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Marek's disease alphaherpesvirus (MDV) RLORF4 is not required for expression of glycoprotein C and interindividual spread

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ABSTRACT

Marek's disease virus (MDV) is a lymphotropic alphaherpesvirus that causes Marek's disease (MD) in chickens. RLORF4 is a MDV-specific gene that is systematically deleted during attenuation of MDV *in vitro*. Concomitantly, the expression of glycoprotein C (gC) is diminished during attenuation, suggesting these two changes may be linked. Original studies in which RLORF4 was deleted utilized an infectious clone that lacked gC expression due to a frame-shift mutation within the gC open reading frame (UL44). Here, we utilized an infectious clone in which gC expression was restored to test our hypothesis that RLORF4 is important for expression of MDV gC, and subsequently, interindividual spread. Contrary to our hypothesis, gC expression was unaltered during both *in vitro* and *in vivo* replication of RLORF4-null MDV and was able to efficiently transmit from chicken to chicken, conclusively showing that RLORF4 does not regulate gC expression and is not required for horizontal transmission.

1. Introduction

Marek's disease (MD) in chickens is caused by *Gallid alphaherpesvirus* 2 (GaHV-2), better known as Marek's disease alphaherpesvirus (MDV). The most prominent sign of MD is the development of solid lymphomas in the viscera and other organs (Calnek, 2001). Natural infection begins through the respiratory route by inhalation of infectious virus where the virus initially infects pulmonary B lymphocytes and/or is taken up by pulmonary macrophages or dendritic cells (Baaten et al., 2009) and transported to lymphoid organs. Following the initiation of primary cytolytic infection in the host, activated T lymphocytes recruited to the sites of infection become the primary cell type infected and latency can be established in these cells, where some can also undergo oncogenic transformation resulting in lymphoma formation. This event is ultimately a dead end for the virus. Important for dissemination in the population, migrating infected cells transport virus to feather follicle (FF) epithelial (FFE) cells in the skin, where infectious virus is shed into the environment and the virus life cycle can repeat in new hosts.

Previously referred to as the “A-antigen,” MDV glycoprotein C (gC) is normally expressed at high levels during *in vitro* and *in vivo* propagation, but its expression is greatly reduced after 30–50 serial passages in tissue culture cells (Bulow and Biggs, 1975; Churchill et al., 1969; Ikuta et al., 1983a, b; Jarosinski and Osterrieder, 2010; Purchase et al., 1971; Tischer et al., 2005). This decreased expression coincides with increased plaque sizes and attenuated characteristics. Wilson et al. (1994) examined the relationship between gC expression and attenuation and found that although gC levels were related to decreased levels of gC mRNA produced by attenuated MDV, there were no alterations in the UL44 (gC) open reading frame or promoter region suggesting a MDV-encoded regulatory protein may regulate its expression.

The RLORF4 gene is unique to MDV and its role in attenuation was originally identified as a gene deleted following serial passage in cell culture cells (Jarosinski et al., 2003; Spatz et al., 2008; Spatz and Silva, 2007; Spatz et al., 2012). Formerly identified as a hypothetical reading frame within the repeat long (RL) regions of the MDV genome, the mRNA of RLORF4 was identified during both lytic and latent infection (Jarosinski et al., 2003) as a single transcript encoding a 142 amino acid protein in wild-type strains. Further studies confirmed its expression at the protein level during lytic replication (Liu et al., 2006). Common among genes expressed within the RL regions of MDV, multiple mRNA splice variants were identified between RLORF4, viral interleukin 8 (vIL-8), and the MDV oncoprotein, Meq (Jarosinski and Schat, 2007); however, the functional importance of these transcripts is currently not known. Following the generation of an infectious clone of the RB-1B strain as a bacterial artificial chromosome (BAC), further experiments confirmed that deletion of RLORF4 within this BAC clone resulted in highly attenuated virus *in vitro* and *in vivo* (Jarosinski et al., 2005). In this report, both wild-type and ΔRLORF4 viruses were unable

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to transmit to naïve contact chickens. This BAC clone was subsequently found to contain numerous frame-shift mutations (Spatz et al., 2007), of which mutations within UL44 (gC) and UL13 protein kinase (CHPK) abrogated MDV spread from chicken to chicken (Jarosinski et al., 2007; Jarosinski and Osterrieder, 2010).

