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Lorf9 deletion significantly eliminated lymphoid organ atrophy induced by *meq*-deleted very virulent Marek's disease virus

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ABSTRACT

Marek's disease virus (MDV) is a highly contagious alphaherpesvirus that causes rapid onset of T cell lymphomas in chickens. MDV continues to break through vaccinal immunity due to the emergence of highly virulent field strains. Earlier studies revealed that deletion of the *meq* gene from MDV results in attenuated vaccines that protect against disease when chickens are infected with highly virulent strains. However, *meq*-deleted viruses still retain the ability to induce lymphoid organ atrophy, which raises safety concerns. In an earlier study, we found that deletion of *lorf9* counteracts this lymphoid organ atrophy. Here, we describe the generation of a double deletion mutant virus lacking virus-encoded *meq* and *lorf9*. *In vitro* studies revealed that during replication, the mutant virus had kinetic characteristics similar to the parental virus; however, *in vivo* the replication capability was significantly reduced. Results of animal studies revealed no obvious MDV-specific symptoms and lesions. Importantly, the double deletion mutant virus lost the capacity to induce lymphoid organ atrophy, which has been the main obstacle during development of a good vaccine candidate.

1. Introduction

Marek's disease (MD) is induced by the highly contagious Marek's disease virus (MDV) and is characterized by severe immunosuppression, neurological symptoms, and rapid onset of T cell lymphoma in the visceral organs (Calnek, 2001). MDV is classified into three serotypes. MDV serotype 1 (gallid herpesvirus type 2, MDV-1) includes all the pathogenic strains and their derivatives. MDV serotype 2 (gallid herpesvirus type 3, MDV-2) and 3 (meleagrid herpesvirus type 1, MDV-3) are non-oncogenic viruses. MD is the first chicken tumor disease that has been successfully controlled by one or bivalent MDV serotype-based vaccine (Reddy et al., 2017). However, the continued evolution of MDV virulence has prompted concern that all the currently available vaccines will soon be irrelevant. Much effort has been devoted to develop more efficacious vaccines in the last few decades. Researchers have concluded that the classical vaccine development method may have approached the biological threshold of vaccine efficacy (Witter and Kreager, 2004). New strategies are needed for the next generation of vaccine development.

The unique genomic structure of MDV includes a unique long (U_L) region flanked by two inverted repeat (IR) regions [*i.e.*, the terminal repeat long (TR_L), and the internal repeat long (IR_L), region]. A unique short (US) region is flanked by two IR regions: the terminal repeat short (TR_S) and the internal repeat short (IR_S) regions (Lee et al., 2000; Tulman et al., 2000). MDV encodes over 100 genes. Improvements in gene editing technologies have greatly facilitated the introduction of mutations into the viral genome. Interestingly, a series of gene deletion mutant viruses have shown great potential to be good vaccine candidates. However, most of them are partially attenuated and retain oncogenicity (Brown et al., 2006; Cui et al., 2005; Lee et al., 2008; Petherbridge et al., 2003). MDV encodes several tumor formation-related genes, such as MDV EcoR I Q (*meq*) and virus-encoded telomerase RNA (*vTR*) (Kaufer et al., 2011, 2010; Osterrieder et al., 2006). A previous report described a Meq deletion mutant virus, rMd5 ΔMeq, with a complete loss of transformation properties in MDV maternal antibody negative (Ab-) chickens (Lupiani et al., 2004). Interestingly, rMd5 ΔMeq provided better protection than CVI988/Rispens against challenge with the most virulent virus (Lee et al., 2010, 2008). Although rMd5 ΔMeq has the potential to be an effective vaccine, it still maintains the ability to induce chicken lymphoid organ atrophy (Dunn and Silva, 2012). This disadvantage has raised safety concerns and has prevented the commercialization and licensing of this candidate

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vaccine. Cell culture attenuation was able to eliminate the rMd5 ΔMeq-induced lymphoid organ atrophy, rendering the mutant virus an effective and safe MD vaccine (Lee et al., 2012). However, the attenuation of virulent viruses using cell culture passage is a time-consuming and expensive process. The attenuated mutant virus also retains the potential to revert to the virulent strain *in vivo*. To improve the safety and usefulness of rMd5 ΔMeq as a vaccine candidate, other molecular factors need to be considered to eliminate the problem of lymphoid organ atrophy.

