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The role of accessory proteins in the replication of feline infectious peritonitis virus in peripheral blood monocytes

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ABSTRACT

The ability to productively infect monocytes/macrophages is the most important difference between the low virulent feline enteric coronavirus (FECV) and the lethal feline infectious peritonitis virus (FIPV). *In vitro*, the replication of FECV in peripheral blood monocytes always drops after 12 h post inoculation, while FIPV sustains its replication in the monocytes from 45% of the cats. The accessory proteins of feline coronaviruses have been speculated to play a prominent role in virulence as deletions were found to be associated with attenuated viruses. Still, no functions have been ascribed to them. In order to investigate if the accessory proteins of FIPV are important for sustaining its replication in monocytes, replication kinetics were determined for FIPV 79-1146 and its deletion mutants, lacking either accessory protein open reading frame 3abc (FIPV-Δ3), 7ab (FIPV-Δ7) or both (FIPV-Δ3Δ7). Results showed that the deletion mutants FIPV-Δ7 and FIPV-Δ3Δ7 could not maintain their replication, which was in sharp contrast to wt-FIPV. FIPV-Δ3 could still sustain its replication, but the percentage of infected monocytes was always lower compared to wt-FIPV. In conclusion, this study showed that ORF7 is crucial for FIPV replication in monocytes/macrophages, giving an explanation for its importance *in vivo*, its role in the development of FIP and its conservation in field strains. The effect of an ORF3 deletion was less pronounced, indicating only a supportive role of ORF3 encoded proteins during the infection of the *in vivo* target cell by FIPVs.

1. Introduction

Feline coronaviruses (FCoVs) are enveloped viruses with a large positive-stranded RNA genome of about 30 kb (Dye and Siddell, 2005). The 5′ two-thirds of the genome encodes for polypeptides that are subsequently cleaved by viral proteinases to yield 16 functional proteins, mainly involved in viral RNA synthesis. The proteins on the 3′-proximal region of the genome are individually expressed from a nested set of subgenomic mRNAs generated by an unique discontinuous transcription mechanism (Enjuanes et al., 2006). These mRNAs cover the four structural proteins; the spike (S), nucleocapsid (N), membrane (M) and envelope (E) proteins, and five accessory proteins 3a, 3b, 3c, 7a and 7b which are most likely produced by leaky scanning from two gene cluster open reading frames ORF3 and ORF7 (Schaecher et al., 2007).

Based on virus antigenicity, two serotypes can be distinguished among FCoVs. Type I feline coronaviruses are most commonly found in the field (Addie et al., 2003; Hohdatsu et al., 1992; Vennema, 1999). Type II feline coronaviruses originate from a double recombination between type I feline coronavirus and canine coronavirus (CCoV) (Herrewegh et al., 1998). Until now, most experiments have been done with type II viruses because type I viruses are difficult to grow *in vitro*. Both serotypes contain two pathotypes. The low virulent feline enteric coronavirus

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(FECV) is endemic in the cat population and causes mostly asymptomatic infections. In young kittens, it can cause mild gastrointestinal infections (Pedersen et al., 1981). The highly pathogenic feline infectious peritonitis virus (FIPV) manifests in a progressive and mostly fatal disease, FIP, characterized by granulomatous lesions (Pedersen et al., 2009). The most prominent factor that determines the difference between the two pathotypes is the ability to establish an efficient and productive infection in monocytes/macrophages (Dewerchin et al., 2005; Rottier et al., 2005). The low virulent FECV mainly infects enterocytes but can also enter and replicate in monocytes (Dewerchin et al., 2005; Meli et al., 2004). However, it was shown *in vitro* that after one replication cycle, the percentage of FECV infected monocytes decreases (Dewerchin et al., 2005). This is probably the reason why only low viral loads of FECV can be found systemically and infection does not lead to FIP. In contrast, the highly pathogenic counterpart, FIPV, has the ability to productively infect monocytes and this sustained infection is probably the basis for the development of FIP (Kipar et al., 2006).

