murphy et al., 2002; Wright et al., 2005). We also found that treatment of cells with TSA or NaBut selectively increased EHV-1 replication in target CD172a+ cells but not in control RK-13 epithelial cells. It is possible that the effectiveness of HDAC inhibitor treatment is cell dependent or that RK-13 cells possess a low level of HDAC, which directly correlates with their increased EHV-1 permissiveness (Cody et al., 2014). Further studies are still needed to determine which individual HDAC(s) might be the most critical for inhibition and which cellular and viral transcription factors are involved in association of the IE promoter with hypoacetylated histones and mediate silencing of the EHV-1 genome within CD172a+ cells. Control of EHV-1 infection by specific HDAC inhibitors is likely to be a key step in the pathogenesis of EHV-1 in vitro and in vivo. We hypothesize that, in vivo, acute EHV-1 infection promotes activation of blood monocytes that subsequently migrate into different tissues (pregnant uterus and/or central nervous system) and differentiate into permissive macrophages, as described for HCMV infection (Smith et al., 2004). The changes in cellular differentiation from monocytes into macrophages may be directly correlated with the association of specific cellular chromatin remodelling factors around the IE promoter and could play a role in controlling EHV-1 infection. Thus, specific HDAC inhibitors may represent useful therapeutic tools, which could serve to reactivate EHV-1 latent virus and/or to clear virus from cellular reservoirs. To date, valproic acid, a carboxylate HDAC inhibitor, has been used to induce Epstein–Barr virus (EBV) lytic-phase gene expression and to sensitize EBV-positive tumour cells to cytotoxicity in the presence of the anti-herpesvirus drug ganciclovir, in the treatment of patients with EBV-positive lymphomas (Feng & Kenney, 2006). Göttlicher et al. (2001) demonstrated that valproic acid relieves the repression of transcription factors, which recruit HDACs, and causes hyperacetylation of the N-terminal tails of histones H3 and H4 in vitro and in vivo.
monocytic cells, as assessed by flow cytometry after indirect immuno-fluorescence staining with a mouse anti-CD172a mAb (clone DH59B, diluted 1:100, IgG1; VMRD) directed against cells from the myeloid lineage, followed by FITC-conjugated goat anti-mouse IgG (diluted 1:100; Molecular Probes).

**Isolation of equine nasal mucosal CD172a<sup>+</sup> cells.** Tissues of the deep intranasal part of the septum were collected from horses between 4 and 7 years old at the slaughterhouse and transported to the laboratory as described previously (Vandekerckhove et al., 2011). The isolation of equine nasal mucosal CD172a<sup>+</sup> cells was adapted from a protocol described by Bannaazadeh Baghi et al. (2014). Mucosal explants were cut into small pieces of 0.5 cm<sup>2</sup> and incubated in digestion medium based on DPBS with 5% FCS, 1% penicillin, 1% streptomycin and 0.5% gentamicin and supplemented with 1 mM EDTA (VWR) at 150 r.p.m. for 30 min at 37 °C on a shaker. The cells were washed twice in RPMI to inactivate EDTA and incubated in digestion medium based on DPBS with 5% FCS, 1% penicillin, 1% streptomycin and 0.5% gentamicin, supplemented with 1 µg DNase type I (Stem Cell Technologies) ml<sup>−1</sup> and 1 mg collagenase type IV (Life Technologies) ml<sup>−1</sup> at 150 r.p.m. for 2 h at 37 °C on a shaker. The cell suspension was passed through a 100 µm cell strainer to remove undigested tissues and centrifuged at 400 g for 10 min at 4 °C. Cells were resuspended in DPBS and layered onto Ficoll-Paque (1.077 g ml<sup>−1</sup>) as described above.

The CD172a<sup>+</sup> cell fraction was sorted from the total mucosal leukocyte population using a magnetic-activated cell sorting approach. Briefly, the isolated mucosal leukocyte population was first incubated with a mAb anti-CD172a for 20 min at 4 °C, followed by a goat anti-mouse IgG antibody-coated magnetic beads MicroBead kit (Miltenyi Biotec). After 20 min incubation at 4 °C, the cells were washed with 1 ml PBS and centrifuged at 300 g for 10 min at 4 °C. The cell pellet was resuspended in 1 ml ice-cold elution buffer (DPBS supplemented with 1 mM EDTA and 2% FCS) and the cell suspension was placed on an LS column. The cell fraction was sorted from the total mucosal leukocyte population using a magnetic-activated cell sorting approach. Briefly, EHV-1-inoculated and mock-inoculated cells were harvested at 12 h p.i. A total of 10<sup>6</sup> cells ml<sup>−1</sup> were 10-fold diluted in leukocyte medium and 0.5 ml of each dilution was added on RK-13 monolayers in a six-well plate and overlaid with a 0.94% carboxymethylcellulose medium (Sigma-Aldrich) prepared in 2 x RPMI and centrifuged at 800 g for 30 min at 18 °C, as described by van der Meulen et al. (2000). Cells were further incubated for 5 days at 37 °C with 5% CO<sub>2</sub>. The cell monolayers were stained with 5% crystal violet and the number of plaques was counted. The percentage of infected cells producing infectious EHV-1 was calculated based on the number of plaques counted and the number of cells seeded ml<sup>−1</sup> according to the volume plated. This experiment was performed three times.

**Indirect immunofluorescence staining of EHV-1 proteins.** To determine which kinetic classes of proteins were expressed in EHV-1-infected cells, a double immunofluorescence staining was performed on cells fixed in 100% methanol at −20 °C for 20 min. Cells were incubated for 1 h at 37 °C with a rabbit polyclonal anti-IEP (diluted 1:1000) (Smith et al., 1994; Jang et al., 2001) to detect IEP expression, and either mouse anti-EICP22 K2 to IR4 (EICP22) protein (1:500), anti-gB (clone 4B6, 1:100) or anti-gC (clone 1B6, 1:100) mAb against early (EICP22) and late gB and gC protein expression, respectively. The IEP and EICP22 antibodies were kindly provided by Dr D. J. O’Callaghan (Louisiana State University, USA). The 4B6 and 1B6 mAbs were provided by Dr N. Osterrieder (Freie Universitat Berlin, Germany) and Dr H. Huemer (University of Innsbruck, Austria), respectively. Subsequently, samples were incubated for 50 min at 37 °C with FITC-conjugated goat anti-rabbit IgG (1:100) or Texas Red-conjugated goat anti-mouse IgG (1:100) antibodies (Molecular Probes). All antibodies were diluted in DPBS. The nuclei were counterstained with Hoechst 33342 (10 µg ml<sup>−1</sup>; Molecular Probes) for 10 min at 37 °C. A negative control, mock-inoculated cells were stained following the above protocols. In addition, appropriate isotype-matched controls were included. The percentage of viral antigen-positive cells was calculated based on 300 cells counted in distinct fields. Samples were analysed by confocal microscopy (Leica TCS SP2 Laser Scanning Spectral Confocal System; Leica Microsystems). All individual images were representative of the infected cell population.

**Statistical analysis.** Data were analysed with GraphPad Prism 5 software (GraphPad Software). Analysed data for statistical significance were subjected to a two-way ANOVA. All results shown represent means ± SD of three independent experiments. Results with a P value of ≤ 0.05 were considered statistically significant.

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