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To cite this version:
Jing Zhao, Katrien C. K. Poelaert, Jolien van Cleemput, Hans J. Nauwynck. CCL2 and CCL5 driven attraction of CD172a+ monocytic cells during an equine herpesvirus type 1 (EHV-1) infection in equine nasal mucosa and the impact of two migration inhibitors, rosiglitazone (RSG) and quinacrine (QC). Veterinary Research, 2018, 48 (1), pp.14. 10.1186/s13567-017-0419-4. hal-01499746

HAL Id: hal-01499746
https://hal.science/hal-01499746v1
Submitted on 31 Mar 2017

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CCL2 and CCL5 driven attraction of CD172a⁺ monocytic cells during an equine herpesvirus type 1 (EHV-1) infection in equine nasal mucosa and the impact of two migration inhibitors, rosiglitazone (RSG) and quinacrine (QC)

Jing Zhao, Katrien C. K. Poelaert, Jolien Van Cleemput and Hans J. Nauwynck*

Abstract
Equine herpesvirus type 1 (EHV-1) causes respiratory disease, abortion and neurological disorders in horses. Besides epithelial cells, CD172a⁺ monocytic cells become infected with EHV-1 in the respiratory mucosa and transport the virus from the apical side of the epithelium to the lamina propria en route to the lymph and blood circulation. Whether CD172a⁺ monocytic cells are specifically recruited to the infection sites in order to pick up virus is unknown. In our study, equine nasal mucosa explants were inoculated with EHV-1 neurological strains 03P37 and 95P105 or the non-neurological strains 97P70 and 94P247 and the migration of monocytic cells was examined by immunofluorescence. Further, the role of monokines CCL2 and CCL5 was determined and the effect of migration inhibitors rosiglitazone (RSG) or quinacrine was analyzed. It was shown that with neurological strains but not with the non-neurological strains, CD172a⁺ cells specifically migrated towards EHV-1 infected regions and that CCL2 and CCL5 were involved. CCL2 started to be expressed in infected epithelial cells at 24 h post-incubation (hpi) and CCL5 at 48 hpi, which corresponded with the CD172a⁺ migration. RSG treatment of EHV-1-inoculated equine nasal mucosa had no effect on the virus replication in the epithelium, but decreased the migration of CD172a⁺ cells in the lamina propria. Overall, these findings bring new insights in the early pathogenesis of EHV-1 infections, illustrate differences between neurological and non-neurological strains and show the way for EHV-1 treatment.

Introduction
Equine herpesvirus 1 (EHV-1) is an important pathogen of horses. It is a member of the subfamily Alphaherpesvirinae with a 150 kb double stranded DNA genome [1]. Alphaherpesviruses of different species have developed in evolution various ways to reach deeper tissues of the upper respiratory tract in order to find lymph and blood vessels for further spread and neurons for inducing latency. Among them, pseudorabies virus (PRV), bovine herpesvirus-1 (BHV-1) and herpes simplex virus-1 (HSV-1) easily spread across the basement membrane (BM) in a plaquewise manner upon the activation of cellular proteases whereas EHV-1 employs a more discrete manner to invade [2–4]. It hitchhikes across the BM using local immune cells, mainly CD172a⁺ cells as Trojan horse [5]. EHV-1 enters CD172a⁺ cells via an endocytic mechanism that requires cholesterol, tyrosine kinase activity, actin, dynamin activity and endosomal acidification, pointing towards a phagocytic mechanism [6]. EHV-1 infection of nasal mucosa epithelial cells leads to an increase of the thickness of the collagen VII and a
during EHV-1 invasion in the respiratory mucosa, inhibi-
tion and transport the virus from the apical side of the epi-
theleium to the lamina propria en route to the lymph
and blood circulation [11]. In general, cytokines and
chemokines are orchestrating the migration of monocy-
cytic cells during viral infections in the airways [12, 13]. It
has been reported that injection of alveolar epithelial cells
with influenza A virus can strongly induce the release of
monocyte chemoattractants CCL2 and CCL5 followed
by a strong recruitment of monocyte transepithelial
migration [14]. Whether EHV-1 infection is activating
the attraction of CD172a+ monocyte cells to the in-
fec-
tion sites and whether CCL2 and CCL5 are driving forces
during this process are largely unknown. In a previous
study, it has been shown that EHV-1 infected PBMC can
up-regulate inflammatory chemokines CCL5, CXCL9
and CXCL10, and down-regulate chemotactic CCL2 and
CCL3 with clear strain differences [15]. During an infec-
tion with another alphaherpesvirus, HSV-1, in mice,
it has been reported that CCL3 attracts NK cells [16],
CCL2 recruits monocytes [17], and CCL5 recruits mon-
cytes, NK cells, and PMNs [18] while CXCL9 recruits
T-cells to the sites of infection [16, 19]. In our study, we
mainly focused on the well-known monokines CCL2 and
CCL5. IL-8/CXCL8 that can specifically induce neutro-
phil recruitment during an EHV-1 infection [20] was also
included.

EHV-1 infection has a significant economical impact
on the equine breeding industry worldwide every year
[21]. Current vaccines do not provide full protection
against severe symptoms induced by EHV-1 and there
is no efficacious antiviral treatment available for EHV-1
infection. As CD172a+ cells function as Trojan horses
during EHV-1 invasion in the respiratory mucosa, inhibi-
tion of the recruitment of these cells may prevent migra-
tion of infected monocyctic cells into the deep tissues.
This might be an effective way to impede the generation
and invasion of infected CD172a+ cells and reduce the
viremia. Phenotypical and functional analysis of the nasal
mucosal CD172a+ cells indicated that they mainly consist
of immature dendritic cells (DC) [22].

