

Guava[®] and Amnis[®]
Flow Cytometers
are Now Part of Luminex.



Luminex
complexity simplified.



Herpesvirus Chemokine-Binding Glycoprotein G (gG) Efficiently Inhibits Neutrophil Chemotaxis In Vitro and In Vivo

This information is current as
of February 16, 2019.

Gerlinde R. Van de Walle, Maeva L. May, Woraporn
Sukhumavasi, Jens von Einem and Nikolaus Osterrieder

J Immunol 2007; 179:4161-4169; ;
doi: 10.4049/jimmunol.179.6.4161
<http://www.jimmunol.org/content/179/6/4161>

References This article **cites 36 articles**, 10 of which you can access for free at:
<http://www.jimmunol.org/content/179/6/4161.full#ref-list-1>

Why *The JI*? [Submit online.](#)

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Fast Publication!** 4 weeks from acceptance to publication

**average*

Subscription Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscription>

Permissions Submit copyright permission requests at:
<http://www.aai.org/About/Publications/JI/copyright.html>

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/alerts>

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2007 by The American Association of
Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Herpesvirus Chemokine-Binding Glycoprotein G (gG) Efficiently Inhibits Neutrophil Chemotaxis In Vitro and In Vivo¹

Gerlinde R. Van de Walle, Maeva L. May, Woraporn Sukhumavasi, Jens von Einem, and Nikolaus Osterrieder²

Glycoprotein G (gG) of alphaherpesviruses has been described to function as a viral chemokine-binding protein (vCKBP). More recently, mutant viruses devoid of gG have been shown to result in increased virulence, but it remained unclear whether the potential of gG to serve as a vCKBP is responsible for this observation. In this study, we used equine herpesvirus type 1 (EHV-1) as a model to study the pathophysiological importance of vCKBP activity. First, *in vitro* chemotaxis assays studying migration of immune cells, an important function of chemokines, were established. In such assays, supernatants of EHV-1-infected cells significantly inhibited IL-8-induced chemotaxis of equine neutrophils. Identification of gG as the responsible vCKBP was achieved by repeating similar experiments with supernatants from cells infected with a gG-negative mutant, which were unable to alter IL-8-induced equine neutrophil migration. Furthermore, rEHV-1 gG was able to significantly reduce neutrophil migration, establishing gG as a *bona fide* vCKBP. Second, and importantly, *in vivo* analyses in a murine model of EHV-1 infection showed that neutrophil migration in the target organ lung was significantly reduced in the presence of gG. In summary, we demonstrate for the first time that EHV-1 gG not only binds to chemokines but is also capable of inhibiting their chemotactic function both *in vitro* and *in vivo*, thereby contributing to viral pathogenesis and virulence. *The Journal of Immunology*, 2007, 179: 4161–4169.

Recently, glycoprotein G (gG)³ of several alphaherpesviruses has been described to function as a broad-spectrum chemokine-binding protein (1, 2). As chemokines play central roles in mediating inflammatory responses during viral infection, it is not surprising that viruses have adopted strategies to evade or modulate their activity. Currently, three viral strategies of chemokine modulation are described, all of which are well-studied in both herpes- and poxviruses (3–5). First, viruses can encode open reading frames with striking homology to genes that encode chemokines. These so-called virus-encoded chemokine homologs or virokinins can act either as agonists or antagonists of host molecules. For example, the alphaherpesvirus Marek's disease virus (MDV) has been shown to encode a biologically active IL-8 homolog (6). MDV viral IL-8 (vIL-8) showed significant similarities with mammalian and chicken IL-8 homologs, with the exception of a highly conserved ELR motif. *In vitro* chemotaxis studies demonstrated that vIL-8 attracts chicken mononuclear cells, and *in vivo* studies with vIL-8 deletion mutants showed a significantly decreased level of lytic infection and virulence (6). Second, a re-

markable number of viral genes have been identified which resemble cellular genes encoding chemokine receptors, and their products have been shown to engage chemokines and induce intracellular signals. Third, several poxviruses, and, more recently, alphaherpesviruses, have been shown to express soluble viral chemokine-binding protein (vCKBP) that do not share sequence similarity with known chemokines or chemokine receptors.

Equine herpesvirus type 1 (EHV-1), a member of the *Alphaherpesvirinae* and a close relative to the causative agents of cold sores and genital herpes in humans, HSV-1 and HSV-2, respectively, is a major pathogen of horses worldwide. EHV-1 is spread via nasal secretions and replicates in the respiratory tract. Initial replication in airway epithelia is followed by a leukocyte-associated viremia, which enables EHV-1 to reach end-vessel endothelia in the uterus and CNS. In these organ systems, viral replication can result in vasculitis and perivascularitis ultimately resulting in abortion and reactive myeloencephalopathy, a highly lethal condition of enormous animal welfare and economic importance (7). A related alphaherpesvirus in horses, EHV-4, is predominantly associated with respiratory disease (8, 9). Both viruses encode gG, a type I integral membrane protein. The N termini of EHV-1 and EHV-4 gG exhibit high sequence similarity, whereas the extracellular C-terminal domains are highly divergent (10). Glycoprotein G is expressed early in the infectious cycle and was shown to be nonessential for virus growth *in vitro* (11–13). Moreover, gG is unusual in that it can exist in three isoforms: a 68-kDa full-length membrane-bound form, a 12-kDa membrane-bound form, and a 60-kDa form, which is secreted from infected cells (14). The latter two isoforms appear to be the result of a proteolytic event from the 68-kDa full-length form resulting in the 12-kDa C- and the 60-kDa N-terminal moiety, respectively.

Because EHV-1 gG has been shown to promiscuously bind a broad range of chemokines of human and murine origin, this glycoprotein has also been implied to function as a vCKBP (1). More

Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853

Received for publication March 1, 2007. Accepted for publication July 9, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by a grant from the Harry M. Zweig Memorial Fund for Equine Research at Cornell University.

² Address correspondence and reprint requests to Dr. Nikolaus Osterrieder, Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853. E-mail address: no34@cornell.edu

³ Abbreviations used in this paper: gG, glycoprotein G; MDV, Marek's disease virus; vIL-8, viral IL-8; vCKBP, viral chemokine-binding protein; EHV, equine herpesvirus; p.i., postinfection; sgG, secreted gG; BAL, bronchioalveolar lavage; BAC, bacterial artificial chromosome.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/\$2.00

Table I. Primers used in this study

Primer	Sequence ^a
gGFCpo	5'-atgc <u>cggtcc</u> gatggccccagatgaactctgttatg-3'
gGRCpo	5'-acgt <u>cgga</u> ccggcgccatacacagttttgagc-3'
CCL2FCpo	5'-agc <u>cggtcc</u> gatgaaggtctctgcagcc-3'
CCL2RCpo	5'-agc <u>cgga</u> ccggcaggctttggagtttgggc-3'

^a Underlined nucleotides indicate the *CpoI* sequence, used for cloning into the pFastBac.

recently, our group constructed a mutant virus devoid of gG, which was tested in a murine EHV-1 infection model. It was observed that infection with such gG-negative virus resulted in increased pathogenicity in mice (13). It remained unclear, however, whether the virulence mechanism of this EHV-1 mutant virus was caused by the absence of vCKBP activity. In the present study, we used EHV-1 as a model to investigate in more detail the pathophysiological importance of gG binding to chemokines. Chemotaxis assays with equine leukocytes and equine chemokines were performed in vitro, and EHV-1 gG was compared with the action of its counterpart in EHV-4, a close relative of EHV-1. Moreover, the in vivo relevance of chemokine binding by gG was tested in a murine infection model. The salient findings of our study are that gG of EHV-1 is not only capable of inhibiting the function of chemokines, such as IL-8, in neutrophil migration in vitro, but also has a significant effect on migration of immune cells in the target organ in vivo. This report, therefore, is the first to link gG binding to chemokines with a virulence phenotype in vivo and this may indicate an important role of gG in viral immune evasion in alphaherpesvirus infection.

Materials and Methods

Cells

Rabbit kidney cells (RK13) or equine fibroblasts (NBL6) were grown and maintained in growth medium (MEM supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin) at 37°C under a 5% CO₂ atmosphere.