Upon infection in susceptible chickens, four phases of MDV-induced pathogenesis are generally recognized: early cytolytic infection, latency, reactivation, and transformation. Early cytolytic infection occurs primarily in B-lymphocytes 3–6 days post-infection. Cytolytic replication then subsides, and latency is established 7–10 days post-infection in activated CD4+ T-lymphocytes. Reactivation (late cytolytic infection) describes the recurrence of cytolytic replication in response to intra- and/or extracellular stimuli. Transformation then takes place in latently infected cells 14 days post-infection (Calnek, 2001). It's worth noting that several genes are necessary for cytolytic infection, but have no effect on transformation. Deleting these cytolytic infection-associated genes could induce a significant reduction in viral pathogenesis, including lymphoid organ atrophy (Cui et al., 2005; Gimeno et al., 2005).

In an earlier study, we successfully deleted the *lorf9* gene, which is located on the right terminus of the Md5 UL region (Lee et al., 2000; Tulman et al., 2000). *Lorf9* deletion significantly reduced the pathogenesis of its parental virus with no obvious lymphoid organ atrophy in infected chickens. These results indicate that LORF9 might play an important regulatory role during MDV-induced pathogenesis (Unpublished data), especially in the early cytolytic replication phase. Thus, to overcome the drawback of lymphoid organ atrophy caused by the *meq* deletion, we introduced a secondary mutation in the MDV genome and deleted *lorf9*, a gene that is important in the early cytolytic replication phase in lymphoid organs. Based on the characteristics of *meq* or *lorf9* deletion mutant viruses, we hypothesized that double deletion of meq and *lorf9* might reduce lymphoid organ atrophy associated with MDV-ΔMeq. We therefore constructed a *meq* and *lorf9* double deletion mutant virus, characterized its *in vitro* and *in vivo* growth kinetics properties, and evaluated its pathogenesis in infected chickens.

2. Material and methods

2.1. Cells, viruses, and reagents

Primary chicken embryonic fibroblasts (CEF) were used for virus propagation, the virus reactivation assay, and the DNA transfection assay. The recombinant viruses Md5BAC ΔMeq and Md5BAC ΔMeqΔLORF9 were generated from Md5BAC. CEF cells were cultured in Leibowitz-McCoy (LM) medium supplemented with 5% bovine calf serum and penicillin-streptomycin at 37 °C.

2.2. Construction of meq and lorf9 double deletion mutant virus

To construct the *meq* and *lorf9* deletion mutants from the Md5BAC, we followed a two-step Red-mediated recombination procedure as previously described (Schumacher et al., 2000; Tischer et al., 2006), using an Md5BAC clone. During the first step, the *Kan^R-I-SecI* cassette was amplified from pEPkan-S using specific primers with homologous sequences for downstream recombination events. The purified PCR product was electroporated into the BAC-containing *E. coli*, where the *meq* sequences were replaced with the *Kan^R-I-SecI* cassette. A second recombination step was carried out by the addition of arabinose to the growth medium, resulting in induction of I-SecI expression and cleavage of Md5BAC-*Kan^R*, and subsequent deletion of *Kan^R* followed by recombination of the BAC sequences to generate one copy of the *meq* deletion mutant. Owing to the presence of two copies of *meq* in the MDV genome, this procedure was repeated to generate the Md5BAC ΔMeq mutant virus. The same procedure was used to construct *lorf9* deletion and double deletion mutant viruses. All deletion mutants were screened by PCR and sequencing to confirm the absence of unexpected mutations. Primer sequences used to construct the mutant viruses will be made available upon request. Transfection of BAC DNA into CEFs resulted in recovery of infectious virus. All the reconstituted viruses were amplified on CEFs less than five passages old and stored in liquid nitrogen.

2.3. Growth kinetics assay

The growth kinetics of Md5BAC ΔMeqΔLORF9, Md5BAC ΔMeq, and Md5BAC viruses were determined as described previously (Lupiani et al., 2004). Briefly, CEF cells seeded on 60 mm plates were infected with 100 PFUs of the different viruses. On days 0, 1, 2, 3, 4, 5, and 6 post-inoculation, the infected cells were trypsinized, fresh CEF cells seeded on 35-mm plates were infected with serial dilutions, and plaques of different dilutions were counted 7 days post-infection.

