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Differential IFN-α/β production suppressing capacities of the leader proteins of mengovirus and foot-and-mouth disease virus

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Summary

Picornaviruses encompass a large family of RNA viruses. Some picornaviruses possess a leader (L) protein at the N-terminus of their polyprotein. The L proteins of encephalomyocarditis virus, a cardiovirus, and foot-and-mouth disease virus (FMDV), an aphthovirus, are both dispensable for replication and their major function seems to be the suppression of antiviral host cell responses. Previously, we showed that the L protein of mengovirus, a strain of encephalomyocarditis virus, inhibits antiviral responses by inhibiting type I interferon (IFN-α/β) gene transcription. The L protein of the FMDV is a protease (L^{pro}) that cleaves cellular factors to reduce cytokine and chemokine mRNA production and to inhibit cap-dependent cellular host mRNA translation, thereby limiting the production of proteins with antiviral activity. In this study, we constructed a viable chimeric mengovirus that expresses FMDV L^{pro} in place of the authentic L protein in order to compare the efficiency of the immune evasion mechanisms mediated by L and L^{pro} respectively. We show that in this mengovirus background the L protein is more potent than FMDV L^{pro} in suppressing IFN-α/β responses. Yet, FMDV L^{pro} is important to antagonize infection-limiting responses both *in vitro* and *in vivo*.

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Introduction

The family Picornaviridae encompasses a large number of small plus-strand RNA viruses. Due to the small size of the genome (7–8 kb), most proteins have dual functions serving both as facilitators of viral replication and modulators of the cellular environment in order to support viral replication. All picornaviruses have a similar genome organization. The viral RNA contains a 5′UTR, which harbours replication signals as well as an internal ribosome entry site, a single polyprotein coding region, and a 3′UTR with a genetically encoded poly(A) tail. The polyprotein coding region is divided into the P1, P2 and P3 regions. The P1 region encodes the capsid proteins while the P2 and P3 regions encode the non-structural proteins involved in replication. Some genera of the Picornaviridae family possess an additional non-structural protein, called the leader (L) protein, at the N-terminal part of their polyprotein. Most is known of the L proteins of members of the genera Cardiovirus (e.g. Encephalomyocarditis virus (EMCV) and Theilovirus) and Aphthovirus (e.g. foot-and-mouth disease virus, FMDV). In contrast to the other non-structural viral proteins, the L proteins of these viruses are dispensable for viral RNA replication and their main function seems to be the suppression of antiviral responses, as such, they have been dubbed viral 'security proteins'(Romanova *et al.*, 2009).

Type I interferons (IFN-α/β) are essential components of the innate immune response against viruses. IFN-α/β causes cells to adopt an antiviral state by inducing production of proteins with antiviral and immunomodulatory properties. Upon infection of cells with cardioviruses, however, little if any IFN-α/β is produced (Zoll *et al.*, 1996; 2002; van Pesch *et al.*, 2001; Hato *et al.*, 2007; Ricour *et al.*, 2009). Through the construction of cardioviruses lacking L or expressing a mutant L, this protein was recognized to inhibit IFN-α/β gene transcription by preventing the activation of Interferon Regulatory Factor 3 (IRF-3), a factor that controls gene transcription of IFN-α/β as well as other IFN-stimulated genes. By consequence, these

mutant viruses were found to be highly attenuated and showed reduced pathogenicity in an *in vivo* mouse model (Hato *et al*., 2007; Zoll *et al*., 1996; 2002).

