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Research article

Differential susceptibility of macrophages to serotype II feline coronaviruses correlates with differences in the viral spike protein.

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Highlights

- Differences in the S protein modulate serotype II FCoV infection of macrophages
- Critical residues in the spike S2 domain of type II FCoV affecting cell tropism
- Cooperativity at 5 positions in the S protein modulates FCoV macrophage entry

Abstract

The ability to infect and replicate in monocytes/macrophages is a critically distinguishing feature between the two feline coronavirus (FCoV) pathotypes: feline enteric coronavirus (FECV; low-virulent) and feline infectious peritonitis virus (FIPV; lethal). Previously, by comparing serotype II strains FIPV 79-1146 and FECV 79-1683 and recombinant chimeric forms thereof in cultured feline bone marrow macrophages, we mapped this difference to the C-terminal part of the viral spike (S) protein (S2). In view of the later identified diagnostic difference in this very part of the S protein of serotype I FCoV pathotypes, the present study aimed to further define the contribution of the earlier observed ten amino acids difference to the serotype II virus phenotype in macrophages. Using targeted RNA recombination as a reverse genetics system we introduced the mutations singly and in combinations into the S gene and evaluated their effects on the infection characteristics of the mutant viruses in macrophages. While some of the single mutations had a significant effect, none of them fully reverted the infection phenotype. Only by combining five specific mutations the infections mediated by the FIPV and FECV spike proteins could be fully blocked or potentiated, respectively. Hence, the differential macrophage infection phenotype is caused by the cooperative effect of five mutations, which occur in five functionally different domains of the spike fusion subunit S2. The significance of these observations will be discussed, taking into account also some questions related to the identity of the virus strains used.

Abbreviations

APN: aminopeptidase N

CCoV: canine coronavirus

CoV: coronavirus

DMEM: Dulbecco's modified Eagle's medium

FCoV: feline coronavirus

FCS: fetal calf serum

FCWF: Felis catus whole fetus

FECV: feline enteric coronavirus

FIPV: feline infectious peritonitis virus

FP: fusion peptide

HR: heptad repeat

MERS: Middle East respiratory syndrome

MHV: mouse hepatitis virus

ORF: open reading flame

PBS: phosphate-buffered saline

S: spike

SARS: severe acute respiratory syndrome

TCID50: median tissue culture infectious dose

TM: transmembrane

Keywords: feline coronavirus, feline infectious peritonitis, spike protein, virus cell entry, macrophage, susceptibility, pathogenesis

1. Introduction

Coronaviruses (CoVs) are enveloped, positive-strand RNA viruses with genomes of about 30 kilobases (kb). Originally considered of veterinary relevance primarily, the outbreaks of Severe Acute Respiratory Syndrome (SARS)-CoV and Middle East Respiratory Syndrome (MERS)-CoV dramatically established their importance as life-threatening human pathogens as well. While the human coronaviruses (HCoVs) mainly target the respiratory system, coronaviruses generally cause respiratory and intestinal tract infections.

Coronaviruses have been detected in almost all animal species. In cats their occurrence is quite ubiquitous, animals often becoming infected already at a very young age. Feline coronaviruses (FCoVs) generally cause enteric infections that are very mild or, more typically, pass unnoticed, particularly in older cats. Yet, the infections by these feline enteric coronaviruses (FECVs) are not efficiently cleared and often persist in cells of the intestinal mucosa, with virus being shed through the feces for weeks or months, sometimes for over a year (Kipar et al., 2010; Pedersen et al., 2008a; Pedersen et al., 2008b; Vogel et al., 2010). Besides this harmless enteric pathotype another feline coronavirus occurs that causes severe and generally fatal systemic infection: feline infectious peritonitis virus (FIPV). This highly virulent but more sporadically occurring pathotype is able to induce lethal immunopathological disease characterized by disseminated pyogranulomas and severe inflammatory damage to serosal membranes, often accompanied by exudation into body cavities (Addie et al., 2009; Campolo et al., 2005; de Groot and Horzinek, 1995; Drechsler et al., 2011; Haijema et al., 2007; Kipar and Meli, 2014; Lorusso et al., 2017; Myrrha et al., 2011; Pedersen, 2009).

FCoVs also come in two serotypes (Pedersen et al., 1984a). Serotype I viruses are most prevalent and responsible for about 70-95% of field infections (Addie et al., 2003; Benetka et al., 2004; Hohdatsu et al., 1992; Kummrow et al., 2005). Serotype II viruses are more rarely observed except in Asia, where they may represent up to about 30% of infections (An et al., 2011; Hohdatsu et al., 1992). Genetic evidence indicates that serotype II viruses arise by recombination between serotype I FCoVs and canine coronaviruses (CCoVs) in doubly-infected animals, as a result of which the feline virus acquires the canine virus spike (S) protein (Herrewegh et al., 1998; Vennema, 1999). Together with the CCoV S-specific antigenic properties the resulting type II FCoV thus also acquires receptor specificity for the aminopeptidase N (APN) protein, a practically important feature as it has enabled investigators to grow and study these type II viruses in fAPN expressing culture cells while such susceptible cell systems were lacking for type I viruses. The S proteins of the two FCoV serotypes are very different. Yet, interestingly, each serotype exhibits both pathotypes, FECV and FIPV (Hohdatsu et al., 1991a; Hohdatsu et al., 1991b; Pedersen et al., 2008a).

Rather than being distinctive circulating viruses (Brown et al., 2009), ample and convincing evidence has accumulated indicating that FIPV arises from FECV by mutation in individual, FECV-infected cats (Chang et al., 2010; Chang et al., 2012; Licitra et al., 2013; Pedersen et al., 2009; Pedersen et al., 2012; Poland et al., 1996; Vennema et al., 1998). Favored by its ability to persist in the gut and by the relatively high mutation rate of RNA viruses, genetic changes in the FECV genome enable this enteric biotype to go systemic. Monocytes/macrophages are considered to be instrumental in this biotope switch as they appear to become susceptible to infection due to the critical mutation(s) (Pedersen, 2014). Infected macrophages subsequently transport the virulent pathotype through the body to the organs where it can cause its devastating pathogenic effects.

Earlier we investigated this macrophage tropism of FCoVs *in vitro* (Rottier et al., 2005). We used the serotype II strains FECV 79-1683 and FIPV 79-1146. The comparative pathogenicity of these strains was originally described in 1984 (Pedersen et al., 1984a), on the basis of which these viruses have subsequently served as the prototypic representatives of the two FCoV pathotypes. Consistent with observations made by others (Dewerchin et al., 2005; Regan et al., 2008; Stoddart and Scott, 1989), we found that FIPV, in contrast to FECV, infects and replicates effectively in cultured bone marrow derived macrophages. By constructing - using reverse genetics (Haijema et al., 2003) - and testing FECV/FIPV chimeric viruses we were able to map this macrophage tropism to the viral spike protein. Surprisingly, but consistent with both viral strains using the fAPN receptor, the property was further mapped to the C-terminal, i.e. membrane-proximal domain of the S protein (S2) (Rottier et al., 2005), which is the domain responsible not for virus-receptor interaction but for viral membrane fusion. The residues responsible for the difference were not further defined.