2.4. Viral genomic copy number measurement

Genomic DNA was extracted from chicken splenocytes using the phenol-chloroform method (Sun et al., 2013). Quantification of MDV genomic copies number was performed by qPCR. Primers specific for the MDV-infected cell protein (*ICP4*) gene (forward primer: 5′-TTATT GCCCCGTACTCACCG-3′, reverse primer: 5′-CATTTAAAGTCTTTCCAT GCCAAAC-3′) and the chicken Ovotransferrin gene (*OVO*) (forward primer: 5′-CACTGCCACTGGGCTCTGT-3′, reverse primer: 5′-GCAATG GCAATAAACCTCCAA-3′) were designed as described before (Baigent et al., 2005; Zhang et al., 2015). For the generation of standard curves, we used Md5BAC and plasmid pcDNA 3.1-*OVO*, respectively, as templates. The standard curves were determined by 10-fold serial dilution, starting from 300,000 copies to 30 copies. The conversion between copy numbers and the mass of plasmids can be calculated using the following formula: Mass of single plasmid = [DNA size (bp)] × [1 mol/ 6.02×10^{23} mol (bp)] × [Average molecular weight of double strand DNA (660 g)/mol]. We plotted the cycle threshold (Ct) values with Md5BAC or plasmid pcDNA 3.1-*OVO* copy numbers to generate each of the standard curves. All qPCR assays were performed on a Quant Studio 5 Detection System (ThermoFisher, USA), and the results were analyzed by Quant Studio™ Design and Analysis Software Version 1.4.3. The 20 µl reaction contained 5 µl (10 ng/µl) of plasmid DNA or cellular genomic DNA, 1 µl of each primer (10 pmol), and 10 µl of FastStart Universal SYBR Green Master (ROX). The thermal cycling conditions were as follows: 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min. The copy numbers of *ICP4* or *OVO* were calculated using the standard curve of each gene. The relative viral genomic copy number is expressed as the ratio of the *ICP4* copy number to the *OVO* copy number.

2.5. Evaluation of Md5BAC ΔMeqΔLORF9 pathogenesis in SPF chickens

Specific pathogen free chickens (SPF) (SPF Egg & Poultry Co, Jinan, China) that were free of maternal antibodies against MDV and specific pathogens were used. Chickens were wing-banded upon hatching, randomly sorted into the experimental groups, housed in modified Horsfall-Bauer isolators, and provided food and water *ad libitum*.

To analyze the pathogenic properties of Md5BAC ΔMeqΔLORF9, three groups of one-day-old SPF chickens were infected subcutaneously with 2000 PFU of Md5BAC ΔMeq, Md5BAC ΔMeqΔLORF9, or Md5BAC. An additional group was not infected with anything and served as the negative control. All chickens that died during the course of the experiment or were sacrificed at the end of the experiment (63 days post-inoculation) were necropsied and examined for gross MD-induced tumors.

