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A Single-Amino-Acid Polymorphism in Reovirus Protein $\mu 2$ Determines Repression of Interferon Signaling and Modulates Myocarditis

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Myocarditis is indicated as the second leading cause of sudden death in young adults. Reovirus induces myocarditis in neonatal mice, providing a tractable model system for investigation of this important disease. Alpha/beta-interferon (IFN-α/β) treatment improves cardiac function and inhibits viral replication in patients with chronic myocarditis, and the host IFN-α/β response is a determinant of reovirus strain-specific differences in induction of myocarditis. Virus-induced IFN-β stimulates a signaling cascade that establishes an antiviral state and further induces IFN-α/β through an amplification loop. Reovirus strain-specific differences in induction of and sensitivity to IFN-α/β are associated with the viral M1, L2, and S2 genes. The reovirus M1 gene-encoded $\mu 2$ protein is a strain-specific repressor of IFN-β signaling, providing one possible mechanism for the variation in resistance to IFN and induction of myocarditis between different reovirus strains. We report here that $\mu 2$ amino acid 208 determines repression of IFN-β signaling and modulates reovirus induction of IFN-β in cardiac myocytes. Moreover, $\mu 2$ amino acid 208 determines reovirus replication, both in initially infected cardiac myocytes and after viral spread, by regulating the IFN-β response. Amino acid 208 of $\mu 2$ also influences the cytopathic effect in cardiac myocytes after spread. Finally, $\mu 2$ amino acid 208 modulates myocarditis in neonatal mice. Thus, repression of IFN-β signaling mediated by reovirus $\mu 2$ amino acid 208 is a determinant of the IFN-β response, viral replication and damage in cardiac myocytes, and myocarditis. These results demonstrate that a single amino acid difference between viruses can dictate virus strain-specific differences in suppression of the host IFN-β response and, consequently, damage to the heart.

Viral infection is the leading cause of myocarditis in North America and Europe (12). This disease can be fatal in infants and, although usually resolved in adults, can lead to dilated cardiomyopathy and cardiac failure. Importantly, myocarditis is indicated as the second leading cause of sudden death in young adults (10). Most virus families are implicated in myocarditis in humans (12), with enteroviruses, such as coxsackievirus B (8, 12), adenoviruses (8, 12), and more recently parvovirus B19 (8, 22, 24) as the most frequently identified. While enterovirus-induced myocarditis in mice is predominantly immune mediated, cardiac damage is also due to direct viral cytopathic effect (CPE). Indeed, immunosuppressive therapy is only minimally beneficial in affected humans (30, 40). Furthermore, adenovirus-positive cardiac sections from patients with myocarditis often lack inflammatory cell infiltrates (29). Therefore, the importance of immune-mediated damage in myocarditis is unclear. Reovirus induction of cardiac lesions in newborn mice reflects direct viral CPE in cardiac myocytes (2, 47) and is virus strain specific (46). Thus, reovirus infection in mice provides a useful experimental system to study the direct effects of viral infection on the heart.

The type I interferon (IFN) response is critical for protection of cardiac cells against reovirus infection *in vitro* (48). Accordingly, nonmyocarditic reoviruses induce myocarditis in mice depleted of alpha/beta IFN (IFN-α/β) (48) or lacking a transcription factor critical for the induction of IFN (15). Reoviruses that are either strong inducers of IFN or are most sensitive to IFN-mediated antiviral effects, such as strain type 3 Dearing (T3D), do not induce myocarditis (48). Conversely, reoviruses that are weak inducers of IFN or are highly resistant to its effects, such as strain type 1 Lang (T1L), induce myocarditis (48). Given that cardiac myocytes are essentially nonreplenishable (4) and thus vulnerable to systemic viral infections, the IFN response provides a critical first-line of protection for these cells. Indeed, cardiac myocytes are pre-armed with higher basal expression of IFN-β than neighboring cardiac fibroblasts (55). Moreover, IFN-α (9, 32) and IFN-β (23) treatment has improved cardiac function and inhibited viral replication in patients with chronic myocarditis.

Viral nucleic acids can be recognized by pattern recognition receptors (PRRs), including RIG-I-like receptors, to stimulate intracellular signaling cascades that result in the induction and secretion of IFN-α/β (52). Through autocrine and paracrine signaling, IFN-α/β induces expression of IFN-stimulated genes (ISGs), including those with antiviral activity (37), and the transcription factor, IRF7, which further amplifies IFN expression (17, 42). Viruses have evolved mechanisms to inhibit the induction of IFN, IFN signaling, and ISG protein function (5, 13, 41, 51). Reovirus subverts IFN signaling by a novel mechanism associated with nuclear accumulation of IRF9 (56). Reovirus strain-specific differences in this subversion are determined by the M1 gene-encoded protein $\mu 2$ (56). While strain-specific differences in reovirus re-

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pression of IFN signaling correlate with induction of myocarditis (56), the role of this repression in disease is not known.

The M1 gene is the primary determinant of reovirus strain-specific differences in murine myocarditis (45, 46). Reovirus strain-specific differences in induction of and sensitivity to IFN are also determined by the M1 gene in concert with the L2 and S2 genes (44). In the present study, we investigated μ2 determinants of IFN induction, repression of IFN signaling, viral replication and CPE in cardiac myocytes, and reovirus-induced myocarditis in mice. We found that μ2 amino acid 208 is a determinant of IFN responses, viral spread, and damage in cardiac cells and that this amino acid modulates myocarditis.

MATERIALS AND METHODS

Cells and mice. Mouse L929 cells were maintained in minimal essential medium (SAFC Biosciences) supplemented to contain 5% fetal calf serum (FCS) (Atlanta Biologicals) and 2 mM L-glutamine (Mediatech, Inc.). L929 cells were plated at 5×10^5 cells per well in 24-well clusters and allowed to adhere 2 h prior to infection.

Timed-pregnant Cr:NIH(S) mice from the National Cancer Institute were maintained as a colony in a facility that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal procedures were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Primary cardiac myocyte and fibroblast cultures were generated from 1-day-old neonatal or term fetal Cr:NIH(S) mice resulting from timed pregnancies. Neonatal or term fetal mice were euthanized, and the apical two-thirds of hearts were excised and treated with trypsin (2). Cardiac myocytes were separated from cardiac fibroblasts by differential adherence to culture wells and resuspended in Dulbecco modified Eagle medium (Gibco) supplemented to contain 7% FCS (Atlanta Biologicals) and 10 μg of gentamicin (Sigma-Aldrich Corp.)/ml. Myocyte culture medium also contained 0.06% thymidine (Sigma-Aldrich Corp.). Myocyte cultures contain $\leq$5% fibroblasts, and fibroblast cultures contain $<$1% myocytes (55). Myocyte cultures were plated at 1.5×10^5 cells per well in 96-well clusters or at 5×10^5 cells per well in 48-well clusters. Fibroblast cultures were plated at one-half those densities in 96-well or 48-well clusters, were confluent by the day of infection, and were assumed to double from the initial plating density. Cells were incubated for 2 days prior to infection. Myocyte and fibroblast cultures were not passaged before use.