Thus, DC migration inhibitors might be an option to
inhibit the EHV-1 deep invasion. It has been demonstra-
ted that rosiglitazone (RSG), which has been used to
treat type 2 diabetes, can specifically impair the depar-
ture of Langerhans cells (LCs) from the epidermis and
moreover can block accumulation of DC in the draining
lymph nodes (DLNs). In the respiratory mucosa, RSG
can also inhibit the migration of DCs from the airway
mucosa to the thoracic lymph nodes (LNs) [23]. Another
DC migration inhibitor, quinacrine (QC), originally used
as an antiprotozoal and anti-rheumatic agent, has also
been reported to inhibit the epidermal DC migration by
blocking NF-κB-dependent production of TNF-α, IL-1β
and CCL21 in the skin [24].

In the current study, equine nasal mucosa explants
were used as a model to study whether CD172a+ mono-
cytic cells are specifically recruited to the EHV-1 infec-
tion sites in order to capture virus and whether the
monokines CCL2 and CCL5 are involved during this
process. In addition, treatment with RSG or QC at dif-
f erent concentrations was performed 12 h prior to or at
the same time of the viral inoculation to test whether the
treatment can impede EHV-1 deeper infection.

Materials and methods

Cells and virus

RK-13 cells were used. They were cultured in Dulbecco’s
Modified Eagle Medium (DMEM) (Invitrogen, Paisley,
UK) supplemented with 10% fetal calf serum (Invitro-
gen), 100 U/mL penicillin, 0.1 mg/mL streptomycin and
1 µg/mL gentamicin.

Four Belgian EHV-1 strains were used in this study.
The neurovirulent strains 95P105 and 03P37 were origi-
nally isolated in 1995 and 2003 from the blood of a para-
lytic horse [25, 26] and the abortigenic strains 97P70
and 94P247 strains were isolated in 1997 and 1994 from
the lungs of an aborted fetus [27]. All the EHV-1 strains
were at the sixth passage, four passages in equine embry-
onic lung cells and two subsequent passages in RK-13
cells, with a titer of 10^6.5 tissue culture infectious dose
with 50% endpoint per milliliter (TCID_{50}/mL). For virus
inactivation, a thin layer of viral suspension was exposed
to short-wave UV light at 1.2 × 10^5 μJ/cm² for 10 min.
Absence of viral infectivity was checked by virus titration
on RK-13 cells.

Equine nasal mucosa explants and inoculation with EHV-1

The nasal mucosa explants were collected from 3 healthy
horses, between 4 and 6 years old at a local slaughter-
house. Firstly, the nasal mucosa explants were stripped
from the nasal surface and divided into pieces of 50 mm².
After 24 h of culture on fine-meshed gauze, explants were washed twice with warm medium and transferred on top of solid agarose. The margins were filled with agarose. Afterwards, the explants were inoculated with 500 μL medium containing 10^6 TCID_{50} EHV-1 of the neurological strains 03P37 and 95P105 or the non-neurological (abortigenic) strains 97P70 and 94P247 for 1 h at 37 °C with 5% CO₂, respectively. After inoculation, the explants were rinsed and further incubated with fresh medium [28]. Mock inoculations, incubated with DMEM medium, were performed in parallel.

Localization and quantification of CD172a⁺ cells in equine nasal mucosa explants

At 0, 24, 48 and 72 h post-incubation (hpi) the explants were collected and snap frozen in methocel™. At each time point, 20 serial 16 μm cryosections were made for immunofluorescence stainings. In brief, biotinylated equine polyclonal anti-EHV-1 IgG (diluted 1:10 in PBS) [26], followed by streptavidin-Texas Red® (Invitrogen, 1:200 in PBS) were added to check for EHV-1 infected cells. Next, mouse monoclonal antibody (mAb) DH59B (VMRD Inc., Pullman) was used as cell marker to detect CD172a⁺ cells, followed by FITC-labeled goat anti mouse IgG. The nuclei were counterstained with Hoechst 33342 (10 μg/mL, Molecular Probes). After staining, the cryosections were rinsed three times in PBS and mounted with glycerin/1,4-diazabicyclo [2.2.2] octane. All IF stainings were analyzed by confocal microscopy (Leica Microsystems GmbH, Wetzlar, Germany). An appropriate isotype-matched, irrelevant control (IgG1) mouse monoclonal anti-PRV gD antibody 13D12 was included for testing the specificity of the stainings [29].

Two regions of interest (ROI) were chosen for the quantification of CD172a⁺ cells and EHV-1⁺ cells. One was the region where the epithelial cells were infected with EHV-1 (ROI_{WI}), and the other was the region without EHV-1 infection in the epithelial cells (ROI_{WOI}) (Figure 1). Three independent replicates were performed and 20 images were taken for each experiment at each time point.

The expression of chemokines CCL2 and CCL5 in EHV-1 infected nasal mucosa explants

To check whether chemokines CCL2 and CCL5 may be involved in the recruitment of CD172a⁺ cells towards the infected epithelial cells, their expression was examined. The equine nasal mucosa explants were collected, cultured and inoculated with EHV-1 as described above. Mock inoculations were carried out in parallel. A scratch-wound assay using a yellow pipette tip to make a straight scratch and simulate a wound was performed to detect the expression of CCL2 or CCL5 in the wounded nasal mucosa explants. Inoculation with UV-inactivated EHV-1 was performed to test whether the production of CCL2 or CCL5 could be induced only by the binding/entry step of EHV-1 to the epithelial cells. Finally, the explants were collected at 0, 24, 48 and 72 hpi, embedded in methocel™, and snap frozen at −70 °C.

Of each explant, 50 serial 16 μm cryosections were made for IF stainings and the aforementioned protocol was followed to detect EHV-1 infected cells. For the detection of CCL2 or CCL5, rabbit polyclonal anti-CCL2 IgG or anti-CCL5 IgG (Biorbyt, 1:100 in PBS) was used as primary antibody, followed by goat anti-rabbit IgG FITC (1:100) (Invitrogen, 1:100 in PBS), respectively. The rabbit polyclonal anti-CXCL8 IgG (Mybiosource, 1:100 in PBS) was used as control. All the results of IF stainings were analyzed by confocal microscopy. By using the software imaging system ImageJ, the percentage of pixels positive for CCL2 or CCL5 was determined. Two ROIs were chosen for the quantification of the expression of CCL2 or CCL5. One was the region where the epithelial cells were infected with EHV-1 (ROI_{WI}) and the other was the region without EHV-1 infection in the epithelial cells (ROI_{WOI}) (Figure 1). Three independent replicates were performed for each experiment.