Equine neutrophils were isolated by density centrifugation of heparinized blood from healthy horses on a discontinuous Percoll gradient as described previously (15). After washing, cells were resuspended in MEM at 1×10^5 cells/ml and used immediately for further experimentation. Equine PBMC were isolated using Histopaque 1077 (Sigma-Aldrich) following the manufacturer's instructions. After two washing steps, cells were resuspended in MEM at 1×10^5 cells/ml and used immediately for further experimentation. To obtain a pure equine monocyte population, PBMC were resuspended in leukocyte medium (RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS, 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, and 1% nonessential amino acids (Invitrogen Life Technologies)), at 2×10^6 cells/ml. Cells were seeded in polystyrene tissue-culture dishes and cultivated at 37°C under a 5% CO₂ atmosphere. After 24 h of incubation, nonadherent cells mainly representing lymphocytes were removed by washing the culture dishes with RPMI 1640, and adherent monocytes were centrifuged, washed, and resuspended in MEM at a density of 1×10^5 cells/ml. Murine neutrophils were isolated from BALB/c mice by peritoneal lavage as described before (16). The purity of each cell population was confirmed on cytopins with modified Wright stain (Sigma-Aldrich).

Viruses

EHV-1 wild-type strain RacL11, the gG deletion mutant (vL11ΔgG), and the gG rescuant virus (vL11ΔgGR) were described previously (13, 17). An EHV-4 isolate (VLS 829) was provided by the Office International d'Epizooties Reference Laboratory (University of Kentucky, Lexington, KY) and was identified as EHV-4 by the restriction fragment length polymorphism of its virus DNA (data not shown). Stocks of EHV-1 and EHV-4 were produced in RK13 or NBL6 cells. For infection experiments, cells were washed twice with MEM and inoculated for 24 h with virus at a multiplicity of infection of 1 in growth medium. At 24-h postinfection (p.i.), supernatants from infected cells were collected. Virus and cellular

debris was removed from supernatants by centrifugation at $100,000 \times g$ for 1 h and cleared supernatants were subsequently used in chemotaxis assays.

Western blotting

Western blot analyses were performed essentially as described (13). To detect gG, polyclonal rabbit anti-gG Abs (1:500) were used that recognize the variable domain of gG of the EHV-1 or EHV-4 protein (both antisera were provided by Drs. M. J. Studdert and C. Hartley, University of Melbourne, Parkville, Australia) (12). Penta-His Abs were obtained from Qiagen and used to detect His-tagged proteins. Anti-mouse (1:7500) or anti-rabbit (1:5000) Ig G (IgG) peroxidase conjugates were obtained from Jackson ImmunoResearch Laboratories.

Plasmids

The pRacL11 bacterial artificial chromosome (BAC) clone has been described previously (18). pRacL11 DNA was isolated using the Maxi-prep kit (Qiagen) and used as a template to amplify by PCR a portion of the gG gene that would encode a truncated, secreted version of the viral protein. The SignalP prediction server (version 1.1) was used to predict signal peptide cleavage sites and membrane-spanning segments. Based on the predictions, a fragment of 1020 bp within the gG gene encoding the extracellular portion of gG was amplified by conventional PCR introducing *CpoI* sites at either end using gGFCpoI and gGRCpoI primers (Table I), and then inserted into pBac11 (Novagen) (pBac11sgG). This cloning strategy fused 1) the extracellular domain of gG in-frame with the baculovirus gp64 signal peptide present in pBac11, and 2) added a polyhistidine tag to the C terminus of secreted gG (His tag) (Fig. 1). The pBac11sgG clones were analyzed by DNA sequencing and subsequently electroporated into DH10Bac cells (Invitrogen Life Technologies) harboring the baculovirus BAC sequence and allowing T7-mediated transposition of gG sequences into the targeted locus of baculovirus DNA. The sequence of equine CCL2 (19) was codon-optimized, synthesized, and cloned into the pLS vector (TOP Gene Technologies). Cloning of equine CCL2 (300 bp) into the pBac11 vector was performed exactly as described for gG but using primer pair CCL2FCpoI and CCL2RCpoI (Table I). Transposition after transformation of DH10Bac cells was done as described above.

Baculovirus expression and protein purification

Recombinant baculovirus bacmid DNA encoding His-tagged secreted gG (sgG-His) or equine CCL2 (eCCL2-His) were transfected into Sf9 insect cells using Cellfectin (Invitrogen Life Technologies). Recombinant viruses were plaque purified twice and verified for expression by Western blotting with Abs against gG and/or the polyhistidine tag. For purification of sgG-His or eCCL2-His from supernatants of Sf9 cells infected with recombinant baculoviruses at a multiplicity of infection of 1.0, TALON resin (BD Clontech) was used exactly as described previously (20). Fractions containing His-tagged proteins were collected and their purity was determined by SDS-PAGE, followed by Coomassie blue staining. Protein concentration was determined with a Bradford assay following the manufacturer's instructions (Bio-Rad).

Chemotaxis assays

For chemotaxis assays, 12-well Costar Transwell plates were used (Corning Costar). Serial dilutions of chemokines were made in MEM. Recombinant equine IL-8 and mouse KC were obtained from Serotec. Recombinant equine CCL-2 was produced as described above. Chemokines were preincubated for 30 min at 37°C with serial dilutions of viral gG, either present in supernatants of infected cells or recombinantly expressed, and 600 μl of each dilution was applied to the lower chambers of the wells. Wells were covered with a polycarbonate membrane with a 3-μm pore size for neutrophils and a 5-μm pore size in the case of mononuclear cells. A 100-μl cell suspension (containing 1×10^4 cells) was added to the top chamber, and assay plates were incubated at 37°C for 45 min for neutrophils or 2 h for mononuclear cells. Dilutions of chemokine and medium

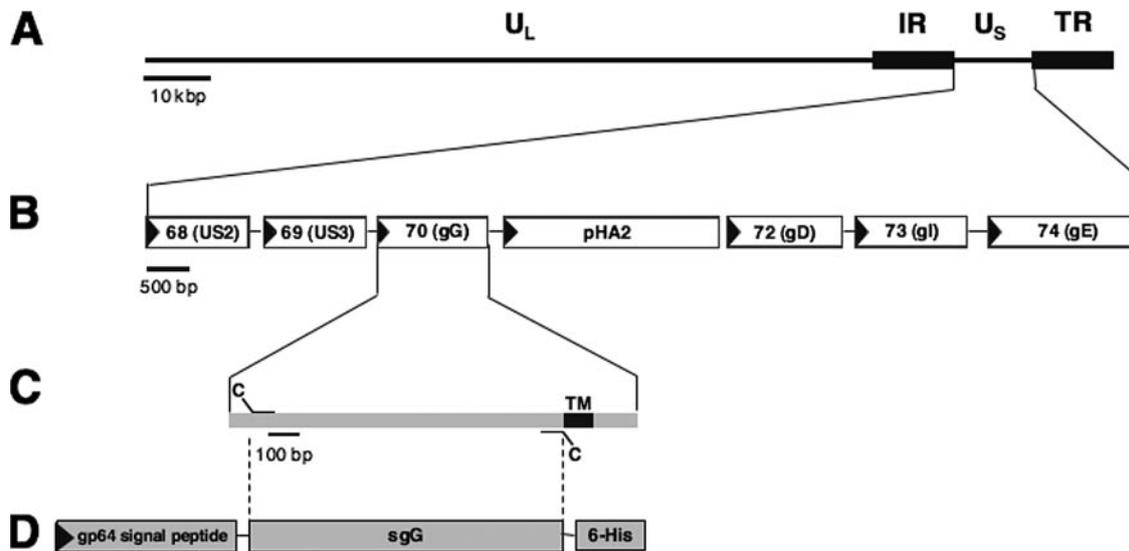


FIGURE 1. Generation of pBac11sgG. *A*, Overall genome organization of the BAC pBacL11 consisting of a unique-long (U_L) and a unique-short (U_S) segment, bracketed by inverted internal repeat (IR) and terminal repeat (TR) sequences. *B*, Organization of the unique-short segment, depicted in *A*. *C*, Schematic representation of gene 70 (gG) with restriction enzyme sites for cloning in pBac11 using primers as described in Table I (TM, transmembrane domain; C, *CpoI*). *D*, Schematic representation of the pBac11sgG containing the extracellular domain of gG in frame with the gp64 signal peptide, with a His tag attached at the C terminus end of gG.

alone were included in each experiment to serve as positive and negative controls, respectively. After incubation, cells in the lower chamber were stained, counted under a light microscope (Zeiss Axiovert 25), and expressed as a percentage of chemotaxis based on the amount of input cells, unless indicated otherwise.