The FMDV L protein is a protease (L^{pro}) that, although structurally not related to cardiovirus L, also has been implicated in restricting antiviral defence mechanisms. The function of FMDV L^{pro} was studied by engineering a FMDV lacking L^{pro} or containing a mutant L^{pro} (Piccone *et al*., 1995; De Los Santos *et al*., 2009). These mutant viruses replicated relatively efficiently in cells lacking a functional IFN-α/β system but were highly attenuated in cells with a functional IFN-α/β system and also *in vivo* (Brown *et al*., 1996; Piccone *et al*., 1995; Mason *et al*., 1997; Chinsangaram *et al*., 1999; De Los Santos *et al*., 2009). Cells infected with wild-type (wt) FMDV produce little IFN-α/β whereas cells infected with a L^{pro}-deleted virus secreted higher amounts of IFN-α/β in the medium (Chinsangaram *et al*., 1999; Chinsangaram *et al*., 2001; De Los Santos *et al*., 2006). The ability of L^{pro} to suppress IFN-α/β production has been linked to its function to cleave eukaryotic initiation factor eIF4G (Devaney *et al*., 1988; Kirchweger *et al*., 1994; Guarne *et al*., 1998), which shuts off cap-dependent translation of cellular mRNAs while leaving the cap-independent, IRES-mediated translation of the viral RNA unaffected (Jang *et al*., 1988). In addition, L^{pro} has been shown to decrease IFN-α/β mRNA production (~3–10-fold) by cleaving NF-κB (De Los Santos *et al*., 2006; De Los Santos *et al*., 2007; De Los Santos *et al*., 2009).

Thus, cardioviruses and aphthoviruses have evolved different L proteins and, by consequence, different mechanisms to suppress innate IFN-α/β responses. Whether both viral mechanisms are equally efficient in suppressing IFN-α/β responses is unknown. Here, we set out to compare the activities of the L proteins of mengovirus and FMDV by comparing functional *in vitro* and *in vivo* IFN-α/β responses induced by wt mengovirus, a strain of EMCV, and a chimeric mengovirus in which L was replaced with FMDV L^{pro}.

Results and discussion

Construction and characterization of chimeric virus

FMDV L^{pro} was cloned in mengovirus cDNA clone pM16.1 in place of mengovirus L. The first six amino acids of mengovirus L were maintained to ensure efficient translation (Kaminski *et al*., 1994). The specific L^{pro} recognition site (KLKG) was introduced at the C terminus to enable its self-cleavage from the polyprotein (Fig. 1A). Transfection of *in vitro* transcribed RNA into BHK-21 cells using DEAE dextran yielded a viable chimeric virus (mengovirus-FL), as observed previously for a chimeric Theiler's virus expressing L^{pro} of FMDV (Piccone *et al*., 1996). Sequence analysis confirmed the integrity of the L^{pro} insert. To confirm L^{pro} activity, protein synthesis and eIF4G cleavage were compared in wt and FL virus-infected cells. To this end, L929 cells were infected at a multiplicity of infection (moi) of 50 $TCID_{50}$ (50% tissue culture infective dose) per cell and incubated for 30 min with methionine-free medium containing [^{35}S]methionine at various times post infection (p.i.). Cells were lysed and protein synthesis was visualized by SDS-PAGE and autoradiography. The results (Fig. 1B) show that from 2 h p.i. little, if any, cellular proteins were synthesized in FL virus-infected cells, while cellular protein production is detectable until 5 h p.i. in mengovirus-infected cells. This indicates that FMDV L^{pro} rapidly inhibits cap-dependent translation. The translation shutdown coincided with the cleavage of eIF4G, as illustrated by the decrease in eIF4G protein levels and the appearance of cleavage products in L929 cells by Western blot analysis (Fig. 1B). Thus, the FL virus expresses a functional L^{pro} that efficiently shuts off cap-dependent mRNA translation.