The genomic mutation(s) responsible for the FCoV virulence switch have not been identified yet. Several genes, including the S gene and the accessory genes 3a, 3b, 3c, 7a, and 7b, have been considered (Balint et al., 2012; Chang et al., 2010; Kennedy et al., 2001; Licitra et al., 2013; Pedersen et al., 2012; Rottier et al., 2005; Vennema et al., 1998). Recently, by comparative full-genome analysis of 11 serotype I viruses of each pathotype and by subsequently zooming in on one region of consistent variance by specifically sequencing this region, we detected an amino acid difference that occurred in more than 95% among 183 FIPVs and 118 FECVs (Chang et al., 2012). Intriguingly, this characteristic difference also mapped to the membrane-proximal S2 part of the S protein. Though the biological significance of this difference, particularly its possible effect on the viral monocyte/macrophage infection phenotype, is still unknown, further studies

indicated it to relate to systemic spread of the virus in the infected animal (Barker et al., 2017; Porter et al., 2014). The identification of this characteristic difference between the serotype I FCoV pathotypes in the C-terminal part of the S protein, the very part to which we earlier mapped the macrophage tropism in the serotype II viruses, prompted us to try to identify the critical tropism determinant(s) in these latter viruses. Despite some concerns relating to the precise identity of the FCoV strains 79-1683 and 79-1146, to be discussed later, but lacking better options to address this critical issue in the pathogenesis of FIP, we continued in the present study with investigating the contributions of the amino acids in the spike S2 domain differing between the prototypic strains to the distinguishing macrophage tropism of these viruses.

2. Materials and methods

2.1. Cells and viruses. Felis catus whole fetus (FCWF) and murine LR7 cells (Kuo et al., 2000) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Lonza group Ltd., Bazel, Switzerland) containing 10% fetal calf serum (FCS), 100 IU penicillin/ml and 100 µg streptomycin/ml (all from Life Technologies, Ltd, Paisley, United Kingdom). The recombinant form of FIPV strain 79-1146 (rec79-1146) and its derivative carrying the S protein of FECV strain 79-1683 (79-1146/1683S) were generated by targeted RNA recombination as described below. All recombinant viruses were propagated and titrated using FCWF cells. The chimeric mFIPV, a FIPV 79-1146 derivative in which the S protein ectodomain is from the mouse hepatitis coronavirus (MHV, strain A59), was propagated in and titrated on LR7 cells (Haijema et al., 2003).

2.2. Targeted RNA recombination. For constructing recombinant mutant FIPVs the targeted RNA recombination method was performed as described previously (Haijema et al., 2003; Rottier et al., 2005) and as illustrated in Fig. 1a. It makes use of the transcription plasmid pBRDI1 (GenBank AY204704), RNA transcripts of which consist of a defective FIPV 79-1146 genome lacking nucleotides (nt) 701-20101, i.e. they are composed of the genomic 5'-terminal 698nt fused to the 3'-terminal 335-nt proximal end of ORF1b and running to the 3' end of the genome (Haijema et al., 2003) (numbers based on AY204704 and DQ010921 sequences). For the preparation of S gene mutants, derivatives of pBRDI1 were constructed in which the S gene was replaced entirely (Fig. 1a) or partially (Fig. 1b) by that of FECV strain 79-1683 (Rottier et al., 2005) and/or in which more subtle mutations were introduced. Nucleotide mutations were inserted in the pBRDI1 plasmid using specific primers and the QuikChange XL site-directed mutagenesis kit (Agilent, Palo Alto, CA, USA) following the provider's instructions. Donor RNA transcripts were synthesized from NotI-linearized pBRDI1 using mMESSAGE mMACHINE T7 (Life Technologies, Carlsbad, CA, USA). Briefly, 1 μg of template plasmid was mixed with 2μl of 10×buffer, 2μl of GTP, 10μl of CAP/NTP, and 2μl of enzyme mix, and incubated at 37ºC for more than 2 hours. LR7 cells were infected with mFIPV at a multiplicity of infection (m.o.i.) of 2. After 4 hours of incubation, cells were trypsinized and washed once with 5% FCS/DMEM and once with PBS (-), and then resuspended in 700μl of PBS(-) in 0.4cm cuvette for Gene Pulser (Bio-rad, Hercules, CA, USA). The RNA transcripts were added to the cuvette and pulsed once at 0.85kV with 50μF using Gene Pulser. After electroporation, the LR7 cells were added to a monolayer of FCWF cells in a T25 flask. After 24 to 48 hours of incubation, supernatants were collected and purified by two rounds of limited dilution on FCWF cells. The resulting virus was subsequently amplified once using FCWF cells and used for the experiments. To confirm the sequences of

recombinants, viral RNAs were isolated after propagation using a QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany), cDNA was synthesized using random primers and SuperScriptII Reverse Transcriptase (Life Technologies) and the relevant S gene region was amplified with specific primers and the Expand High Fidelity PCR system (Roche, Basel, Switzerland). The sequencing analysis was performed by Macrogen Corp. Europe (Amsterdam, The Netherlands).

2.3. Macrophage culture and infection. Feline macrophages were grown as previously described (Rottier et al., 2005; van der Meer et al., 2007) with some modifications. Briefly, the stocks of bone marrow-derived mononuclear cells (2×10^7 cells/ml) stored at -150ºC were thawed, the cells were washed and resuspended in 10 ml of RPMI1640 (Life Technologies) containing 10% FCS, penicillin, streptomycin, L-glutamine, and 50ng/ml of feline GM-CSF (987-FL: R&D Systems, Minneapolis, MN, USA), and then seeded in 96-well plates. After 5 days of incubation, non-adherent cells were removed by aspirating the medium and fresh RPMI1640 medium containing 100ng/ml of GM-CSF was added. After an additional 6 days of incubation, the majority of the cells had differentiated into macrophages (0.5-1.0×104 cells/well) and were used for infection experiments. Macrophages were inoculated with viruses at an m.o.i. of 10 or 50. After 3 hours of incubation, inoculum viruses were removed by washing the cells twice with PBS after which 10% FCS RPMI1640 medium containing penicillin, streptomycin and L-glutamine was added and incubation at 37ºC continued. At the indicated hours of post-infection (h.p.i.), culture supernatants were collected and the virus titers were determined on FCWF cells by TCID50 assay. FCWF cells were also used to analyze the replication kinetics of viruses. To this end the viruses were inoculated onto FCWF cells at an m.o.i. of 1. After 1h of virus adsorption the cells were washed twice with

PBS and DMEM containing 5% FCS, penicillin and streptomycin was added. Cells were incubated further at 37ºC and culture supernatants were collected at indicated h.p.i. for titration on FCWF cells. To detect viral antigens, macrophages were infected with recombinant viruses as described above and were fixed at 10 and 24h p.i. with methanol/acetone. The cells were first incubated with a 1:500 dilution in PBS of ascites 9912, derived from a FIPV 79-1146-infected cat (a gift from Dr. H. Glansbeek and E. te Lintelo), and then stained with FITC-conjugated goat anti-cat IgG (55291, MP Biomedicals, Santa Ana, CA, USA) and DAPI (D21490, Invitrogen). The cell images were captured by EVOS fl (AMG, Bothell, WA, USA) and the number of nuclei and fluorescence positive cells were counted by Image J software (1.46r, National Institute of Health, USA).

2.4. Statistical analysis. Unpaired t test was used to detect the statistical significance of the difference. A p value of <0.05 was considered to be statistically significant. The significance levels were expressed as *, $p<0.01$ or **, $p<0.05$.

3. Results

3.1. The replication kinetics of recombinant FIPV 79-1146 carrying 1146/1683 hybrid S proteins.

First, we verified our earlier conclusions by comparing, both in FCWF cells and in feline bone marrow macrophages, the replication of four FIPV 79-1146-based recombinant viruses: the recombinant wild-type virus (rec79-1146), the virus carrying the FECV 79-1683 S protein (79-1146/1683S), and the two viruses carrying chimeric S proteins of both strains (79-1146/1683S-N and 79-1146/1683S-C; Fig. 1b-i). As Fig.1b-ii shows, these viruses grew similarly and to comparable titers of around 10^6 TCID50/100μl in FCWF cells. In feline macrophages, however, only rec79-1146 and 79-1146/1683S-N replicated well; the replication of viruses 79-1146/1683S and 79-1146/1683S-C was very poor (Fig.1b-iii), similar to the original 79-1683 virus (Haijema et al., 2003; Rottier et al., 2005). These observations confirmed that the poor growth phenotype of strain 79-1683 in feline macrophages maps to the C-terminal part of its S protein.