Since RLORF4 is consistently deleted during serial passage and expression of MDV gC is significantly reduced, we postulated that RLORF4 is important for gC expression and subsequently, interindividual spread of MDV. However, this hypothesis could not be tested in the original studies due to a frame-shift mutation in UL44 (gC). Here, we tested this hypothesis using recombinant (r)MDV that expresses gC and is transmission-competent. Contrary to our hypothesis, deletion of RLORF4 within a fully restored rMDV did not affect gC expression *in vitro* and *in vivo*, and RLORF4 played no role during interindividual spread of MDV. These results directly address an important question in gC regulation *in vitro* and *in vivo* and conclusively show that RLORF4 is not involved in gC expression as originally hypothesized. In addition, the level of attenuation was not as dramatic as previously seen; indicating the severe attenuation previously reported was most likely due to compounding mutations within the rMDV used in that study.

2. Materials and methods

2.1. Cell culture and cells

Chicken kidney cells (CKCs) were prepared from 2 to 4 weeks-old specific-pathogen-free (SPF) chickens obtained from the University of Illinois at Urbana-Champaign (UIUC) Poultry Farm following standard methods (Schat and Sellers, 2008). Briefly, primary CKCs were seeded in growth medium consisting of Medium 199 (Cellgro, Corning, NY, USA) supplemented with 10% tryptose-phosphate broth (TPB), 0.63% $NaHCO_3$ solution, antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and 4% fetal bovine serum (FBS). Confluent CKCs were maintained in F10.199 medium consisting of a 1:1 mixture of Ham's F10 (Cellgro) and Medium 199 supplemented with 7.5% TPB, 0.63% $NaHCO_3$, 0.2% FBS, and antibiotics. CKCs were maintained at 38 °C in a humidified atmosphere of 5% CO_2.

The chicken DF-1-Cre fibroblast cell line (Niikura et al., 2011) was cultivated in a 1:1 mixture of Leibovitz L-15 and McCoy 5A (LM) media (Gibco, Gaithersburg, MD, USA) supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), and maintained in 50 µg/ml Zeocin (Invitrogen, Carlsbad, CA). DF-1-Cre cells were maintained at 38 °C in a humidified atmosphere of 5% CO_2.

2.2. Generation of ΔRLOF4 and ΔgC rMDVs

Coding sequences of RLORF4 and UL44 (gC) were deleted from a previously described, fully virulent BAC clone (Jarosinski et al., 2012) using two-step Red-mediated mutagenesis (Tischer et al., 2010). Briefly, the I-*Sce*I-*aphAI* cassette from pEP-KanSII (Tischer et al., 2006) was amplified by PCR with Thermo Scientific Phusion Flash High-Fidelity PCR Master Mix using primers shown in Table 1 and used for mutagenesis in GS1783 *Escherichia coli* cells. Restriction fragment length polymorphism (RFLP) analysis, analytical PCR, and DNA sequencing confirmed all clones were correct. Primers used for sequencing have been previously published (Jarosinski et al., 2003; Jarosinski and Osterrieder, 2012; Jarosinski and Schat, 2007).

rMDVs were reconstituted by transfecting DF-1-Cre cells, which efficiently remove the mini-F BAC sequences from the viral genome, with purified BAC DNA plus Lipofectamine 2000 (Invitrogen) using the manufacturers' instructions. Transfected DF-1-Cre cells were mixed with fresh primary CKCs until plaques formed, then further propagated in CKCs until virus stocks could be stored. All rMDVs were used at ≤ 5 passages for *in vitro* and *in vivo* studies.

2.3. Measurement of plaque areas

Plaque areas were measured as previously described (Jarosinski et al., 2005). Briefly, CKCs were seeded on 6-well dishes and infected with 100 plaque-forming units (PFU) per well. After 5 days, cells were washed once with phosphate buffered saline (PBS), fixed and permeabilized with PFA buffer (2% paraformaldehyde, 0.1% Triton X-100) for 15 min, and washed twice with PBS. Immunofluorescence assays (IFAs) were performed as previously described (Jarosinski et al., 2005) using anti-MDV chicken sera and goat anti-chicken IgY-Alexa Fluor® 568 secondary antibody (Molecular Probes, Eugene, OR). Digital images of 50–75 individual plaques were collected using an EVOS™ FL Cell Imaging System (Thermo Fisher Scientific) and compiled using Adobe® Photoshop® CC 2015 version 7 SP1. Plaque areas were measured using ImageJ (Abramoff et al., 2004) version 1.51j8 software, and means were determined for each plaque population. Significant differences in mean plaque areas were determined using Student's *t* tests assuming equal variances in Microsoft® Excel 2016.