Viral growth curves showed that the FL virus replicated efficiently but that the end titre was always 10–20 times lower than that of wt virus, both in BHK-21 cells and in L929 cells (Fig. 1C). To investigate whether the lower end titres of mengo FL were due to a reduction in the amount of viral RNA, we determined the amount of viral RNA at different times p.i. To this end, total RNA was isolated from infected cells, spotted on a nitrocellulose membrane, and hybridized to a radioactive probe, pM16.1 (full-length mengovirus cDNA clone), labelled with [α-^{32}P]dATP by nick translation. The data showed that the FL virus produced equal amounts of RNA, or even more, than the wt virus (Fig. 1D). A possible explanation for the lower end titre of the FL virus may therefore be that there are not enough capsid proteins produced in the later stage of infection, when maximal viral RNA synthesis takes place (i.e. at 6 h p.i.), to encapsidate all newly synthesized viral RNAs.

In vitro *IFN-α/β responses*

Previously, we showed that mengovirus L inhibits dimerization and thereby activation of IRF-3, a transcription factor whose activity is crucial for the induction of IFN-α/β gene transcription (Hato *et al*., 2007). Recent studies indicate that this function of L may be coupled to its ability to inhibit nucleocytoplasmic trafficking of proteins and mRNAs (Delhaye *et al*., 2004; Lidsky *et al*., 2006; Porter *et al*., 2006; Bardina *et al*., 2009; Porter and Palmenberg, 2009; Ricour *et al*., 2009). In contrast, the FL virus was unable to inhibit IRF-3 dimerization (Fig. 2A), as demonstrated by native PAGE electrophoresis, and to completely abrogate immediate early IFN-α/β gene transcription (Fig. 2B), as observed for the wt virus by