RNA viruses, such as FCoVs, generally have very high mutation rates compared to DNA viruses, because viral RNA polymerases lack proof-reading. This, together with the close genetic similarity of FECV to FIPV and the low incidence of FIP, despite the high prevalence of FCoV seropositive cats, led to the widespread acceptance of the "internal mutation theory". Numerous publications supported that FIPV, unlike FECV, is not transmitted from cat to cat but arises from FECV by mutation within an individual cat (Chang et al., 2010; Herrewegh et al., 1995; Pedersen et al., 2012; Rottier et al., 2005; Vennema et al., 1998). Up till now, it is not yet completely clarified what these FIP-inducing mutations in FECV might be. The C-terminal domain of the FIPV spike protein has already been shown to be important for efficient macrophage infection (Chang et al., 2012; Rottier et al., 2005). In addition, accessory proteins have been proposed to be potential targets for mutations and as such important for virulence. These accessory proteins are species-specific and are dispensable for *in vitro* replication. During passaging of CoVs in cell cultures, viral mutants with deletions in accessory ORFs arise spontaneously and show growth advantages (Lorusso et al., 2008), suggesting that loss of accessory gene expression increases viral fitness *in vitro*. Despite their redundancy *in vitro*, deletion of accessory ORF clusters from FIPV 79-1146, mouse hepatitis virus (MHV) and transmissible gastroenteritis virus (TGEV) genome resulted in attenuated viruses (de Haan et al., 2002; Haijema et al., 2004; Ortego et al., 2003). Thus, these proteins are of key importance for virus–host interactions and critically contribute to viral virulence and pathogenesis. Nevertheless, a clear function could not be attributed so far to any of the accessory proteins of FCoVs.

Based on their sequence, both 3a and 3b proteins are predicted which consist of 71 amino acids (AA). None of these predicted proteins have a hydrophobic segment that can serve as a signal peptide or transmembrane domain, so they are probably located in the cytosol where they exert an intracellular function. Their sequences are well conserved within the two pathotypes, while more differences have been seen between the two serotypes (Volker, 2007). Comparative sequence analysis with CCoV and FCoV strains revealed that type II FCoV has received these genes from CCoV.

Translating the consensus nucleotide sequence, the 3c protein appears to be 238–244 AA in length. Hydrophobicity analysis predicts that 3c is a class III triple-membrane spanning protein suggesting a topology that is quite similar to the one of the M protein of FCoVs and the 3a protein of the 'severe acute respiratory syndrome' coronavirus (SARS-CoV) (Narayanan et al., 2008; Oostra et al., 2006). In the first quarter of the amino acid sequence of the 3c gene, big differences are observed between type I and type II FCoVs. Again the double recombination between type I FCoV and CCoV could explain these differences. Remarkably, in studies of Pedersen et al. (2012) and Chang et al. (2010) 60–71.4% of FIP isolates, had a truncated 3c protein, while in FECV isolates from feces this protein was intact. Chang et al. launched the hypothesis that 3c could be necessary for replication in enterocytes but dispensable or even a burden during FIPV infection in monocytes. This may also explain the restricted transmission of FIPV from cat to cat (Addie et al., 1996). A recent study added to these findings that a truncated ORF3 was important for efficient *in vitro* replication of FIPV type II in feline monocytes (Balint et al., 2012).

The 7a protein, a small hydrophobic protein of 101 AA (~10 kDa) with a N-terminal cleavable signal sequence and a C-terminal transmembrane domain, is more or less well conserved among FCoVs (Volker, 2007). It has a 72% homology with TGEV protein 7, which has been demonstrated to function against host antiviral responses (Cruz et al., 2011).

Most knowledge has been gathered about the 7b protein, which is a soluble glycoprotein of ~24 kDa (207 AA). A lot of single amino acid polymorphisms are present between FCoV strains (Herrewegh et al., 1995). Antibodies against 7b protein, found in serum of asymptomatic FCoV-seropositive and FIP cats, imply expression of this protein during natural infections (Kennedy et al., 2008). The 7b protein contains a KDEL-like endoplasmic reticulum (ER) retention signal (=KTEL) at its C-terminus (Vennema et al., 1992). The 7b protein is initially retained in the ER, whereupon it is secreted from infected cells. As 7b is released extracellular, it might function as a virokine which acts as an immune-modulator of host immune responses (Herrewegh et al., 1995; Rottier, 1999). Again the real function has not yet been elucidated.

Knowing that the deletion of accessory genes is associated with attenuated viruses (Haijema et al., 2004) and only virulent FIPV can sustain its replication in monocytes (Dewerchin et al., 2005), the hypothesis was created that one or more accessory proteins may be needed by FIPV for productive replication in feline monocytes. In the present study, this hypothesis was investigated by establishing *in vitro* replication kinetics of FIPV 79-1146 and its accessory ORF deletion mutants in feline peripheral blood monocytes. Results were compared with kinetics in fcwf cells, which is one of the often used model cell lines for infection studies of type II FCoVs. In this way, the function

of the accessory proteins was evaluated in its natural host cell.

2. Materials and methods

2.1. Cats

Three purpose-bred FCoV, FeLV and FIV antibody-negative cats were used as blood donors for the infection kinetics study. The cats were maintained in a temperature-controlled closed household (approved by Local Ethical Committee).

