JOURNAL OF VIROLOGY, June 2011, p. 6091–6095 Vol. 85, No. 12
0022-538X/11/$12.00 doi:10.1128/JVI.02113-10
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

Partial Functional Complementation between Human and Mouse Cytomegalovirus Chemokine Receptor Homologues▽

Helen E. Farrell,[1] Alexander M. Abraham,[1] Rhonda D. Cardin,[2] Alexander H. Sparre-Ulrich,[3] Mette M. Rosenkilde,[3] Katja Spiess,[4] Tine H. Jensen,[4] Thomas N. Kledal,[4] and Nicholas Davis-Poynter[1]*

The University of Queensland, Clinical Medical Virology Centre, and Royal Children's Hospital, Sir Albert Sakzewski Virus Research Centre, Queensland, Australia[1]; Cincinnati Children's Hospital Medical Center, Division of Infectious Diseases, Cincinnati, Ohio[2]; Laboratory for Molecular Pharmacology, Faculty of Health Sciences, University of Copenhagen, Copenhagen, Denmark[3]; and INAGEN, Lyngby, Denmark[4]

Received 5 October 2010/Accepted 31 March 2011

The human cytomegalovirus (CMV) proteins US28 and UL33 are homologous to chemokine receptors (CKRs). Knockout of the mouse CMV M33 protein (UL33 homologue) results in substantial attenuation of salivary gland infection/replication and reduced efficiency of reactivation from tissue explants. M33-mediated G protein-coupled signaling is critical for the salivary gland phenotype. In this report, we demonstrate that US28 and (to a lesser degree) UL33 restore reactivation from tissue explants and partially restore replication in salivary glands (compared to a signaling-deficient M33 mutant). These studies provide a novel small animal model for evaluation of therapies targeting the human CMV CKRs.

Chemokine receptor (CKR) homologues and other seven-transmembrane receptor (7TMR) homologues are characteristic of beta- and gammaherpesviruses and are potential therapeutic targets (7, 14, 16). Human cytomegalovirus (HCMV) encodes four 7TMRs: UL33 and UL78 are conserved in all betaherpesviruses, whereas US28/US27 homologues are restricted to primate betaherpesviruses.

Clues to the roles of HCMV 7TMRs have come from *in vivo* studies of mouse and rat CMVs (MCMV and RCMV, respectively). Acute-phase replication in primary organs is followed by dissemination to secondary sites, such as salivary glands, where virus may replicate for several days, attaining high titers. Following immune clearance of productive infection, latently infected cells remain distributed in a number of organs. In the absence of M33 (UL33 homologue), MCMV failed to attain detectable levels of infectious virus in salivary glands, with similar observations for R33 of RCMV (1, 5), suggesting evolutionarily conserved functions. The mechanisms underlying salivary gland attenuation may include defects in dissemination to, initial infection of, or maintenance of productive infection within that organ. Definitive studies are lacking, but there is some evidence for attenuation at a postdissemination step; the M33-null mutant was defective for replication following direct intrasalivary gland inoculation, and the R33-null mutant was detectable in salivary glands (by PCR), similar to the wild type at early times postinfection (1, 5). We and others have demonstrated the importance of constitutive M33-induced signaling for the salivary gland phenotype (4, 15). We further demonstrated that an M33-null mutant was deficient for reactivation in an *ex vivo* tissue explant model (2), although whether M33 plays a specific role during the establishment of, maintenance of, or reactivation from latency is not yet known.

An *in vivo* model for HCMV-encoded 7TMR function would provide a useful system to determine potential therapeutic targets. The viral CKRs M33, UL33, and US28 all exhibit constitutive signaling, suggesting potential functional conservation (6, 9). Preliminary studies suggested partial complementation of M33 function (salivary gland phenotype) via substitution with UL33 (4).

In this report, we evaluated MCMV as a model for HCMV-encoded CKR function with regard to tissue-specific replication and explant reactivation. We also investigated whether M33-induced G protein-mediated signaling, critical for salivary gland infection/replication, was important for replication in other organs and for the reactivation phenotype.