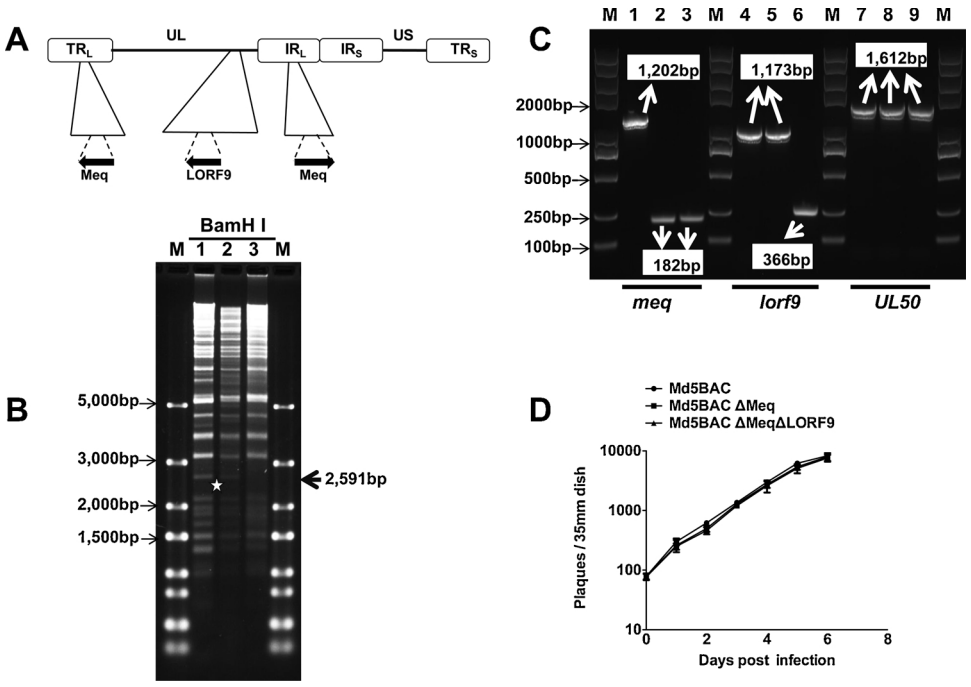

Fig. 1. LORF9 deletion virus construction, identification, and *in vitro* characterization. (A) The MDV genome consists of a unique lone (UL) region flanked by two inverted repeats regions, including the terminal repeat long (TR_L) and internal repeat long (IR_L) regions, and a unique short region (U_S) flanked by two inverted repeats regions, including the terminal repeat short (TR_S) and internal repeat short (IR_S) regions. Locations of the *meq* and *lorf9* genes used to generate double deletion mutant virus. (B) PCR analysis of viral genomes with the use of primers specific to *meq, lorf9*, and *UL50*. Lane 1 represents amplification of *meq* from Md5BAC; Lane 2 represents amplification of *meq* from Md5BAC ΔMeq; Lane 3 represents amplification of *meq* from Md5BAC ΔMeqΔLORF9; Lane 4 represents amplification of *lorf9* from Md5BAC; Lane 5 represents amplification of *lorf9* from Md5BAC ΔMeq; Lane 6 represents amplification of *lorf9* from Md5BAC ΔMeqΔLORF9; Lane 7 represents amplification of *UL50* from Md5BAC; Lane 8 represents amplification of *UL50* from Md5BAC ΔMeq; Lane 9 represents amplification of *UL50* from Md5BAC ΔMeqΔLORF9. (C) RLFP analysis of all constructs. DNA were digested with *BamH* I. Digestion products were separated by gel electrophoresis on a 1% agarose gel and stained with ethidium bromide. Asterisk represents fragment size differences between Md5BAC (lane 1), Md5BAC ΔMeq (lane 2), and Md5BAC ΔMeqΔLORF9 (lane 3). M: DNA marker ladder. (D) *In vitro* growth kinetics. CEF were infected with 100 PFUs of the indicated virus separately, and on days 0, 1, 2, 3, 4, 5, and 6 post-inoculation, the cells were trypsinized and seeded on fresh CEF. Virus titers were determined 7 days post-inoculation on fresh CEF. The experiment was performed in duplicate, and virus titer is indicated as PFUs for each 35-mm dish. Results represent mean values with error bars showing the standard error of the mean.

2.6. Hematoxylin and Eosin (H&E)-staining assay

For H&E staining, tissues were embedded in optimal cutting temperature compound, frozen in liquid nitrogen, and stored at -80 °C until use. 6–8 μm-thick cryostat sections of tissue blocks were prepared and fixed with cold acetone for 10 min, and then air-dried. H&E staining was carried out following standard protocols. Lymphocyte infiltration was examined under a microscope (Nikon Eclipse E100).

2.7. Evaluation of lymphoid organ atrophy in Md5BAC ΔMeqΔLORF9-infected SPF chickens

To evaluate the effect of virus replication on lymphoid organ atrophy, one-day-old SPF chickens were infected subcutaneously with 2000 PFU of Md5BAC, Md5BAC ΔMeq, or Md5BAC ΔMeqΔLORF9. An additional group was not infected with anything and served as the negative control. Five chickens from each group were euthanized 14 days post-infection, and their lymphoid organs (thymus and bursa) were collected and weighed. Lymphoid organ atrophy was determined by relative lymphoid organ weight, and was expressed as the weight of lymphoid organs divided by the body weight of each chicken multiplied by one hundred.