2.2. Viruses and cells

Crandell Rees feline kidney (CrFK) cells were used to obtain third passages of type II FIPV strain 79-1146. FIPV strain 79-1146 was kindly provided by Dr. Rottier (Faculty of Veterinary Medicine, Utrecht University, The Netherlands)

Felis catus whole foetus (fcwf) cells were used to obtain fifth passages of FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7. These three viruses are deletion mutants from type II FIPV strain 79-1146, kindly provided by Dr. Rottier (Faculty of Veterinary Medicine, Utrecht University, The Netherlands). They are, respectively, deleted in open reading frames (ORFs) 3abc, 7ab or both 3abc and 7ab, using reverse genetics (Haijema et al., 2004).

Blood monocytes were isolated from feline blood and seeded on glass coverslips in a 24-well dish, as described previously (Dewerchin et al., 2005).

2.3. Inoculation of fcwf cells and monocytes

Forty-five hours after seeding, fcwf cells and feline monocytes were inoculated with FIPV 79-1146, FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7 at a multiplicity of infection (m.o.i.) of 0.02. After 1 h incubation at 37 °C with 5% CO_2, cells were washed 3 times with RPMI-1640 and further incubated in medium.

2.4. Detection of viral nucleocapsid protein expression in FCoV infected monocytes and fcwf cells

Monocytes or fcwf cells were fixed at 0, 6, 12 and 24 h post inoculation (hpi) with 4% paraformaldehyde and further permeabilized with 0.1% Triton X-100 (Sigma–Aldrich GmbH, Steinheim, Germany). To identify FCoV-infected cells, viral cytoplasmic antigens were stained with mouse IgG1 monoclonal antibody (mAb) 10A12 (produced and characterized in our laboratory) which recognizes FCoV nucleocapsid (N) protein, followed by fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Molecular Probes). Nuclei were visualized with Hoechst 33342 (Molecular Probes). Finally, glass coverslips were mounted on microscope slides using glycerin–PBS solution (0.9:0.1, v/v) with 2.5% 1,4-diazabicyclo(2,2,2)octane (Janssen Chimica, Beerse, Belgium). The percentage of infected cells at each time point and for every virus was determined by fluorescence microscopy (Leica Microsystems DMRBE).

2.5. Growth curves of feline coronaviruses in fcwf cells and monocytes

At different time points post inoculation, culture medium was harvested and centrifuged at $400 \times g$ for 10 min. Supernatant was stored at −70 °C until titration of extracellular virus. Intracellular virus was obtained by scraping the remaining cells in 1 ml fresh medium. Cells were transferred to the eppendorf with the pellet of the centrifuged extracellular virus and subjected to two freeze–thaw cycles. Virus titers were assessed by a 50% tissue culture infective dose ($TCID_{50}$) assay using fcwf cells. The 50% end-point was calculated according to the method of Reed and Muench (1938).

2.6. Statistical analysis

Statistical analysis was performed by Mann–Whitney *U* tests with SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Significant differences were considered if $p \leq 0.1$.

3. Results

3.1. Viral nucleocapsid protein expression kinetics in FCoV-infected fcwf cells

After infection of fcwf cells at a m.o.i. of 0.02, viral antigen positive cells were first detected at 6 hpi and the percentage kept increasing afterwards (Fig. 1). FIPV always gave the highest percentage of infected fcwf cells at 24 hpi (56.6 ± 15.6%), followed in order by FIPV-Δ3 (41.5 ± 12.3%), FIPV-Δ7 (37.0 ± 11.4%) and FIPV-Δ3Δ7 (18.2 ± 7.6%) (Figs. 1 and 2). At that time point, the percentage of fcwf cells infected with FIPV-Δ7 and FIPV-Δ3Δ7 was significantly lower compared to wt-FIPV indicating a role of ORF7 during FIPV replication.

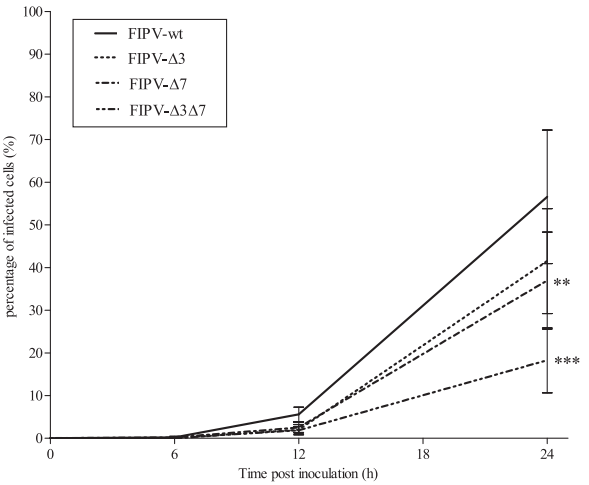

Fig. 1. Kinetics of percentage of infected cells in FCoV-inoculated fcwf cells. Cells were inoculated with FIPV 79-1146, FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7 at a m.o.i. of 0.02. At different time points post inoculation, cells were fixed and cytoplasmic nucleocapsid protein was visualized with an immunofluorescence staining. The data represent means ± SD of five replicate assays. Significant difference with FIPV 79-1146 is indicated with $^{*}p < 0.1$; $^{**}p < 0.05$; or $^{***}p < 0.01$.