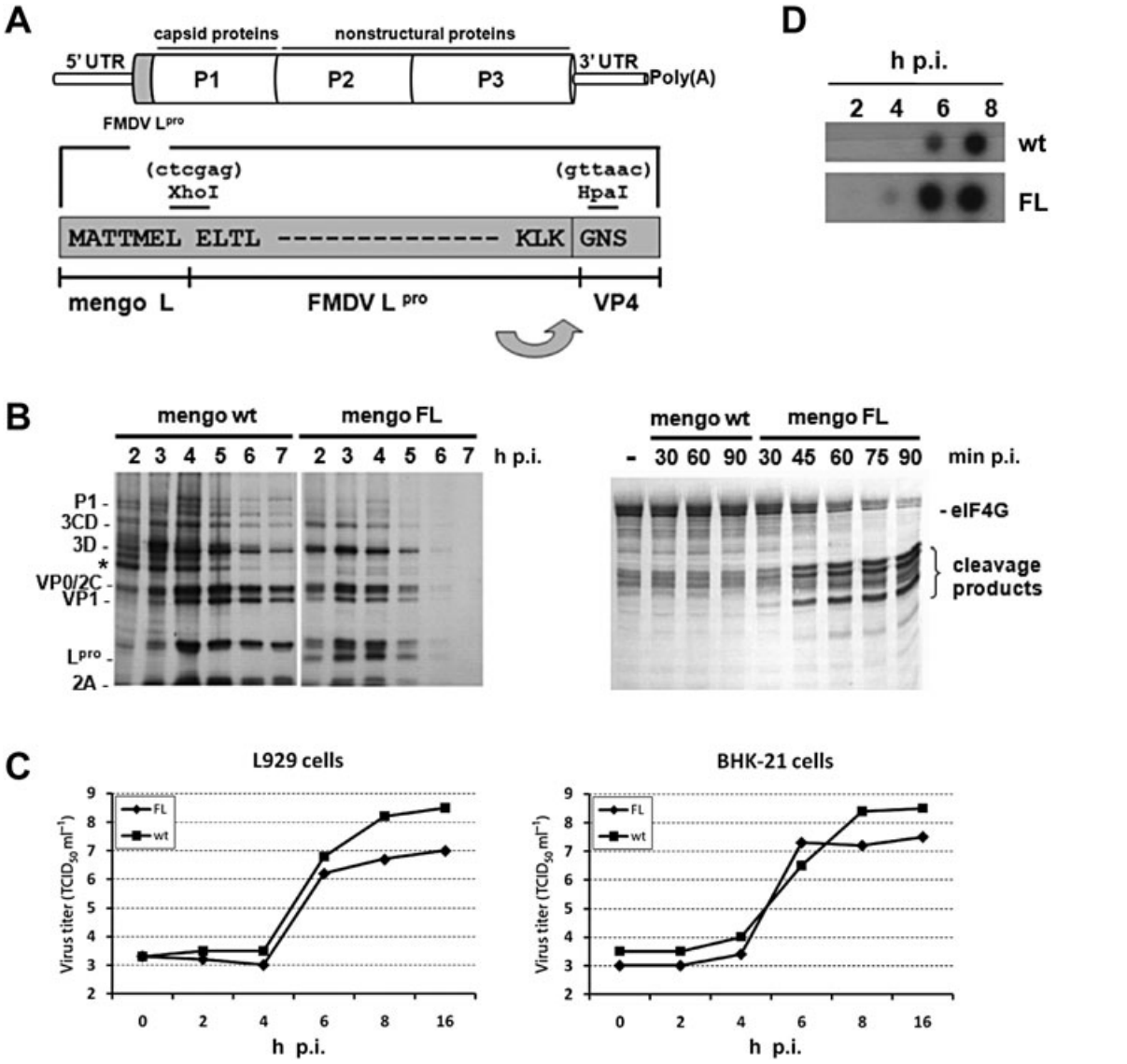

Fig. 1. Construction and characterization of FMDV leader-mengovirus chimera.
A. Schematic representation of the chimera showing the sequence for self-cleavage from the poly protein (KLK*G).
B. Left: Protein synthesis in L929 cells infected with wt and FL virus. Proteins were labelled with [^{35}S]methionine at the indicated hours p.i. The asterisk indicates an unknown host protein. Right: Western blot analysis showing the cleavage of eIF4G in L929 cells infected with the FL chimera at various times (in minutes p.i.).
C. Viral growth curves of wt and FL virus on L929 and BHK-21 cell. Cells were infected at a moi of 1. At the indicated hours p.i., cells were lysed and virus titres were determined by titration on BHK-21 cells.
D. Amount of viral RNA in L929 cells infected with wt or FL virus at the indicated times. RNA was visualized by hybridization to a radioactive probe.

RT-PCR. Unlike the wt virus (Lidsky *et al.*, 2006), the FL virus was unable to modify nucleocytoplasmic transport, as shown by a normal distribution of a nucleus-targeted GFP (GFP-NLS) (Fig. 2C). This observation is in agreement with the observed inability of wt FMDV to alter nucleocytoplasmic transport (De Los Santos *et al.*, 2007). This finding suggests that it is unlikely that L^{pro} cleaves essential nucleoporins to disrupt nuclear pore function, a mechanism that has been demonstrated for the 2A proteases of enteroviruses and rhinoviruses (Belov *et al.*, 2000; Gustin and Sarnow, 2001; 2002; Belov *et al.*, 2004; Park *et al.*, 2008) with which L^{pro} shares a number of similarities [e.g. cleavage of eIF4G (Krausslich *et al.*, 1987) and NF-κB (Neznanov *et al.*, 2005)].

Our data clearly demonstrate that FMDV L^{pro} has less profound effects on IFN-α/β gene transcription than mengovirus L. To test effects of L^{pro} on the production and secretion of bioactive IFN-α/β, a bioassay was performed.