2.4. Ethics statement

All animal work was conducted at UIUC according to national regulations. The animal care facilities and programs of UIUC meet the requirements of the law (89–544, 91–579, 94–276) and NIH regulations on laboratory animals and are in compliance with the Animal Welfare Act, PL 279. UIUC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All experimental procedures were in compliance with approval of UIUC's Institutional Animal Care and Use Committee.

2.5. In vivo experiment

Commercial Pure Columbian × New Hampshire chickens were obtained from the UIUC Poultry Farm (Urbana, IL) and were from MD-vaccinated parents; therefore, considered to be maternal antibody positive. All experimental procedures were conducted in compliance with approved Institutional Animal Care and Use Committee protocols. Water and food were provided *ad libitum*. Five-day old chicks were experimentally infected by intra-abdominal inoculation of 2000 PFU for each rMDV and housed in separate rooms (n = 5/group). For each group, another 11 chickens were left uninfected to act as contacts to determine whether rMDVs were able to naturally infect naïve chickens by interindividual spread. Chickens were evaluated for symptoms of

Table 1
Primers used for generation of recombinant Marek's disease virus (rMDV).

Primer Name	Direction[a]	Sequence (5' → 3')[b]
ΔRLORF4	Forward	GTATATAGCGCAAGCGCGCAGGGCTGGTTCGGGTAAGGCGTTCACGC**TAG**TTTATGCCCCATCGTAGGGATAACAGGGTAATCGATTT
	Reverse	GATGCATTTTGTTTATTGAAAATTTCCATTCGATGGGGCATAAA**CTA**GCGTGAACGCCTTACCCGCCAGTGTTACAACCAATTAACC
ΔgC	Forward	CATCCCGAAGAGACACCAAACGTAACCCTCTACATATCTTCCCTC**TAA**TCTCATTGTTATGTAGTTTAGGGATAACAGGGTAATCGATTT
	Reverse	GAGTTATAAAAAATATGTTTAATAAATCACAACTACATAACAATGAGA**TTA**GAGGGAAGATATGTAGCCAGTGTTACAACCAATTAACC

[a] Directionality of the primer.
[b] Bold nucleotides indicate stop codons for each respective gene and underlined sequences are priming sites within the mutagenesis template plasmid pEP-KanS.