3.2. Amino acid substitutions in the S protein of recombinant FIPV 79-1146/1683S.

There are eleven amino acid differences in the C-terminal domain of the S proteins of FIPV 79-1146 and FECV 79-1683 to which we mapped these viruses' differential ability to infect macrophages (Fig.1c). Due to the rare occurrence of type II viruses in the field (Addie et al., 2003; An et al., 2011; Hohdatsu et al., 1992), only few S protein sequences, all from FIPV's, are available for comparison. Alignment of these sequences indicated three amino acids in the C terminal S2 domain [974(A), 1096(K), and 1239(P)] to be unique to FECV 79-1683 (Fig. 1c).

We started studying the significance of these unique differences by individually replacing each one of these amino acids in the 79-1683 S protein of the chimeric 79-1146/1683S virus to the corresponding residue of FIPV S using site directed mutagenesis. The mutant viruses were generated by targeted RNA recombination. They all replicated indistinguishable from their parents in FCWF cells to titers of 10^6 to 10^7 TCID50/100µl at 24h p.i. (Fig. 2a). In macrophages a slight but significant enhancement of replication was observed only by the substitutions A974V and K1096Q, with titers reaching around 10^2 TCID50/100µl (Fig. 2b). Clearly, the growth difference between viruses 79-1683 and 79-1146 in feline macrophages is not caused by a single point mutation in the S protein. Thus, we also created double-mutants by combining substitutions. Though the resulting three viruses all seemed to exhibit increased replication in macrophages relative to the parental 79-1146/1683S virus, the effect was only significant for the virus carrying the combined substitutions A974V and K1096Q (Fig. 2b). Yet, the titer this virus reached was still much lower than that of FIPV rec79-1146.

In a next round of mutagenesis, we combined additional substitutions by including in our analysis also "non-unique" amino acid differences between the S2 proteins of strains 79-1683 and 79-1146. We were particularly interested in the effect of substitutions in the protein's C-terminal domain, in view of the atypical amino acid pattern of strain Tokyo/cat/130627 in this domain relative to that of all other FIPVs in our sequence collection (see Fig. 1c). Thus, we combined substitutions of the three 79-1683-specific amino acids with substitutions at positions 1407 and 1436. All 9 new viruses grew equally well in FCWF cells to titers of 10^6 to 10^7 TCID50/100µL (Fig. 2c). In macrophages, replication of most viruses appeared to be enhanced to some extent relative to virus 79-1146/1683S. Among the 7 viruses combining 2 substitutions, significant enhancement was

observed for the combination of mutations A974V/I1407V and P1239S/M1436I (Fig. 2d). In the latter case it is of note that each of these mutations individually had not revealed any effect (Fig. 2b and 2d). Additional enhancement of replication was achieved when the mutations at these different sites were further combined. Thus, the combination of three (positions 974, 1096 and 1407) and five (positions 974, 1096, 1239, 1407 and 1436) amino acid substitutions increased virus yields extra by 1-2 log10 units (Fig. 2d).

3.3. Amino acid substitutions in the S protein of recombinant FIPV 79-1146.

Next, we studied the effects of the reciprocal mutations. Thus, corresponding substitutions were made into recFIPV 79-1146 and evaluated for their effect on virus replication in macrophages (Fig. 3). As before, all recombinant viruses showed unaffected growth to 10^6 to 10^7 TCID50/100μL in FCWF cells (fig. 3a). Consistent with our earlier observations (Fig. 2), single amino acid substitutions at positions 972 (V to A) and 1094 (Q to K) reduced virus multiplication in feline macrophages slightly (Fig. 3b). When combining these substitutions, viruses carrying mutations V972A/Q1094K and V972A/Q1094K/V1405I grew statistically less efficient in macrophages than rec79-1146 thought the difference was only about 1 log unit (Fig. 3b). Finally, combining substitutions of all five amino acids resulted in a strong and significant virus growth reduction to a level approximating that of virus 79-1146/1683S (Fig. 3b). Collectively, the observations in figures 2 and 3 indicate that the combined amino acid substitutions in the C-terminal part of S protein can revert the FCoV macrophage infection phenotype.

3.4. Evaluation of additional amino acid differences in the S2 domain of viruses 79-1146 and 79-1683 S protein

There are six other differences between FIPV 79-1146 and FECV 79-1683 in the C-terminal part of their S proteins (Fig. 1c; note that FIPV strains DF-2 and 79-1146 have the same origin). Among these differences, the A to D substitution at position 1014 in 79-1146 S has been reported not to affect FCoV infection of feline macrophages (Rottier et al., 2005). We therefore evaluated the effect of mutations at the remaining five positions in the 79-1146 S protein (Fig. 4). Once again, all the resulting recombinant viruses grew to the usual titers of 10^6 to 10^7 TCID50/100μL in FCWF cells (fig. 4a). Of the five single amino acid substitutions, only mutation F1416C caused a significant, about 3 $\log_{10}$ decrease in virus yield in feline macrophages. Consistently, the reciprocal mutation C1418F introduced into the 79-1146/1683 virus increased replication significantly as well (Fig. 4b).

We also introduced this mutation into two rec FIPV 79-1146 viruses already carrying mutations that decreased replication in macrophages (V972A/Q1094K/V1405I and V972A/Q1094K/S1237P/V1405I/I1434M) as well as into the corresponding two 79-1146/1683S viruses that already carried the inverse mutations causing increased replication in macrophages (A974V/K1096Q/I1407V and A974V/K1096Q/P1239S/I1407V/M1436I). However, when studied for its effect, no additional decrease or increase in virus yield was observed (Fig. 4c).

3.5. Effects of S protein substitutions on FCoV entry and spread in macrophages.

Finally, we evaluated the effects of the mutations on the susceptibility of macrophages to infection and on virus spread. Primary infection (virus entry) was measured by immunofluorescence staining of the cells at 10h.p.i. and virus spread was measured by staining the cells at 24h.p.i. Feline macrophages were inoculated at an m.o.i. of 10 (note that all virus stocks were titrated on FCWF cells), at which dose about 2% of the cells became antigen-positive at10 h.p.i. after inoculation with FIPV rec79-1146 while almost no cells became positive after inoculation with virus 79-1146/79-1683 (Fig. 5), consistent with observations using the parental viruses FIPV 79-1146 and FECV 79-1683 (Rottier et al., 2005). For this study we selected three 79-1146/1683S mutant viruses based on their significantly increased growth phenotypes in macrophages: mutants C1418F, A974V/K1096Q/I1407V and A974V/K1096Q/P1239S/I1407V/M1436I. All three recombinant viruses showed increased primary infection at 10h as well as increased infection spread in macrophages at 24h after inoculation (Fig. 5a). The reciprocal substitutions, introduced into FIPV rec79-1146 S, had generally less dramatic effects on virus entry. Only the substitution of all five amino acids caused a significantly decreased infection at 10h.p.i. (Fig. 5b). However, when measured at 24h.p.i., all three mutant viruses exhibited a significant decrease in secondary spread (Fig. 5b). The results indicate that the observed differences in viral growth in feline macrophages are caused by differences in virus entry and spread (i.e. secondary infection) rather than by differences in virus replication *per se*.