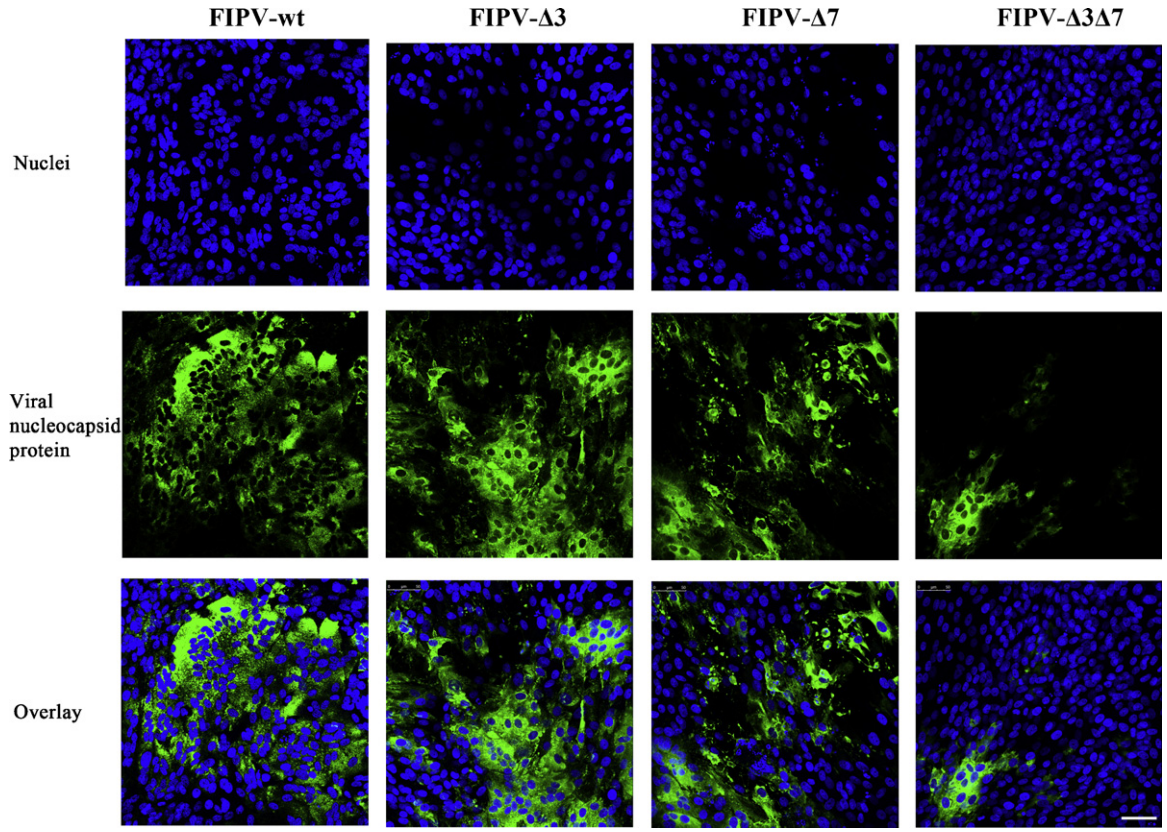

Fig. 2. Confocal microscopy images of cytoplasmic expression of the nucleocapsid proteins (green) in fcwf cells 24 h after inoculation with FIPV 79-1146, FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7 at a m.o.i. of 0.02. Nuclei were visualized with Hoechst 33342 (blue). Bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. Growth kinetics of FCoV in fcwf cells

Growth curves for the different viruses in fcwf cells are given in Fig. 3. Production of progeny virus was detected between 6 and 12 hpi and kept increasing up till 24 hpi. Concerning the intracellular titer, the increase between 12 and 24 hpi was significant stronger for FIPV 79-1146 and FIPV-Δ3 (up to 7.35 $\log_{10}$ $TCID_{50}/10^6$ cells at 24 hpi) than for FIPV-Δ7 and FIPV-Δ3Δ7 (up to 5.7 and 5.1 $\log_{10}$ $TCID_{50}/10^6$ cells, respectively, at 24 hpi). The extracellular virus titers showed a similar pattern with slightly lower titers for FIPV 79-1146 and FIPV-Δ3 (up to 6.8 $\log_{10}$ $TCID_{50}/10^6$ cells at 24 hpi). FIPV-Δ7 and FIPV-Δ3Δ7 reached the same extracellular as intracellular titers.