Evaluation of tissue toxicity of rosiglitazone (RSG) and quinacrine (QC) on nasal mucosa explants

As DC migration inhibitors might be useful to inhibit the deep invasion of EHV-1 in respiratory mucosa via infected monocytic cells, two DC migration inhibitors RSG or QC (Sigma-Aldrich) were used to treat equine nasal mucosa explants. TUNEL staining was performed to assess the tissue toxicity of RSG or QC (1, 3, 10, 30 μM) [23] on the nasal mucosa explants. Briefly, after 24 h culture on fine-meshed gauze, these explants were transferred into 24-well plate and immersed with RSG or QC at different concentrations (1, 3, 10, 30 μM) for 1 h at
37 °C with 5% CO₂. Afterwards, the explants were transferred back to the gauze and cultured within the medium in the presence of RSG or QC at corresponding concentrations. Untreated explants were immersed in and incubated with medium in the absence of RSG or QC. At 0, 24, 48 and 72 hpi, the explants were collected and snap frozen in methocel™ at −70 °C. Cryosections were made and the TUNEL reaction was performed according to the manufacturer’s guidelines. TUNEL-positive cells were counted in five randomly chosen fields of 100 cells in the epithelium as well as in the lamina propria with confocal microscopy.

The effect of RSG or QC on EHV-1 infection of nasal mucosa explants

The equine nasal mucosa explants were inoculated with EHV-1 neurological strains 03P37, 95P105 or non-neurological strains 97P70, 94P247 and treated with RSG or QC (1, 3, 10, 30 μM), respectively. Mock inoculations and treatments were carried out in parallel. The RSG or QC treatment was performed 12 h prior to or at the same time of EHV-1 inoculation, respectively. The supernatant of cultured explants was collected at 2, 24, 48, 72 hpi for viral titration. The explants were collected and snap frozen at 0, 24, 48, 72 hpi. Cryosections were made and IF stainings were performed to analyze EHV-1 infection by confocal microscopy. In the epithelium, the plaque formation was analyzed. In the lamina propria of the mucosa, two regions of interest (ROI) were chosen for analysis of EHV-1 replication. One was the region where the epithelial cells were infected with EHV-1 (ROIWI) and the other was the region without EHV-1 infection in the epithelial cells (ROIWOI). Three independent replicates were performed for each experiment.

The effect of RSG treatment on the distribution and numbers of CD172a⁺ cells in nasal mucosa explants infected with EHV-1

The EHV-1 infected cell types in the lamina propria of which their recruitment was affected by treatment with RSG were identified by a double IF staining. For the staining of EHV-1, the protocol described above was followed. To detect CD172a⁺ cells, CD5⁺ T lymphocytes or B lymphocytes (IgM⁺), mAbs DH59B, HT23A or 1.9/3.2 (VMRD Inc., Pullman) were used as primary antibodies and FITC-labeled goat anti-mouse IgG was used as secondary antibody. Proper controls were included for testing the specificity of the stainings. The nuclei were counterstained with Hoechst 33342. All the stained cryosections were analyzed by confocal microscopy. Three independent replicates were performed for each experiment.

Data analysis

Three independent experiments were performed and the data are presented as means ± standard deviations (Givens and Marley). ANOVA was used to calculate statistical significance among multiple groups. Data were classified: $P > 0.05$, not significantly different; $P ≤ 0.05$ (*), significantly different; $P ≤ 0.01$ (**), very significantly different; $P ≤ 0.001$ (***) extremely significantly different.

Results

The localization and quantification of CD172a⁺ cells in the equine nasal mucosa explants infected with EHV-1

In mock-inoculated nasal mucosa, the CD172a⁺ cells were mainly localized in the lamina propria underneath the BM. In the epithelium, the CD172a⁺ cells were present in a scattered manner (Figure 2A). Cultivation for 72 h had no impact on the distribution and number of CD172a⁺ cells. For the neurological EHV-1 strain 03P37 inoculated nasal mucosa explants, a basal to apical migration in the infected nasal mucosa area was observed. At 24 hpi, EHV-1 infected CD172a⁺ cells were observed mainly in the epithelium and less frequently underneath the BM. There was no significant difference for the number of total CD172a⁺ cells in ROIWI or ROIWOI compared with the mock. At 48 hpi, more EHV-1 infected CD172a⁺ cells were found in the epithelium and a few underneath the BM. Compared with the mock, the number of total CD172a⁺ cells in the ROIWI was 30.5 ± 21.6% ($P < 0.05$) higher, while in the ROIWOI there was no significant difference ($P > 0.05$) (Figure 2B). At 72 hpi, nearly no EHV-1 infected CD172a⁺ cells were found in the epithelium while there were much more EHV-1 infected CD172a⁺ cells observed underneath the BM. The number of total CD172a⁺ cells in the ROIWI was 44.2 ± 19.5% ($P < 0.01$) higher than that in the mock (Figure 2B). The percentage of EHV-1 positive cells in the population of CD172a⁺ positive cells in the ROIWI was 77.8 ± 31.5% ($P < 0.01$) higher than ROIWOI at 48 hpi and 76.8 ± 27.9% ($P < 0.01$) higher than ROIWOI at 72 hpi compared with the ROIWOI (Figure 2C). Similar results were observed when inoculated with another EHV-1 neurological strain 95P105 (Additional file 1). For the EHV-1 non-neurological strains 97P70 and 94P247, there were no significant differences for the number and distribution of CD172a⁺ cells between ROIWI or ROIWOI and the mock at all time points (Figure 2; Additional file 1).