The wild-type (wt) MCMV strain K181 (Perth) was used. Recombinant MCMV lacking M33 expression, expressing mutated M33, or expressing HCMV CKR (US28 or UL33), via the endogenous M33 promoter, are described in Table 1. All recombinants replicated normally in mouse embryonic fibroblasts (MEF) (data not shown). Green fluorescent protein (GFP)-tagged constructs enabled expression of the various viral CKRs to be confirmed in virus-infected MEF (2 days postinfection [p.i.]; multiplicity of infection [MOI], 0.01) by immunofluorescence (Fig. 1), and the results indicated a predominantly intracellular distribution, with evidence for cell surface localization. Cell surface expression and ligand binding of US28 were confirmed (Fig. 1) via competition binding studies using soluble fractalkine (CX3CL) (8) at 48 h postinfection of NIH 3T3 cells (MOI, 1) by ΔM33/US28. Reagents for detection of cell surface M33 and UL33 were not available.

Replication kinetics were determined in 6-week-old, female BALB/c mice following intraperitoneal (i.p.) infection (10^6 PFU; $n = 4$). M33(R131Q) replicated similarly to wt MCMV in spleen, liver, lung, and pancreas, demonstrating that ablation of G protein-coupled signaling of M33 had no discernible

* Corresponding author. Mailing address: Sir Albert Sakzewski Virus Research Centre, Bldg. C28, Royal Children's Hospital, Herston, QLD 4029, Australia. Phone: 617 36368138. Fax: 617 36361401. E-mail: n.davispoynter@uq.edu.au.
▽ Published ahead of print on 13 April 2011.

Downloaded from https://journals.asm.org/journal/jvi on 04 October 2024 by 34.239.150.167.

TABLE 1. Descriptions of recombinant viruses

Virus	Description	Source (reference)
Untagged constructs		
ΔM33/Z[a]	M33-null via *lacZ* insertion	Reported previously, KΔ33B_{T2} (5)
ΔM33/STOP	M33-null via premature stop codon	Recombination plasmid JF495130[b]
M33(R131Q)	M33 mutant defective for G protein-coupled signaling	Reported previously (4)
ΔM33/UL33	M33 replaced by UL33 (HCMV strain AD169)	Reported previously (4)
ΔM33/US28	M33 replaced by US28 (HCMV strain AD169)	Recombination plasmid HQ895841[b]
GFP-tagged constructs		
M33-GFP	Wild-type M33/C-terminal GFP fusion	Reported previously (4)
M33(R131Q)-GFP	M33 signaling-deficient mutant (R131Q)/C-terminal GFP fusion	Reported previously (4)
ΔM33/UL33-GFP	M33 replaced by UL33/C-terminal GFP fusion	Recombination plasmid HQ895843[b]
ΔDM33/US28-GFP	M33 replaced by US28/C-terminal GFP fusion	Recombination plasmid HQ895842[b]

[a] ΔM33/Z was the parent virus for all other recombinants.
[b] GenBank accession number of the plasmid used to generate the virus recombinant, according to published methods (4, 5).

phenotype in these organs (Fig. 2A). The M33-null (ΔM33/Z) recombinant had enhanced clearance (spleen) and reduced replication (pancreas) compared with wt MCMV, as reported previously (2). To determine whether the *lacZ* cassette of ΔM33/Z contributed to these phenotypes, a $lacZ^-$ M33-null virus was generated (ΔM33/STOP) and compared with wt and ΔM33/Z in a second study (1.5×10^6 PFU; $n = 3$ to 4) (Fig. 2B). Both M33-null viruses exhibited rapid initial clearance from the spleen, whereas ΔM33/STOP replicated similarly to wt MCMV in the pancreas. These results suggest that the *lacZ* cassette of ΔM33/Z has an attenuating effect upon pancreatic replication, but the spleen phenotype is attributable to a function of M33 that is not disrupted by the R131Q mutation.

ΔM33/UL33 and ΔM33/US28 replication levels were similar overall to wt MCMV in all organs except for salivary glands (Fig. 2A). Whereas wt MCMV attained high titers, infectivity was not detected for the M33-null viruses or M33(R131Q) at any time, consistent with previous studies (2, 4, 5). Notably, low levels of infectious virus were detected for ΔM33/US28 (all time points) and ΔM33/UL33 (7, 17, and 32 days p.i.), although UL33 titers were significantly higher than baseline on day 17 only (Fig. 2A).