Two-day-old mice from timed litters were injected in the left hind limb with 20 μl of gel saline (46) containing 10^6 PFU of various virus strains or gel saline as a mock infection. At 7 days postinjection, mice were euthanized, and hearts were removed, fixed in 10% buffered formalin, and sectioned for hematoxylin and eosin (H&E) staining. Sections were photographed with a Nikon AZ100 zoom microscope at $\times$75 magnification. A minimum of eight hearts (with a minimum of 15 sections per heart) were scored as blinded cardiac sections for each virus. Mice were considered "positive" if more than a single lesion was detected in the $\geq$15 sections examined. Lesions in adjacent sections were not scored as independent lesions.

Viruses. All viruses were generated from plasmids by reverse genetics as described previously (20, 38). The recombinant viruses were plaque purified, amplified using L929 cells, purified using CsCl gradients (49), and stored as diluted aliquots at -80°C.

Virus replication. Primary cardiac myocyte and cardiac fibroblast cultures were plated in 96-well clusters. Cells were infected at a multiplicity of infection (MOI) of 3 PFU per cell for single-cycle assays and 0.1 PFU per cell for multicycle assays. After 1 h of incubation at 37°C, additional supplemented medium was added to each well. For some experiments, this overlay also contained 640 neutralizing units per ml (final concentration) of anti-IFN-β antibody (catalog no. 32400-1; PBL, Inc., Piscataway, NJ) and, at 2 days postinfection, these cells were again treated with anti-IFN-β antibody (320 neutralizing units per ml). Cells were incubated for various times before freezing at -80°C. Plates of cells were frozen and thawed twice more, and cells were lysed in 0.5% Nonidet P-40. For plaque assays, serial dilutions of the lysates were used to infect monolayers of L929 cells, which were subsequently overlaid with agar and stained with neutral red as described previously (44). In each experiment, duplicate or triplicate wells of cardiac cells were each assessed for viral replication by duplicate plaque assay wells.

SDS-PAGE and immunoblotting. Primary cardiac myocyte cultures were plated in 24-well clusters, infected at an MOI of 10 PFU per cell, incubated for 24 h, and lysed to generate total cellular protein extracts using radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented to contain 1% SDS and a cocktail of protease and phosphatase inhibitors (Sigma-Aldrich, catalog numbers P8340 and P2850, respectively). Cells were rocked on ice for 25 min and centrifuged at $10{,}000 \times g$ for 10 min at 4°C to remove cellular debris. Protein concentrations were determined by using a bicinchoninic acid protein assay (Thermo Fisher Scientific, catalog no. 23227), and 10 μg of protein from each lysate was boiled for 5 min in 1$\times$ Laemmli sample buffer and resolved using 10% SDS-polyacrylamide gel electrophoresis (PAGE). After protein transfer onto nitrocellulose, membranes were blocked for 1 h in phosphate-buffered saline (PBS) containing 5% bovine serum albumin. Membranes were incubated overnight at 4°C with a rabbit anti-μ2 polyclonal antibody (1:500; generated against two μ2 peptides by Open Biosystems, Huntsville, AL). Membranes were washed three times for 5 min each in PBS containing 0.05% Tween 20 (PBS-T) and then incubated for 1 h at room temperature with a goat anti-rabbit horseradish peroxidase-conjugated polyclonal (secondary) antibody (1:2,000; Millipore, catalog no. AP132P). The membranes were washed, and proteins were visualized using enhanced chemiluminescence (GE Healthcare, catalog no. RPN2108) according to the manufacturer's instructions. Membranes were exposed to film and converted to digital format using an HP Scanjet G4050.

CPE. Primary cardiac myocyte and cardiac fibroblast cultures were plated in 96-well clusters. Cells were infected at an MOI of 10 PFU per cell (single-cycle assays) or 0.1 PFU per cell (multicycle assays) in five replicate wells. After 1 h of incubation at 37°C, additional supplemented medium was added to each well. At 7 days (multicycle assay), the cultures were photographed using a Nikon TE-200 inverted microscope (phase contrast). At 2 days (single-cycle assays) or 5 or 7 days (multicycle assays) postinfection, an MTT assay was performed to quantify cell viability (48). An automated microplate reader (TECAN Sunrise Microplate Reader) was used to determine the optical density at 570 (OD_{570}) and the OD_{650}. The absorbance was determined by subtracting OD_{650} from OD_{570}.

Quantitative (real-time) reverse transcription-PCR (qRT-PCR). Duplicate wells were plated in 24-well (L929 cells) or 48-well (primary cardiac cell cultures) clusters. Cells were infected at an MOI of 25 PFU per cell (L929 cells) or 10 PFU per cell (primary cardiac cell cultures). After 1 h of incubation at 37°C, additional supplemented medium was added to each well. L929 cells were incubated for 20 h, treated with 100 or 1,000 U of IFN-β (catalog no. 12400-1; PBL, Inc.)/ml, and incubated for an additional 5 h. Primary cardiac myocyte and cardiac fibroblast cultures were incubated for 8 and 24 h postinfection. All cells were lysed with an RNeasy kit (Qiagen, Inc.), and total RNA was harvested and treated with RNase-free DNase I (Qiagen, Inc.). From each harvested well, one-third of the total RNA was used to generate cDNA by reverse transcription in a 100-μl reaction containing 5 μM oligo(dT) (Invitrogen Corp.), 1$\times$ *Taq* buffer (Promega Corp.), 7.5 mM $MgCl_2$ (Promega Corp.), 1 mM dithiothreitol (Promega Corp.), 1 mM concentrations of each deoxynucleoside triphosphate (Roche), 0.67 U of RNasin (Promega Corp.)/μl, and 0.20 U of avian myeloblastosis virus reverse transcriptase (Promega Corp.)/μl. Then, 5% of the reverse transcription product was amplified on an iCycler iQ fluorescence thermocycler (Bio-Rad Laboratories) in 96-well plates. Each duplicate 25-μl reaction contained 1$\times$ Quantitech SYBR green master mix (Qiagen, Inc.), 10 mM fluorescein, and 0.3 μM concentrations of each

forward and reverse primer. Primer sequences were as previously published (50). The relative abundance of IRF7, IFN-β, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was determined by comparison to a standard curve generated from serial dilutions of a DNA standard; IRF7 and IFN-β expression and IRF7 fold repressions were normalized to GAPDH expression.

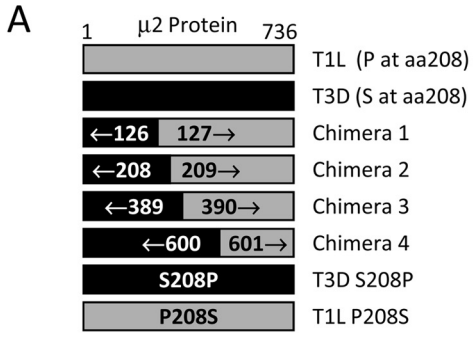
Statistical analysis. A Student two-sample *t* test (pooled variance; Systat 9.0) was applied to all data except those for Fig. 6, where a chi-square test was applied. The results were considered significant if the *P* value was <0.05.