2.8. Data and statistical analysis

For virus growth kinetics analysis, two-way ANOVA statistical analysis was used at individual time points. Each data point derived from the plaque counting assay represents an average of duplicates. The relative organ weight ratios were averaged in each group and analyzed with the Student *t* test. All statistical analysis was performed with Graphpad Prism version 5.01 software (GraphPad Software, Inc. La Jolla, CA). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. The Md5BAC ΔMeqΔLORF9 double mutant was successfully generated

We constructed a mutant virus, Md5BAC ΔMeqΔLORF9, in which the entire coding sequences of the *meq* and *lorf9* genes were deleted (Fig.1A). PCR amplification was used to confirm that the deletions were successful. The MDV *UL50* gene was used as an internal control because it was not affected by the deletion. The results indicated that the *meq* gene was amplified in Md5BAC (lane 1), but not in Md5BAC ΔMeq (lane 2) or Md5BAC ΔMeqΔLORF9 (lane 3). The *lorf9* gene was amplified in Md5BAC (lane 4) and Md5BAC ΔMeq (lane 5), but not in Md5BAC ΔMeqΔLORF9 (lane 6). The *UL50* gene could be amplified in Md5BAC (lane 7), Md5BAC ΔMeq (lane 8), and Md5BAC ΔMeqΔLORF9 (lane 9). The PCR results indicate that all of the deletion mutant constructs were correct (Fig. 1B). To confirm that there were no major rearrangements in the BAC clones, Md5BAC, Md5BAC ΔMeq, and Md5BAC ΔMeqΔLORF9 were subjected to restriction fragment length polymorphism (RFLP) analysis. There was a *BamH* I restriction fragment change of 2591 bp in Md5BAC ΔMeqΔLORF9 compared to Md5BAC and Md5BAC ΔMeq (Fig. 1C). These results further indicate that we successfully generated a double deletion recombinant construct.

3.2. The double mutant has a significantly reduced viral replication rate in vivo

All of the deletion mutant viruses were rescued and amplified in CEF cells. The growth kinetics of Md5BAC, Md5BAC ΔMeq, and Md5BAC ΔMeqΔLORF9 were compared to determine whether the double deletion affected viral replication properties *in vitro*. Viral titers at each time point were similar for all viruses (Fig. 1D). This result suggests that the double deletion mutant did not affect viral replication *in vitro*.

The early cytolytic infection occurs on days 3–6 post MDV infection. The latent phase begins 7–8 days post-infection, or slightly later. To

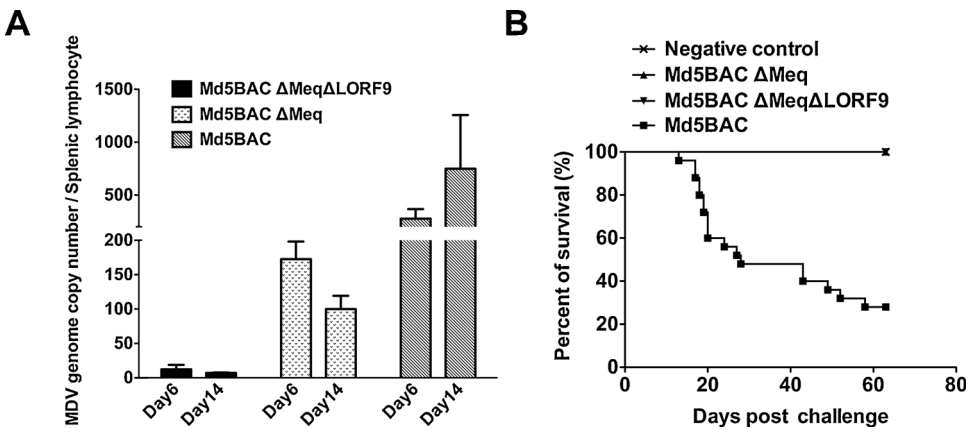

Fig. 2. *In vivo* characterization of mutant virus replication and pathogenic experiment on SPF chickens.
(A) On day 6 post-infection, the genomic DNA of infected splenic cells was extracted and viral genomic copies were measured by qPCR. Results are presented as the mean values with error bars showing the standard error of the mean. (B) SPF chickens were infected subcutaneously with 2000 PFU of Md5BAC ΔMeq, Md5BAC ΔMeqΔLORF9, or Md5BAC. Mortality rates (expressed as survival percentages on each day post-infection) are shown for each group.

examine whether the double deletion mutant virus has an altered viral load in the spleen during the early cytolytic and latent stages of infection, one-day-old SPF chickens were infected with the same dose of Md5BAC ΔMeqΔLORF9, Md5BAC ΔMeq, or Md5BAC virus. The replication rates were compared in spleen samples taken on days 6 and 14 post-inoculation. On day 6, the Md5BAC ΔMeq chickens had lower genomic copy numbers than the Md5BAC chickens ($p > 0.05$), but significantly higher copy numbers than the Md5BAC ΔMeqΔLORF9 chickens. On day 14, the Md5BAC ΔMeq animals had significantly reduced genomic copy numbers. However, the genomic copy number was low on day 6, and even lower on day 14, in the Md5BAC ΔMeqΔLORF9 group compared to the Md5BAC ΔMeq group (Fig. 2A). The *in vivo* viral genomic copy number results suggested that double deletion of Meq and LORF9 significantly reduced the viral replication rate.