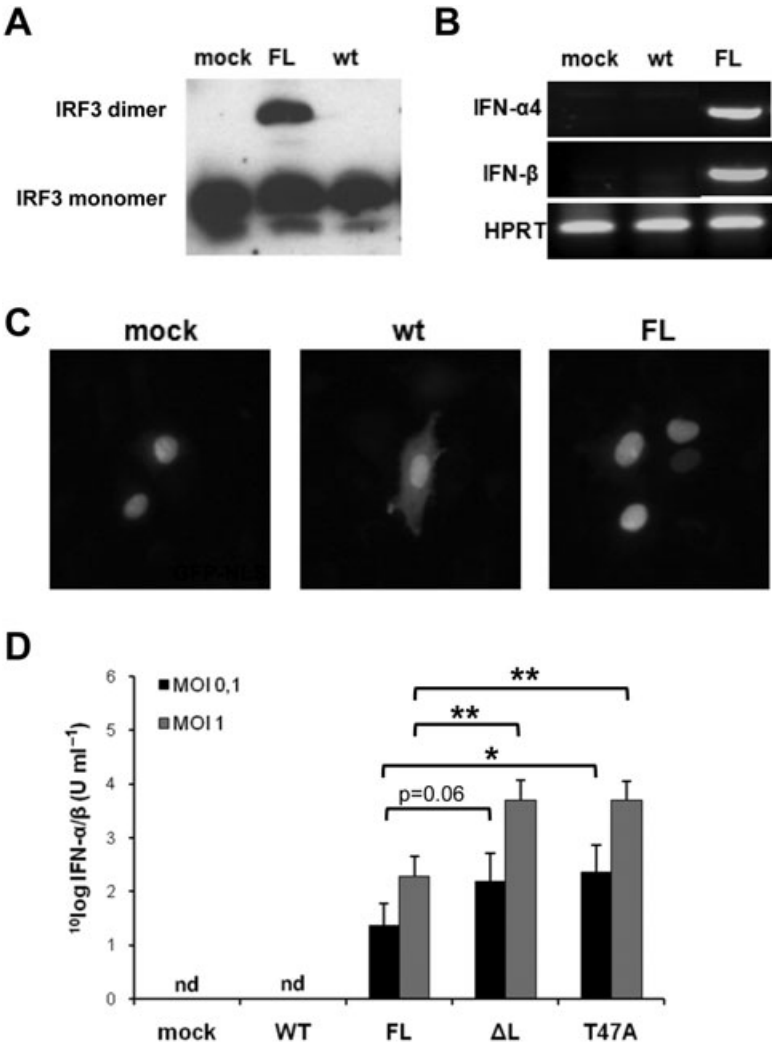

Fig. 2. Suppression of IFN-α/β production *in vitro*.
A. IRF-3 dimerization in cells infected with mengo wt or FL at 8 h p.i., visualized by native PAGE.
B. IFN-α/β gene transcription in L929 cells infected with wt or FL virus at 6 h p.i.
C. Nucleocytoplasmic traffic disorder in infected cells. Cells were transfected with GFP-NLS and then infected with either wt or FL virus. The fate of GFP-NLS was monitored at 5 h p.i.
D. Analysis of IFN-α/β production by bioassay. L929 cells were infected with the indicated virus at the indicated moi for 48 h. Cell culture supernatants were treated for 24 h at pH 2, neutralized, and used for priming of fresh L929 cells, which were then infected with wt virus and scored for the production of cytopathic effect. IFN-α/β concentrations were calculated by using a standard curve generated with dilutions of commercially available mouse IFN-α/β. $^{*}P < 0.05$; $^{**}P < 0.01$.

In short, cells were infected with wt virus, FL virus, an L-deleted virus (ΔL), or a mutant virus carrying a deleterious amino acid alteration in L (T47A), at a moi of 0.1 or 1 for 48 h. Cell culture supernatants were treated for 24 h at pH 2, neutralized, and used for priming of fresh L929 cells. For comparison, a standard curve was made by priming cells with serial dilutions of commercially available mouse IFN-α/β. The primed cells were subsequently infected with serial dilutions of wt mengovirus. The amount of IFN-α/β produced in the cell culture supernatants was determined by comparing the protective effects with those observed with the standard curve. The results (Fig. 2D) showed that unlike the wt virus, the FL virus was unable to completely inhibit IFN-α/β production. However, the amount of IFN-α/β produced was about 10-fold reduced compared with that produced in cells infected with the mutant viruses lacking (ΔL) or containing an inactive L protein (T47A). Thus, the L^{pro}-mediated shut-off of cap-dependent mRNA translation reduces, but does not completely inhibit, IFN-α/β production *in vitro*.