The amount of infectious virus assembled per cell was calculated based on virus titers and percentages of infected cells. All viruses formed a similar low amount of infectious viruses per infected cell (less than 5) at 12 hpi.

3.3. Viral nucleocapsid protein expression kinetics in FCoV-infected monocytes

The first viral antigen positive monocytes appeared around 6 hpi (Fig. 4). Monocytes from all three cats showed an increase in percentage of infected cells for FIPV 79-1146 up till 24 hpi. When ORF3 was deleted from this virus, it was still able to produce infectious virus which kept on infecting exponentially new cells up till 24 hpi, though, the percentage of infected cells was 5–20 times lower in comparison with wt-FIPV. The observed difference in percentage infected monocytes between wt-FIPV and FIPV-Δ3 was more pronounced than the difference in percentage infected fcwf cells (which was only 1.3 times).

More remarkable were the results when ORF7 (FIPV-Δ7), or both ORF3 and ORF7 (FIPV-Δ3Δ7) were deleted from FIPV. These mutant viruses initially infected the monocytes similarly as wt-FIPV but from 12 hpi the percentage of infected cells declined. Although FIPV-Δ7 and FIPV-Δ3Δ7 reached also a lower percentage of infected cells in fcwf at 24 hpi compared to wt-FIPV (1.5 and 3.3 times less, respectively), the difference in infected cells was far more prominent in monocytes (for both deletion mutants 100–3000 times less infected cells in comparison with wt-FIPV).

3.4. Growth kinetics of FCoV replication in feline monocytes

Both wt-FIPV and FIPV-Δ3 kept producing new progeny virus between 12 and 24 hpi in monocytes of all three cats but virus titers were always higher for wt-FIPV. In contrast, growth curves of both FIPV-Δ7 and FIPV-Δ3Δ7 reached a plateau or even decreased from 12 hpi (Fig. 5). Knowing the total production of infectious progeny virus and the number of infected cells, it can be calculated that wt-FIPV

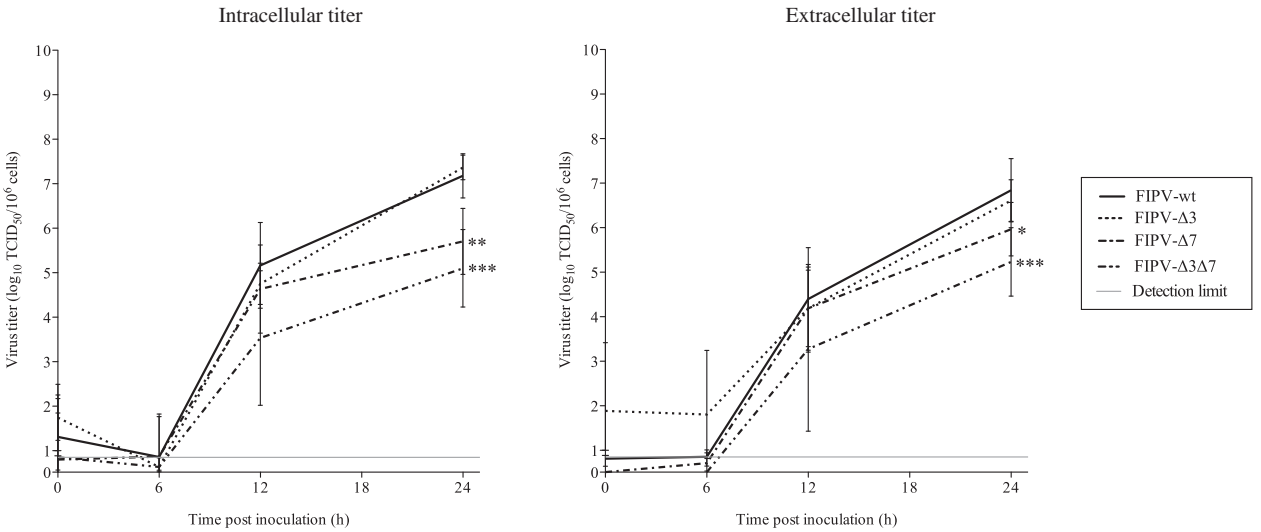

Fig. 3. Kinetics of FCoV replication in fcwf cells. Cells were inoculated with FIPV 79-1146, FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7 at a m.o.i. of 0.02. At different time points post inoculation, the intracellular and extracellular virus titers were determined. The data represent means ± SD of five replicate assays. Significant difference with FIPV 79-1146 is indicated with $^{*}p < 0.1$; $^{**}p < 0.05$; or $^{***}p < 0.01$.

infected monocytes have produced 50–250 infectious viruses per cell, at 12 hpi. Monocytes infected with the three deletion mutants produced a somewhat lower amount of infectious viruses per infected cell (FIPV-Δ3: 4–170 viruses/cell; FIPV-Δ7: 4–50 viruses/cell and FIPV-Δ3Δ7: 1–90 viruses/cell).