4. Discussion

Serotype II FCoV strains 79-1683 and 79-1146 grow similarly well in feline culture cells like FCWF and CrFK but propagate remarkably different in cultured feline bone marrow macrophages, with strain 79-1683 multiplying very poorly in these macrophage cultures. Interesting as these observations are already by themselves, their impact seems even more relevant considering that the differential ability to infect macrophages is the main hallmark distinguishing the harmless enteric from the lethal systemic FCoV pathotype (Pedersen, 2009; Pedersen, 2014). Hence the observations described in this paper are of peculiar interest as the FECV 79-1683 and FIPV 79-1146 strains studied have historically served as the prototypes of both pathotypes, though their precise origin and identity have recently been questioned, as discussed below.

Earlier we mapped the differential efficiency of macrophage infection by the two prototypic serotype II FCoV strains to the S2 subunit of the viral spike protein. Coronavirus spike proteins, trimers of which form the typical virion projections, are class I fusion proteins of which the N-terminal S1 subunit functions in receptor binding and the C-terminal S2 subunit in membrane fusion. Functional domains within the S2 protein are a proteolytic cleavage site (S2') for fusion activation, located just upstream of a fusion peptide (FP), downstream of which two heptad repeats (HR1 and HR2) occur, followed by a transmembrane (TM) domain and a C-terminal tail. Of the 5 amino acid differences in the S2 subunit that distinguish FECV 79-1683 from almost all known FIPVs one is located within the putative FP, one in HR1, one in the domain between HR1 and HR2, one in the TM domain and one in the C-terminal tail (Fig. 1c). When analyzing these differences by introducing the relevant mutations into the S2 domains of our chimeric test viruses it appeared that none of the 5 differences individually could account for the

difference in macrophage tropism. Thus, though some of the single mutations (A974V, K1096Q) significantly enhanced infection mediated by the FECV 79-1683 S protein, the effect of the others was only marginal. Reciprocally, none of the single mutations introduced into FIPV 79-1146 significantly reduced infection of macrophages. Rather, combinations of the 5 mutations were required, and only the collective effect of all 5 mutations elicited the maximal, almost complete phenotype reversal, both ways.

As the tropism difference between the two FCoV strains results from multiple amino acid differences in multiple functional domains of the S2 subunit, a clear mechanistic interpretation is difficult. Since infection by both viruses is mediated by binding of their S1 subunit to the same fAPN receptor (Rottier et al., 2005), the individually insignificant differences in their S2 subunit somehow cooperatively determine whether or not feline macrophages, not other feline cells, can be infected. If not at the level of receptor recognition, these differences must act at another level of cell entry, one that manifests itself specifically upon infection of macrophages. Considering their distribution across the S2 polypeptide various aspects can then be considered including co-receptor usage, fusion activation and membrane fusion *per se*. Except for the potential involvement of sialic acids and C-type lectins as attachment factors - mediated by the S1 subunit (Li et al., 2016) and by S protein-linked sugars (Regan et al., 2010; Regan and Whittaker, 2008; Van Hamme et al., 2011), respectively - there are no indications for an involvement of an additional receptor in FCoV infection. Yet, it cannot be excluded that such co-receptor, interacting with the S2 subunit, is essentially required for infection of feline macrophages and specifically exposed by these cells. Similarly, also conditions for membrane fusion *per se* might be typically different in macrophages as compared to other cells. Specific circumstances such as the membrane lipid composition might require adaptations in the S2 protein to enable efficient fusion and infection. Activation of

coronavirus spike proteins for membrane fusion occurs by proteolytic cleavage at two sites, the S2' site located just upstream of the FP and the S1/S2 site (Millet and Whittaker, 2015). Cleavage at the latter site is typically mediated by furin-like enzymes. This cleavage seems, however, not essential; a furin cleavage motif is absent in the S proteins of many coronaviruses. Interestingly, unlike serotype I FCoVs the S proteins of which do have a furin cleavage site (de Haan et al., 2008), such a site is lacking in FCoVs of serotype II. The implications hereof are presently unclear. In the comparable situation of SARS-CoV, introduction of a furin cleavage motif at the S1/S2 site was shown to enhance cell-cell fusion mediated by the expressed spike protein (Belouzard et al., 2009).

Proteolytic activation at the S2' site liberates the fusion peptide, which seems critical for efficient initiation of membrane fusion. Cleavage is achieved by enzymes occurring at the cell surface or in the endosomes. The repertoire of these proteases can vary significantly among different cell types, which hence potentially represents a host tropism determinant. Consistently, the tropism difference between the serotype II FCoV pathotypes was found by Regan et al. (Regan et al., 2008) to be accompanied by distinctive differences in the sensitivities of FECV 79-1683 and FIPV 79-1146 to inhibitors of endo-/lysosomal enzymes and endosomal acidification. Thus, while infection of various feline cell lines (CrFK, AK-D and Fc2Lu) with FECV was highly dependent on cathepsin B and L as well as on low endosomal pH, infection by FIPV was independent on cathepsin L and low pH condition but dependent on cathepsin B activity only. Notably, this dependence of FIPV on cathepsin B was also found to be critical for infection of primary feline blood monocytes (Regan et al., 2008). The primary sequences of the two viruses at and around their S2' site do not offer an obvious explanation for these different entry requirements. Though there is one sequence difference occurring immediately upstream of the FP, where FIPV has a G

instead of the R^{963} that occurs in the FECV S sequence, this difference is not consistent when considering other available FIPV sequences. Besides in strain DF2, which is nearly identical to FIPV 79-1146 and which is perhaps just another descendant of the same viral isolate from which FIPV 79-1146 originated, all other FIPVs have an R at this position (Fig. 1c). We can only speculate about the reasons for the different macrophage entry requirements of FECV 79-1683 and FIPV 79-1146, such as long-range structural effects on the exposure of the S2' loop that might be caused by other sequence differences in the S2 subunit. With regard to the R/G difference observed at the S2' site of FCoV strains 79-1683 and 79-1146 it is interesting to mention an R to G mutation at exactly the same S2' position that occurred at least twice independently during adaptation of PEDV field viruses to Vero cells (Park et al., 2011; Wicht et al., 2014). Whereas infection of cultured cells with PEDV requires trypsin to activate the spike protein for membrane fusion, this particular mutation appeared to render PEDV infection trypsin-independent (Wicht et al., 2014).

Of the sequence differences between the FCoV strains 79-1683 and 79-1146 S2 proteins those at FECV positions 974, 1096 and 1407 generally contributed most strongly to the macrophage entry phenotype. Of these, residue A^{974} occurs in the putative FP. The mechanistic consequences of a change to V as it occurs in FIPV S2 are hard to imagine. This holds as well for a similar change in hydrophobic residues - I^{1407} to V - occurring in the TM domain. Residue K^{1096} is located in the HR1 domain. Its positive charge is likely to be involved in stabilizing the prefusion spike structure and in facilitating the proper assembly of the 6-helix bundle during the membrane fusion reaction. A change into the polar residue Q is likely to affect these processes though it remains hard to understand why this is beneficial for infection of macrophages. The other two unique differences - P^{1239} vs S and M^{1436} vs I – exhibited their effects only in combination with the others. Though the P to S change certainly has structural consequences, the effects of either of

these changes cannot be easily appreciated without more knowledge about the FCoV spike structure as well as about the molecular details of the membrane fusion process.