These data suggest that salivary gland replication of MCMV is highly sensitive to disruption of M33 signaling, whereas replication in other organs is apparently unaffected. This function of M33 was partially complemented by US28 or UL33. M33, US28, and UL33 all signal constitutively by a variety of pathways, predominantly involving Gαq (3, 10, 11, 18). However, the signaling properties of M33 are not identical to those of US28 and UL33, and these differences may influence salivary

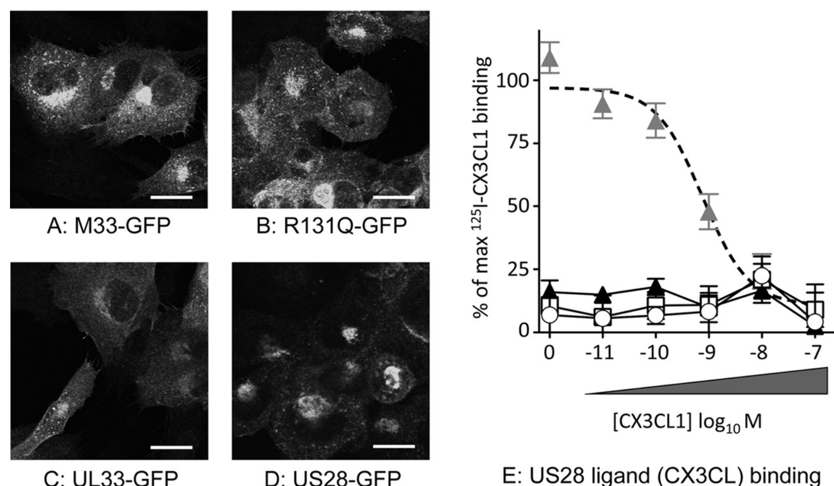

FIG. 1. Expression of vCKR in cells infected with recombinant MCMV. (A to D) Immunofluorescence detection of GFP-tagged vCKR. MEF coverslip cultures, 2 days postinfection (MOI, 0.01), were fixed and permeabilized, and GFP was detected by using a rabbit polyclonal anti-GFP primary antibody and AlexaFlour-488 conjugated goat anti-rabbit secondary antibody, according to published methods (4). Confocal images are shown (63× objective; 4× zoom). Bar, 10 μM. (E) Competition binding experiments with wild-type and recombinant MCMV-infected fibroblasts (3T3), using [^{125}I]CX3CL1 as a tracer (12 pM) in the presence of various concentrations of unlabeled CX3CL1 (0 to 10^{-7}M). Uninfected cells (white squares) and cells infected with the wild-type MCMV (white circles) or ΔM33/Z (black triangles) revealed no specific binding of CX3CL1. Fibroblasts infected with ΔM33/US28 (gray triangles) revealed significant binding of CX3CL1. Thus, cells infected with ΔM33/US28 express functional US28 on the infected cell surface, binding CX3CL1 with a 50% effective concentration (EC_{50}) of 0.75 nM. Data are presented as means ± standard deviations from three independent experiments. Nonlinear regression and EC_{50} values were determined using GraphPad Prism 5 software (San Diego, CA).

Downloaded from https://journals.asm.org/journal/jvi on 04 October 2024 by 34.239.150.167.