Animal experiments

All animal experiments were performed in accordance with the U.S. Animal Welfare Act, under the supervision of the Cornell Institutional Animal Care and Use Committee, and were conducted as described previously with some modifications (21). Briefly, 4-wk-old female BALB/c mice (16 mice per group) were infected with vL11 Δ gG or vL11 Δ gGR by the intranasal route at 4×10^4 PFU/mouse. Virus was suspended in 20 μ l of cell growth medium. Control mice were infected with growth medium alone. Individual weights of mice were determined on the day of infection (day 1) until day 14 and percentages of body weight loss were determined. Two mice in each group were euthanized to collect lungs, which were homogenized to determine virus titers by standard titration on RK13 cells. On days 1, 2, 4, and 14 p.i., four mice in each group were euthanized and inflammatory cells infiltrating the airways were harvested by bronchioalveolar lavage (BAL) exactly as described previously (22). BAL cells were washed twice and finally resuspended in 1 ml of ice-cold PBS. Total cell numbers were determined based on the volume of recovered BAL fluid. To analyze the differential cell counts both cytopins and flow cytometric analyses were performed. Cytopins were obtained by centrifuging 100 μ l of the cell suspension onto microscope slides at 700 rpm for 4 min. Cytopins were air dried and stained with modified Wright stain (Sigma-Aldrich). In total, at least 300 cells per BAL were counted by light microscopy and the different immune cells and their absolute numbers were determined.

Flow cytometry

To analyze the composition of immune cells in the airways of lungs of infected animals, BAL cells were stained with fluorescently labeled anti-mouse mAbs against surface markers on neutrophils (Gr-1⁺-PerCP; BD Biosciences), macrophages (F4/80-allophycocyanin; Invitrogen Life Technologies), B lymphocytes (B220-PE; BD Biosciences), or different T lymphocytes (CD3-allophycocyanin and CD4-FITC obtained from eBioscience; CD8-PECy7 obtained from BD Biosciences). All Abs were used at a final concentration of 2 μ g/ml, with the exception of the Gr-1⁺-PerCP, which was used at a 1 μ g/ml final concentration. BAL cells were incubated with ammonium chloride for 1 min at room temperature to lyse erythrocytes (RBC lysis buffer Hybri-Max; Sigma-Aldrich) and preincubated with normal mouse serum for 30 min at 4°C to block FcRs. Subsequently, cells were stained with labeled Abs for 15 min at 4°C. After two washing steps, flow cytometric analysis was performed and individual cell populations were identified using the specific fluorescence-labeled Abs. At least 10,000

events were recorded on a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed using Cell Quest software (BD Biosciences).

Statistical analysis

The Student *t* test for paired data was used to test statistically significant differences. Data given are the mean \pm SD. For the in vivo pathogenesis study, body weights were examined by ANOVA and Bonferroni adjustments.

Results

Chemotaxis assays demonstrate IL-8-induced migration of equine neutrophils

Recently, secretion of vCKBPs by alphaherpesviruses, including the equine alphaherpesvirus EHV-1, was described. Using radio-labeled human and murine chemokines in cross-linking assays, the formation of chemokine-vCKBP complexes using EHV-1 gG was demonstrated (1). To investigate whether gG-chemokine complex formation is functionally important and to assess the putative mechanism of vCKBP action, chemotaxis assays were established using equine immune cells and the equine chemokine IL-8. This type of assay was selected because chemokines play a key role in the directional attraction of immune cells during lytic infection (23). IL-8 was chosen as the preferential chemokine as human IL-8 has been described to form a complex with EHV-1 gG (1). As shown in Fig. 2A, equine neutrophils readily migrated in response to recombinant equine IL-8. Around 37% of neutrophils migrated along the cytokine gradient when 10 ng/ml IL-8 was used and migration increased up to 52% in the presence of 50 ng/ml IL-8. Similar results were observed using equine basophils (data not shown). However, when using equine PBMC in the chemotaxis assay, no cell migration could be observed for all concentrations tested (Fig. 2A), indicating the specificity of the equine molecule for cells of the neutrophil lineage.

Supernatants of EHV-1-infected cells inhibit IL-8-induced chemotaxis of equine neutrophils

To study the possible interference of the EHV-1 vCKBP with the migration of immune cells in vitro, IL-8 was preincubated with 300 μ l of supernatant from EHV-1-infected cells for 30 min before

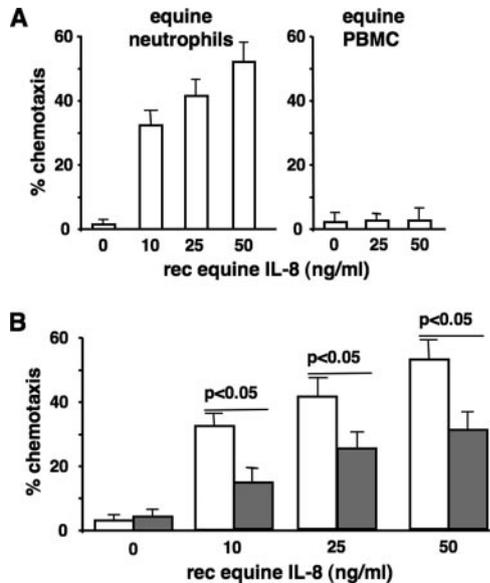


FIGURE 2. *A*, Chemotaxis assays studying IL-8-induced migration of equine immune cells. For neutrophils, chemotaxis was performed using 3 μ M pore-size Transwell plates with the indicated concentrations of equine IL-8 at 37°C for 45 min; for equine PBMC, 5 μ M pore-size Transwell plates were used and the incubation was increased to 2 h. Data are expressed as the mean \pm STDEV of at least three independent experiments. *B*, Supernatant of EHV-1-infected cells inhibits IL-8-induced chemotaxis of equine neutrophils. Chemotaxis assays with equine neutrophils and equine IL-8 were performed with 300 μ l of supernatant of mock-infected (□) or EHV-1-infected cells (▨). Data are expressed as the mean \pm STDEV of at least three independent experiments.

performing the chemotaxis assay. The supernatants had been cleared of any cell debris and virus particles by ultracentrifugation, and, therefore, only proteins secreted from infected cells were used as a protein source in the assays. As shown in Fig. 2*B*, a significant reduction up to 50% in the migration of equine neutrophils was observed in the presence of supernatant recovered from EHV-1-infected cells ($p < 0.05$). Infected cell culture supernatant by itself, similar to the observations made in the absence of IL-8, did not have any effect on equine neutrophil migration under unstimulated conditions (Fig. 2*B*).

Viral gG of EHV-1 is responsible for the inhibition of IL-8-induced chemotaxis

To determine whether gG in the supernatants of EHV-1-infected cells was responsible for the reduction of IL-8-induced chemotaxis, the assays were repeated with supernatants of cells that had been infected with a gG deletion mutant (vL11 Δ gG). As a control, supernatants of cells infected with a rescuant virus (vL11 Δ gGR), in which gG expression was restored, were also included. These viruses have been intensively characterized both in vitro and in vivo in a previous study (13). First, Western blot analysis using polyclonal Abs directed against EHV-1 gG demonstrated indeed the presence of secreted gG as a 55- to 60-kDa protein in supernatants of wild-type- and vL11 Δ gGR-infected cells (Fig. 3*A*). As expected, no secreted gG was present in the supernatant of mock- and vL11 Δ gG-infected cells (Fig. 3*A*). Supernatants of vL11 Δ gG-infected cells, which are devoid of secreted gG, did not alter the neutrophil migration in the chemotaxis assay and migration was virtually identical with that in the presence of IL-8 alone (Fig. 3*B*). In contrast, supernatants of cells infected with wild-type or the gG rescuant virus, both of which contained secreted gG, were shown to significantly reduce neutrophil migration (Fig. 3*B*), similar to