An interesting example of how subtle mutations can alter the entry pathway and thereby also the susceptibility of cells to viral infections was recently reported for another alphacoronavirus, HCoV-229E, known to cause common cold. Primary isolates of this virus were found to prefer cell entry via the cell surface by using the transmembrane protease serine 2 [TMPRSS2]) as the cellular protease for cleavage activation of their S protein (Shirato et al., 2017). Upon serial passaging in culture cells, the virus appeared to rapidly adapt to preferentially using the endosomal pathway by the acquisition of just a single mutation (S577D) that allows fusion activation of the S protein by cathepsin L. Consistently, the highly passaged classical HCoV-229E isolate (ATCC-VR740) was already shown earlier to use this endosomal enzyme to infect cells (Kawase et al., 2009). In this case, sequence comparison of the S protein of this laboratory strain with S proteins of clinical isolates led to the identification of two other responsible mutations. Their substitutions (R642M and N714K) in the S protein of the laboratory strain reduced HeLa cell infection and rendered the virus less sensitive to the action of cathepsin L inhibitor while the converse was observed after introduction of the reciprocal mutations in the S protein of a clinical isolate, suggesting these amino acids to be critical for the cathepsin L recognition of the HCoV-229E S protein (Shirato et al., 2017). Thus, different amino acid substitutions, all in the S2 part of the viral spike protein, gave rise to the same outcome. Notably, none of these residues is part of the protease recognition site itself as deduced from 3D structural homology modeling, suggesting their substitutions induce structural changes that alter the S protein's accessibility to cathepsin L. Similar effects might also explain the differential abilities of different feline coronaviruses to infect feline macrophages, not only for the serotype II strains investigated in the current study but perhaps

as well for the two serotype I pathotypes that we showed earlier to generally differ by just one amino acid (M1058L or S1060A) at a position just upstream of the FP (Chang et al., 2012).

Lacking a suitable cell culture system for the more relevant serotype I FCoVs, studies into the molecular biology of FCoVs have for decades been performed almost exclusively with serotype II viruses, out of necessity. Strains 79-1683 and 79-1146 always served as the pathotypic prototypes, particularly after a comparative evaluation of their pathogenicity in kittens had reproduced the distinctive characteristics of the intestinal and systemic infections, respectively, including the typical replication in mature apical columnar epithelial cells by the enteric virus which was also observed for serotype I FECV strain UCD (Pedersen et al., 1984b). More recently, however, concerns have been raised regarding the precise identities of these serotype II viruses (Pedersen, 2009; Pedersen, 2014). FIPV 79-1146 was originally reported to be isolated from a 4-day-old kitten (McKeirnan et al., 1981). It has about 99% homology to FIPV strain DF2 (except in its ORF3abc region) (Balint et al., 2012), which was reported to originate from the spleen of a cat that died from FIP (Evermann et al., 1981). The FECV 79-1683 virus was originally isolated from tissue of an adult cat with fatal peracute hemorrhagic enteritis (McKeirnan et al., 1981), yet caused mild to inapparent enteritis in SPF cats (Pedersen et al., 1984b). All these viruses were isolated around the same time and in the same laboratory, leading to speculations about their possible relationships. Thus, it was suggested that the three virulent viruses might share a common ancestor (Balint et al., 2012) and that the avirulent virus might actually represent a tissue culture-attenuated derivative of a virulent precursor (Pedersen, 2009; Pedersen, 2014). The latter concern stems in particular from the observed occurrence of deletions in FECV 79-1683's genes 3c and 7b, genes that are normally intact in avirulent field strains. These deletions may have been acquired during in vitro passaging of the virus as these genes are known to be dispensable for viral

replication in cultured cells; actually, during propagation in cell culture FCoV mutants with deletions in the 7b gene are even selected for, suggesting that functional loss of the gene increases viral fitness *in vitro* (Herrewegh et al., 1995). Of note, avirulent field viruses with (generally small) deletions in gene 7b have been described (Desmarets et al., 2016; Lin et al., 2009a). It is also worrisome that strain 79-1683 thus far is the only serotype II enteric coronavirus isolated. Though such viruses have been detected in feces from clinically healthy cats by RT-PCR (Lin et al., 2009b), no successful isolations have been described and, thus, no alternative enteric pathotypes are yet available.

In our previous study (Rottier et al., 2005) we described that neither the functional deletion of gene 7b nor the restoration of gene 3c in FIPV 79-1146 affected the mutant virus' infection efficiency of bone marrow-derived macrophages. Obviously, limitations intrinsic to this in vitro system preclude evaluation of contributions from these as well as from other viral genes to biological features that exhibit their phenotype only in the context of the infected animal. Rather, our observations with this system primarily relate to the differential in vitro macrophage entry characteristics of the two strains used. Hence, the general implications of our observations will await further studies using more and better FECV and FIPV representatives, preferably including viruses of serotype I.

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6. Conflicts of interest

The authors declare that they have no competing interests.

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Figure legends

Figure 1. a) Schematic representation of the targeted RNA recombination method used for the generation of FIPV 79-1146 mutants in which the gene for the S protein was replaced either entirely or in part by the corresponding gene or gene segment of FECV 79-1683. Donor RNA transcribed from linearized pBRDI1 plasmids is transfected into murine LR7 cells infected with mFIPV, a FIPV 79-1146 derivative carrying the murine coronavirus (MHV) S protein. Homologous recombination within the 3' domain of ORF1b (indicated by X) can produce a recombinant virus carrying the FCoV S protein, which can then be selected and amplified in feline FCWF cells. b) The replication kinetics of FIPVs carrying chimeric S proteins. i) Schematic representations of the recombinant virus genomes. In the chimeric viruses either the N-terminal 874 residues (79-1146/79-1683S-N) or the residues downstream thereof (79-1146/79-1683S-C) were from FECV 79-1683. Viruses were inoculated onto ii) FCWF cells at m.o.i. of 1 or iii) feline macrophages at m.o.i. of 50. At the indicated times samples were taken from the culture supernatants and titrated on FCWF cells (FCWF cells, n=3; feline macrophages, n=4). c) Schematic image of the amino acid differences in the C-terminal part of the S proteins of FCoV strains 79-1146 and 79-1683 (top) and comparison at the critical positions with the amino acid identities of known type II FIPVs. Sequences deposited in GenBank were used (FECV 79-1683, X80799; FIPV 79-1146, DQ010921; FIPV DF2, JQ408981; FIPV M91-267, AB781788; FIPV KUK-H/L, AB781789; FIPV NTU156P07, GQ152141; FIPV Tokyo/cat/130627, AB907624; FIPV HABER, DQ122859). FIPV 831 and 1c are Dutch type II FIPV isolates of which only partial sequences are available (unpublished). FP, fusion peptide; HR, heptad repeat; TM, transmembrane domain.

Figure 2. Replication of chimeric 79-1146/1683S mutant viruses in FCWF cells (a,c) and in feline macrophages (b,d). FCWF cells were inoculated at an m.o.i. of 1 for 1h after which the cells were washed with PBS twice and incubated in 5%FCS-DMEM. After 24 hours of incubation, supernatants were collected and titrated by TCID50 assay using FCWF cells (n=3). Macrophages were inoculated at an m.o.i. of 10 for 3h; cells were then washed with PBS twice and incubated in 10%FCS-RMPI1640 medium. After 48 hours of incubation, supernatants were collected and titrated by TCID50 assay using FCWF cells (n=6; *, $p<0.01$).

Figure 3. Replication of recombinant FIPV 79-1146 mutant viruses in FCWF cells (a) and feline macrophages (b). Viruses were inoculated onto FCWF cells or feline macrophages as described in the legend to figure 2. (FCWF cells, n=3; feline macrophages, n=6; *, $p<0.01$).