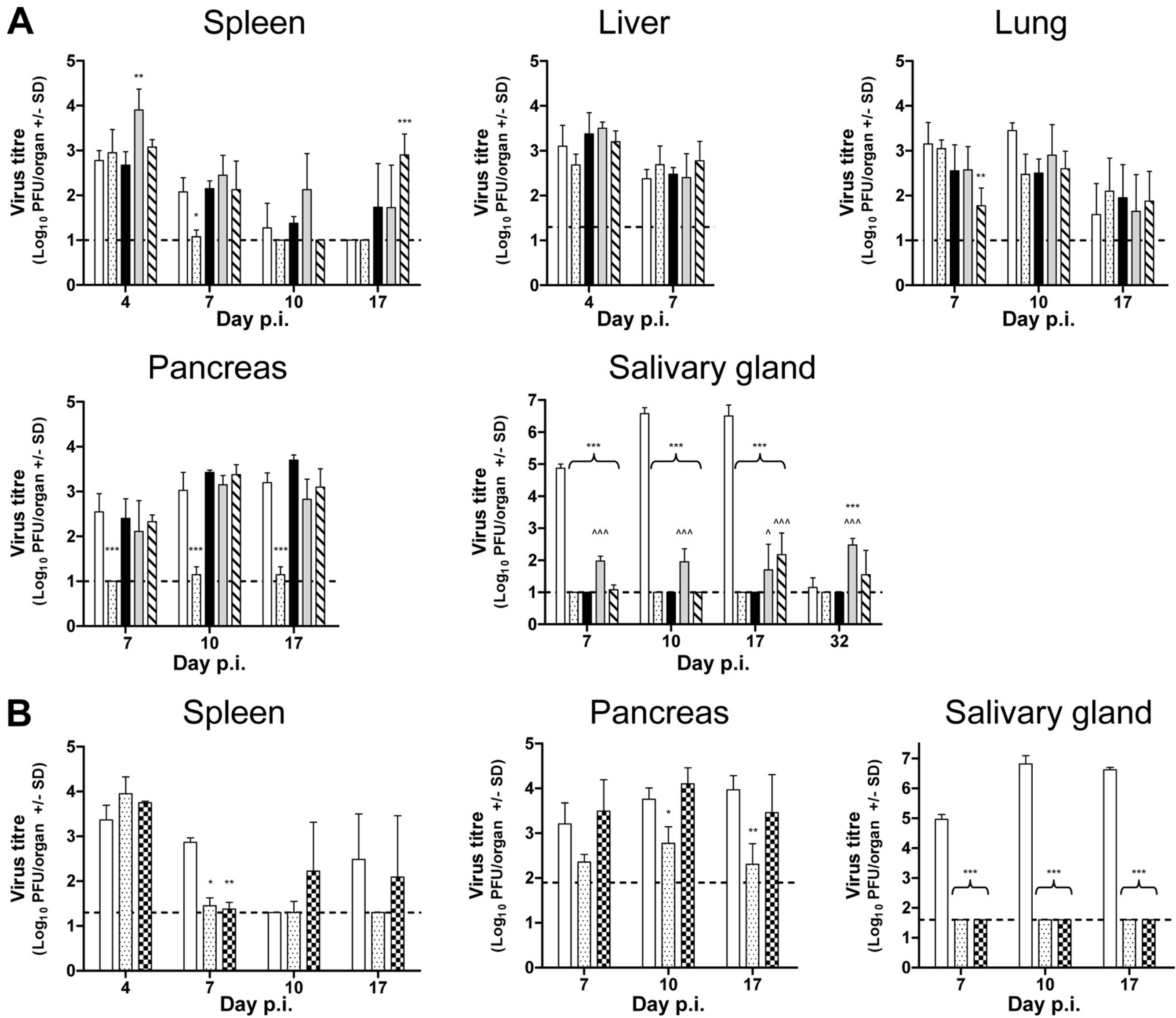

FIG. 2. Acute-phase replication of wild-type and recombinant MCMV in BALB/c mice. (A) Six-week-old mice were inoculated i.p. with 10^6 PFU of each virus [wild-type MCMV (white bars), ΔM33/Z (stippled), M33(R131Q) (black), ΔM33/US28 (gray), and ΔM33/UL33 (hatched)] and euthanized at the indicated time (days p.i.). Titers of individually homogenized organs (n = 4) were assayed on MEF; the mean virus titers ($\log_{10}$ PFU/organ) and standard deviations are shown. Samples with no detectable virus were assigned a titer equivalent to the threshold of detection for the plaque assay. The detection threshold for each organ is indicated as a dashed line; organ titers presented in the absence of error bars indicate that the virus was not detected in any samples. Comparisons of virus titers were made using ANOVA (GraphPad Prism version 5.0), with a Bonferroni posttest determination of statistically significant differences at each time point between wt MCMV and the MCMV recombinant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) and between M33(R131Q) and the other recombinants (^, $P < 0.05$; ^^^, $P < 0.001$). (B) Six-week-old mice were inoculated i.p. with 1.5×10^6 PFU of each virus (wild-type MCMV [white], ΔM33/Z [stippled], and ΔM33/STOP [checked]). Titers of individually homogenized organs are shown (n = 3 to 4), and data are presented as described for panel A.

gland replication. Furthermore, although G protein-coupled signaling of M33 is clearly important, other activities of M33 that are not fully complemented by US28 or UL33 may influence the salivary gland phenotype.