3.3. Md5BAC ΔMeqΔLORF9 was fully attenuated

Meq gene deletion in the MDV genome induced a complete loss of oncogenicity, and *lorf9* gene deletion significantly reduced tumorigenesis (data not shown). Therefore, we hypothesized that double deletion would result in an attenuated virus. To test the pathogenic properties of the double mutant virus, one-day-old SPF chickens were infected with Md5BAC, Md5BAC ΔMeq, or Md5BAC ΔMeqΔLORF9 and observed for mortality for a 63-day duration. All of the chickens that died during the experiment or upon termination of the experiment were examined for MDV-specific lesions, including gross tumors and nerve lesions. MDV-associated mortality began 13 days after infection in the Md5BAC group chickens; 7 out of 25 (28%) chickens survived the entire duration of the experiment (Fig. 2B). All the chickens in the Md5BAC groups had MDV-specific gross lesions. However, there was no MDV-specific mortality or tumors apparent in the Md5BAC ΔMeqΔLORF9 group. These results indicate that the Md5BAC ΔMeqΔLORF9 virus was fully attenuated (Table 1). To further evaluate the pathogenicity on day 63, histological lesions were examined in the spleen. The results showed lymphocyte infiltration in the spleens of Md5BAC- infected chickens, but not in Md5BAC ΔMeqΔLORF9-infected chickens (Fig. 3).

Table 1
Comparison of MDV mortality and lesions incidence in SPF chickens.

Virus[a]	No. of chickens that died/No. tested (%)	No. of chickens with MD lesion/No. tested (%)
None	0/24 (0)	0/24 (0)
Md5BAC	18/25 (72)	25/25 (100)
Md5BAC ΔMeq	0/23 (0)	0/23 (0)
Md5BAC ΔMeqΔLORF9	0/21 (0)	0/21 (0)

[a] All chickens were inoculated with 2,000PFU of the indicated viruses. None: means no virus inoculation.

3.4. Md5BAC ΔMeqΔLORF9 does not induce lymphoid organ atrophy in infected SPF chickens

MDV infection can induce immunosuppression and cause lymphoid organ atrophy. Severity of disease depends on viral virulence and susceptibility of the host. We therefore examined whether Md5BAC ΔMeqΔLORF9 induced lymphoid organ atrophy. One-day-old SPF chickens were infected with Md5BAC, Md5BAC ΔMeq, or Md5BAC ΔMeqΔLORF9. Lymphoid organs were collected and examined 14 days post-infection. The relative lymphoid organ weight results indicated that Md5BAC ΔMeq and Md5BAC induced severe bursa and thymus atrophy ($p < 0.05$), compared to the negative control. This change did not occur in the Md5BAC ΔMeqΔLORF9 chickens (Fig. 4). These results indicate that the double deletion mutant virus did not cause lymphoid organ atrophy.

4. Discussion

MDV encodes several unique genes that are involved in the early cytolytic replication phase, such as pp38 (phosphorylated protein 38 KD) and vIL8 (virus-encoded interleukin 8). The *pp38* gene spans the junction between the IR_L and U_L regions. The *vIL8* gene is located in the repeat long regions of the MDV genome and consists of three exons separated by two introns, and is a homolog of cellular IL-8. Either *pp38* or *vIL8* deletion could significantly reduce the virulence of a parental virus (Engel et al., 2012; Osterrieder et al., 2006). Interestingly, the *meq* and *vIL8* double deletion mutant was fully attenuated and conferred better protection than CVI988/Rispens against a vv + MDV challenge (Sun et al., unpublished data). These findings strongly suggest that MDV-encoded cytolytic replication-related genes could be an alternative choice for the introduction of secondary mutations in the MDV genome.