The expression of chemokine CCL2 and CCL5 in EHV-1 infected nasal mucosa explants

Both CCL2 and CCL5 positive cells were scattered underneath the epithelial cells of mock-inoculated nasal mucosal tissues at all the time points of cultivation, whereas CXCL8 positive cells were not observed
Figure 2 The localization and quantification of CD172a<sup>+</sup> cells in equine nasal mucosa. The distribution (A) and the number (B) of CD172a<sup>+</sup> cells in mock inoculated and EHV-1 neurological strain 03P37 and abortigenic strain 97P70 inoculated nasal mucosa at 0, 24, 48 and 72 hpi. C. The percentage of EHV-1 infected cells in the population of CD172a<sup>+</sup> cells in the nasal mucosa. ROI<sub>W</sub> is the region including the epithelium and the lamina propria with EHV-1 infection in the epithelium whereas ROI<sub>WOI</sub> is the region without EHV-1 infection in the epithelium (two-way ANOVA, *P < 0.05; **P < 0.01). EHV-1 infected cells (red), CD172a<sup>+</sup> cells (green). The white line drawn on the image represents the BM. Scale bar: 50 µm.
(Figures 3A and B). Upon inoculation with the EHV-1 neurological strain 03P37, the expression of CCL2 and CCL5 strongly increased and colocalized with the EHV-1 infected epithelial cells (Figures 3A and B). The total expression of CCL2 was much higher than that of CCL5. The expression of CCL2 in nasal mucosa at ROIWI increased eightfold \( (P < 0.01) \) compared to the mock at 48 hpi, ninefold \( (P < 0.01) \) at 48 hpi and 11-fold \( (P < 0.001) \) at 72 hpi (Figure 3A). For CCL5, its expression in the ROIWI of the nasal mucosa explant inoculated with EHV-1 was fourfold \( (P < 0.01) \) and eightfold \( (P < 0.01) \) higher than the mock at 48 hpi and 72 hpi, respectively (Figure 3B). There were no significant differences \( (P > 0.05) \) for CCL2 and CCL5 production between EHV-1 (ROIWoI), UV-EHV-1 and mock-inoculated explants at all the time points mentioned. The neurological strain 95P105 showed similar results as strain 03P37 (Additional file 2).

For nasal mucosa inoculated with the non-neurological EHV-1 strains 97P70 and 94P247, the expression of CCL2 and CCL5 was not significantly different compared with mock-inoculated nasal mucosa (Figure 3; Additional file 2). CCL2 and CCL5 positive cells were distributed in a scattered way underneath the epithelium in the nasal mucosa in scratch-wound assay and their expression was similar with the mock (data not shown).

**Viability analysis during treatment with RSG or QC in in vitro cultures**

Apoptotic cell death was detected at different concentrations \( (1, 3, 10, 30 \mu M) \) with RSG or QC. The number of apoptotic cells in the epithelium had a slight, but not significant increase during the 72 h cultivation period with both products. The number of apoptotic cells in the epithelium did not increase significantly for either RSG or QC (Table 1, only the data at 72 hpi were shown).

**The effect of RSG or QC on EHV-1 infection in nasal mucosa explants**

During RSG or QC 12 h pre-treatment or treatment at the same time with the neurological EHV-1 strain 03P37 inoculation, the viral titers in the supernatant of cultured explants and the plaque formation in the epithelium were similar, so only the data of RSG or QC treatment at the same time with EHV-1 inoculation were shown. Viral titers (Figure 4A) and plaque formation among different concentrations of RSG and QC treatment did not differ significantly (Figure 4B). In the lamina propria of nasal mucosa infected with the neurological 03P37 strain, the number of EHV-1 infected cells was remarkably lower at 72 hpi when treated with RSG 12 h before inoculation or at the same time, with 41.2 ± 11.7% \( (P < 0.01) \) or 30.3 ± 8.2% \( (P < 0.05) \) at 10 μM and 67.2 ± 9.8% \( (P < 0.001) \) or 54.5 ± 16.8% \( (P < 0.01) \) at 30 μM for the ROIWI (Figure 4C). This lower number of EHV-1 infected cells was not observed when treated with QC. The neurological strain 95P105 showed similar results as strain 03P37. For strain 97P70 and 94P247, pre-treatment and treatment with RSG or QC did not change virus replication in the epithelium and the number of EHV-1 infected cells in the lamina propria (Additional file 3, only the data for strain 97P70).

**The RSG treatment inhibited the migration of CD172a+ cells in the EHV-1 inoculated nasal mucosa explants**

In the mock-inoculated explants and EHV-1-inoculated explants, the RSG pretreatment or treatment at the same time had no effect on the number of CD5+ T and B-lymphocytes in the lamina propria. Within the mock-inoculated explants, the RSG pretreatment or treatment at the same time had no effect on the number of CD172a+ cells in the lamina propria (Figure 5B). In the EHV-1-inoculated explants, at 72 hpi, in the lamina propria of ROIWI the number of total CD172a+ cells decreased with 28.9 ± 6.5% \( (P < 0.01) \) or 41.3 ± 9.6% \( (P < 0.01) \) at a pretreatment with 10 or 30 μM. When treated with RSG at the same time of EHV-1 inoculation, the number of total CD172a+ cells in the lamina propria of ROIWI was reduced with 31.1 ± 7.6% \( (P < 0.01) \) at the concentration of 30 μM (Figure 5C). The neurological strain 95P105 showed similar results as strain 03P37 (Additional file 4).