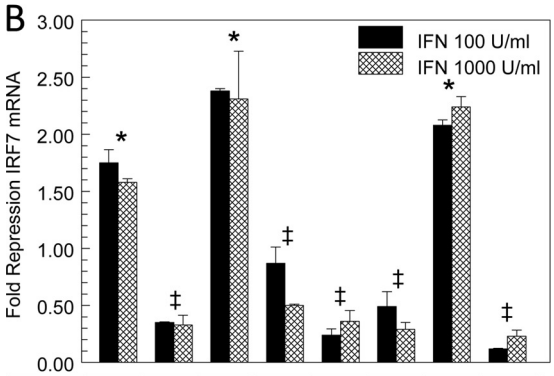
RESULTS

Amino acid (aa) 208 in reovirus protein μ2 determines reovirus repression of IFN signaling. The M1 gene product, μ2, of reovirus strain T1L but not that of strain T3D represses IFN signaling in L929 cells (56). To identify the μ2 amino acids responsible for repression of IFN signaling, viruses containing T1L-T3D chimeric M1 genes or viruses containing mutations in the M1 gene (Fig. 1A) were tested for the capacity to repress IFN signaling in L929 cells (Fig. 1B). A recombinant virus containing nine genes from T3D and a chimeric M1 gene expressing the N-terminal 126 μ2 amino acids from T3D and remaining amino acids from T1L (Ch1) was as effective as T1L at repressing IFN induction of IRF7. In contrast, viruses containing a chimeric M1 gene expressing the N-terminal 208 μ2 amino acids (Ch2) or more (Ch3 and Ch4) from T3D failed to repress IFN signaling. Strains T1L and T3D differ at only aa 208 in the μ2 region spanning aa 127 through aa 208. Remarkably, a T3D virus mutated at only μ2 residue 208 (T3D-S208P), changing it from the T3D serine (S) to a T1L proline (P), repressed IFN signaling as effectively as T1L. Conversely, a virus containing nine T3D genes and the T1L M1 gene mutated to T3D serine at μ2 residue 208 (T3D-T1LM1-P208S) failed to repress IFN signaling. Together, these data demonstrate that T1L μ2 aa 208 is both required and sufficient for reovirus repression of IFN signaling.

μ2 aa 208 modulates reovirus induction of IFN-β in cardiac cells. As anticipated from previous reports (48, 55), in cardiac myocytes, T3D induced IFN-β well and T1L induced IFN-β poorly (Fig. 2A, $P < 0.001$ at 8 and 24 h). Differences in T3D and T1L induction of IFN in cardiac myocytes segregate with the M1, L2, and S2 genes (48). Reovirus infection stimulates PRRs to induce IFN-β (16, 18, 26). While strain-specific differences in induction of IFN could reflect differences in this stimulation, they also could reflect differences in repression of IFN signaling since reovirus-mediated induction of IFN involves the positive feedback loop in cardiac myocytes (50, 56). T3D-T1LM1 induced markedly less IFN-β than did T3D (Fig. 2A, $P < 0.001$ at 8 and 24 h), a finding consistent with the association of the T1L M1 gene with poor induction of IFN (48). T1L-T3DM1 also induced minimal IFN-β ($P < 0.001$ relative to T3D at 8 and 24 h), which is consistent with the association of the T1L L2 and S2 genes with poor induction of this cytokine (48). Thus, the T3D M1 gene is not sufficient for strong induction of IFN. Instead, a full response requires the T3D L2 and S2 genes in addition to the T3D M1 gene.

To determine the effect of μ2 aa 208 on reovirus induction of IFN, viruses with single aa substitutions at that site were tested. At 8 h postinfection in cardiac myocytes (Fig. 2A), viral induction of IFN-β was decreased almost 3-fold by changing T3D μ2 aa 208 to that of T1L (T3D-S208P; $P < 0.001$), indicating that μ2 aa 208 modulates reovirus induction of IFN-β at this early time. However, by 24 h postinfection, T3D and T3D-S208P induction of

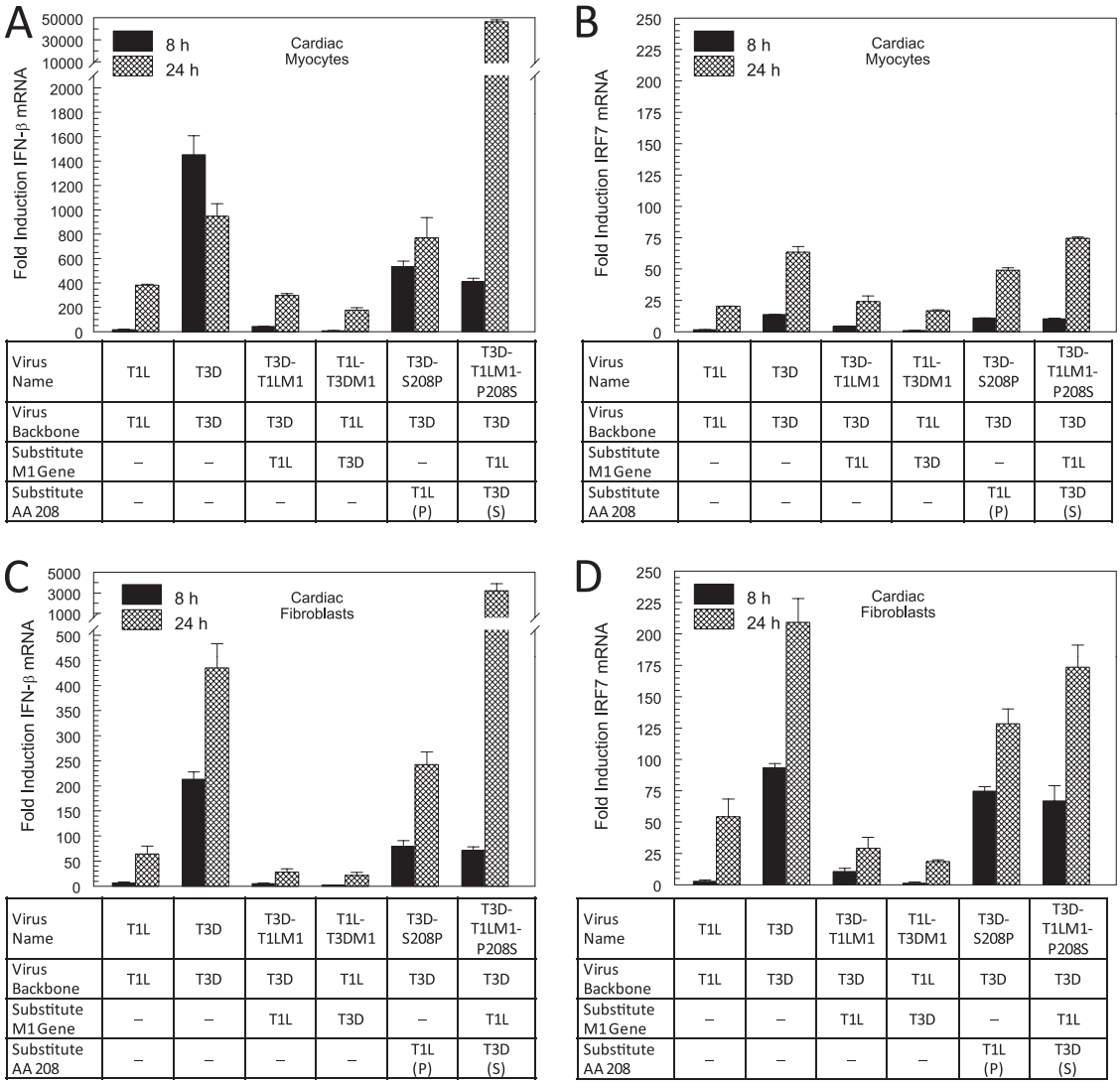




Virus Name	T1L	T3D	Ch 1	Ch 2	Ch 3	Ch 4	T3D-S208P	T3D-T1LM1-P208S
9 Genes	T1L	T3D	T3D	T3D	T3D	T3D	T3D	T3D
Substitute M1 Gene	–	–	Ch 1	Ch 2	Ch 3	Ch 4	–	T1L
T1L μ2 Amino Acids	–	–	127-736	209-736	390-736	601-736	208	all except 208