As a unique gene, *lorf9* shares some identity between three MDV serotypes, but not with other alphaherpesvirus, although its function in MDV pathogenesis is still unknown. We previously generated a *lorf9* deletion mutant virus named Md5BAC ΔLORF9. This mutant virus has attenuated virulence and infected animals have a significantly reduced risk (*i.e.* incidence) of gross lesion development and mortality during pathogenesis of the disease (Unpublished data). These results indicate that LORF9 might play an important regulatory role during MDV pathogenesis. In the present study, we generated a *meq* and *lorf9* double deletion mutant virus, which shared characteristics of both the *meq* and *lorf9* single deletion mutants. Like the single deletion mutant viruses, Md5BAC ΔMeqΔLORF9 replicated well *in vitro* (Fig. 1D). Further characterization of this virus *in vivo* showed that it replicates at significantly lower levels than Md5BAC ΔMeq in the spleen, as measured by real-time PCR on days 6 and 14 post-inoculation (Fig. 2A). These results show that the double gene deletion did not affect virus replication *in vitro*, but significantly reduced its replication *in vivo*.

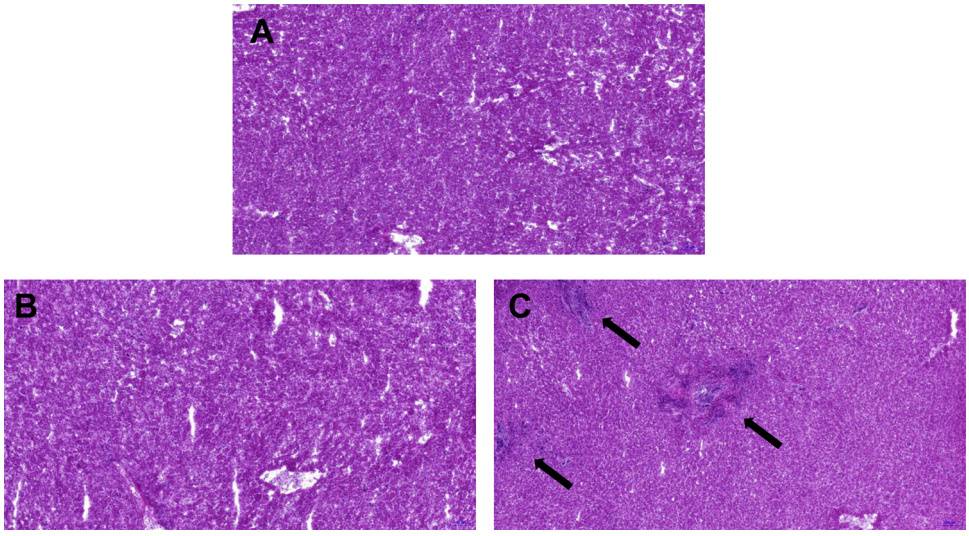

Fig. 3. Histological analysis of lesions in virus-infected spleens.
Hematoxylin and eosin (H&E)-stained sections of spleens. (A) Uninfected chickens. (B) Md5BAC ΔMeqΔLORF9. (C) Md5BAC, 63 days post-inoculation. 6–8 μm-thick cryostat sections of the tissue were stained with H&E (Scale bar is 100 μm).

The severity of lymphoid organ atrophy correlates with MDV virulence. Witter et al. found that the relationship between lymphoid organ atrophy and MDV virulence is not always consistent in all viral strains, but there is a tendency for vMDV to induce less severe atrophy compared with vv and vv+ (Witter et al., 2005). The *meq* deletion mutant virus has lytic infection *in vivo* comparable to that of the parental virus, which induces severe lymphoid organ atrophy (Fig. 4) (Lupiani et al., 2004). In an earlier study, we found that deletion of *lorf9* significantly reduces the virulence of the early cytolytic replication phenotype (Unpublished data). Comparing the viral genomic copy number in chickens at the early cytolytic infectious phase (day 6), Md5BAC ΔMeq had significantly higher copy numbers compared to Md5BAC ΔMeqΔLORF9. This result suggests that the lymphoid organ atrophy might be the direct result of robust cytolytic viral replication in the lymphoid organ. Dunn et al. reported that virus replication is highly correlated with relative thymus weight between 2 and 8 weeks post-infection (Dunn and Silva, 2012). However, the correlation between early cytolytic infection and lymphoid organ atrophy was not determined, because the latent phase occurs 2 weeks post-infection. Lee et al. reported that rMd5 and rMd5 ΔMeq/19 (cell-culture passage 19) viruses have robust replication in lymphoid organs and reduced lymphoid organ atrophy severity. Neither detectable viral DNA nor lymphoid organ atrophy occurred in chickens infected with rMd5 ΔMeq/40 and rMd5 ΔMeq/50 (cell-culture passage 40 and 50) (Lee et al., 2012). This result also revealed the association between viral replication and lymphoid organ atrophy. In the present study, the lower viral replication in the lymphoid organ of chickens infected with Md5BAC ΔLORF9ΔMeq was likely the reason that lymphoid organ atrophy did not occur (Fig. 2A and Fig. 4).