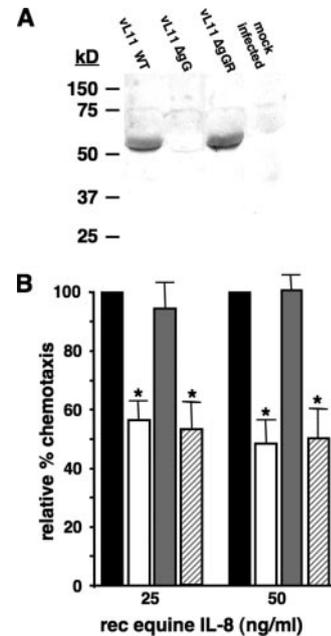


FIGURE 3. IL-8-induced chemotaxis of equine neutrophils is not altered in the absence of secreted gG in supernatant of EHV-1-infected cells. *A*, Cell- and virion-free supernatants of EHV-1 wild-type (WT), vL11 Δ gG- or vL11 Δ gGR-infected cells were separated by SDS-12% PAGE, transferred to a nitrocellulose membrane, and incubated with anti-EHV-1 gG Abs (1:500). Supernatant of mock-infected cells was included as a negative control. *B*, Chemotaxis assays with equine neutrophils were performed with IL-8 alone (■), 300 μ l of supernatant of EHV-1 WT- (□), vL11 Δ gG- (▨), or vL11 Δ gGR-infected cells (▩). Data are expressed as the mean \pm STDEV of at least three independent experiments. *, A significant difference compared with IL-8 alone. The 100% response equals $42 \pm 5.2\%$ and $54 \pm 3.7\%$ chemotaxis for 25 and 50 ng/ml IL-8, respectively.

the observation documented in Fig. 2*B*. These results were a strong indication that secreted gG functions as the vCKBP responsible for the interference with neutrophil chemotaxis. Finally, to unequivocally identify gG as the vCKBP and also to exclude possible interactions of other viral and/or cellular proteins secreted from cells with chemokine-mediated attraction of neutrophils, the secreted form of EHV-1 gG was expressed in insect cells using a baculovirus expression system. A 6xHis tag was introduced to facilitate purification of sgG by resin affinity chromatography. Coomassie blue staining of fractions eluted from the affinity column revealed a major protein of ~ 58 kDa present in fractions 2 and 3 (Fig. 4*A*). Western blot analyses of sgG-His using EHV-1 gG-specific polyclonal Abs showed a major protein band of ~ 58 kDa and also a minor band of 130 kDa, possibly representing sgG-His dimers (Fig. 4*A*) (24). Western blot analysis with anti-Penta-His Abs was also performed and gave identical results, i.e., proteins of 58 and 130 kDa in size were detected (data not shown). When performing the IL-8 chemotaxis assays using 50 ng/ml IL-8 and serial dilutions of sgG-His, we observed a significant reduction in cell migration, which reached a plateau of $78 \pm 5.2\%$ reduction, starting at 0.6 μ g/ml sgG-His (Fig. 4*B*). These data confirmed the importance of EHV-1 gG for inhibition of IL-8-induced chemotaxis and, therefore, its identity as a major, physiologically, and functionally important vCKBP encoded by this herpesvirus.

EHV-1 and EHV-4 are two closely related viruses with sequence similarities of proteins reaching 80%, and importantly, the gGs of both viruses share a high sequence identity of 72% (10, 25). Despite the similarities between the viral genomes, EHV-4 is unable to induce a systemic infection and remains confined to the

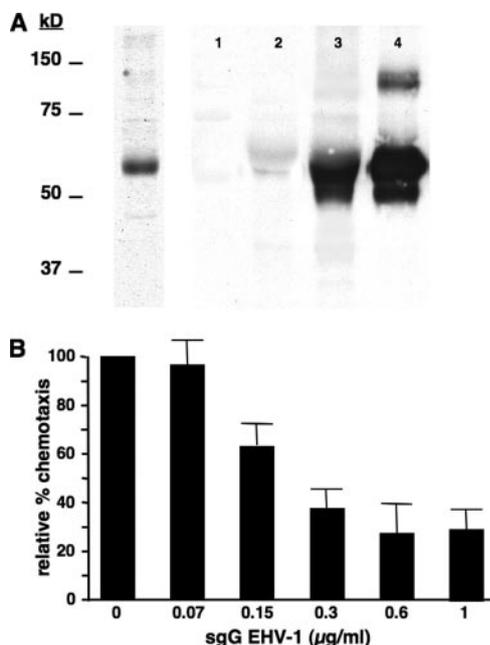


FIGURE 4. gG of EHV-1 is responsible for the inhibition of IL-8-induced chemotaxis of equine neutrophils. *A*, sgG-His from a baculovirus expression system was purified with TALON columns and the different elution fractions were separated by SDS-10% PAGE and stained with Coomassie blue. *Left panel*, Elution fraction; *right panel*, results of a Western blot where sgG-His was detected using polyclonal anti-gG Abs: 1, supernatant mock-infected cells; 2, supernatant mock-infected Sf9 insect cells; 3, supernatant EHV-1-infected cells; 4, purified sgG-His. *B*, Chemotaxis assays with equine neutrophils and 50 ng/ml equine IL-8 were performed with increasing concentrations of purified sgG of EHV-1. Data are expressed as the mean \pm STDEV of at least three independent experiments. The 100% response equals $42 \pm 5.7\%$ chemotaxis.

upper respiratory tract. We tested in our chemotaxis assay whether anti-chemotactic activity was observed when supernatants of EHV-4-infected cells were used (1). The chemotaxis assays using recombinant equine IL-8 and supernatants of EHV-4-infected cells revealed that no significant interference with neutrophil migration was observed (Fig. 5A). This failure to block neutrophil migration was not caused by an absence of EHV-4 gG, as confirmed by Western blot analysis using polyclonal Abs directed against EHV-4 gG (Fig. 5B). The Ab is specific for EHV-4 gG and did not recognize gG of EHV-1, consistent with results obtained in previous studies (12) (Fig. 5B). These results clearly indicated that gG of EHV-4, despite high sequence and structural similarity with its EHV-1 counterpart, does not exhibit properties consistent with a vCKBP interfering with at least IL-8-induced chemotaxis. This observation may be of considerable interest in the light of the different lifestyles of EHV-1 and EHV-4 gG and might at least in part explain the failure of the latter to establish a systemic infection (9).

gG of EHV-1 does not inhibit CCL2-induced chemotaxis of equine monocytes in vitro

It was demonstrated that an excess of human CCL2 is unable to competitively inhibit binding of EHV-1 vCKBP to IL-8, implying that the vCKBP does not bind CCL2 (1). We here examined whether EHV-1 gG could interfere with CCL2-induced chemotaxis. As equine CCL2 was not commercially available, recombinant, His-tagged equine CCL2 was expressed in insect cells using a recombinant baculovirus system. The equine CCL2 sequence introduced into the baculovirus genome was based on the pub-

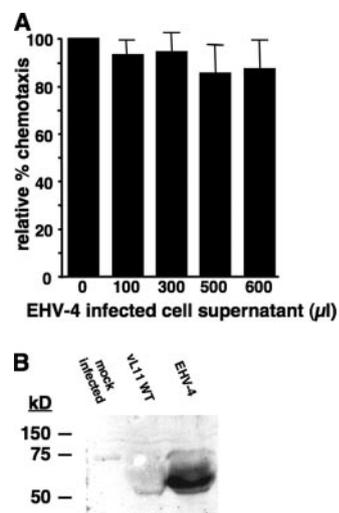


FIGURE 5. Supernatant of EHV-4 does not interfere with IL-8-induced chemotaxis of equine neutrophils. *A*, Chemotaxis assays with equine neutrophils and 50 ng/ml equine IL-8 were performed with increasing concentrations of supernatant of EHV-4-infected cells. Data are expressed as the mean \pm STDEV of at least three independent experiments. The 100% response equals $47 \pm 7.3\%$ chemotaxis. *B*, Cell- and virion-free supernatants of EHV-4-, EHV-1-, or mock-infected cells were separated by SDS-12% PAGE, transferred to a nitrocellulose membrane, and incubated with anti-EHV-4 gG Abs (1:500).

lished equine CCL2 sequence (19). The presence of CCL2-His in the supernatant of insect cells infected with the CCL2-expressing baculovirus was demonstrated by Western blot analysis using Penta-His Abs (Fig. 6A). Its biological activity was shown in a chemotaxis assay where CCL2 was able to attract equine monocytes in a dose-dependent fashion. Dependent on the input of cells, the maximum percentage of chemotaxis of monocytes in response to equine CCL2 was $\sim 32 \pm 5.2\%$, using 300 μl of supernatants of Sf9 insect cells expressing equine CCL-2 (Fig. 6B). The activity of equine CCL-2 decreased with increasing volumes of supernatant, which is consistent with previous reports that chemokine receptors are down-regulated after full saturation (26). Preincubation of equine CCL2 with 0.6 $\mu\text{g/ml}$ sgG-His for 30 min, however, did not alter CCL-2-induced migration of monocytes, indicating that the vCKBP of EHV-1 indeed does not interfere with the biological function of CCL2 in monocyte recruitment (Fig. 6C).