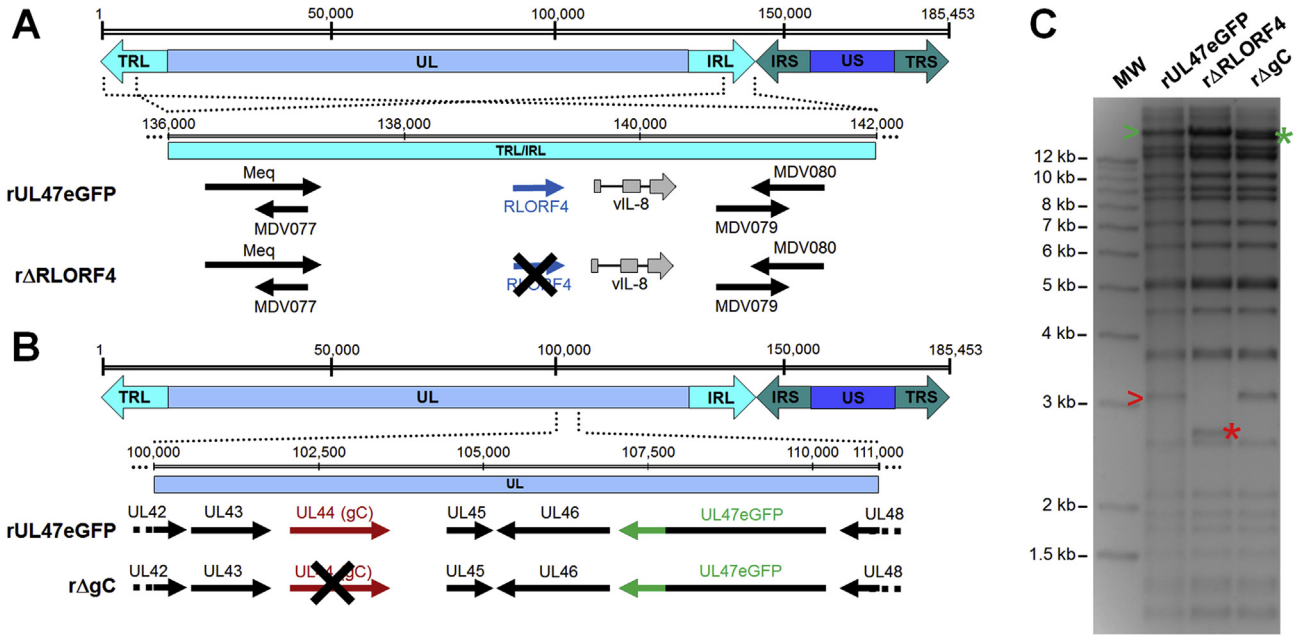

Fig. 1. Generation of rMDVs. (A) Schematic representation of the MDV genome depicting the locations of the terminal repeat long (TRL) and short (TRS), internal repeat short (IRS), and unique long (UL) and short (US) regions. The TRL/IRL region is expanded to show the relevant genes within this region and deletion of RLORF4 in the rΔRLORF4 rMDV. (B) Schematic representation of the MDV genome with the UL region spanning nucleotides 100,000 to 111,000 expanded to show relevant genes within this region, including eGFP tagged UL47 (UL47eGFP) and removal of gC (vΔgC). (C) BAC DNA obtained for rUL47eGFP, rΔRLORF4, and rΔgC were digested with BamHI and examined using RFLP analysis. Integrates are excluded for simplicity. Removal of both copies of RLORF4 results in shifting of the BamHI-L fragments of rUL47eGFP (>) from 3098 bp to 2672 bp (*) in vΔRLORF4. Removal of the UL44 ORF (gC) results in a shift of the BamHI-A fragment from 18,748 in rUL47eGFP (>) down to 17,245 bp in rΔgC (*). The molecular weight marker used was the 1 kb Plus DNA Ladder from Invitrogen, Inc. (Carlsbad, CA). No extraneous alterations are evident.

MD daily, euthanized when birds showed clinical signs of MD (*e.g.*, lethargy, depression, paralysis, torticollis, etc.), and examined for gross MD lesions by a veterinarian blinded to the groups. Chickens positive for MD included birds succumbing to disease prior to the experimental termination date and birds positive for MD-related lesions at termination of the experiment. Fisher's exact tests were used to determine statistical differences between groups of chickens for MD incidence at a significance level of $p < 0.05$.

2.6. DNA extraction from blood cells and qPCR assays

Whole blood was collected as previously described (Jarosinski et al., 2007), and DNA was extracted using the E.Z. 96 blood DNA kit from Omega Bio-tek, Inc. (Norcross, GA). To quantify MDV genomic copies in blood, 2 × Power SYBR® Green PCR Master Mix (Thermo Fisher Scientific) was performed as previously described (Ponnuraj et al., 2019). Standard curves were generated for MDV ICP4 and chicken GAPDH using previously described templates (Jarosinski et al., 2005) starting with approximately 500 pg of DNA. Total copy numbers were determined as previously described (Jarosinski et al., 2002) using the C_T value for that sample. The coefficient of regression was always > 0.99 for standard curves. Thermal cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. All qPCR assays were performed using an Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) and the results were analyzed using QuantStudio™ Design & Analysis Software v1.4.2 supplied by the manufacturer. Significant differences in MDV genomic copies at each time point were determined using Student's *t* tests assuming equal variances at a significance level of $p < 0.05$ using Microsoft® Excel 2016.

2.7. Monitoring rMDV in feather follicles (FFs)

To track the time at which each rMDV reached the FFs, two flight feathers were plucked from the right and left wings (4 total) of inoculated birds weekly beginning at 7 dpi, and UL47eGFP expression was examined using a Leica M205 FCA fluorescent stereomicroscope with a Leica DFC7000T digital color microscope camera (Leica Microsystems, Inc., Buffalo Grove, IL). Feather plucking for all birds was discontinued after 48 dpi, since only a few experimentally infected birds remained.

2.8. Fluorescent microscopy of FFs

Whole feathers were plucked from chickens infected with different rMDVs and the FFs were fixed using PFA buffer, washed twice with PBS, and then blocked in 10% neonatal calf serum. Fixed FFs were stained with primary anti-gC monoclonal A6 (kindly provided by Jean-Francois Vautherot, INRA, Nouzilly, France) antibody (Tischer et al., 2005) and anti-mouse Ig Alexa Fluor 568 (Molecular Probes, Eugene, OR) was used as secondary antibody. The Leica M205 FCA fluorescent stereomicroscope with a Leica DFC7000T digital color microscope camera (Leica Microsystems, Inc., Buffalo Grove, IL) was used to analyze stained FFEs. All images were compiled using Adobe® Photoshop® CC, 2017.0.1 release.