FIG 1 μ2 aa 208 determines reovirus repression of IFN signaling. (A) Viruses with a chimeric M1 gene or viruses with a mutated amino acid at position 208 of μ2 were generated by reverse genetics (21, 38). (B) L929 cells were infected with the indicated viruses, cells were treated with IFN, and mRNA was harvested for qRT-PCR. The fold repression was calculated as ([copies IRF7/GAPDH in IFN-treated mock-infected cells]/[copies IRF7/GAPDH in IFN-treated virus-infected cells]). Viruses that induce high levels of IFN resulted in values <1, but the relative repression between viruses was the same (56). The results are means $\pm$ the standard deviation (SD) (average of replicate wells; representative of four experiments). *, Different from T3D ($P < 0.05$); ‡, different from T1L ($P < 0.05$).

IFN-β (Fig. 2A, $P > 0.05$) and IRF7 (Fig. 2B, $P > 0.05$) were similar, indicating that repression of IFN signaling is not the primary determinant of strain-specific differences in reovirus induction of IFN-β. The similar induction of IRF7 by T3D and T3D-S208P (Fig. 2B) contrasts with T3D-S208P repression of exogenous IFN induction of IRF7 (Fig. 1B), likely reflecting the difference between induction of IRF7 concomitant with (Fig. 2B) or after (Fig. 1B) the majority of μ2 expression. Interestingly, other sequences in the T1L M1 gene also affected induction of IFN-β. Specifically, in contrast to the minimal effect of changing T3D μ2 aa 208 to that of T1L (T3D-S208P), induction of IFN-β and IRF7 was significantly increased by changing T1L μ2 aa 208 of T3D-T1LM1 to that of T3D (T3D-T1LM1-P208S, Fig. 2A and B, $P < 0.01$ at 8 and 24 h for both IFN-β and IRF7). In addition, the substantial increase in the induction of IFN-β at 24 h by T3D-T1LM1-P208S relative to T3D (Fig. 2A, $P < 0.001$) suggests that



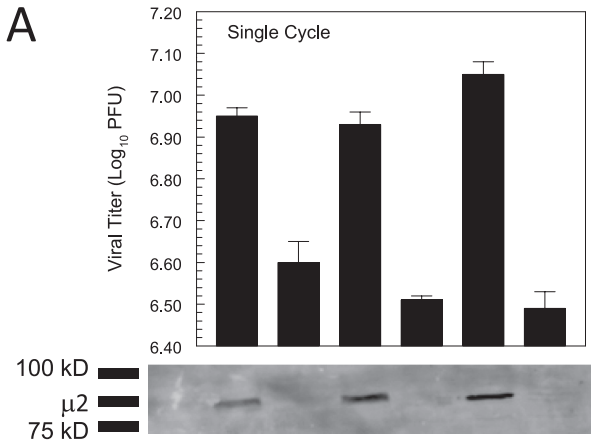
Virus Name	T1L	T3D	T3D-T1LM1	T1L-T3DM1	T3D-S208P	T3D-T1LM1-P208S
Virus Backbone	T1L	T3D	T3D	T1L	T3D	T3D
Substitute M1 Gene	–	–	T1L	T3D	–	T1L
Substitute AA 208	–	–	–	–	T1L (P)	T3D (S)

FIG 2 μ2 aa 208 modulates reovirus induction of IFN in cardiac cells. Primary cardiac myocyte (A and B) or fibroblast (C and D) cultures were infected with the indicated viruses, and mRNA was harvested for qRT-PCR using primers specific for IFN-β (A and C) or IRF7 (B and D) and GAPDH. The fold induction was calculated as copies IFN-β or IRF7/GAPDH in infected cultures relative to uninfected cultures. (A and C) Results are means ± the standard errors of the mean (SEM) (average of two experiments); (B and D) results are means ± the SD (average of replicate wells; representative of two experiments). See the statistical analyses in the text.

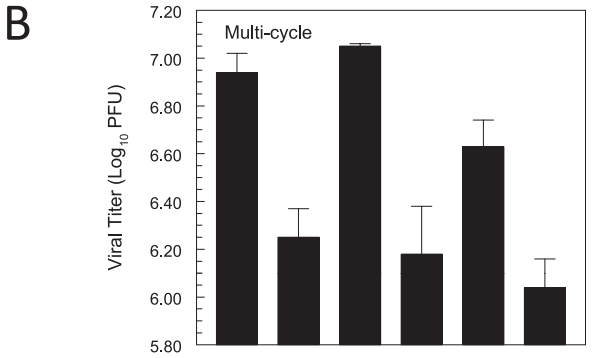
T1L M1 sequences other than aa 208 influence induction of this cytokine. The results for cardiac fibroblasts (Fig. 2C and D) were similar to those for cardiac myocytes, with the notable and expected difference (50, 55) that reovirus induction of IFN-β is much higher in cardiac myocytes than in cardiac fibroblasts (compare scales between Fig. 2A and C), but the opposite is true for reovirus induction of ISGs (Fig. 2B and D). Thus, reovirus μ2 proline 208 (the T1L residue) represses the induction of IFN-β in both cardiac myocytes and cardiac fibroblasts, but predominantly when expressed in the context of the entire T1L M1 gene.

***μ2* aa 208 determines reovirus single-cycle and multicycle replication in cardiac myocytes.** As expected (48, 50), T1L achieved higher titers in cardiac myocytes than did T3D after single or multiple cycles of replication (Fig. 3A and 3B; $P < 0.005$ for both cases). Differences between T1L and T3D could reflect IFN-dependent, IFN-independent, or both mechanisms. After single or multiple cycles of replication, T1L-T3DM1 titers were as low ($P > 0.05$) or lower than those for T3D, whereas T3D-T1LM1 titers were as high ($P > 0.05$) as those for T1L, demonstrating that the T1L M1 gene is both required and sufficient for reovirus to replicate maximally in cardiac myocytes. After single (Fig. 3A) or multiple (Fig. 3B) cycles of replication, the titers for T3D-T1LM1-P208S were as low ($P > 0.05$) or lower than those for T3D, demonstrating that T1L μ2 aa 208 is required for production of high titers in cardiac myocytes. Conversely, after a single round of replication, titers for T3D-S208P were even higher ($P < 0.05$) than those for T1L, demonstrating that T1L μ2 aa 208 is sufficient for high titers in cardiac myocytes. As expected, levels of μ2 protein paralleled levels of infectious virus (Fig. 3A).