Viral gG of EHV-1 interferes with neutrophil migration in vivo

In the final set of experiments, we tested the relevance of expression of EHV-1 gG, with emphasis on neutrophil migration, in a murine model of EHV-1 infection. Therefore, BALB/c mice were infected intranasally with 4×10^4 PFU of vL11 Δ gG or the revertant virus vL11 Δ gGR, in which expression of gG had been restored. After infection, mice were monitored daily for clinical signs of infection and body weight loss to investigate the pathogenesis of the gG deletion mutant in general. Infected mice in both groups started to lose weight as early as day 1 p.i., which continued to day 4 p.i., and reached preinfection weights by day 10. However, throughout the whole observation period, mice infected with the gG deletion mutant vL11 Δ gG lost more weight (statistically significant on day 3 p.i.) and recovered less rapidly compared with the group infected with the rescuant virus vL11 Δ gGR (Fig. 7A). Also, virus titers were determined in lungs of infected mice on days 1, 2, and 4 p.i. At days 1 and 2 p.i., viral titers in lungs of mice infected with the vL11 Δ gG were indistinguishable from those infected with vL11 Δ gGR ($p = 0.5$ and $p = 0.2$, respectively). These results

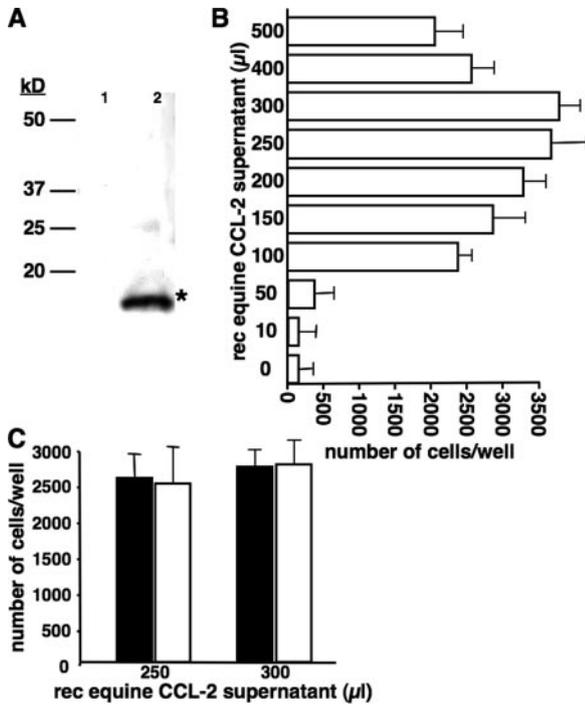


FIGURE 6. Supernatant of EHV-1-infected cells does not interfere with CCL-2-induced chemotaxis of equine monocytes. *A*, Cell-free supernatant from Sf9 insect cells infected with eCCL-2-His recombinant baculovirus was prepared and separated by SDS-10% PAGE, transferred to a nitrocellulose membrane, and incubated with anti-Penta-His Abs (1:1000). This resulted in a major protein band with a size of ~10 kDa (lane 2, *). Lane 1, Supernatant from mock-infected insect cells. *B*, Chemotaxis was performed using 5 μ M pore-size Transwell plates with the indicated dilutions of supernatant containing equine CCL-2 at 37°C for 2 h. Data are expressed as the mean \pm STDEV of at least three independent experiments. *C*, Chemotaxis assays with equine monocytes and the indicated supernatant dilution containing equine CCL-2 were performed in the presence (■) or absence (□) of 0.6 μ g/ml sgG-His. Data are expressed as the mean \pm STDEV of at least three independent experiments.

indicated that, during the first 2 days of infection, gG is completely dispensable for EHV-1 replication in the lungs (Fig. 7*B*). In contrast, at day 4 p.i. when declining viral titers indicated a clearance of the virus, the gG deletion mutant replicated to higher titers than wild-type virus ($p < 0.05$), which may indicate a possible delay in viral clearance in the absence of gG (Fig. 7*B*).

The next logical step was to study neutrophil migration in infected mice in more detail. First, *in vitro* chemotaxis assays were performed to determine whether gG is able to interfere with murine neutrophil migration. As no mouse homolog of IL-8 has been described, we used murine KC, a potent murine neutrophil attractant, in our chemotaxis assay (27, 28). Murine neutrophils migrated in response to KC (0.1 μ g/ml) and migration was significantly reduced in the presence of EHV-1 gG (Fig. 8*A*, $p < 0.05$), indicating that gG can interfere with KC-induced murine neutrophil migration *in vitro*. After confirming EHV-1 vCKBP activity on murine neutrophils *in vitro*, mice from each group were sacrificed to collect the inflammatory cells infiltrating the airways by BAL at days 1, 2, 4, and 14 p.i.. As shown in Fig. 8*B*, the total amount of BAL cells differed significantly between groups. Compared with the control group, significantly more immune cells could be recovered from lungs of infected mice at days 1, 2, and 4 p.i. At day 14 p.i., the number of BAL cells from infected mice had dropped drastically and was indistinguishable from that of mock-infected mice (Fig. 8*B*). Total numbers of BAL cells were increased to

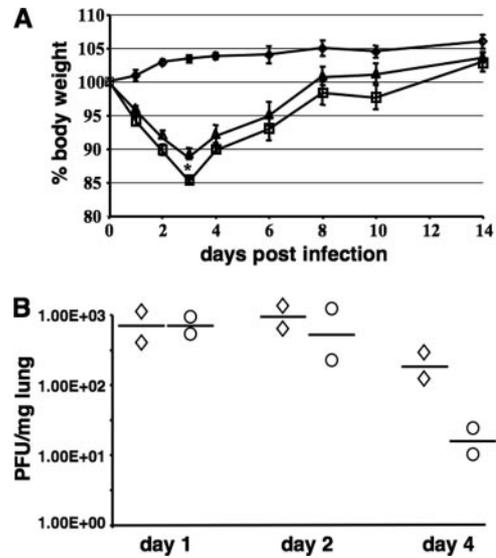


FIGURE 7. Pathogenesis of EHV-1 infection in a murine infection model in the absence of gG. *A*, Mice (16 per group) were inoculated intranasally with 10^4 PFU of vL11 Δ gGR (Δ), vL11 Δ gG (\square), or medium alone as a negative control (\diamond). Mean body weights were determined on the day of infection (day 0) and daily up to day 14 p.i. Mean body weights and SD are shown. *, Statistically significant differences ($p < 0.05$) between vL11 Δ gGR- and vL11 Δ gG-inoculated mice. *B*, Viral titers were determined in two mice of each group at days 1, 2, and day 4 p.i. Titers in lungs of vL11 Δ gG- (\diamond) or vL11 Δ gGR-inoculated mice (\circ) and means are shown. The limit of detection was 10 PFU/mg lung and for the control mice, no virus from lungs was recovered.