2.9. Western blot analysis

Western blot analyses were performed as previously described (Tischer et al., 2005). To detect relative level of MDV infection, MAb H19 (Ikuta et al., 1983a) was used at 1:10,000 dilution to detect MDV pp38 protein. To detect gC, MAb A6 was used at a 1:500 dilution. For protein loading control, anti-β-actin (ACTNO5; Abcam, Cambridge, MA) MAb was used at its recommended dilution. Secondary anti-mouse IgG peroxidase conjugate was purchased from GE Healthcare (Piscataway, NJ). The SuperSignal West Pico Chemiluminescent Substrate kit from Thermo Fischer Scientific (Rockford, IL) was used to detect antigens using the manufacturer's instructions.

3. Results

3.1. Generation of rΔRLORF4 and rΔgC rMDV

In order to test the ability of RLORF4 to regulate gC expression, the complete RLORF4 open reading from was deleted from a previously described fluorescent rMDV (vUL47eGFP) that is fully transmissible among chickens (Jarosinski et al., 2012) using two step Red-mediated recombination (Tischer et al., 2006). Since there are two copies of RLORF4 located in the RL regions, two rounds of integration and resolution were required. First, RLORF4 was removed within the internal RL (IRL) and then subsequently removed from the terminal RL (TRL) to generate vΔRLORF4 (data not shown). Fig. 1A shows a schematic representation of parental clone rUL47eGFP and the RLORF4-null (rΔRLORF4) clone. As a control for gC expression and downstream studies, rΔgC was generated in which the complete UL44 gene encoding gC was removed using two-step Red-mediated recombination (Fig. 1B). RFLP analysis of rUL47eGFP, rΔRLORF4, and rΔgC confirmed the integrity of the BAC clones as the predicted banding patterns were observed (Fig. 1C). PCR and DNA sequencing was used to confirm that each clone was correct at the nucleotide level (data not shown) using previously described primers (Jarosinski et al., 2003; Jarosinski and Schat, 2007).

3.2. Deletion of RLORF4 results in increased MDV replication in tissue culture

Following reconstitution of rUL47eGFP and rΔRLORF4 resulting in vUL47eGFP and vΔRLORF4, respectively, we first tested *in vitro* growth properties using plaque size assays. We hypothesized that deletion of RLORF4 would result in increased plaque sizes, similar to former studies using rMDV that lacked expression of gC and UL13 protein kinase (Jarosinski et al., 2005). Consistent with the former work, RLORF4-null rMDV generated increased plaque sizes compared to the vUL47eGFP (Fig. 2). Also consistent with former studies (Jarosinski et al., 2007; Jarosinski and Osterrieder, 2010, 2012), vΔgC generated significantly larger plaques than vUL47eGFP, but there was no difference between

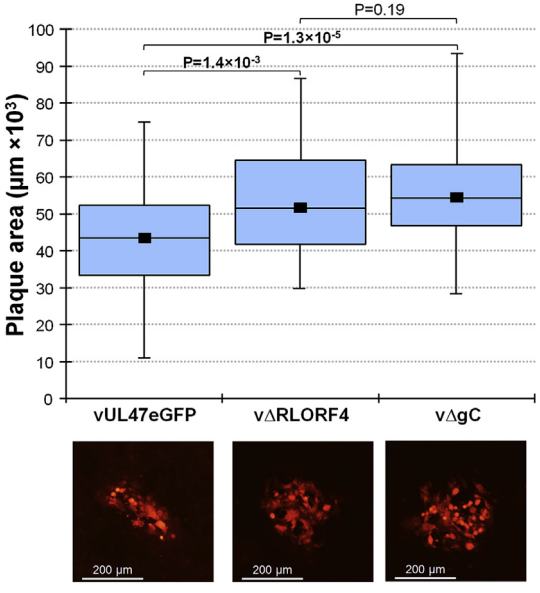

Fig. 2. Replication in tissue culture cells. Plaque areas were measured for viruses reconstituted from rUL47eGFP (vUL47eGFP), rΔRLORF4 (vΔRLORF4), and rΔgC (vΔgC). Both vΔRLORF4 and vΔgC generated significantly larger plaque areas than the parental vUL47eGFP using Student's *t* tests. Representative plaques induced by vUL47eGFP, vΔRLORF4, and vΔgC are shown below each group.