In vivo *IFN-α/β responses and pathogenicity*

We also tested the functional activity of L^{pro} *in vivo*. For this, IFN-α/β gene transcription as well as pathogenicity were analysed in mice. To monitor IFN-α/β gene transcription, we used IFN-α/β receptor knock out (IFNAR-KO) mice because the ability of the viruses to infect and spread is unhindered by the antiviral activity caused by IFN because the mice cannot respond to IFN-α/β. Four-week-old 129/Sv IFNAR-KO mice were infected intracranially with 10^3 $TCID_{50}$ of wt or FL virus. We then measured viral load and cytokine gene expression in the brain of the infected mice. To this end, total RNA was extracted at 3 days p.i. and real-time RT-PCR was used to quantify viral RNA as well as IFN-β, RANTES, and β-actin transcripts. The results showed that FL virus infection resulted in higher mRNA levels of IFN-β and RANTES than wt virus infection, while there was no difference in replication (Fig. 3A). The mRNA levels induced by FL virus infection were similar as those observed in mice infected with

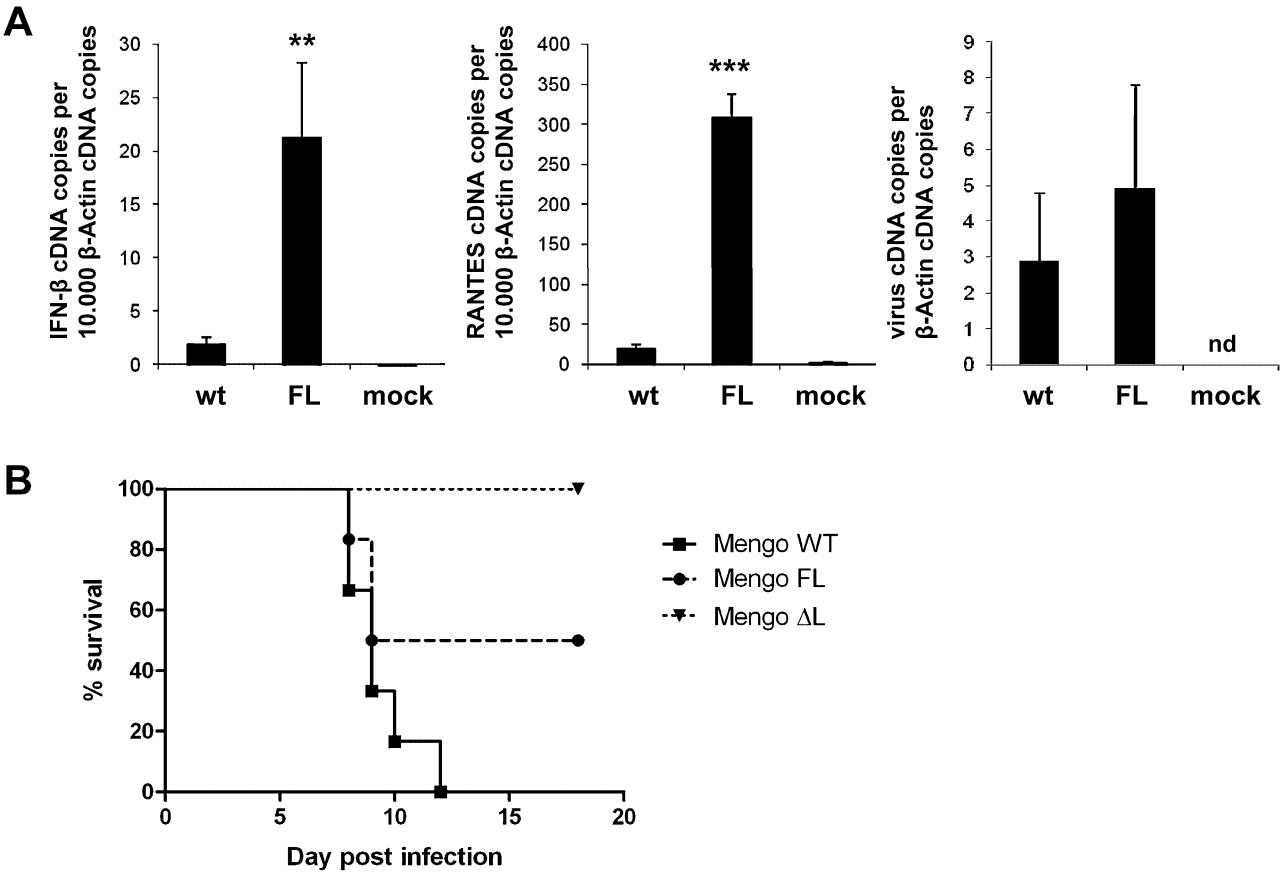

Fig. 3. Suppression of IFN-α/β production and virus pathogenicity *in vivo*.
A. Four-week-old 129/Sv IFNAR-KO mice were infected intracranially with wt or FL virus. IFN-β and RANTES transcripts as well as mengovirus and β-actin transcripts were quantified by real-time RT-PCR in RNA extracted from brains at 3 days p.i. Histograms show mean ± SD of cDNA copy numbers normalized to β-actin cDNA copies ($\times 10^{-4}$ in the case IFN-b and RANTES). $^{**}P < 0.01$; $^{***}P < 0.001$.
B. Four-week-old 129/Sv mice were infected intraperitoneally with 10^4 $TCID_{50}$ of the indicated virus and survival was charted.

mengovirus ΔL and T47A mutants (Hato *et al.*, 2007), which were determined in a parallel experiment. Notwithstanding the observation that L^{pro} has been shown to cause a three- to five-fold reduction in transcription of cytokine and chemokine genes *in vitro* (De Los Santos *et al.*, 2007), it is clear that the FL virus is far less potent in inhibiting cytokine and chemokine gene transcription *in vivo* than wt mengovirus.