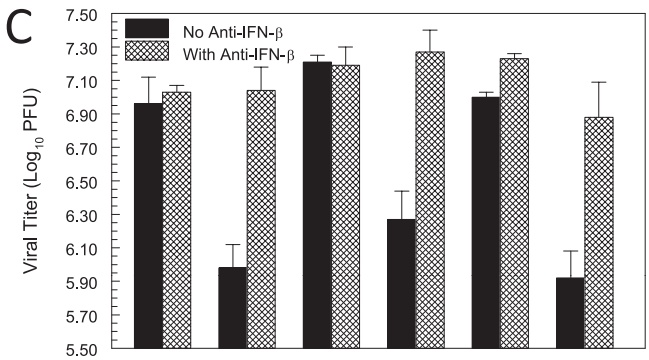
To determine whether T1L μ2 aa 208 regulates replication in cardiac myocytes solely by regulating the IFN response, titers were compared after multiple cycles of replication in the absence or



Virus Name	T1L	T3D	T3D-T1LM1	T1L-T3DM1	T3D-S208P	T3D-T1LM1-P208S
Virus Backbone	T1L	T3D	T3D	T1L	T3D	T3D
Substitute M1 Gene	–	–	T1L	T3D	–	T1L
Substitute AA 208	–	–	–	–	T1L (P)	T3D (S)



Virus Name	T1L	T3D	T3D-T1LM1	T1L-T3DM1	T3D-S208P	T3D-T1LM1-P208S
Virus Backbone	T1L	T3D	T3D	T1L	T3D	T3D
Substitute M1 Gene	–	–	T1L	T3D	–	T1L
Substitute AA 208	–	–	–	–	T1L (P)	T3D (S)



Virus Name	T1L	T3D	T3D-T1LM1	T1L-T3DM1	T3D-S208P	T3D-T1LM1-P208S
Virus Backbone	T1L	T3D	T3D	T1L	T3D	T3D
Substitute M1 Gene	–	–	T1L	T3D	–	T1L
Substitute AA 208	–	–	–	–	T1L (P)	T3D (S)

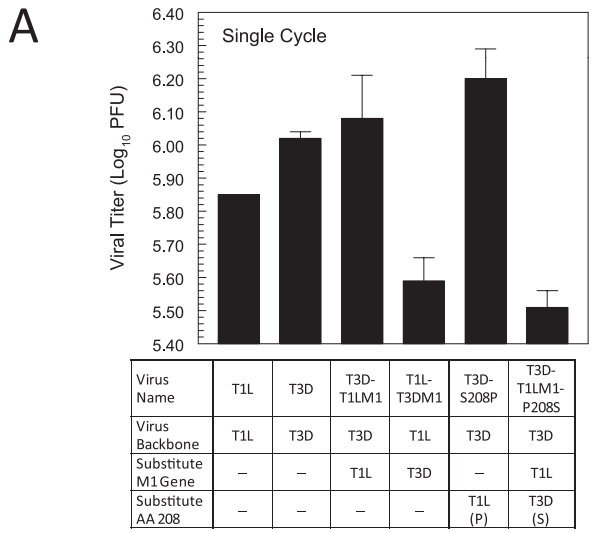
presence of anti-IFN-β antibody (Fig. 3C). As anticipated (48), elimination of the IFN response in cardiac myocytes increased T3D replication 11.5-fold ($P = 0.001$), which is consistent with evidence that the IFN response dictates the replication efficiency of this strain in these cells. Furthermore, elimination of the IFN response increased replication of all viruses tested to the level of T1L ($P > 0.05$) or higher, demonstrating that μ2 aa 208 regulates reovirus multicycle replication in cardiac myocytes by regulating the IFN response.

μ2 aa 208 influences reovirus single-cycle and multicycle replication in cardiac fibroblasts. In contrast to results obtained in experiments using cardiac myocytes, differences in yields of T1L and T3D were apparent in cardiac fibroblasts only after multiple cycles of replication, suggesting that the IFN response has little effect on initial infection in cardiac fibroblasts (Fig. 4A and B). This finding is consistent with the dramatically lower induction of IFN by reovirus in cardiac fibroblasts (Fig. 2C) than in cardiac myocytes (Fig. 2A). Indeed, in contrast to cardiac myocytes, the T1L M1 gene did not result in a higher yield of T3D (T3D-T1LM1) after a single cycle of replication in cardiac fibroblasts (Fig. 4A, $P > 0.05$). Replication of T3D-T1LM1-P208S was markedly reduced relative to the parent T3D-T1LM1 virus, most likely reflecting the effect of the single aa 208 substitution on induction of IFN (Fig. 2C. $P = 0.002$) and suggesting that the IFN response can modulate reovirus replication in cardiac fibroblasts if the IFN response is sufficiently strong. Replication of T1L-T3DM1 was reduced relative to both T1L and T3D after a single cycle of replication (Fig. 4A, $P < 0.001$), but intermediate between these two viruses after multiple cycles (Fig. 4B, $P < 0.001$). While reproducible, the underlying mechanism is unclear but may reflect heterologous gene combinations that are detrimental only in certain cell types (33). After multiple cycles of replication in cardiac fibroblasts, T3D replicated less well than T1L ($P < 0.001$), a property determined by the M1 gene (Fig. 4B). T3D-T1LM1-P208S produced the lowest titers of the viruses tested, a finding consistent with its dramatically higher induction of IFN (Fig. 2C).

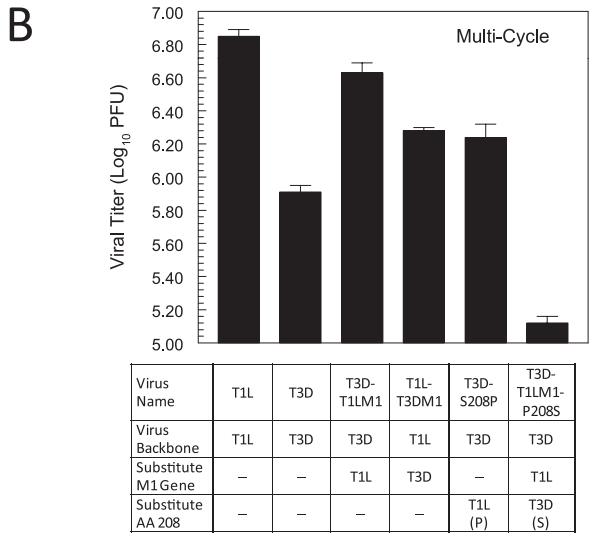
To test the role of IFN in μ2 aa 208 effects on reovirus replication in cardiac fibroblasts, virus titers in the presence or absence of anti-IFN-β antibody were compared (Fig. 4C). In comparison to results using cardiac myocytes, all viruses achieved only slightly higher titers when IFN was eliminated, consistent with the low level of IFN induced in cardiac fibroblasts relative to cardiac myocytes (Fig. 2).

μ2 aa 208 determines reovirus CPE in cardiac myocytes only after spread. Results thus far suggested that T1L μ2 aa 208 influences the IFN response to reovirus in both cardiac myocytes and cardiac fibroblasts but that the effect on viral replication is greater in cardiac myocytes than in cardiac fibroblasts. To determine the

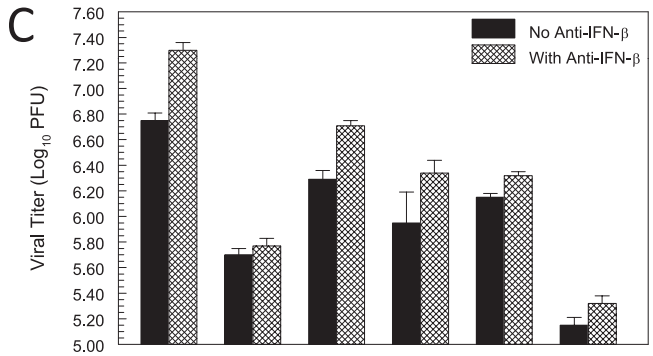
FIG 3 μ2 aa 208 determines reovirus single-cycle and multicycle replication in cardiac myocytes. (A) Primary cardiac myocyte cultures were infected with the indicated viruses at an MOI of 3 PFU per cell and harvested for plaque assay after 24 h or infected at 10 PFU per cell and harvested for immunoblotting after 24 h. The leftmost lane contains mock-infected sample. (B) Primary cardiac myocyte cultures were infected at an MOI of 0.1 PFU per cell and harvested for plaque assay after 5 days. (C) Primary cardiac myocyte cultures were infected as in panel B, treated with anti-IFN-β antibody where indicated, and harvested for plaque assay. All results are means ± the SD (average of replicate wells; representative of two to four experiments). See the statistical analyses in the text.