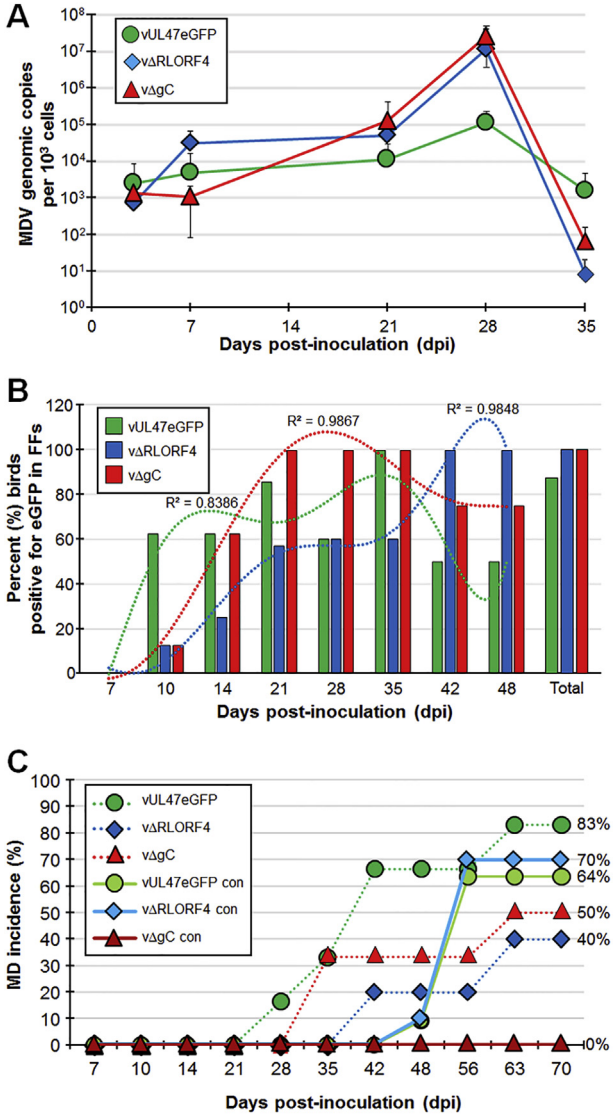

Fig. 3. Replication, MD incidence, and transmission of rMDVs in chickens. Pure Columbian × New Hampshire chickens were experimentally infected with vUL47eGFP, vΔRLORF4, or vΔgC as described in the Materials and Methods (n = 5/group) for 70 days. (A) Replication was monitored in experimentally infected chickens by quantification of genomic MDV genomes in the blood over the first 5 weeks of infection. Shown is the mean MDV genomic copies per 10^3 blood cells ± standard error of means. No significant differences were observed between all groups and time points using Student's *t* tests. (B) Quantitative analysis of the percent of birds with rMDV in the FFs (UL47eGFP) over the course of the experiment, including the total number of birds over the course of the experiment. Using One-Way ANOVA, there was no significant difference in the total number of chickens positive for rMDV in the FFs (*f*-ratio value = 1.20103; *p*-value = 0.327724). Trend lines for each group were added using Microsoft Excel using the polynomial setting at an order of 6 and R^2 values for each trend line are shown. (C) Total MD incidence was determined by identification of gross lesions in euthanized chickens in both experimentally- and naturally- (contact) infected chickens. Although MD incidence induced by vΔRLORF4 (40%) and vΔgC (50%) in experimentally chickens was much lower than vUL47eGFP (83%), these differences were determined to be not significant using Fisher's exact test at $p < 0.05$ (vUL47eGFP vs. vΔRLORF4, $P = 0.2424$; vUL47eGFP vs. vΔgC, $P = 0.5455$). In contact chickens naturally infected, 64 and 70% of chickens housed with vUL47eGFP (vUL47eGFP con) or vΔRLORF4 (vΔRLORF4 con) developed MD, while 0% of contact chickens housed with vΔgC (vΔgC con) developed MD. There was no significant difference between vUL47eGFP con and vΔRLORF4 con groups, while the vΔgC con group was significantly different between vUL47eGFP ($P = 0.0039$) and vΔRLORF4 ($P = 0.0031$).

vΔRLORF4- and vΔgC-generated plaque sizes. These results show that even in the restored RB-1B rMDV, deletion of RLORF4 results in increased plaque sizes (replication) that is indicative of attenuation *in vitro* (Jarosinski et al., 2005; Spatz et al., 2008; Spatz and Silva, 2007).