The MDV genome copy numbers in the spleens of infected chickens are a good measure of early cytolytic infection and latency. Md5BAC ΔMeqΔLORF9 replicated poorly during early cytolytic infection (day 6) resulting in low viral load compared to parental Md5BAC and Md5BAC ΔMeq viruses (Fig. 2A). However, since Md5BAC ΔLORF9 virus may cause lymphocyte transformation, by day 14 there was a four-fold increase in virus genome copy number (data not shown). On the other hand, at the same time point, the genome copy number in the Md5BAC ΔMeq group decreased two-fold in agreement with a lack of transformation (Fig. 2A). As a result of the role of Meq and LORF9 in virus replication and transformation, the virus genome copy number of Md5BAC ΔMeqΔLORF9 virus was low during early cytolytic infection and even lower during the latency phase (Fig. 2A).

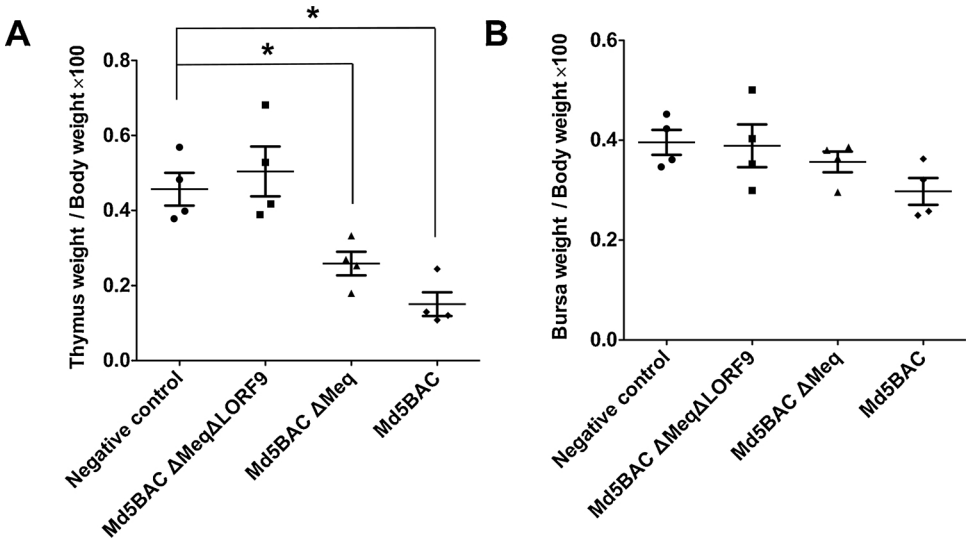

Fig. 4. Evaluation of lymphoid organ atrophy of chickens infected with Md5BAC, Md5BAC ΔMeq, and Md5BAC ΔMeqΔLORF9 viruses.
Body and lymphoid organ weights of negative control chickens and those infected with Md5BAC, Md5BAC ΔMeq, and Md5BAC ΔMeqΔLORF9 were measured and calculated in each group 14 days post-infection. (A) Ratio of thymus weight to body weight. (B) Ratio of bursa weight to body weight. Results represent mean values with error bars showing the standard error of the mean. * indicates $P < 0.05$.

5. Conclusion

The double deletion mutant virus overcame the disadvantage of Meq-deleted mutant virus, which induced lymphoid organ atrophy. Whether this double deletion mutant virus has the potential to be an excellent MDV vaccine needs to be further investigated. In our laboratory, vaccinal experimentation on the double deletion mutant virus is ongoing. This new strategy will pave the way for the next generation of MDV vaccines.

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