**Discussion**

Monocytic cells play an important role in the pathogenesis of EHV-1 infection [6]. CD172a+ monocytes become infected with EHV-1 in the nasal mucosa (the initial infection site), and transport the virus from the apical side of the epithelium into the deep lamina propria [11]. Our data demonstrate that a basal to apical side migration of non-infected CD172a+ cells was present during the early stage of the EHV-1 neurological strain infection in the epithelial cells. Indeed, at 24 and 48 hpi, CD172a+ cells migrated towards the infected epithelium region as fewer CD172a+ cells migrated into the deep lamina propria underneath the epithelium toward the BM while more infected CD172a+ cells were present in the epithelium at these time points. In the infected regions more CD172a+ cells were found at 48 and 72 hpi, most probably due to migration from deeper tissues, as there was no reduction of CD172a+ cells gathered in the lamina propria at 72 hpi, which indicated that after getting in contact with the virus, the infected CD172a+ cells migrated into the deep regions for further invasion. It has also been reported that a basal-to-apical monocyte transepithelial migration in vitro can be elicited by influenza A virus infection of primary alveolar epithelial cells and resident alveolar...
Figure 3 The expression of CCL2 and CCL5 in EHV-1 infected nasal mucosa. The expression of CCL2 (A) and CCL5 (B) in mock inoculated, EHV-1 neurological strain 03P37 and abortigenic strain 97P70 inoculated (at ROIWI and ROIWOI) as well as their UV inactivated viruses (UV-03P37 and UV-97P70) inoculated nasal mucosa at 0, 24, 48 and 72 hpi (two-way ANOVA, **P < 0.01; ***P < 0.001). EHV-1 infected cells (red); CCL2 or CCL5 (green). The white line drawn on the image represents the BM. Scale bar: 50 µm.
macrophages, which can be induced by monocyte chemotactants CCL2 and CCL5 [14, 30]. Chemokines are secreted in response to signals such as proinflammatory cytokines where they play an important role in selectively recruiting monocytes, neutrophils, and lymphocytes. Monocyte chemoattractant protein-1 (MCP-1/CCL2) can be secreted by epithelial cells and many immune cells including monocytes and DC and is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages [31, 32]. CCL5, previously known as RANTES (regulated upon activation, normal T cell expressed and secreted) is a member of the CC chemokine family. It can be produced by T lymphocytes, tumor cells and fibroblasts and recruits monocytes, T cells, basophils and eosinophils [33]. The current data in our study showed that the neurological EHV-1 infected nasal mucosa explants displayed a release of both CCL2 and CCL5. The CCL2 started to be expressed in the epithelial cells at 24 hpi, which is corresponding with the time point when the number of CD172a+ cells in the lamina propria decreased. This indicates that the secreted CCL2 is most probably involved in the recruitment of CD172a+ cells during an infection with EHV-1 neurological strains. The basal to apical migration and chemokine production were shown for neurological strains but not for the non-neurological strains. This strain specificity may be linked with the cell tropism of EHV-1. For the neurological strain 03P37, the majority of individual infected cells were CD172a+ cells while for the non-neurological strain 97P70, individual infected cells were equally identified as CD5+ T lymphocytes and CD172a+ cells [5]. For CD5+ T lymphocytes attraction, other chemokines such as, CXCL9 and CXCL10 [16, 19] may be involved. Current research is looking into the involvement of these T lymphocytic chemokines in the early pathogenesis of non-neurological EHV-1 strains. For the difference between neurological strains and non-neurological strains, there is a hypothesis that the mutation in ORF30 (DNA polymerase, A2254/G2254), which results in a modified DNA polymerase activity, enhances the virulence of this viral strain [10]. However, there is compelling evidence that this nucleotide substitution is not the only determinant for the induction of neurological disease by EHV-1 [34]. Not only ORF30, but also other ORFs may have an impact on the viral replication rate, with potential concomitant effects on neuropathogenicity [35]. This still needs to be further examined.

Absence of CCL2 and CCL5 expression in the wound-scratch assay indicated that cell death induced by wound ing was not responsible for or at least, had no direct impact on their expression. CCL2 and CCL5 were not detected when inoculated with UV inactivated EHV-1. This demonstrated that the step of binding to and entry into host cells is not sufficient for the production of CCL2 and CCL5. A further stage, including viral gene expression and replication is necessary for their expression. The production of CCL2 and CCL5 was first detected at 24 hpi in the nasal mucosa infected with EHV-1. At 12 hpi, there was nothing observed (data not shown). This indicates that the chemokines CCL2 and CCL5 become expressed between 12 and 24 hpi. This is the time period during which some late viral proteins become expressed in epithelial cells and CD172a+ cells [36, 37]. It seems to indicate that some late viral proteins or transcription factors are involved in the induction of CCL2 and CCL5 expression. It has been reported that human cytomegalovirus (HCMV) tegument protein pp71 [38] and the viral G protein coupled receptor (vGPCR) of human herpesvirus 8 (HHV-8) lead to an increased expression of CCL2 during infection [39]. In addition, HHV-8 upregulates activating transcription factor 4 (ATF4) expression, which induces CCL2 production in endothelial cells [40]. RNase protection analyses revealed increased expression of CCL2 at 8 and 12 hpi with EHV-1 strain KyARgp2F (an EHV-1 recombinant strain from KyA, expressing the full-length gp2 protein) compared to EHV-1 strain KyA (harbors part of gp2 protein) when infecting mice [41]. It is very well possible that the full-length gp2 protein is involved in the induction of CCL2 in the early stage of infection. The difference of the time points may be explained by the fact that our detection was on the protein level, which is later than

### Table 1 Absence of toxicity of RSG and QC in nasal mucosa explants

| Localization (nasal mucosa) | Products | Percentage (%) of TUNEL-positive cells treated at… μM at 72 hpi |
|-----------------------------|----------|---------------------------------------------------------------|
|                             |          | 0 (mock) | 1 | 3 | 10 | 30 |
| Epithelium                  | RSG      | 0.3 ± 0.2 | 0.4 ± 0.3 | 0.7 ± 0.6 | 0.6 ± 0.5 | 1.1 ± 0.6 |
|                             | QC       | 0.4 ± 0.1 | 0.7 ± 0.3 | 1.2 ± 0.6 | 1.4 ± 0.6 | 1.6 ± 0.7 |
| Lamina propria              | RSG      | 0.5 ± 0.6 | 1.4 ± 0.8 | 3.0 ± 0.7 | 2.8 ± 1.9 | 4.0 ± 1.1 |
|                             | QC       | 0.6 ± 0.2 | 1.2 ± 0.7 | 2.7 ± 0.9 | 3.2 ± 0.7 | 4.7 ± 1.6 |