3.3. RLORF4 is not required for interindividual spread

Although RLORF4 has been shown to be important for pathogenesis of transmission-deficient MDV (Jarosinski et al., 2005), its importance during *in vivo* attenuation and interindividual spread has not been evaluated in transmission-competent MDV. To test *in vivo* replication, chickens were inoculated with 2000 PFU of vUL47eGFP, vΔRLORF4, or vΔgC and naïve contact chickens were housed with experimentally infected birds to evaluate interindividual spread through natural infection. A third vΔgC group was included as a negative control for interindividual spread and gC expression.

MDV genomic copies were measured in blood from experimentally infected chickens over 35 days pi and showed no significant difference between all groups at each time point (Fig. 3A). In order to determine whether there was a difference in each virus reaching the FFs required for transmission, FFs were plucked from each infected bird and expression of vUL47eGFP in FFs was observed using a fluorescent stereoscope. Fig. 3B shows that each virus readily reached the FFs with 100% of vΔRLORF4 and vΔgC infected chickens having positive FFs by 48 days, while 88% of vUL47eGFP birds were positive. There was no significant difference between each group, though vUL47eGFP tended to reach the FFs faster than both vΔRLORF4 and vΔgC.

MD incidence was determined for each group in both experimentally infected and contact chickens. MD was induced in 83, 40, and 50% of chickens experimentally infected with vUL47eGFP, vΔRLORF4, and vΔgC, respectively (Fig. 3C); however, these were not significant using Fisher's exact test. These results show that there were no significant differences between each group in virulence when birds were experimentally infected.

No contact chickens housed with vΔgC (vΔgC con) developed MD over the course of 70 days and these birds were determined to be uninfected based on a lack of viral DNA in the blood using qPCR assays and anti-MDV antibodies in their serum using IFAs at termination of the experiment (data not shown). This is consistent with the requirement of MDV gC for interindividual spread in chickens (Jarosinski et al., 2007; Jarosinski and Osterrieder, 2010, 2012). In contrast, vUL47eGFP and vΔRLORF4 were able to efficiently infect contact chickens with 64 and 70%, respectively, developing MD. These result show that RLORF4 is not required for interindividual spread or natural infection of MDV. In all, these results show that deletion of RLORF4 in a fully virulent, transmission-restored rMDV is not required for interindividual spread of MDV.

3.4. RLORF4 is not required for gC expression in FFE cells

Our data thus far show that RLORF4 is not important for replication and chicken-to-chicken transmission of MDV. We originally hypothesized that RLORF4 was important for gC expression and subsequently host-to-host transmission; however, our data suggests this is not the case. To address our original question directly, we used IFA and western blotting to evaluate gC expression in FFE cells of infected chickens. Feathers plucked from infected chickens were fixed and stained using anti-MDV gC antibody. Fig. 4A shows both vUL47eGFP- and vΔRLORF4-infected FFs abundantly express gC, while vΔgC-infected FFs were negative for gC protein. Fig. 4B shows western blotting for gC in FFE cells scraped from FFs, consistent with IFA staining of FFs. Thus, RLORF4 does not affect gC expression in FFs which is consistent with it being dispensable for interindividual spread in chickens.

4. Discussion

In this report, we tested the importance of RLORF4 for expression of gC and interindividual spread of MDV. Based on former data from multiple laboratories, we hypothesized that the decrease in expression of MDV gC and inability of attenuated MDV to transmit from chicken-to-chicken following passage *in vitro* was directly due to deletion of RLORF4. However, the most direct study of RLORF4 in attenuation used a rMDV that lacked gC expression and was unable to transmit (Jarosinski et al., 2005). To address the role RLORF4 has on gC expression and, consequently, chicken-to-chicken transmission, gC and transmission competent rMDV was used in this report.

Deletion of both copies of RLORF4 resulted in increased plaque sizes *in vitro*, which was consistent with attenuated nature of MDV (Jarosinski et al., 2005) and rMDV lacking gC (vΔgC) generated similar plaque sizes as vΔRLORF4. In former studies, it was presumed the increased plaque sizes seen with attenuated MDV was because these viruses lacked RLORF4, and subsequently, gC expression was ablated, resulting in larger plaques sizes. However, our results show that the increased plaque sizes induced by viruses lacking RLORF4 or gC are independent of each other, thus RLORF4 enhances replication in tissue culture cells through a mechanism independent from gC.