~200% in lungs harvested from mice infected with the gG deletion mutant vL11 Δ gG when compared with vL11 Δ gGR-infected mice at the early times p.i. (Fig. 8*B*, $p < 0.05$). First, cytopins

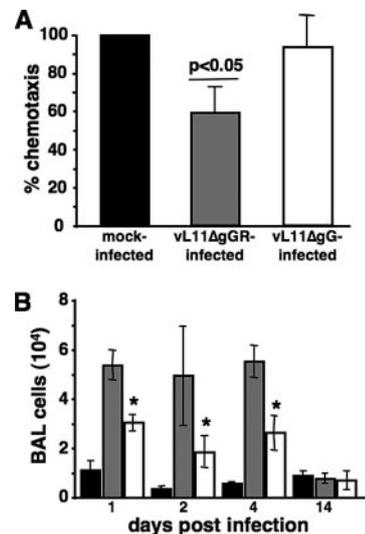


FIGURE 8. *A*, gG of EHV-1 inhibits KC-induced chemotaxis of murine neutrophils. Chemotaxis assays with murine neutrophils were performed with 0.1 μ g/ml murine KC, preincubated with 300 μ l supernatant of mock-infected, vL11 Δ gGR- or vL11 Δ gG-infected cells. Data are expressed as the mean \pm STDEV of at least three independent experiments. The 100% response equals $37 \pm 8.4\%$ chemotaxis. *B*, *In vivo* relevance of gG on the influx of immune cells in a murine model of EHV-1 infection. Total amount of immune cells from mice infected intranasally with vL11 Δ gGR (\square) or vL11 Δ gG (\blacksquare), or mock infected (\blacksquare). At different days p.i., inflammatory cells were harvested by BAL, washed, and counted. Data are presented as the mean \pm SD; *, statistically significant differences ($p < 0.05$).

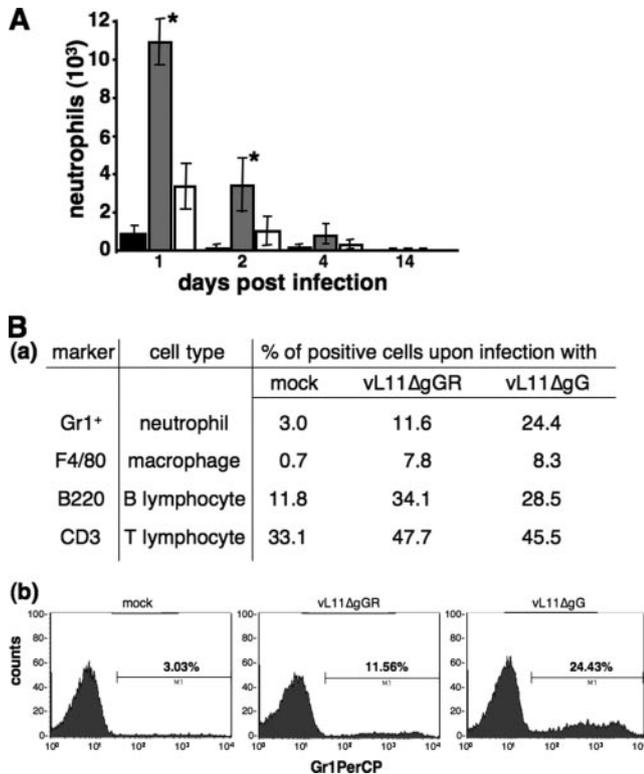


FIGURE 9. In vivo relevance of gG on neutrophil migration in a murine model of EHV-1 infection. **A**, Absolute number of neutrophils in BAL cells of mice infected intranasally with vL11ΔgGR (□) or vL11ΔgG (▨), or mock infected (■). At different days p.i., BAL cells were harvested, stained, and the number of neutrophils were determined. Data are presented as the mean \pm SD; *, statistically significant differences ($p < 0.01$). **B**, Flow cytometric analysis of BAL cells of mice infected intranasally with vL11ΔgGR, vL11ΔgG, or medium (negative control). At day 2 p.i., BAL cells from at least four mice per group were preincubated with normal mouse serum for 30 min at 4°C and subsequently stained with labeled Abs against cells of the innate immune system or the adaptive immune system for 15 min at 4°C. Percentages of leukocyte subpopulations were determined (**a**) and representative histograms showing the percentage of neutrophils for each group of infected mice are given (**b**).

were prepared which showed that macrophages and mainly lymphocytes were the predominating cell types in the pulmonary airways of mock-infected control mice, whereas neutrophils represented $<5\%$ of total cells (data not shown). Within 24 h of EHV-1 infection, neutrophil counts rose sharply, and, thereafter, returned equally rapidly to preinfection levels starting at day 4 p.i. At days 1 and 2 p.i., neutrophil counts in mice infected with the gG deletion mutant were between 3- and 4-fold higher when compared with those in vL11ΔgGR-infected mice (Fig. 9A). The statistically significant difference ($p < 0.01$) between these two groups confirmed that viral gG interfered with neutrophil influx in the target organ, the lung, during EHV-1 infection. Second, to exactly quantify the phenotypes of immune cells present in the airways of infected mice, BAL cells were analyzed by flow cytometry. At day 2 p.i., the percentage of neutrophils was increased in response to infection. The percentage of neutrophils in BAL of mice infected with the gG deletion mutant reached up to 24%, whereas neutrophil percentages remained lower in vL11ΔgGR-infected mice and did not exceed 12% (Fig. 9B). Also the percentage of macrophages was clearly increased in infected, when compared with mock-infected, animals, but no difference was found in relative numbers of BAL cells of animals infected with the gG deletion mutant or the gG-expressing virus vL11ΔgGR at that particular time point p.i.

(Fig. 9B). At day 4 p.i., however, the total amount of macrophages was significantly higher in vL11ΔgG-infected mice compared with mice infected with vL11ΔgGR, $5.5 \times 10^4 \pm 7.1 \times 10^3$ vs $1.5 \times 10^4 \pm 6.1 \times 10^3$ ($p < 0.001$), indicating that viral gG can also modulate migration of macrophages in lungs of infected mice. For B cells and T cells, no difference could be observed between BAL of vL11ΔgG- and vL11ΔgGR-infected mice at 2 days p.i. (Fig. 9B), and the CD4⁺/CD8⁺ T cell subpopulations likewise were very similar between different groups of mice: 50% CD4⁺ vs 50% CD8⁺, 47% CD4⁺ vs 53% CD8⁺, and 48% CD4⁺ vs 52% CD8⁺ for mock-infected, vL11ΔgGR-, and vL11ΔgG-infected mice, respectively. Taken together, the flow cytometry results confirmed those obtained after the microscopic inspection of the BAL cells in the cytopins and indicated interference of EHV-1 gG with neutrophil and macrophage traffic at different times p.i., whereas no detectable modulation of lymphocytes was observed.

Discussion

In several members of the large herpesvirus family, open reading frames were identified, which encode vCKBP that are capable of binding to chemokines with high affinity. Viral gG was identified as the vCKBP produced by some of the alphaherpesviruses, including EHV-1, and it had been proposed to function as a chemokine modulator and possibly an entry receptor into immune cells in its membrane-bound form (1). Moreover, recent studies using a mutant virus devoid of gG, identified viral gG as a virulence factor in vivo (13, 29). Because 1) binding of vCKBPs to target molecules does not necessarily imply functional activity, and 2) it had never been investigated thus if the function of gG as a vCKBP was responsible for the different phenotypes observed for mutant viruses in vivo, we aimed at investigating the pathophysiological importance of gG binding to chemokines both in vitro and in vivo. We could demonstrate here for the first time that gG of an alphaherpesvirus not only functions as a vCKBP in vitro, but also has a significant effect in vivo as demonstrated by the reduction of the recruitment of immune cells to sites of infection, thereby significantly altering the inflammatory response of the host.