Viability of nasal mucosa explants treated with RSG or QC at different concentrations was determined in the TUNEL assay at 72 hpi. Values are given as mean ± SD of 3 different experiments.
Figure 4: Viral production and plaque formation of EHV-1 in equine nasal mucosa. Viral production (A) at 0, 2, 24, 48 and 72 hpi and plaque formation (B) at 72 hpi in nasal mucosa explants inoculated with EHV-1 neurological strain 03P37 and treated at the same time with RSG or QC at different concentrations (1 μM, 3 μM, 10 μM, 30 μM). The number of individual EHV-1 infected cell in the lamina propria of nasal mucosa explants (C) treated with RSG or QC 12 h prior to or at the same time of EHV-1 inoculation. ROI_WI is the region of interest including the epithelium and the lamina propria with EHV-1 infection in the epithelium whereas ROI_WOI is the region of interest without EHV-1 infection in the epithelium (two-way ANOVA, **P < 0.01; ***P < 0.001)
**Figure 5** The localization and quantification of CD172a⁺ cells in the lamina propria treated with RSG. The distribution (A) and the number of CD172a⁺ cells in the lamina propria of mock-inoculated (B) or EHV-1 neurological strain 03P37 inoculated (C) nasal mucosa explants at 72 hpi and treated with RSG 12 h prior to or at the same time of the mock or viral inoculation at a concentration of 10 μM or 30 μM. ROIWI is the region of interest including the epithelium and the lamina propria with EHV-1 infection in the epithelium whereas ROIWOI is the region of interest without EHV-1 infection in the epithelium (two-way ANOVA; *P < 0.05; **P < 0.01). EHV-1 infected cells (red); CD172a⁺ cells (green). The white line drawn on the image represents the BM. Scale bar: 50 μm.
the detection of RNA transcripts. Except for gp2 protein, other EHV-1 viral proteins and transcription factors involved in the CCL2 and CCL5 induction also need to be further identified.

As we have observed the migration of CD172α⁺ cells during the EHV-1 infection in the nasal mucosa, migration inhibitors were used to treat the nasal mucosa. The treatment with RSG of equine nasal mucosa had no impact on the virus replication in the epithelium while the number of infected CD172α⁺ cells in the lamina propria decreased when treated with RSG at 10 and 30 μM. A possible explanation is that RSG treatment can inhibit the migration of CD172α⁺ cells in a dose-dependent manner during an EHV-1 infection. It showed that the migration of CD172α⁺ cells from the deeper tissues towards the infected region was inhibited in our study. This is in agreement with the research of Kintscher et al. [42], demonstrating that RSG function as peroxisome proliferator-activated receptor (PPAR) γ agonist and the activation of PPAR γ could impede DC migration from the peripheral sites of antigen capture to the DLNs. PPAR γ activation could also play a role in the spontaneous migration of intramucosal DCs and reduced migration of mice lung DCs under steady state conditions without infection [23, 43]. Our data showed that only in the condition with EHV-1 infection, the RSG treatment could inhibit the migration of CD172α⁺ cells, whereas no inhibitory impact was observed with mock-inoculation and RSG treatment. This may be due to the species specificity and the difference between in vivo and ex vivo cultures.

In conclusion, we have demonstrated that a basal to apical migration of CD172α⁺ cells in nasal mucosa was present during infections with neurological EHV-1 strains, with CCL2 and CCL5 involved in the attraction of CD172α⁺ cells towards the infected regions. RSG treatment efficiently inhibited the CD172α⁺ cells migration in a dose-dependent manner while it had no effect on the virus replication in the epithelium. A better understanding of the viral and cellular factors during this process could give new insights into the early pathogenesis of an infection with neurological EHV-1 and provide future therapeutic strategies.

Additional files

**Additional file 1. The quantification of CD172α⁺ cells in equine nasal mucosa**. (A) The number of CD172α⁺ cells in mock inoculated and EHV-1 neurological strain 9SP105 and abortigenic strain 94P247 inoculated nasal mucosa at 0, 24, 48 and 72 hpi. (B) The percentage of EHV-1 infected cells in the population of CD172α⁺ cells in the nasal mucosa. ROIWI is the region including the epithelium and the lamina propria with EHV-1 infection in the epithelium whereas ROIWOI is the region without EHV-1 infection in the epithelium (Two-way ANOVA, *: P < 0.05, **: P < 0.01).

**Additional file 2. The expression of CCL2 and CCL5 in EHV-1 infected nasal mucosa**. The expression of CCL2 (A) and CCL5 (B) in mock-inoculated EHV-1 neurological strain 9SP105 and abortigenic strain 94P247 inoculated (at ROIWI and ROIWOI) and their UV inactivated viruses 9SP105 and 94P247 inoculated nasal mucosa at 0, 24, 48 and 72 hpi (Two-way ANOVA, **: P < 0.01; ***: P < 0.001).

**Additional file 3. Viral production and plaque formation of EHV-1 in nasal mucosa**. Viral production (A) at 0, 2, 24, 48 and 72 hpi and plaque formation (B) at 24 hpi in nasal mucosa explants inoculated with EHV-1 abortigenic strain 97P170 and treated at the same time with RSG or QC at different concentrations (1 μM, 5 μM, 10 μM, 30 μM). The number of individual EHV-1 infected cells in the lamina propria of nasal mucosa explants (C) treated with RSG or QC 12 h prior to or at the same time of EHV-1 inoculation. ROIWI is the region of interest including the epithelium and the lamina propria with EHV-1 infection in the epithelium whereas ROIWOI is the region of interest without EHV-1 infection in the epithelium (Two-way ANOVA, **: P < 0.01; ***: P < 0.001).