Figure 4. Replication of recombinant 79-1146 and 79-1146/1683S viruses containing additional S protein mutations in FCWF cells (a) and feline macrophages (b, c). Inoculations were performed as described in the legend to figure 2. (FCWF cells, n=3; feline macrophages, n=6; *, $p<0.01$).

Figure 5. Effect of the S protein mutations on viral entry and spread in feline macrophages. Recombinant viruses were inoculated onto feline macrophages as described in the legend to figure 2. At 10h and 24 h p.i. cells were fixed with methanol/acetone. They were then treated with ascites 9912 antibodies and subsequently stained with FITC-conjugated goat anti-cat IgG and DAPI. The cell images were captured and the numbers of nuclei and fluorescence positive cells were counted by Image J software (a, 79-1146/1683S mutants; b, 79-1146 mutants). The data are expressed as the percentage of the FITC-positive of the DAPI-positive cells. (n=6; *, $p<0.01$; **, $p<0.05$).

Fig.1

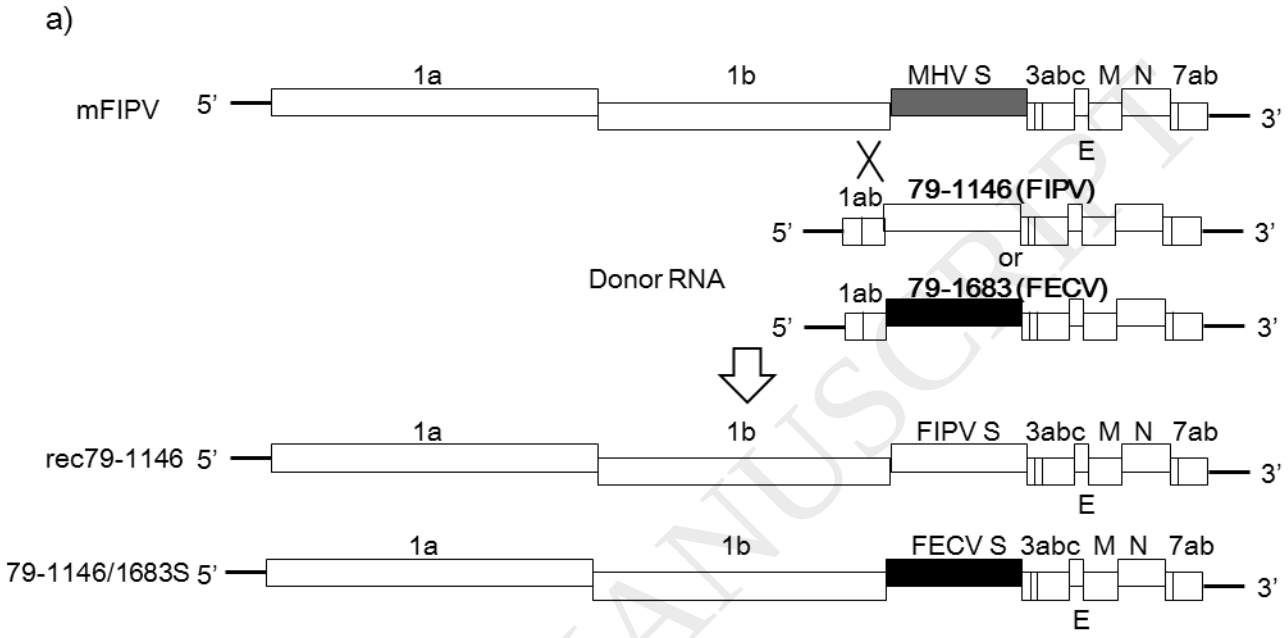


Fig.1

b)

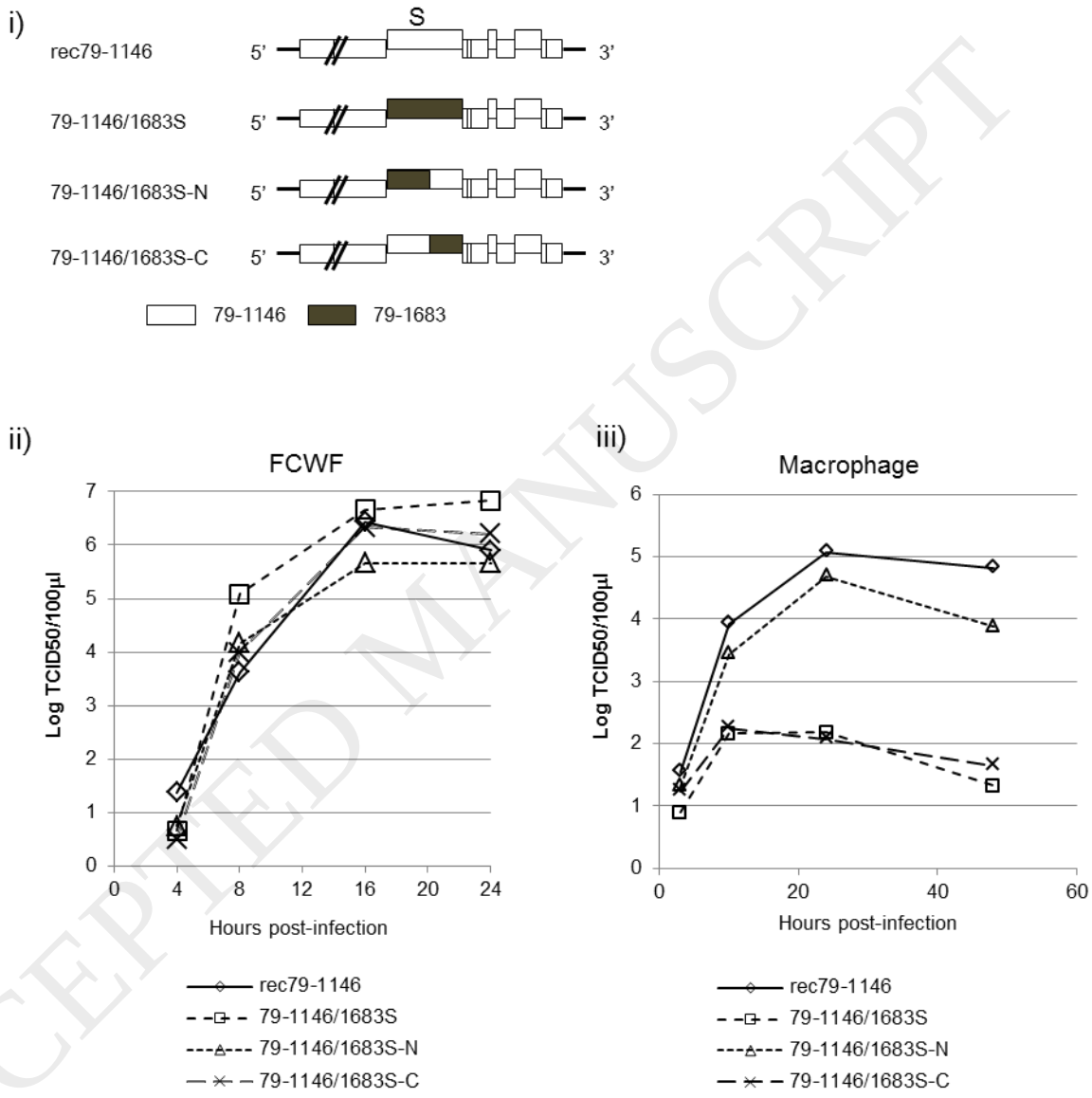
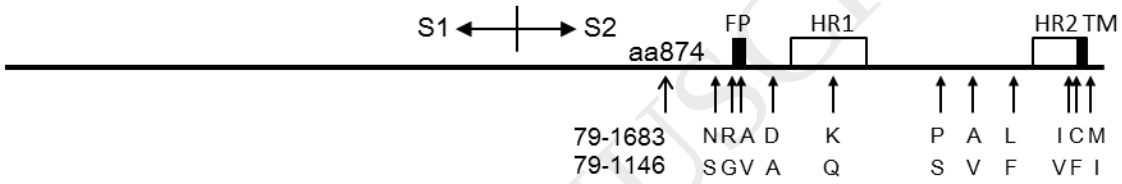