4. Discussion

In the present article, the role of both accessory ORF encoded proteins of FIPV in the replication in its *in vivo* target cell, the feline blood monocyte, was investigated for the first time. This was done by comparing *in vitro* replication kinetics of different accessory gene-deletion mutants of FIPV strain 79-1146 with the kinetics of this wt-FIPV strain in peripheral blood monocytes from three cats. All the viruses were able to initially infect monocytes but only wt-FIPV and FIPV-Δ3 sustained their replication while the replication kinetics of FIPV-Δ7 and FIPV-Δ3Δ7 dropped after one cycle.

Our results indicate that newly produced viruses deleted in ORF7 were not able to enter new monocytes or that virus protein synthesis was inhibited (by an antiviral response) in monocytes cultured for some time in an infected environment. Because currently, accessory proteins are not found in the envelope of feline coronaviruses, it is unlikely that they play a role during binding with and entry of the target cell. Counteraction on the antiviral response is a far more plausible function for proteins 7a and/or 7b. When cells are infected, they can warn neighboring cells of viral presence by releasing interferon (IFN). This induces production of several antiviral proteins that have roles in combating viruses or blocking protein synthesis in response to new viral infection (Goodbourn et al., 2000). As ORF7 is located at the 3′ far end of the viral genome, where transcription starts (Dye and Siddell, 2005), 7a and 7b mRNA is produced at the very beginning of replication, indicating an early translation of proteins 7a and 7b. It is likely that these proteins interfere quickly with one or more effects of IFN in newly infected cells and as such inhibiting the antiviral response. As a consequence, viruses deleted in ORF7 may not counteract with IFN induced antiviral responses anymore and virus replication may be blocked. The crucial role of ORF7 in FIPV replication in monocytes *in vitro*, as shown in our study, could also give an explanation for the attenuated phenotype of ORF7 deletion mutants *in vivo*, observed by Haijema et al. (2004).

In contrast to ORF7 proteins, ORF3 proteins were less important for virus replication in monocytes because FIPV-Δ3 was still capable of sustaining infection in feline monocytes up till 24 hpi. However, the increase in infected monocytes and the resulting virus titer was less pronounced for FIPV-Δ3 than for wt-FIPV. These results showed that at least one of the ORF3 encoded accessory proteins should be intact for optimal replication. FIPV-Δ3 is distinguished from wt-FIPV 79-1146 by a deletion of only the 3a and 3b genes, since the 3c gene of wt-FIPV 79-1146 encodes for a truncated 3c protein (Volker, 2007), which also appears in 60–71.4% of *in vivo* FIPV strains (Chang et al., 2010; Pedersen et al., 2012). Thus, the somewhat lower replication capacity of FIPV-Δ3 in monocytes is most likely due to the lack of protein 3a and/or 3b. The use of the truncated wt-FIPV in the present study can also explain the, at first sight, contrasting results obtained by Balint et al. (2012). They showed that an ORF3 truncation was needed for efficient FIPV type II replication in monocytes/macrophages. Our results demonstrate that ORF3c-truncated wt-FIPV 79-1146 indeed efficiently replicates in monocytes/macrophages, but that at least one of the other proteins (3a and 3b) should be intact to support FIPV replication in monocytes.

Not surprisingly, infection with the double deletion mutant FIPV-Δ3Δ7 showed an additive effect of both ORF3