A tissue explant model was used to assess reactivation from latency (2, 17). Reduced detection of infectious (reactivated) virus in this assay could be due to a reduced ability to establish, maintain, or reactivate from latency. Previously, we found that ΔM33/Z had a diminished capacity to reactivate from spleen and lung explants (2), which was associated with a marked reduction in the viral DNA load within explanted tissue. In this study, we determined whether ablation of M33-induced G protein-coupled signaling affected explant reactivation and whether explant reactivation efficiencies could be restored by US28 or UL33. Three-week-old BALB/c mice were infected i.p. with either 10^4 or 10^5 PFU of virus. At 12 to 16 weeks p.i., when no infectious virus was detected in salivary gland homogenates (data not shown), spleens and lungs were dissected into small pieces and cultured in complete medium (RPMI containing 2 mM GlutaMAX-1, 100 U/ml penicillin, 100 μg/ml

Downloaded from https://journals.asm.org/journal/jvi on 04 October 2024 by 34.239.150.167.

streptomycin, and 10% [vol/vol] heat-inactivated fetal calf serum). Each organ was distributed among 6 wells of a 24-well plate (1 ml/well), with medium changed twice weekly for 7 to 8 weeks. At days 4 and 10 and weekly thereafter, 80 μl of supernatant/well was assayed for infectivity on MEF in 96-well trays by using centrifugal enhancement (13) and scoring visually for cytopathic effect after 1 week of incubation. Cultures positive during the first 10 days postexplant (potential persistent [lytic] infection) were excluded from the analyses.

M33(R131Q) had a lower reactivation rate (spleen and lung) than wt MCMV at both doses (P values of <0.05 to <0.001), suggesting that G protein-mediated signaling of M33 is a contributory factor toward efficient establishment, maintenance, and/or reactivation of MCMV latency (Fig. 3A). Notably, ΔM33/US28 reactivated with similar efficiency as wt MCMV, and UL33 was also shown to compensate, albeit with a trend to reduced efficiency.

The defective explant reactivation phenotype observed for M33(R131Q) may have been due to a lower viral load that resulted in a reduced opportunity to establish latency. However, there was no evidence for attenuation of M33(R131Q) compared to wild-type virus in those tissue types used for explantation (spleen and lung) (Fig. 2A). A direct assessment of viral DNA load at the time of explant was made using quantitative PCR (qPCR) (Fig. 3B). DNA was extracted from tissues (10 to 20 mg) by using DNAzol (Invitrogen). An MCMV-specific qPCR, targeting open reading frame M38, used primers from GeneWorks (South Australia) qM38f (TG GCCGCGGAAATGAG) and qM38r (CCAAGGAGAAGA GGCAGAAGTC) and the qM38 probe (6-carboxyfluorescein–CATGAGGCGATTGCACTTGAGAGGCA–Black Hole Quencher 1).

Reaction mixtures contained 5 μl DNA sample and 15 μl of reaction mix (FastStart TaqMan probe mix [Roche]), 900 nM (each) qM38f and qM38r, and 250 nM qM38 probe; reactions were run (on a RotorGene 6000) at 95°C for 10 min, then 45 cycles of (i) 95°C for 10 s and (ii) 60°C for 1 min. Cellular DNA was quantified using EXPRESS SYBR GreenER (Invitrogen) and primers for mouse Nidogen (12). Wild-type and recombinant qPCRs (in duplicate) were run in parallel, using a standard curve of control virus-infected cell DNA. Viral DNA levels were normalized according to the total cellular DNA for each sample. The mean level of viral DNA in the wild-type MCMV-infected group was determined, and the viral DNA load was expressed as a ratio relative to this value. Viral DNA levels in lung samples of all groups were below the detection threshold. Viral DNA was detected in all ($n = 8$) spleen samples, except for UL33 ($n = 7$), for which one sample failed for both virus and cellular DNA PCR. The mean DNA levels detected for the US28 and UL33 recombinants were similar to the wild type. Although there was a trend toward lower levels for M33(R131Q), there was no statistically significant difference overall between the groups (analysis of variance [ANOVA], $P = 0.09$).