Fig.1

c)



	Amino acid position										
Name (Acession No.)	940	963	**974**	1016	**1096**	**1239**	1281	1335	1407	1418	1436
FECV 79-1683 (X80799)	N	R	A	D	K	P	A	L	I	C	M
	938	961	**972**	1014	**1094**	**1237**	1279	1333	1405	1416	1434
FIPV 79-1146 (DQ010921)	S	G	V	A	Q	S	V	F	V	F	I
FIPV DF2 (JQ408981)	S	G	V	A	Q	S	V	F	V	F	I
FIPV M91-267 (AB781788)	N	R	V	D	Q	S	A	L	V	C	I
FIPV KUK-H/L (AB781789)	N	R	V	D	Q	S	V	L	V	C	I
FIPV NTU156P07 (GQ152141)	N	R	V	D	Q	S	A	L	V	C	I
FIPV Tokyo/cat/130627 (AB907624)	N	R	V	D	Q	S	A	F	I	L	L
FIPV 831 (unpublished)	N	R	V	D	Q	S	A	L	ND	ND	ND
FIPV 1c (unpublished)	ND	ND	ND	D	Q	S	A	L	ND	ND	ND
FIPV HABER(DQ122859)	ND	ND	ND	ND	ND	S	V	L	ND	ND	ND