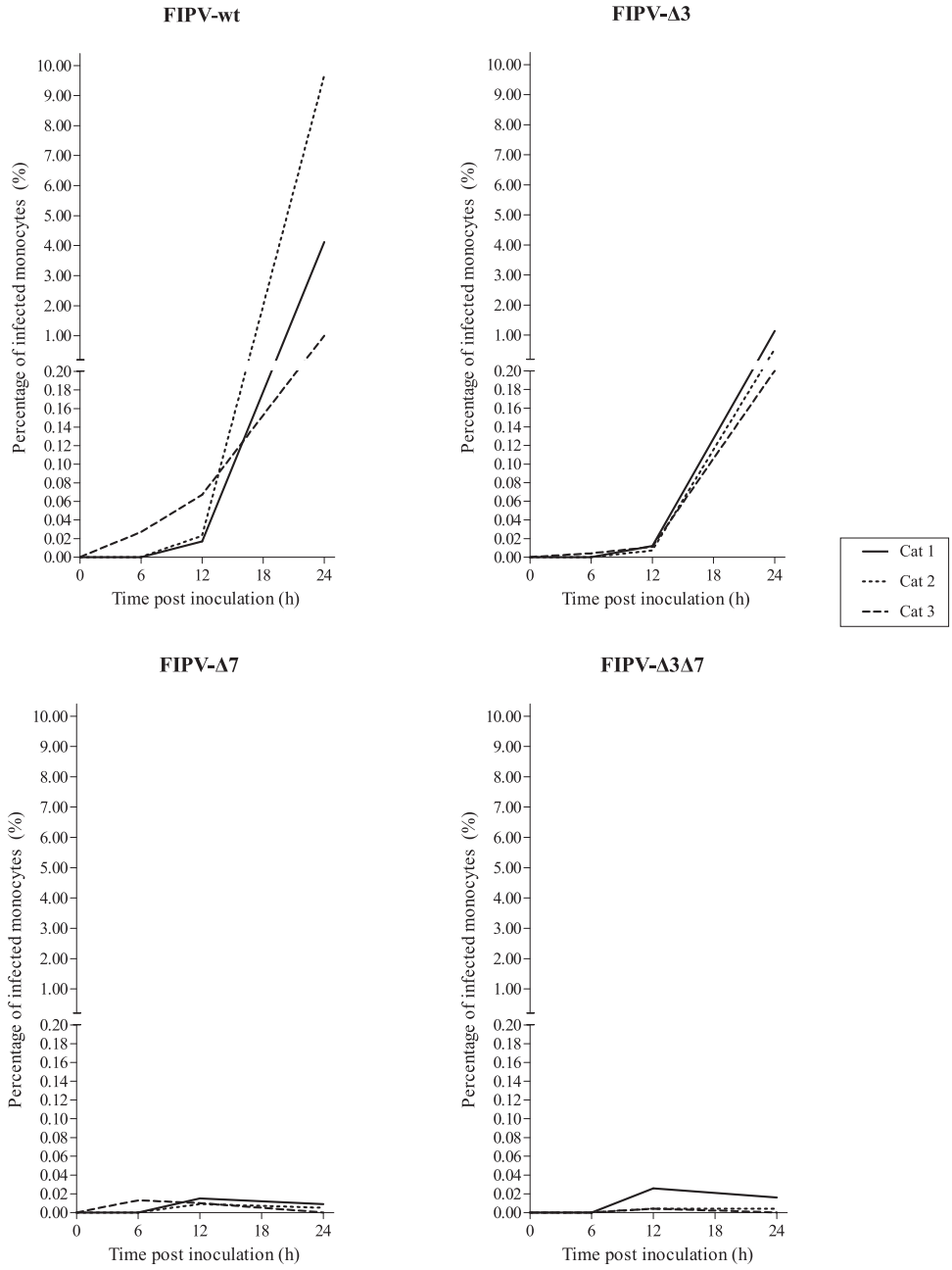

Fig. 4. Kinetics of percentage of infected cells in FCoV inoculated monocytes isolated from the blood of three independent cats. Cells were inoculated with FIPV 79-1146, FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7 at a m.o.i. of 0.02. At different time points post inoculation, cells were fixed and cytoplasmic nucleocapsid protein was visualized with an immunofluorescence staining.

and ORF7 deletions. Consequently, FIPV-Δ3Δ7 reached lowest titers and lowest percentages of infected cells at 24 hpi.

Finally, in this study it was observed that in fcwf cells, FIPV-Δ7 and FIPV-Δ3Δ7 infection resulted in lower virus titers compared to wt-FIPV, whereas these viruses reached similar titers in CrFK cells (data not shown). The significant lower titer obtained for FIPV-Δ7 and FIPV-Δ3Δ7 was explained by a lower production capacity of these viruses in fcwf cells (10 times less infectious virus than wt-FIPV) compared to CrFK cells. This phenomenon could be ascribed to macrophage features of the fcwf cells (Jacobse-Geels and Horzinek, 1983). The impaired replication of FIPV-Δ7 and FIPV-Δ3Δ7 in fcwf cells, seen in this study, confirmed the role of ORF7 in efficient replication of FIPV in its host cells (monocytes/macrophages), suggesting a function in typical macrophage pathways.