These studies demonstrated that, despite having a profound effect upon salivary gland replication, disruption of M33 signaling activity, exhibited by M33(R131Q), did not affect replication in other organs tested, including the lung and spleen. However, M33(R131Q) was attenuated for reactivation from lung and spleen tissue explants. US28 and, to a lesser degree, UL33 were able to functionally complement activities of M33 for the reactivation phenotype and partially rescue the salivary gland phenotype. Further studies are required to determine whether G protein-coupled signaling induced by UL33 and

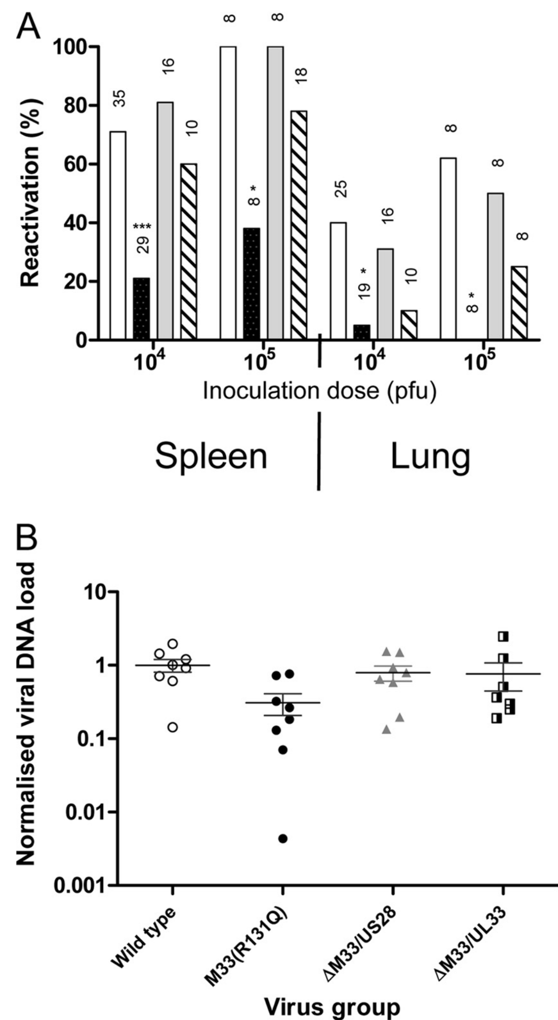

FIG. 3. (A) Reactivation of wild-type and recombinant MCMV from spleen and lung explant cultures. Three-week-old BALB/c mice were infected i.p. with the indicated dose of each virus [wt MCMV (white), M33(R131Q) (black), ΔM33/US28 (gray), and ΔM33/UL33 (hatched)]. Organs were obtained between 12 and 16 weeks p.i., finely dissected, and cultured in 24-well cluster plates (6 wells per organ). Supernatants were assayed for infectious virus up to 7 weeks postexplantation; individual wells with infectious virus by 10 days postexplantation were excluded from the analyses. Results for each group are derived from 1 to 4 experiments; each bar indicates the percentage of mice positive for virus reactivation from either spleen or lung explants, with the numbers of mice in each group shown above the bars. Determination of statistically significant differences between groups and wt was made using Fisher's exact test (*, $P < 0.05$; ***, $P < 0.001$). (B) Relative levels of viral DNA in spleen samples at the time of explantation. DNA was recovered from a representative sample of spleen at the time of tissue explantation for reactivation studies (inoculation dose, 10^5 PFU), with 8 [wild type, M33(R131Q), and ΔM33/US28] or 7 (ΔM33/UL33) mice per group. DNA quantification was carried out by qPCR, and viral DNA levels are expressed relative to the mean level of wild-type MCMV DNA. The individual values for each spleen are plotted, with horizontal lines indicating the group mean and error bars indicating the standard deviations. There were no statistically significant differences between the means (ANOVA, $P > 0.05$).

Downloaded from https://journals.asm.org/journal/jvi on 04 October 2024 by 34.239.150.167.

US28 is responsible for complementation. Moreover, although these results are consistent with a shared biological function between human and rodent CMV viral CKR (vCKR), this hypothesis has yet to be tested, for example, via a tissue culture model of HCMV latency. Notwithstanding these caveats, the recombinant MCMVs expressing UL33 and US28 provide novel reagents for evaluation of antiviral drugs active against human CMV in a mouse model.

N.D.-P. and H.E.F. were supported by grants from the NHMRC (456137 and 631401) and Queensland Health. R.D.C. was supported by grant AHA 0665211B.