**Additional file 4. The quantification of CD172α⁺ cells in the lamina propria treated with RSG**. The number of CD172α⁺ cells in the lamina propria of EHV-1 neurological strain 9SP105 inoculated nasal mucosa explants at 72hpi treated with RSG 12 h prior to (A) or at the same time (B) of the mock or viral inoculation at a concentration of 10 μM or 30 μM. ROIWI is the region of interest including the epithelium and the lamina propria with EHV-1 infection in the epithelium whereas ROIWOI is the region of interest without EHV-1 infection in the epithelium (Two-way ANOVA, *: P < 0.05, **: P < 0.01).

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

JS designed and performed the experiments, analyzed the data and helped to draft the manuscript. KP and JC helped to draft the manuscript. HN conceived and designed the study and helped in writing the manuscript. All authors read and approved the final manuscript.

Acknowledgements

This research was supported by China Scholarship Council (201208250001). Katrien CK Poelaert is a researcher funded by the Institute for the promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) (141627). Jolien Van Cleemput is a researcher funded by the Research Foundation Flanders (FWO) (11Y5415N). We thank Carine Boone, Melanie Bauwens, Zeger Vandenabeele, Lieve Sys, Chantal Vanmaercke and Nele Dennequin for their excellent technical assistance.

Received: 6 September 2016 Accepted: 2 February 2017 Published online: 27 February 2017