ND= not determined

Fig.2

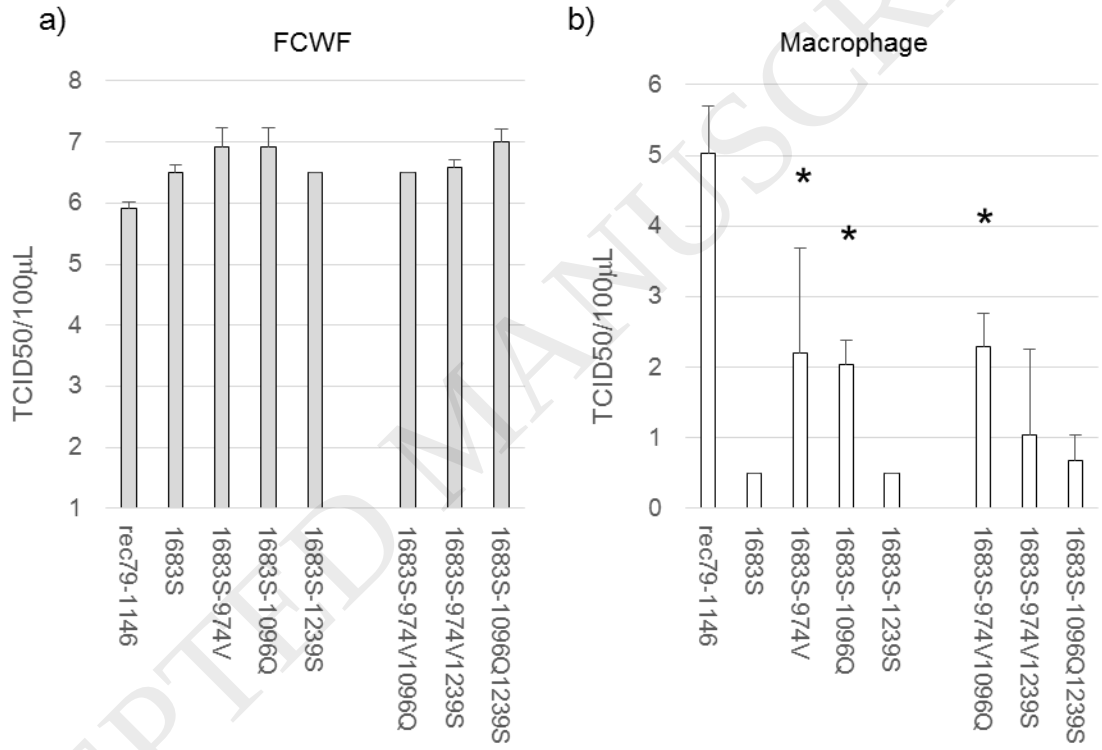


Fig.2

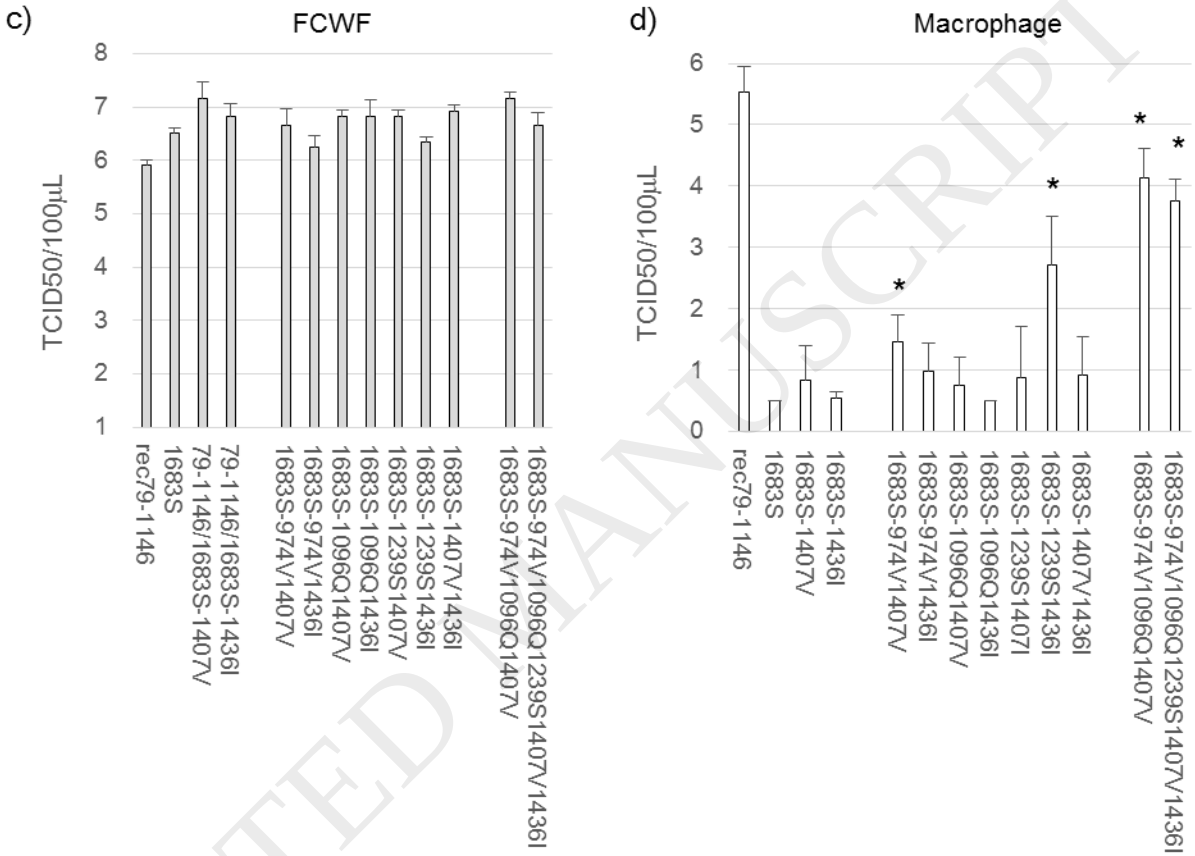


Fig.3

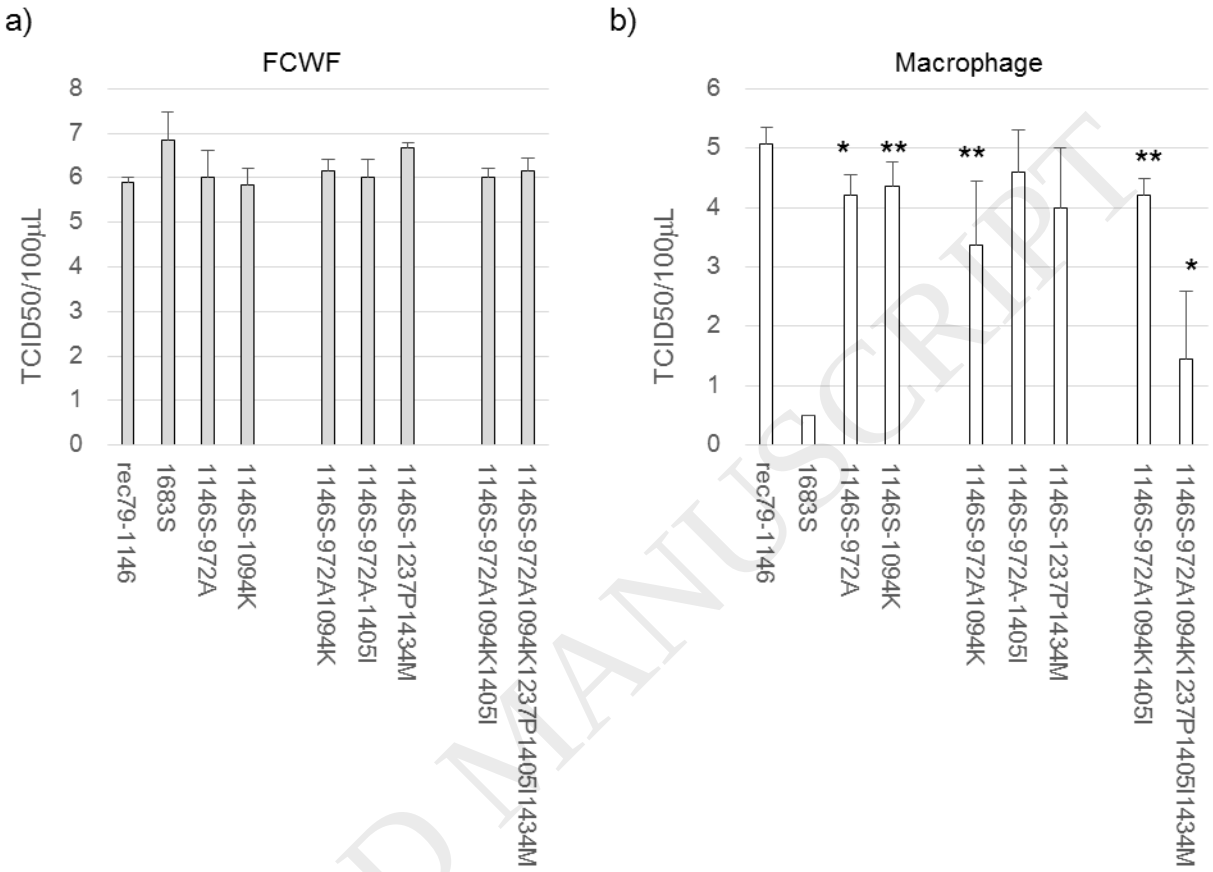


Fig.4

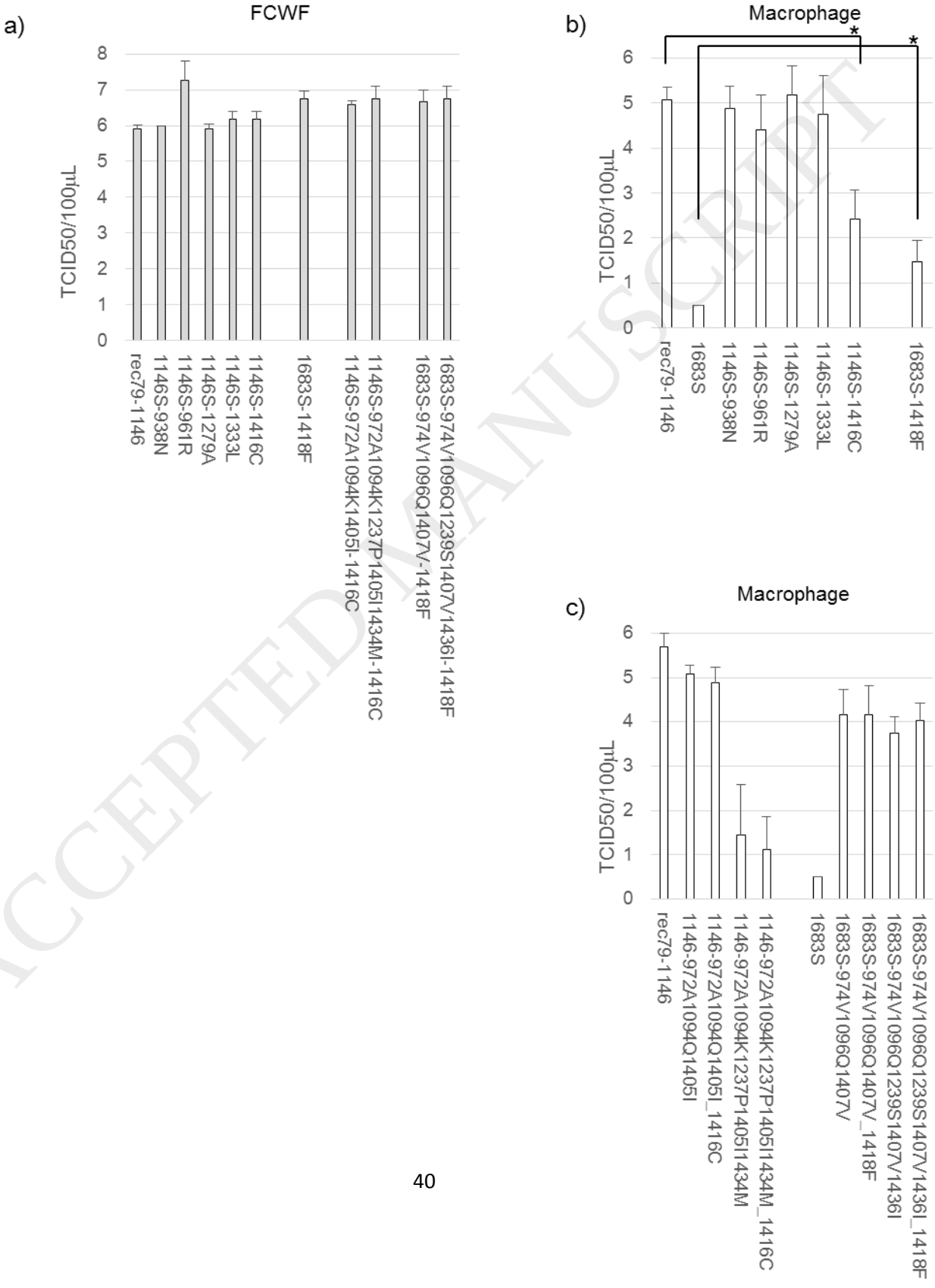


Fig.5