The equine alphaherpesviruses EHV-1 and EHV-4 were used as model organisms, and in vitro chemotaxis assays with equine leukocytes and equine chemokines were first established. Chemotaxis assays were conducted to study the functional role of vCKBP, as the attraction of immune cells is a significant property of chemokines determining the outcome of viral infections (4). We mainly focused on IL-8-induced chemotaxis of equine neutrophils because 1) human IL-8 has been described to bind to gG of EHV-1 (1) and 2) viral infections, including those by EHV-1, have been shown to be regularly accompanied by an up-regulation of cellular IL-8 production (30–32). We were able to demonstrate that equine neutrophils, but not equine PBMC, readily migrated in response to recombinant equine IL-8. This cell type-specific attraction is due to the tri-amino acid motif ELR present in equine IL-8, which is essential for the specific recruitment of neutrophils and is found in most IL-8-like molecules, with some notable exceptions such as the potent B cell chemoattractant, viral vIL-8, encoded by another alphaherpesvirus, MDV (6, 23, 33). When chemotaxis assays were performed in the presence of supernatants of EHV-1-infected cells, a significant inhibition of neutrophil migration was observed and additional experiments unequivocally identified secreted EHV-1 gG as the factor responsible for inhibition of neutrophil chemotaxis. Interestingly, when using supernatants of cells infected EHV-4, a close relative of EHV-1, no inhibition of neutrophil chemotaxis was observed despite the presence of secreted gG. Future

experiments are needed to determine the reason for this discrepancy in biological function. We currently hypothesize that the extracellular part of EHV-1 gG in close proximity to the membrane-anchoring region is important for binding to chemokines and hence, inhibition of chemotaxis. Our hypothesis is based on the observation that ~100 aa of the extracellular part of EHV-4 and EHV-1 gG directly neighboring the transmembrane domain are highly divergent and possess strong type-specific epitopes (10). Regardless, linking the observations that 1) EHV-1 gG shows vCKBP properties whereas gG of EHV-4 does not, with 2) EHV-1 can lead to multiorgan clinical signs whereas EHV-4 is predominantly associated with mild upper respiratory disease in the natural host (8, 9), leads to the hypothesis that the ability of the gG of alphaherpesvirus to interfere with chemokine action might contribute to virulence and wide organ dissemination. One may speculate that the inability of gG to stop the first line of the host defense, likely in concert with a less productive replication in the airways when compared with EHV-1 (9), may help confine EHV-4 to the upper airways.

Another important feature of gG is the induction of a strong Ab response in response to infection (10, 34). Consequently, one has to consider the effect of Abs directed against gG for its ability to properly function as a vCKBP. Preliminary *in vitro* data indicate that the inhibitory effect of gG on neutrophil migration sharply declines in the presence of an EHV-1-specific equine hyperimmune serum, and future experiments are necessary to study in more detail the effect of anti-gG Abs on vCKBP activity. It is conceivable that the interference of Abs with vCKBP function might influence the efficacy and safety of vaccines. This is important as EHV-1 gG deletion mutants have been proposed as useful modified-live virus marker vaccines that allow distinction between vaccinated and infected horses (12). This approach would seem less ideal if Abs against gG indeed down-modulated vCKBP function and therefore, might be an important regulator preventing the virus from evading the immune system. Accordingly, thorough testing of such engineered vaccines with respect to vaccine safety and efficacy will be all the more important.

Besides studying IL-8-induced chemotaxis of equine neutrophils and the effects on this process by EHV-1 gG, we also evaluated the effect of the vCKBP on the functionality of a chemokine that was shown not to be bound by gG (1). We chose to analyze CCL-2 because it is the only chemokine among the non-gG-binding chemokines, which has been described in horses. Expression of equine CCL-2 (19) using the baculovirus system allowed us to study its functionality in a chemotaxis assay. CCL-2 produced in insect cells was able to attract equine monocytes in a dose-dependent manner, but EHV-1 gG was not able to interfere with chemotaxis. These data therefore indicate that, as had been expected, failure of a chemokine to interact with gG will result in an absence of functional interference by viral gG with chemokine-induced chemotaxis.

To address the biological importance of interference of EHV-1 gG with chemokine signaling, we used a murine model of EHV-1 respiratory disease to analyze the influx of inflammatory cells infiltrating the main target organ, the lung. First, functional interference of viral gG with murine chemokines was demonstrated in our *in vitro* chemotaxis assay using murine KC. Next, at several time points during the acute phase of EHV-1 infection, BALs were performed on mice, which had been infected with either a gG-negative deletion mutant or the gG-expressing revertant virus. The rationale for performing BALs was to facilitate examination of the different types of immune cells migrating into the airways and to correlate cellular influx with severity in disease development based on the notion that in diffuse lung disease caused by virus infection

and other noxae, cytology of BAL correlates well with lung immunocytology (35). The *in vivo* experiments showed that, following EHV-1 infection, a significant increase in total cell numbers in BALs was observed when compared with control mice. The majority of infiltrating cells were macrophages and lymphocytes, but at day 1 p.i., a sharp and transient increase in neutrophil counts was evident. These data are in good agreement with immunohistological studies of EHV-1-infected murine lung tissue where an influx of neutrophils was observed on days 1 and 2 p.i. (36). When BAL cells were compared between mice infected with the gG deletion mutant and animals infected with a gG-expressing virus, both the total number of immune cells as well as the percentage of neutrophils was significantly higher in the absence of the vCKBP. Interestingly, not only an effect on the migration of neutrophils between both groups of infected mice was observed, but there was also an effect on the influx of macrophages. At day 4 p.i., the percentage of macrophages was significantly lower in BAL cells from mice infected with the gG revertant virus. This observation is consistent with earlier findings that EHV-1 gG can bind to a broad range of chemokines, including members of the C-C subfamily, which generally attract mononuclear cells (1).

In line with a previous study, it was observed that, especially at lower doses of infection ($<10^5$) and during the acute phase of infection, more severe clinical signs and a stronger inflammatory infiltration was observed in histological lung sections of mice infected with a gG-negative virus (13). Following these initial observations, our data now clearly demonstrate that expression of EHV-1 gG can inhibit the recruitment of inflammatory cells *in vitro* and *in vivo*, which is in good agreement with what has been described for secreted vCKBPs of poxviruses (5). As such, our data are the first to unravel the mechanisms of action of EHV-1 gG on murine chemokines *in vivo*, but it still remains elusive whether EHV-1 gG will display similar effects on inflammation and cell migration in its natural host, the horse. Future experiments are planned to close this gap and investigate the behavior of gG-negative mutants in horses.

Interference with chemokine signaling, thereby perturbing a fine-tuned network of the host's most immediate protection system, the innate response, is one of the many defenses viruses use to evade or manipulate the host's arsenal to cope with intruders. In general, viruses must survive their encounter with the host's immune system to establish a successful infection. This requires that the virus avoids immediate immune elimination, but not to an extent that the host succumbs to infection. With this in mind, it is reasonable to speculate that gG has played an important role in the establishment of a mutually acceptable relationship between virus and host that allows the virus to replicate, albeit at suboptimal levels, but at the same time does not cause too severe or even irreversible damage to the target host tissue. As such, immune modulators might constitute a prerequisite for sustained maintenance of alphaherpesviruses within host populations and play an essential role in these delicate coevolutionary relationships.

Acknowledgments

We are grateful to Drs. Michael Studdert and Carol Hartley for supplying Abs. We thank Jeremy Kamil and Neil Margulis for help with the baculovirus system. While we were working on this manuscript, Dr. Patrick Smith, a dedicated and talented immunologist, who helped us with the initial design of the animal experiments and BAL, passed away quite unexpectedly. We dedicate this article to his memory.

Disclosures

The authors have no financial conflict of interest.