S. Bialasiewicz assisted with the qPCR.

REFERENCES

1. **Beisser, P. S., et al.** 1998. The R33 G protein-coupled receptor gene of rat cytomegalovirus plays an essential role in the pathogenesis of viral infection. J. Virol. **72:**2352–2363.
2. **Cardin, R. D., G. C. Schaefer, J. R. Allen, N. J. Davis-Poynter, and H. E. Farrell.** 2009. The M33 chemokine receptor homolog of murine cytomegalovirus exhibits a differential tissue-specific role during in vivo replication and latency. J. Virol. **83:**7590–7601.
3. **Casarosa, P., et al.** 2001. Constitutive signaling of the human cytomegalovirus-encoded chemokine receptor US28. J. Biol. Chem. **276:**1133–1137.
4. **Case, R., et al.** 2008. Functional analysis of the murine cytomegalovirus chemokine receptor homologue M33: ablation of constitutive signaling is associated with an attenuated phenotype in vivo. J. Virol. **82:**1884–1898.
5. **Davis-Poynter, N. J., et al.** 1997. Identification and characterization of a G protein-coupled receptor homolog encoded by murine cytomegalovirus. J. Virol. **71:**1521–1529.
6. **Hughes, A. L., and M. Yeager.** 1999. Coevolution of the mammalian chemokines and their receptors. Immunogenetics **49:**115–124.
7. **Hulshof, J. W., et al.** 2006. Synthesis and pharmacological characterization of novel inverse agonists acting on the viral-encoded chemokine receptor US28. Bioorg. Med. Chem. **14:**7213–7230.
8. **Kledal, T. N., M. M. Rosenkilde, and T. W. Schwartz.** 1998. Selective recognition of the membrane-bound CX3C chemokine, fractalkine, by the human cytomegalovirus-encoded broad-spectrum receptor US28. FEBS Lett. **441:**209–214.
9. **Luttichau, H. R., and T. W. Schwartz.** 2000. Validation of chemokine receptors as drug targets. Curr. Opin. Drug Discov. Dev. **3:**610–623.
10. **McLean, K. A., P. J. Holst, L. Martini, T. W. Schwartz, and M. M. Rosenkilde.** 2004. Similar activation of signal transduction pathways by the herpesvirus-encoded chemokine receptors US28 and ORF74. Virology **325:**241–251.
11. **Miller, W. E., D. A. Houtz, C. D. Nelson, P. E. Kolattukudy, and R. J. Lefkowitz.** 2003. G protein-coupled receptor (GPCR) kinase phosphorylation and beta-arrestin recruitment regulate the constitutive signaling activity of the human cytomegalovirus US28 GPCR. J. Biol. Chem. **278:**21663–21671.
12. **Morrison, T. B., Y. Ma, J. H. Weis, and J. J. Weis.** 1999. Rapid and sensitive quantification of Borrelia burgdorferi-infected mouse tissues by continuous fluorescent monitoring of PCR. J. Clin. Microbiol. **37:**987–992.
13. **Osborn, J. E., and D. L. Walker.** 1968. Enhancement of infectivity of murine cytomegalovirus in vitro by centrifugal inoculation. J. Virol. **2:**853–858.
14. **Rosenkilde, M. M., and T. N. Kledal.** 2006. Targeting herpesvirus reliance of the chemokine system. Curr. Drug Targets **7:**103–118.
15. **Sherrill, J. D., et al.** 2009. Activation of intracellular signaling pathways by the murine cytomegalovirus G protein-coupled receptor M33 occurs via PLC-β/PKC-dependent and -independent mechanisms. J. Virol. **83:**8141–8152.
16. **Smit, M. J., et al.** 2003. Virally encoded G protein-coupled receptors: targets for potentially innovative anti-viral drug development. Curr. Drug Targets **4:**431–441.
17. **Stoddart, C. A., et al.** 1994. Peripheral blood mononuclear phagocytes mediate dissemination of murine cytomegalovirus. J. Virol. **68:**6243–6253.
18. **Waldhoer, M., T. N. Kledal, H. Farrell, and T. W. Schwartz.** 2002. Murine cytomegalovirus (CMV) M33 and human CMV US28 receptors exhibit similar constitutive signaling activities. J. Virol. **76:**8161–8168.

Downloaded from https://journals.asm.org/journal/jvi on 04 October 2024 by 34.239.150.167.