References

1. Telford EA, Watson MS, McBride K, Davison AJ (1992) The DNA sequence of equine herpesvirus-1. Virology 189:304–316
2. Glorieux S, Favoreel HW, Steukers L, Vandekerckhove AP, Nauny wrench HJ (2011) A trypsin-like serine protease is involved in pseudorabies virus invasion through the basement membrane barrier of porcine nasal respiratory mucosa. Vet Res 42:58
3. Glorieux S, Bachert C, Favoreel HW, Vandekerckhove AP, Steukers L, Rekecki A, Van den Broeck W, Goossens J, Croubels S, Clayton RF (2011) Herpes simplex virus type 1 penetrates the basement membrane in human nasal respiratory mucosa. PLoS One 6:e22160
4. Steukers L, Vandekerckhove AP, Van den Broeck W, Glorieux S, Nauny wrench HJ (2012) Kinetics of BoHV-1 dissemination in an in vitro culture of bovine upper respiratory tract mucosa explants. ILAR J 53:43–54
5. Vandekerckhove AP, Glorieux S, Gryseelert D, Steukers L, Duchateau L, Osterrieder N, Van de Walle G, Nauny wrench H (2010) Replication kinetics of
neurovirulent versus non-neurovirulent equine herpesvirus type 1 strains in equine nasal mucosal explants. J Gen Virol 91:2019–2028
6. Laval K, Favoreel HW, Poelaert KC, Van Cleemput J, Nauwynck HJ (2015) Equine herpesvirus type 1 enhances viral replication in CD172a+ monocytic cells upon adhesion to endothelial cells. J Virol 89:10912–10923
7. Baghi HB, Nauwynck HJ (2016) Effect of equine herpesvirus type 1 (EHV-1) infection of nasal mucosa cellular cells on integrin alpha 6 and on different components of the basement membrane. Arch Virol 161:103–110
8. Lunn D, Davis-Poynter N, Flamino M, Horroh D, Osterrieder K, Pusterla N, Townsend H (2009) Equine herpesvirus 1 consensus statement. J Vet Intern Med 23:450–461
9. Pusterla N, Wilson WD, Madigan JE, Ferraro GL (2009) Equine herpesvirus-1 myeloencephalopathy: a review of recent developments. Vet J 180:279–289
10. Nugent J, Birch-Machin I, Smith K, Mumford J, Swain Z, Newton J, Bowden R, Allen G, Davis-Poynter N (2006) Analysis of equid herpesvirus 1 strain variation reveals a point mutation of the DNA polymerase strongly associated with neuropathogenic versus nonneuropathogenic disease outbreaks. J Virol 80:4047–4060
11. Baghi HB, Nauwynck HJ (2014) Impact of equine herpesvirus type 1 (EHV-1) infection on the migration of monocytic cells through equine nasal mucosa. Comp Immunol Microbiol Infect Dis 37:321–329
12. Vareille M, Kieninger E, Edwards MR, Regamey N (2011) The airway epithelium: soldier in the fight against respiratory viruses. Clin Microbiol Rev 24:210–229
13. Rudrajaraj R, Sealy RE, Surman SL, Thomas PG, Dayton BH, Hurwitz JL (2013) Non-random lymphocyte distribution among virus-infected cells of the respiratory tract. Viral Immunol 26:378–384
14. Herold S, von Wufffen W, Steinmueller M, Pleschka S, Kuzel WA, Mack M, Siivastava M, Seeger W, Maus UA, Lohmeyer J (2006) Alveolar epithelial cells direct monocyte transepithelial migration upon influenza virus infection: impact of chemokines and adhesion molecules. J Immunol 177:1817–1824
15. Wimer CL, Damiani A, Osterrieder N, Wagner B (2011) Equine herpesvirus type 1 modulates CCL2, CCL3, CCL5, CXCL9, and CXCL10 chemokine expression. Vet Immunol Immunopathol 140:266–274
16. Wuest TR, Carr DJ (2008) The role of chemokines during herpes simplex virus-1 infection. Front Biosci 13:4862
17. Rebenko-Moll NM, Liu L, Cardona A, Ransohoff RM (2006) Chemokines, mononuclear cells and the nervous system: heaven (or hell) is in the details. Curr Opin Immunol 18:683–689
18. Wuest T, Farber J, Luster A, Carr DJ (2006) CD4+ T cell migration into the cornea is reduced in CXCL9 deficient but not CXCL10 deficient mice following herpes simplex virus type 1 infection. Cell Immunol 243:83–89
19. Van de Walle GR, May ML, Sukhumwasi W, von Einem J, Osterrieder N (2007) Herpesvirus chemokine-binding glycoprotein G (gG) efficiently inhibits neutrophil chemotaxis in vitro and in vivo. J Immunol 179:4161–4169
20. Bazanová B, Jackulač N, Fracka A, Staroniewicz C (2014) Abortogenic viruses in horses. Equine Vet Educ 26:48–55
21. Baghi HB, Laval K, Favoreel H, Nauwynck HJ (2014) Isolation and characterization of equine nasal mucosal CD172a+ cells. Vet Immunol Immunopathol 157:155–163
22. Angeli V, Hammad H, Staels B, Capron M, Luster A, Carr DJ (2006) CD4+ T cells mediate the inflammation, chemokines and viral infection. Springer, Berlin, pp 47–65
23. Angeli V, Hammad H, Staels B, Capron M, Lambrecht BN, Toretten F (2003) Peroxisome proliferator-activated receptor y inhibits the migration of dendritic cells: consequences for the immune response. J Immunol 170:5295–5301
24. Gorbachev AV, Gasparyan AV, Gurova KY, Gudkova AV, Fairchild RL (2007) Quinacrine inhibits the epidural dendritic cell migration initiating T cell mediated skin inflammation. Eur J Immunol 37:2257–2267
25. Garel B, Gyspleerdt A, Croubels S, De Backer P, Nauwynck H (2009) Evaluation of orally administered valacyclovir in experimentally EHV1-infected ponies. Vet Microbiol 135:214–221
26. Van der Meulen K, Vercauteren G, Nauwynck H, Pensaert M (2003) A local epidemic of equine herpesvirus 1-induced neurological. Vlaams Diergeneeskd Tijdschr 72:366–372
27. Van der Meulen KM, Nauwynck HJ, Buddaert W, Pensaert MB (2000) Replication of equine herpesvirus type 1 in freshly isolated equine peripheral blood mononuclear cells and changes in susceptibility following mitogen stimulation. J Gen Virol 81:21–25
28. Vairo S, Van den Broeck W, Favoreel H, Sciglianini A, Nauwynck H (2013) Development and use of a polarized equine upper respiratory tract mucosal explant system to study the early phase of pathogenesis of a European strain of equine arteritis virus. Vet Res 44:22
29. Nauwynck H, Pensaert M (1995) Effect of specific antibodies on the cell-associated spread of pseudorabies virus in monolayers of different cell types. Arch Virol 140:1137–1146
30. Rosseau S, Selhorst J, Wiechmann K, Leissner K, Maus U, Mayer K, Grimminger F, Seeger W, Lohmeyer J (2000) Monocyte migration through the alveolar epithelial barrier: adhesion molecule mechanisms and impact of chemokines. J Immunol 164:427–435
31. Deshmule SL, Kremlev S, Amini S, Sawaya BE (2009) Monocyte chemotactant protein-1 (MCP-1): an overview. J Interferon Cytokine Res 29:313–326
32. Standiford TJ, Kunkel S, Phan S, Rollins B, Strieter R (1991) Alveolar macrophage-derived cytokines induce monocyte chemotactant protein-1 expression from human pulmonary type II-like epithelial cells. J Biol Chem 266:9912–9918
33. Vilela MC, Mansur DS, Lacerda Queiroz N, Rodrigues DH, Lima GK, Arantes RME, Kroon EG, Da Silva Campos MA, Teixeira MM, Teixeira AL (2009) The chemokine CCL5 is essential for leukocyte recruitment in a model of severe Herpes simplex encephalitis. Ann NY Acad Sci 1153:256–263
34. Perkins GA, Goodman LB, Tsujimura K, Van de Walle GR, Kim SG, Dubovi EJ, Osterrieder N (2009) Investigation of the prevalence of neurologic equine herpesvirus type 1 (EHV-1) in a 23-year retrospective analysis (1984–2007). Vet Microbiol 139:375–378
35. Liu S, Knafel JD, Chang JS, Waszk GA, Baldwin ET, Deibel MR, Thomsen DR, Homa FL, Wells PA, Tory MC (2006) Crystal structure of the herpes simplex virus 1 DNA polymerase. J Biol Chem 281:18193–18200
36. Laval K, Favoreel HW, Nauwynck HJ (2015) Equine herpesvirus type 1 replication is delayed in CD172a+ monocytic cells and controlled by histone deacetylases. J Gen Virol 96:118–130
37. Gyspleerdt AC, Vandekerckhove AP, Baghi HB, Van de Walle GR, Nauwynck HJ (2012) Expression of late viral proteins is restricted in nasal mucosal leukocytes but not in epithelial cells during early-stage equine herpesvirus-1 infection. Vet J 193:576–578
38. Naing Z, Webel R, Hamilton S, Schmeiser C, Scott G, Marshall M, Rawlinson W (2015) Stimulatory effects of human cytomegalovirus tegument protein pp71 lead to increased expression of CCL2 (monocyte chemotactic protein-1) during infection. J Gen Virol 96:1855–1862
39. Choi YB, Nicholas J (2010) Induction of angiogenic chemokine CCL2 by human herpesvirus 8 chemokine receptor. Virology 397:369–378
40. Caselli E, Benedetti S, Grigolato J, Caruso A, Di Luca D (2012) Activating transcription factor 4 (ATF4) is upregulated by human herpes virus-8 infection, increases virus replication and promotes proangiogenic properties. Arch Virol 157:63–74
41. Smith PM, Kahn SM, Rorex CB, von Einem J, Osterrieder N, O’Callaghan DJ (2005) Expression of the full-length form of g22 of equine herpesvirus 1 (EHV-1) completely restores respiratory virulence to the attenuated EHV-1 strain KYa in CBA mice. J Virol 79:5105–5115
42. Kintscher U, Goetze S, Wasko S, Kim S, Nagpal S, Chandraratna RA, Graf K, Fleck E, Hsueh WA, Law RE (2000) Peroxoxosome proliferator-activated receptor and retinoid X receptor ligands inhibit monocyte chemotactic protein-1-directed migration of monocytes. Eur J Pharmacol 401:259–270
43. Holt PG, Haining S, Nelson DJ, Sedgwick JD (1994) Origin and steady-state turnover of class II MHC-bearing dendritic cells in the epithelium of the conducting airways. J Immunol 153:256–261