References

- Bryant, N. A., N. Davis-Poynter, A. Vanderplassen, and A. Alcami. 2003. Glycoprotein G isoforms from some alphaherpesviruses function as broad-spectrum chemokine binding proteins. *EMBO J.* 22: 833–846.
- Costes, B., M. B. Ruiz-Arguello, N. A. Bryant, A. Alcami, and A. Vanderplassen. 2005. Both soluble and membrane-anchored forms of Felid herpesvirus 1 glycoprotein G function as a broad-spectrum chemokine-binding protein. *J. Gen. Virol.* 86: 3209–3214.
- McFadden, G., A. Lalani, H. Everett, P. Nash, and X. Xu. 1998. Virus-encoded receptors for cytokines and chemokines. *Semin. Cell Dev. Biol.* 9: 359–368.
- Lusso, P. 2000. Chemokines and viruses: the dearest enemies. *Virology* 273: 228–240.
- Lalani, A. S., J. W. Barrett, and G. McFadden. 2000. Modulating chemokines: more lessons from viruses. *Immunol. Today* 21: 100–106.
- Parcells, M. S., S. F. Lin, R. L. Dienglewicz, V. Majerciak, D. R. Robinson, H. C. Chen, Z. Wu, G. R. Dubyak, P. Brunovskis, H. D. Hunt, et al. 2001. Marek's disease virus (MDV) encodes an interleukin-8 homolog (vIL-8): characterization of the vIL-8 protein and a vIL-8 deletion mutant MDV. *J. Virol.* 75: 5159–5173.
- Allen, G. P., and J. T. Bryans. 1986. Molecular epizootiology, pathogenesis, and prophylaxis of equine herpesvirus-1 infections. *Prog. Vet. Microbiol. Immunol.* 2: 78–144.
- van Maanen, C. 2002. Equine herpesvirus 1 and 4 infections: an update. *Vet. Q.* 24: 58–78.
- Patel, J. R., and J. Heldens. 2005. Equine herpesviruses 1 (EHV-1) and 4 (EHV-4)—epidemiology, disease and immunoprophylaxis: a brief review. *Vet. J.* 170: 14–23.
- Crabb, B. S., and M. J. Studdert. 1993. Epitopes of glycoprotein G of equine herpesviruses 4 and 1 located near the C termini elicit type-specific antibody responses in the natural host. *J. Virol.* 67: 6332–6338.
- Colle, C. F., III, and D. J. O'Callaghan. 1995. Transcriptional analyses of the unique short segment of EHV-1 strain Kentucky A. *Virus Genes* 9: 257–268.
- Huang, J., C. A. Hartley, N. P. Ficorilli, B. S. Crabb, and M. J. Studdert. 2005. Glycoprotein G deletion mutants of equine herpesvirus 1 (EHV1; equine abortion virus) and EHV4 (equine rhinopneumonitis virus). *Arch. Virol.* 150: 2583–2592.
- von Einem, J., P. M. Smith, G. R. Van de Walle, D. J. O'Callaghan, and N. Osterrieder. 2007. In vitro and in vivo characterization of equine herpesvirus type 1 (EHV-1) mutants devoid of the viral chemokine-binding glycoprotein G (gG). *Virology* 362: 151–162.
- Drummer, H. E., M. J. Studdert, and B. S. Crabb. 1998. Equine herpesvirus-4 glycoprotein G is secreted as a disulphide-linked homodimer and is present as two homodimeric species in the virion. *J. Gen. Virol.* 79: 1205–1213.
- Pycock, J. F., W. E. Allen, and T. H. Morris. 1987. Rapid, single-step isolation of equine neutrophils on a discontinuous Percoll density gradient. *Res. Vet. Sci.* 42: 411–412.
- Bennouna, S., W. Sukhumavasi, and E. Y. Denkers. 2006. *Toxoplasma gondii* inhibits Toll-like receptor 4 ligand-induced mobilization of intracellular tumor necrosis factor α to the surface of mouse peritoneal neutrophils. *Infect. Immun.* 74: 4274–4281.
- Hubert, P. H., S. Birkenmaier, H. J. Rziha, and N. Osterrieder. 1996. Alterations in the equine herpesvirus type-1 (EHV-1) strain RacH during attenuation. *Zentralbl. Veterinarmed. B.* 43: 1–14.
- Rudolph, J., D. J. O'Callaghan, and N. Osterrieder. 2002. Cloning of the genomes of equine herpesvirus type 1 (EHV-1) strains KyA and racL11 as bacterial artificial chromosomes (BAC). *J. Vet. Med. B Infect. Dis. Vet. Public Health* 49: 31–36.
- Benarafa, C., F. M. Cunningham, A. S. Hamblin, D. W. Horohov, and M. E. Collins. 2000. Cloning of equine chemokines eotaxin, monocyte chemoattractant protein (MCP)-1, MCP-2 and MCP-4, mRNA expression in tissues and induction by IL-4 in dermal fibroblasts. *Vet. Immunol. Immunopathol.* 76: 283–298.
- Kamil, J. P., B. K. Tischer, S. Trapp, V. K. Nair, N. Osterrieder, and H. J. Kung. 2005. vLIP, a viral lipase homologue, is a virulence factor of Marek's disease virus. *J. Virol.* 79: 6984–6996.
- von Einem, J., J. Wellington, J. M. Whalley, K. Osterrieder, D. J. O'Callaghan, and N. Osterrieder. 2004. The truncated form of glycoprotein gp2 of equine herpesvirus 1 (EHV-1) vaccine strain KyA is not functionally equivalent to full-length gp2 encoded by EHV-1 wild-type strain RacL11. *J. Virol.* 78: 3003–3013.
- Smith, P. M., Y. Zhang, W. D. Grafton, S. R. Jennings, and D. J. O'Callaghan. 2000. Severe murine lung immunopathology elicited by the pathogenic equine herpesvirus 1 strain RacL11 correlates with early production of macrophage inflammatory proteins 1 α , 1 β , and 2 and tumor necrosis factor α . *J. Virol.* 74: 10034–10040.
- Baggiolini, M. 1998. Chemokines and leukocyte traffic. *Nature* 392: 565–568.
- Fiebig, B. L., B. Jager, C. Schollmann, K. Weindel, J. Wiltling, G. Kochs, D. Marme, H. Hug, and H. A. Weich. 1993. Synthesis and assembly of functionally active human vascular endothelial growth factor homodimers in insect cells. *Eur. J. Biochem.* 211: 19–26.
- Telford, E. A., M. S. Watson, J. Perry, A. A. Cullinane, and A. J. Davison. 1998. The DNA sequence of equine herpesvirus-4. *J. Gen. Virol.* 79(Pt. 5): 1197–1203.
- Luscinskas, F. W., J. M. Kiely, H. Ding, M. S. Obin, C. A. Hebert, J. B. Baker, and M. A. Gimbrone, Jr. 1992. In vitro inhibitory effect of IL-8 and other chemoattractants on neutrophil-endothelial adhesive interactions. *J. Immunol.* 149: 2163–2171.
- Zlotnik, A., and O. Yoshie. 2000. Chemokines: a new classification system and their role in immunity. *Immunity* 12: 121–127.
- Lin, M., E. Carlson, E. Diaconu, and E. Pearlman. 2006. CXCL1/KC and CXCL5/LIX are produced selectively by corneal fibroblasts and mediate neutrophil infiltration to the corneal stroma in LPS keratitis. *J. Leukocyte Biol.* 81: 786–792.
- Devlin, J. M., G. F. Browning, C. A. Hartley, N. C. Kirkpatrick, A. Mahmoudian, A. H. Noormohammadi, and J. R. Gilkerson. 2006. Glycoprotein G is a virulence factor in infectious laryngotracheitis virus. *J. Gen. Virol.* 87: 2839–2847.
- Oakes, J. E., C. A. Monteiro, C. L. Cubitt, and R. N. Lausch. 1993. Induction of interleukin-8 gene expression is associated with herpes simplex virus infection of human corneal keratocytes but not human corneal epithelial cells. *J. Virol.* 67: 4777–4784.
- Inagi, R., R. Guntapong, M. Nakao, Y. Ishino, K. Kawanishi, Y. Isegawa, and K. Yamanishi. 1996. Human herpesvirus 6 induces IL-8 gene expression in human hepatoma cell line, Hep G₂. *J. Med. Virol.* 49: 34–40.
- Pusterla, N., W. D. Wilson, P. A. Conrad, S. Mapes, and C. M. Leutenegger. 2006. Comparative analysis of cytokine gene expression in cerebrospinal fluid of horses without neurologic signs or with selected neurologic disorders. *Am. J. Vet. Res.* 67: 1433–1437.
- Legler, D. F., M. Loetscher, R. S. Roos, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1998. B cell-attracting chemokine 1, a human CXC chemokine expressed in lymphoid tissues, selectively attracts B lymphocytes via BLR1/CXCR5. *J. Exp. Med.* 187: 655–660.
- Crabb, B. S., C. M. MacPherson, G. H. Reubel, G. F. Browning, M. J. Studdert, and H. E. Drummer. 1995. A type-specific serological test to distinguish antibodies to equine herpesviruses 4 and 1. *Arch. Virol.* 140: 245–258.
- Shanley, J. D., and Z. K. Ballas. 1985. Alteration of bronchoalveolar cells during murine cytomegalovirus interstitial pneumonitis. *Am. Rev. Respir. Dis.* 132: 77–81.
- Bartels, T., F. Steinbach, G. Hahn, H. Ludwig, and K. Borchers. 1998. In situ study on the pathogenesis and immune reaction of equine herpesvirus type 1 (EHV-1) infections in mice. *Immunology* 93: 329–334.