Virus Name	T1L	T3D	T3D-T1LM1	T1L-T3DM1	T3D-S208P	T3D-T1LM1-P208S
Virus Backbone	T1L	T3D	T3D	T1L	T3D	T3D
Substitute M1 Gene	–	–	T1L	T3D	–	T1L
Substitute AA 208	–	–	–	–	T1L (P)	T3D (S)



Virus Name	T1L	T3D	T3D-T1LM1	T1L-T3DM1	T3D-S208P	T3D-T1LM1-P208S
Virus Backbone	T1L	T3D	T3D	T1L	T3D	T3D
Substitute M1 Gene	–	–	T1L	T3D	–	T1L
Substitute AA 208	–	–	–	–	T1L (P)	T3D (S)

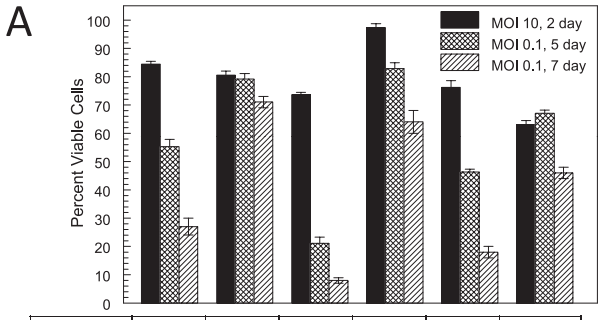


Virus Name	T1L	T3D	T3D-T1LM1	T1L-T3DM1	T3D-S208P	T3D-T1LM1-P208S
Virus Backbone	T1L	T3D	T3D	T1L	T3D	T3D
Substitute M1 Gene	–	–	T1L	T3D	–	T1L
Substitute AA 208	–	–	–	–	T1L (P)	T3D (S)

FIG 4 μ2 aa 208 influences reovirus single-cycle and multicycle replication in cardiac fibroblasts. Primary cardiac fibroblast cultures were infected with the indicated viruses at an MOI of 3 PFU per cell and incubated for 24 h (A) or at an MOI of 0.1 PFU per cell and incubated for 5 days (B) and harvested for plaque assay. (C) Primary cardiac fibroblast cultures were infected as in panel B, treated with anti-IFN-β where indicated, and harvested for plaque assay. All results are means ± the SD (average of replicate wells; representative of two to four experiments). See the statistical analyses in the text.

contribution of μ2 aa 208 to viral CPE, cardiac cells were infected at a high MOI (10 PFU per cell) and cell viability was quantified after a single cycle of replication (2 days) or the cells were infected at a low MOI (0.1 PFU per cell) and cell viability was quantified after multiple cycles of replication (5 days and 7 days). PFU are quantified using L929 cells, and virus is less infectious in cardiac cells than in L929 cells (data not shown). Therefore, even at an MOI of 10 PFU per cell, not all cardiac cells are infected (44). In cardiac fibroblasts, none of the viruses were cytopathic after a single cycle of replication, and CPE increased only minimally after multiple cycles (data not shown). As expected (48), T1L and T3D were similarly cytopathic in cardiac myocytes after a single cycle of replication (Fig. 5). However, after multiple cycles of replication, T1L and T3D clearly differed in the induction of CPE ($P < 0.001$ at 5 and 7 days), as did the entire panel of viruses. The overall trend inversely correlated with viral replication in cardiac myocytes (Fig. 3A and B): viruses that replicated to the highest titers left the fewest cells viable. More specifically, after 5 or 7 days, T3D-T1LM1 was even more cytopathic than T1L ($P < 0.001$), while T1L-T3DM1 was as noncytopathic as T3D at 5 days ($P > 0.05$) and only slightly more cytopathic at 7 days. Moreover, changing only T3D μ2 aa 208 to that of T1L (T3D-S208P) increased cytopathicity to even greater than that of T1L ($P < 0.002$). Finally, T3D-T1LM1-P208S cytopathicity was markedly reduced relative to the parent T3D-T1LM1 virus ($P < 0.001$). Gross CPE paralleled MTT results (Fig. 5B). Together, these results demonstrate that T1L μ2 aa 208 determines CPE after viral spread, likely through repression of the IFN signaling that is required for the positive feedback loop of induction of IFN-β.

***μ2* aa 208 modulates myocarditis.** To determine the effect of μ2 aa 208 on viral myocarditis, mice were inoculated with the panel of recombinant viruses, and cardiac lesions were quantified. As expected (45), T1L induced cardiac lesions in all mice, while T3D induced few lesions (Fig. 6). Reovirus strain-specific differences in the capacity to induce myocarditis segregate with the M1, L1, and L2 genes (45, 46), but the role of the M1 gene alone has not been previously determined. A virus containing the T1L M1 gene in the T3D genetic background (T3D-T1LM1) induced a higher frequency of myocarditis than did T3D ($P < 0.05$), demonstrating that the T1L M1 gene when introduced into the genetic background of T3D is sufficient to cause myocarditis. The T3D M1 gene in the T1L genetic background (T1L-T3DM1) induced a lower frequency of myocarditis than did T1L ($P < 0.05$), confirming the importance of the T1L M1 gene in this phenotype. Furthermore, although not statistically significant, T1L-T3DM1 appeared to induce myocarditis more efficiently than did T3D, providing evidence to support previous conclusions that genes in T1L in addition to M1 contribute to myocarditis (45, 46). Finally, changing T3D μ2 aa 208 to that of T1L (T3D-S208P) dramatically increased the frequency of myocarditis ($P < 0.05$) compared to that caused by T3D ($P < 0.05$), suggesting that repression of IFN signaling alone can modulate this disease. T3D-T1LM1-P208S appeared to be less myocarditic than the parental T3D-T1LM1 virus but more myocarditic than T3D (not statistically significant), supporting the importance of repression of IFN signaling but indicat-



Virus Name	T1L	T3D	T3D-T1LM1	T1L-T3DM1	T3D-S208P	T3D-T1LM1-P208S
Virus Backbone	T1L	T3D	T3D	T1L	T3D	T3D
Substitute M1 Gene	–	–	T1L	T3D	–	T1L
Substitute AA 208	–	–	–	–	T1L (P)	T3D (S)