To investigate the potential of FMDV L^{pro} to suppress infection-limiting IFN-α/β responses *in vivo*, we compared the pathogenic potential of wt, FL and ΔL virus in normal mice. Six 4-week-old 129/Sv mice were infected intraperitoneally with 10^4 $TCID_{50}$ virus. Mice infected with wt virus exhibited severe paralysis and 6/6 died by day 12 (Fig. 3B). Mice infected with FL virus all developed paralysis but only 3/6 died. The other three mice were still partially paralysed by day 18 but were recovering. In contrast none of the mice infected with ΔL virus died or showed signs of paralysis. These data indicate that, similar as observed *in vitro*, the function mediated by FMDV L^{pro} *in vivo* suppresses antiviral responses to a certain extent, but not as efficiently as that of mengovirus L.

Concluding remarks

Collectively, our results show that the cardiovirus and aphthovirus L proteins have a different mechanism and capacity to limit IFN-α/β production both *in vitro* and *in vivo*. The suppression of antiviral responses by FMDV L^{pro}, which is mediated by its ability to decrease cytokine and chemokine gene transcription and to suppress host cell translation by cleaving cellular proteins, is less effective than the mengovirus L-mediated inhibition of cytokine and chemokine gene transcription that is mediated through the blockage of IRF-3 activation. It should be emphasized that we cannot exclude that mengovirus L is more potent in suppressing IFN-α/β production only in a mengovirus background and that different results would have been obtained with a reciprocal FMDV chimera in

which mengovirus L was substituted for L^{pro}. Notwithstanding this, the data that demonstrate that the FL chimera is much more virulent than the ΔL virus in normal mice show that the FMDV L^{pro} plays an important function in virus virulence. Thus, our data clearly indicate that the distinct, structurally unrelated picornavirus security proteins are partially interchangeable at the functional level.