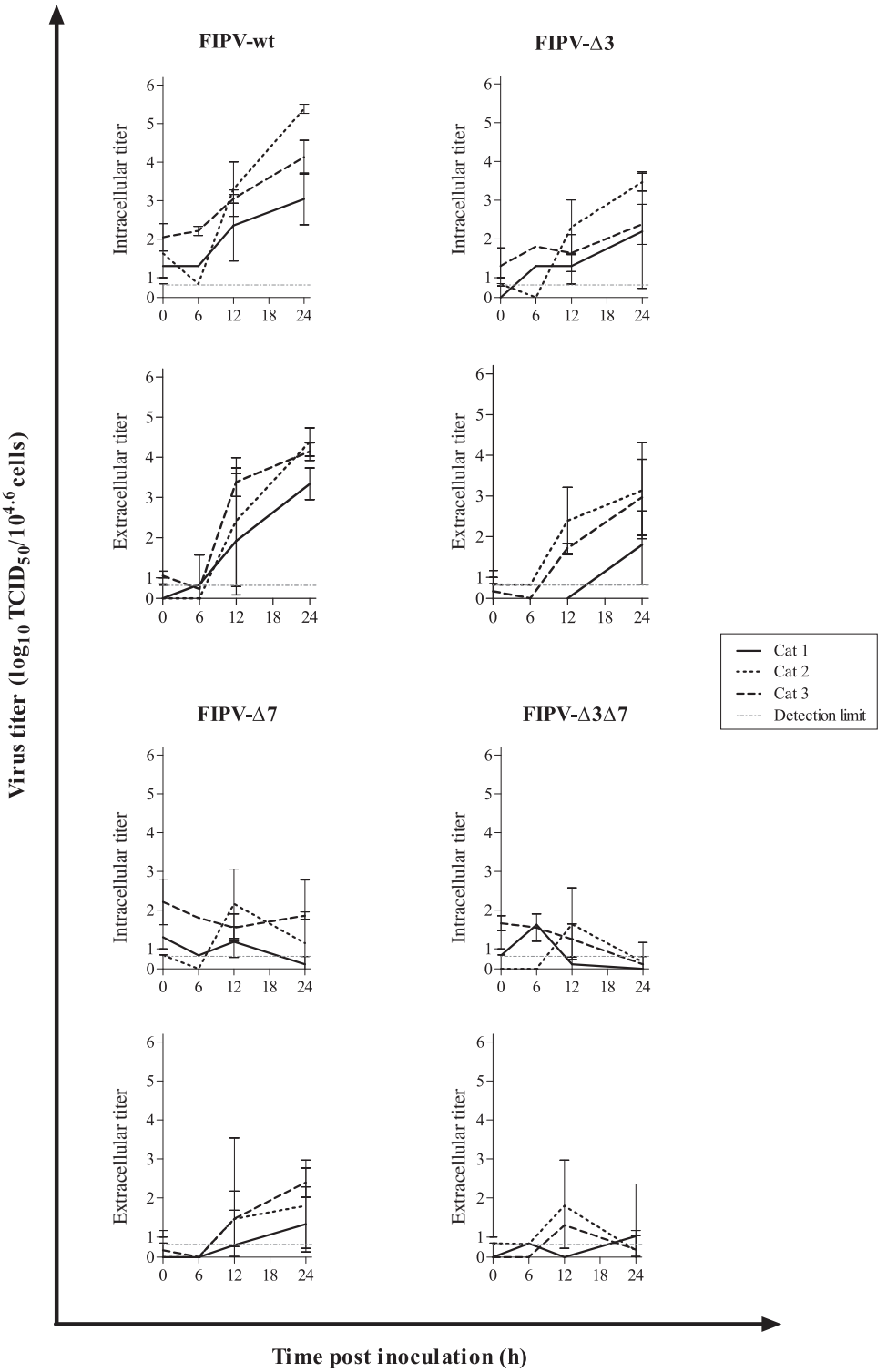

Fig. 5. Kinetics of FCoV replication in feline monocytes of three cats. Cells were inoculated with FIPV 79-1146, FIPV-Δ3, FIPV-Δ7 and FIPV-Δ3Δ7 at a m.o.i. of 0.02. At different time points post inoculation, intracellular and extracellular virus titers were determined. The data represent means ± SD of triplicate assays.

In conclusion, it was found that deleting ORF7 from type II FIPV 79-1146 had a negative impact on the replication kinetic in feline monocytes. Thus, proteins encoded by ORF7 play a decisive role in sustaining the replication of type II FIPV in its *in vivo* target cell. This role is most probably a general function for the 7a/7b proteins in both type I (most prevalent in the field) and type II FCoVs. Sequence analysis revealed that 7a gene is well conserved among both serotype FCoV strains. Although, the 7b gene shows more single amino acid polymorphisms (~5-8%), these are not serotype related. The moderate effect of an ORF3 deletion from type II FIPV, indicates that proteins encoded by ORF3 have only a supportive role in the replication. Despite the homology of ORF3 within the two serotypes, clear differences are present in between them. This is due to the fact that type II FCoVs received their ORF3 (together with the spike gene) from CCoVs by double recombination with type I FCoVs. It remains to be determined if the ORF3 is of the same importance for the replication of type I field isolates in feline monocytes. The results of this study will aid in the understanding of the role of accessory proteins in FCoV infections of monocytes. Although it is still only a tip of the iceberg, our discoveries are a step forward in unraveling the function(s) of the accessory proteins of FCoVs and give indications for further research in the involvement of the accessory proteins (such as 7a and/or 7b) in antiviral pathways.

Conflict of interest

The authors declare that they have no conflict of interests.

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