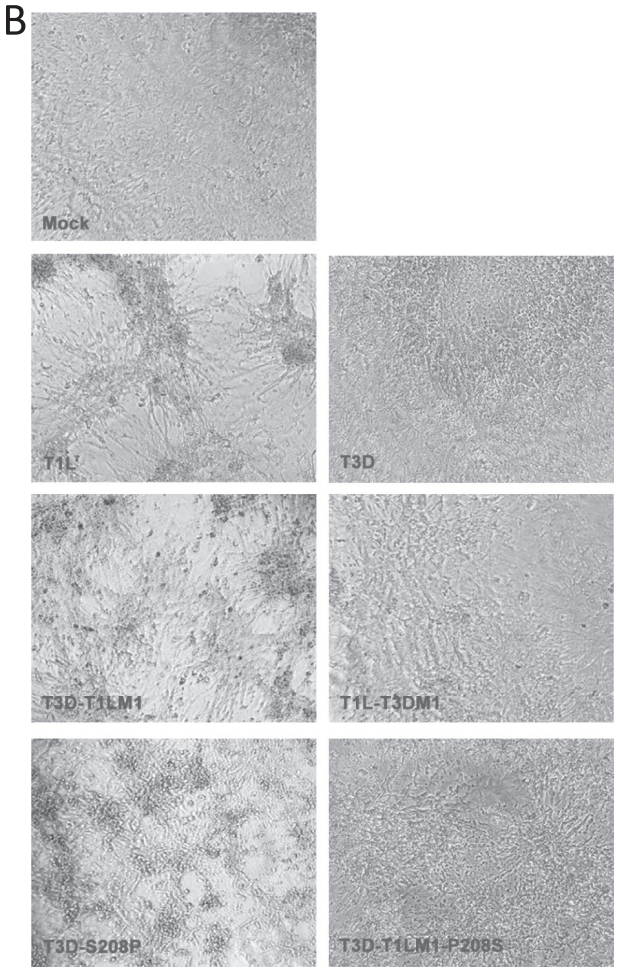


FIG 5 μ2 aa 208 determines reovirus CPE in cardiac myocytes only after spread. (A) Primary cardiac myocyte cultures were infected with the indicated viruses at an MOI of 10 PFU per cell and incubated for 2 days (single cycle) or at an MOI 0.1 PFU per cell and incubated for 5 or 7 days (multicycle) and harvested for MTT assay. The percent viable cells was calculated relative to mock-infected cells. The results at 2 and 5 days are means ± the SEM (average of at least two experiments); results at 7 days are means ± the SD (average of replicate wells for a single experiment). See the statistical analyses in the text. (B) Primary cardiac myocyte cultures were infected at an MOI of 0.1 PFU per cell, incubated for 7 days, and photographed at a final magnification of ×100 (representative fields shown).

ing that other T1L M1 functions are also determinants of myocarditis. Collectively, these results demonstrate that μ2 aa 208 modulates myocarditis, suggesting that repression of IFN signaling is a determinant of reovirus-induced cardiac disease.

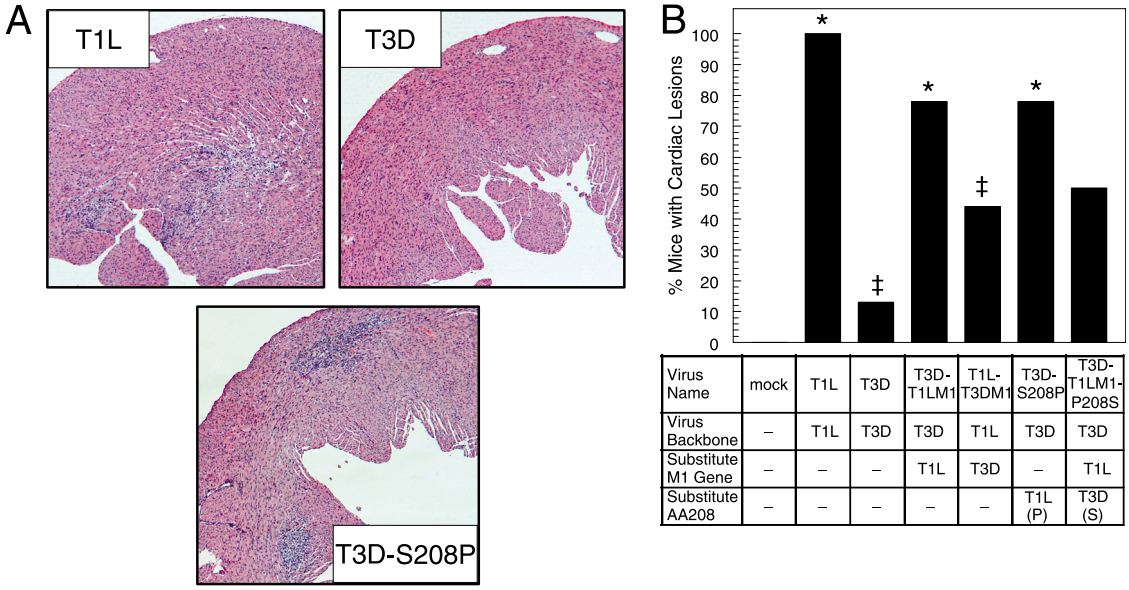
DISCUSSION

The IFN-α/β response is essential for protection against reovirus infection of cardiac cells *in vitro* and reovirus-induced myocarditis in mice (48). We demonstrated here that sequence polymorphisms at reovirus protein μ2 aa 208 are both required and sufficient for strain-specific differences in reovirus repression of IFN-β signaling. Furthermore, μ2 aa 208 modulation of IFN expression and signaling determines viral replication and spread in cardiac myocytes and modulates myocarditis in mice.

Virus-induced IFN-β stimulates the expression of antiviral ISGs, as well as transcription factor IRF7, which results in further induction of IFN-β in a positive amplification loop. Viral repression of IFN signaling could: (i) limit IFN expression to that initiated by PRRs and (ii) suppress IFN-induced expression of antiviral genes. Viruses have evolved many mechanisms to antagonize IFN signaling, including inactivation of JAKs, degradation or sequestration of STATs, and inhibition of karyopherins required for ISGF3 nuclear translocation (41). Only two viruses are known to repress IFN signaling through IRF9: human papillomavirus type 16 sequesters IRF9 to inhibit its nuclear translocation (1), and adenovirus infection leads to a decrease in IRF9 levels (25). The μ2 protein from reovirus strain T1L but not strain T3D represses IFN-β signaling, and this repression is associated with nuclear accumulation of IRF9 (56). However, it is not known whether alterations in IRF9 mediate repression or are merely a consequence of that event. In the present study, we found that sequence variation in μ2 aa 208 determines these reovirus strain-specific differences in repression of IFN-β signaling (Fig. 1).

The M1 gene sequences of nine field isolate reovirus strains are available, and while all nine contain a proline or serine at μ2 residue 208, eight contain a proline-like T1L, suggesting a selective advantage (53). Interestingly, μ2 aa 208 governs several differences displayed by strains T1L and T3D. Reovirus replicates and assembles in viral inclusion bodies (VIBs). While T3D induces formation of globular VIBs, T1L induces formation of filamentous VIBs, reflecting T1L μ2-stimulated hyperacetylation and stabilization of microtubules (39). While these T1L μ2-stimulated events are disrupted when the T1L proline at aa 208 is substituted with the T3D serine (39), substitutions at μ2 aa 383 also prevent T1L formation of filamentous VIBs (21). Importantly, aa 208 mediates greater ubiquitylation and aggregation of T3D μ2 than T1L μ2, suggesting that the capacity of T1L μ2 to stabilize microtubules and induce filamentous VIBs may be a consequence of virus strain-specific posttranslational μ2 modifications (31). The results presented here identify T1L μ2 aa 208 as both required and sufficient for reovirus repression of IFN signaling and suggest two possible models: (i) repression of IFN signaling requires the cytoskeletal changes that accompany formation of filamentous VIBs or (ii) ubiquitylated and aggregated μ2 cannot function to repress IFN signaling. Future studies are planned to discriminate between these possibilities.