Experimental procedures

Cells

Baby Hamster Kidney cells (BHK-21) and L929 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum as described (Hato *et al*., 2007).

Viruses

Wild-type and mutant mengoviruses (ΔL and T47A) have been described previously (Hato *et al*., 2007). For the construction of the chimeric mengovirus expressing FMDV L^{pro}, first a unique XhoI site was introduced at position 737 with oligonucleotide 5′-GGA ATG AGC ACA AAT CTC GAG TTC CAT GGT TGT AGC-3′ and a unique HpaI site was introduced at position 920 with oligonucleotide 5′-ATC GGA TGA GGT TGA GTT AAC TTG TGT CTC GAA CAC-3′ by site-directed mutagenesis (restriction sites are underlined). FMDV L^{pro} was amplified by PCR using cDNA clone pMR15 [FMDV type O1K (Ryan *et al*., 1989)] as template with forward primer 5′-ACA GGG AAA CTC GAG CTG ACA CTG TAC AAC GG-3′ (XhoI site underlined) and reverse primer 5′-GGA TTG TCC GGC GCC TTT GAG CTT GCG TTG-3′ (EheI site underlined). The PCR product was cut with the indicated restriction enzymes and cloned between the XhoI and HpaI restriction sites that were introduced in mengovirus cDNA clone pM16.1. Chimeric FL virus was produced by DEAE-mediated transfection of BHK-21 cells with T7 RNA polymerase generated RNA transcripts as described (Zoll *et al*., 2002). Virus titres were determined using serial 10-fold dilutions of eight replicates in 96 well plates containing BHK-21 monolayers. $TCID_{50}$ were calculated as described (Zoll *et al*., 2002).

Western blot analysis

Cells were infected at moi of 50 for 30 min, grown at 37°C, and lysed at the indicated time points. Proteins were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membrane. The membrane was probed with a rabbit antiserum against amino acid residues 327–342 of eIF-4G (kindly provided by R. Rhoads).

In vivo *labelling*

Cells were infected at moi 50 and incubated with methionine-free medium containing 10 μCi ml^{-1} [^{35}S]methionine at various times p.i. After a 1 h labelling period, the cells were lysed and protein synthesis was analysed by 12.5% SDS-PAGE gel electrophoresis and autoradiography as described (Zoll *et al*., 2002).

Viral growth analysis

Cells were infected at the indicated moi for 30 min and grown at 37°C for the indicated times. Viruses were released by three successive cycles of freezing and thawing and titrated on BHK-21 cells as described above.

Analysis of IRF-3 dimerization by native PAGE

Preparation of cell extracts, native PAGE, and Western blotting with a goat polyclonal anti-IRF-3 antibody (C-20) (Santa Cruz) were performed as described (Hato *et al*., 2007).

RT-PCR analysis

Total cellular RNA was harvested using GenElute™ mammalian total RNA Kit (Sigma) according to the manufacturer's instructions. RNA was treated with DNase I prior to reverse transcription and amplification by PCR. Primer sequences have been described (Hato *et al*., 2007).

Interferon bioassay

Infection of cells, harvesting and acid treatment of supernatants to inactivate virus, neutralization, priming of fresh L929 monolayers were all performed as described (Zoll *et al*., 2002). For comparison, a standard curve was made by priming cells with serial dilutions of commercially available mouse IFN-α/β (Sigma).

Mouse experiments and real-time RT-PCR

Infection of mice, extraction of total RNA and real-time RT-PCR were performed as described (Paul and Michiels, 2006). Handling of mice and experimental procedures were conducted in accordance with national and institutional guidelines for animal care and use.

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