In chickens experimentally infected with rMDV, no significant differences were observed between each group for replication or MD incidence (Fig. 3). This is consistent with former studies on MDV gC playing no role in replication and MD incidence in experimentally infected chickens (Jarosinski et al., 2007; Jarosinski and Osterrieder, 2010, 2012). However, our previous studies showed that RLORF4 was important for replication and disease induction in experimentally infected chickens (Jarosinski et al., 2005). Although disease induction was reduced from 83% for vUL47eGFP- down to 40% for vΔRLORF4-infected chickens (Fig. 3C), this was not a significant decrease and therefore suggest no attenuation. There are at least three potential reasons why the level of MD incidence was not significant. First, it could be due to the low number of experimentally infected birds for each group (n = 5), since our primary goal was to examine transmission and used more birds for this group (n = 11). Secondly, the lack of attenuation could be due to using different chicken lines in the former and current studies, as P2a chickens (Cole, 1968) were used previously, while Pure Columbian × New Hampshire chickens were used in the current study. The PC × NH line has not been characterized for MD resistance/susceptibility to date. Thirdly, the rMDV used previously had numerous mutations (Spatz et al., 2007), including frame-shifts within the UL13 protein kinase, UL44 (gC), and US6 (gD) genes that generated truncated proteins, and lacked the US2 gene; while the rMDV used in the current study was fully repaired. Most likely, these additional mutations contributed to the severely attenuated nature of RLORF4-null virus in the former studies, while the current report suggests only minor attenuation based on the combined *in vitro* and *in vivo* data. In all, these three reasons could explain the insignificant result in MD induction (Fig. 3).

Consistent with abundant gC expression in FFs (Fig. 4), vΔRLORF4 was able to efficiently transmit from chicken-to-chicken as wild type virus (vUL47eGFP) conclusively showing that RLORF4 is not involved in gC expression nor interindividual transmission of MDV. Currently, it is not known why expression of gC is severely affected following serial passage of MDV in tissue culture cells, but the data presented here conclusively show that RLORF4 is not involved in this process. Recently, the role of the *Herpesviridae* conserved ICP27 regulatory protein, encoded by MDV UL54 (ICP27) has been shown to be essential for expression of MDV gC in cell culture (Ponnuraj et al., 2019). Future work in our laboratory focuses on the role ICP27 plays in attenuation and gC expression.

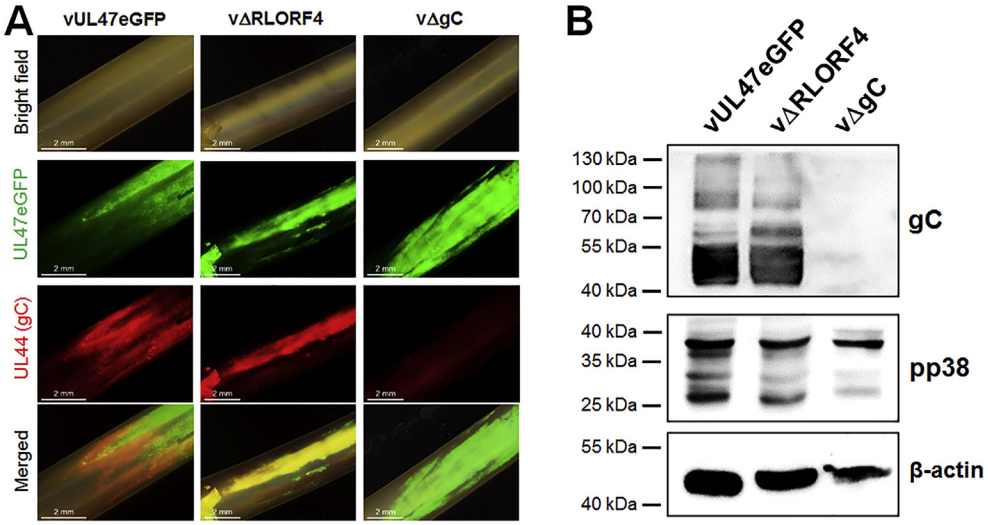

Fig. 4. Expression of gC in FFs of infected chickens. (A) Feathers were plucked from vUL47eGFP-, vΔRLORF4-, and vΔgC-infected birds at 28 dpi, fixed, then stained using anti-MDV gC antibody. FFs obtained from vUL47eGFP- and vΔRLORF4-infected chickens were positive for gC protein, while FFs from vΔgC-infected birds were negative. (B) Western blot analysis for gC in FFEs. (A) Whole cell protein lysates were collected from FFE cells scraped from FFs infected with vUL47eGFP, vΔRLORF4, or vΔgC, electrophoresed through a 15% SDS-PAGE gel, transferred to nitrocellulose membranes, and probed for MDV gC as described in the Materials and Methods. Anti-β-actin antibody was used as internal cellular control.

Conflicts of interest

The authors declare no conflicting financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.06.008.

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