Strain-specific differences in reovirus induction of IFN-α/β genetically segregate with the reovirus M1, L2, and S2 genes (48). While T3D induces IFN well and T1L induces IFN poorly (48), it is unclear whether this polymorphism reflects differences in PRR-



Virus Name	mock	T1L	T3D	T3D-T1LM1	T1L-T3DM1	T3D-S208P	T3D-T1LM1-P208S
Virus Backbone	–	T1L	T3D	T3D	T1L	T3D	T3D
Substitute M1 Gene	–	–	–	T1L	T3D	–	T1L
Substitute AA208	–	–	–	–	–	T1L (P)	T3D (S)

FIG 6 μ2 aa 208 modulates myocarditis. Neonatal mice were inoculated with the indicated viruses and euthanized 7 days postinjection. Hematoxylin-eosin-stained cardiac sections were scored for lesions. (A) Representative sections. (B) A minimum of eight hearts (minimum of 15 sections per heart) were scored for each virus. Lesions in adjacent sections were not scored as independent lesions. Hearts were considered "positive" if more than a single lesion was detected in the $\geq$ 15 sections examined. *, Different from T3D; ‡, different from T1L (chi square, $P < 0.05$). The results are representative of two independent experiments.

stimulated induction, IRF7-mediated amplification, or both effects. The roles of the L2-encoded λ2 and S2-encoded σ2 proteins in these processes have not been reported, but one possible role for the M1-encoded μ2 protein could be to suppress IFN induction of IRF7, thereby inhibiting further induction of IFN. Indeed, substitution of only μ2 aa 208 in T3D to provide a repressor of IFN signaling (T3D-S208P), significantly reduced reovirus induction of IFN in both cardiac myocytes and cardiac fibroblasts at 8 h postinfection (Fig. 2). Interestingly, the converse substitution, in which only μ2 aa 208 in T3D-T1LM1 is replaced to remove the repressor of IFN signaling (T3D-T1LM1-P208S), had a much more substantial effect on reovirus induction of IFN in both cell types (Fig. 2). Specifically, T3D-T1LM1-P208S induced 10-fold more IFN-β than did T3D-T1LM1 at 8 h and a remarkable 150-fold more at 24 h (Fig. 2A) with a concomitant increase in induction of IRF7 (Fig. 2B). Therefore, in the context of the T1L M1 gene, T1L μ2 aa 208 repression of IFN signaling dramatically affected reovirus induction of IRF7 and consequently IFN-β. Importantly, viruses with a T1L M1 gene display enhanced kinetics of viral RNA synthesis in cardiac myocytes in comparison to those with a T3D M1 gene (44). Thus, T1L μ2 should be synthesized earlier than T3D μ2, offering T3D-T1LM1 greater opportunity than T3D-S208P to repress induction of IRF7. The substantial increase in the induction of IFN-β at 24 h by T3D-T1LM1-P208S relative to T3D (Fig. 2A) is consistent with both the absence of the T1L μ2 aa 208 repressor of IFN signaling and the T1L M1-associated rapid RNA synthesis phenotype facilitating RNA-stimulated activation of IRF7 (44). Together, these data indicate that reovirus induction of IFN in cardiac cells involves a positive amplification loop and that repression of IFN signaling reduces reovirus induction of IFN-β.

The reovirus M1-, L2-, and S2-encoded proteins also could modulate reovirus activation of PRRs. Depending on the cell type or perhaps infection stage (14), reovirus activates the PRRs RIG-I, MDA5, or both molecules to induce IFN (16, 18, 26), although the specific nucleic acid stimulus remains unclear. While reovirus particles contain genomic double-stranded RNA (dsRNA), virions are not thought to fully uncoat during viral replication (43), and thus genomic dsRNA should be protected from PRRs. Instead, reovirus cores synthesize single-stranded RNA, which is extruded and then complexed into new subviral particles for RNA second-strand synthesis (43). The μ2 protein is found at the icosahedral vertices of viral cores as part of a probable RNA-synthesizing complex (19, 54), has both NTPase (19, 35) and RNA-binding activity (6, 19, 35), and determines the rate of viral RNA synthesis in cardiac myocytes (44). However, reoviruses with a T1L M1 gene synthesize RNA more rapidly than those with a T3D M1 gene (44), the opposite of what would be predicted if μ2-stimulated viral RNA synthesis solely determined the level of IFN induction. It is possible that the location of both μ2 and λ2 at the icosahedral vertices (7, 11) influences particle stability. Virions contain not only genomic dsRNA, a potential RIG-I and MDA5 ligand, but also short oligonucleotides with free 5′ triphosphates (3, 34, 43), an additional potential RIG-I ligand, although shorter than other RIG-I agonists (27, 28). Therefore, T3D particles may aberrantly disassemble more readily than T1L particles, resulting in commensurately greater PRR activation and induction of IFN. We found that T3D induction of IFN in cardiac cells was reduced when its M1 gene was replaced with that of T1L (T3D-T1LM1, Fig. 2A and C). However, the striking increase in induction of IFN when the T1LM1 repressor of IFN signaling was removed (T3D-T1LM1-P208S), particularly later during infection, suggests that the main function of μ2 in the IFN response is to repress IFN signaling (as in T1L) rather than to shield PRR ligands from the innate immune surveillance machinery.

The protective effects of secreted IFN should be greater in cells that have not been infected, i.e., as a paracrine signal, than in cells already infected, i.e., as an autocrine signal, since the former have

more time to induce antiviral proteins. In addition, IFN-mediated reductions in viral yield should result in fewer secondary infections. Therefore, the effect of the IFN response on viral yield should be amplified following viral spread. The IFN response determines reovirus yields in primary cardiac myocyte cultures and, as expected, the effect is increased following viral spread (48). We found that μ2 aa 208 determines strain-specific differences in viral replication in cardiac myocytes even after a single cycle of replication (Fig. 3A), suggesting that IFN can reduce viral replication in these cells even after infection is initiated. As expected, the effect was enhanced after multiple cycles (Fig. 3B), and all viruses achieved equivalent titers when IFN was removed (Fig. 3C). Together, the data demonstrate that μ2 aa 208 regulation of the IFN response determines reovirus replication and spread between cardiac myocytes.

Virus strain-specific differences in the induction of myocarditis correlate with differences in CPE in primary cardiac myocyte cultures only after viral spread (2, 48), which is consistent with the IFN response regulating viral spread *in vitro* and *in vivo* (15, 36, 48). Many investigations have used IFN-deficient mice or viruses that are defective in repressing the IFN response to demonstrate the importance of the IFN response in protection against virus-induced disease, but few have pinpointed the populations of differentiated cells in which IFN critically acts to limit progression of viral infection. Here, we found that a serine-proline polymorphism at aa 208 in the reovirus μ2 protein influences CPE after multiple cycles of replication in cardiac myocytes (Fig. 5) and modulates myocarditis (Fig. 6), providing evidence that viral repression of IFN signaling in a target differentiated cell can determine disease outcome.

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