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# INFECTIOUS DISEASE

## Short Title: Immune Response to Feline Coronavirus

### Inflammatory Mediators in the Mesenteric Lymph Nodes, Site of a Possible Intermediate Phase in the Immune Response to Feline Coronavirus and the Pathogenesis of Feline Infectious Peritonitis?

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## Summary

Feline infectious peritonitis (FIP) is an almost invariably fatal feline coronavirus (FCoV)-induced disease thought to arise from a combination of viral mutations and an overexuberant immune response. Natural initial enteric FCoV infection may remain subclinical, or result in mild enteric signs or the development of FIP; cats may also carry the virus systemically with no adverse effect. This study screened mesenteric lymph nodes (MLNs), the presumed first site of FCoV spread from the intestine regardless of viraemia, for changes in the transcription of a panel of innate immune response mediators in response to systemic FCoV infection and with FIP, aiming to identify key pathways triggered by FCoV. Cats with and without FIP, the latter with and without FCoV infection in the MLN, were compared. Higher expression levels in FIP were found for toll-like receptors (TLRs) 2, 4 and 8. These are part of the first line of defence and suggest a response to both viral structural proteins and viral nucleic acid.

Expression of genes encoding inflammatory cytokines and chemokines including interleukin (IL)-1 $\beta$ , IL-6, IL-15, tumour necrosis factor (TNF)- $\alpha$ , CXCL10, CCL8, interferon (IFN)- $\alpha$ , IFN- $\beta$ , and IFN- $\gamma$ , was higher in cats with FIP, consistent with inflammatory pathway activation. Expression of genes encoding transcription factors STAT1 and 2, regulating signalling pathways, particularly of the interferons, was also higher. Among cats without FIP, there were few differences between virus-positive and -negative MLNs; however, TLR9 and STAT2 expression were higher with infection, suggesting a direct viral effect. The study provides evidence for TLR involvement in the response to FCoV. This could open up new avenues for therapeutic approaches.

*Keywords:* feline coronavirus; toll-like receptors; cytokines; mesenteric lymph nodes

## Introduction

Feline infectious peritonitis (FIP) is a well-known and widely distributed coronavirus-induced disease of felids. With as yet no effective vaccine or viable treatment options, FIP is almost invariably fatal, and understanding the pathogenetic and immunological mechanisms involved in disease development is crucial to aiding chances of combatting FIP and identifying novel avenues for possible treatment.

After initial enteric infection, feline coronavirus (FCoV) may spread beyond the intestine resulting in a monocyte-associated viraemia, with or without the development of FIP. In cases progressing to FIP, which may have a time lag of weeks to years, viral and host factors combine to turn an initial, usually subclinical, enteritis into an overt immune-mediated disease (Pedersen *et al.*, 1981; Kipar and Meli, 2014). Much research has focussed on viral mutations and has partially elucidated the function of various viral proteins in the pathogenesis of FIP. The viral spike (S) protein gene has been of particular interest, and a switch from methionine (M) to leucine (L) at amino acid residue 1,058 (M1058L) has been strongly associated with a gain of virulence (Chang *et al.*, 2012). A second switch from serine (S) to alanine (A) at amino acid residue 1,060 (S1060A) distinguished tissue-associated FCoV in a further small subset of FIP cases from FCoV shed with the faeces by healthy cats (Chang *et al.*, 2012). These mutations have since been associated with systemic spread of FCoV, rather than providing proof of virulence (Porter *et al.*, 2014; Barker *et al.*, 2017; Felten *et al.*, 2017a) so the two forms are subsequently referred to here as ‘systemic’ and ‘enteric’ FCoV.

Early experiments demonstrated that not all cats are susceptible to FCoV infection, even with known pathogenic strains (Pedersen and Boyle, 1980), indicating the importance of host genetic factors/immune mechanisms in disease development. More recently it was shown that cultured monocytes from different cats vary in their ability to sustain viral replication, again suggesting that there is a subset of animals who can resist disease (Dewerchin *et al.*,

2005; Tekes *et al.*, 2010). Monocytes/macrophages are not the only cell type beyond enterocytes that may be infected by FCoV, but are also key cells in the innate immune defence system. They are able to detect pathogen associated molecular patterns (PAMPs), triggering a number of intracellular signalling pathways leading to activation of an antiviral state in the host (Abbas *et al.*, 2017). Chief amongst these pathways are those triggered by engagement of toll-like receptors (TLRs); highly evolutionarily conserved, membrane-bound pathogen recognition receptors (PRRs) (Lester and Li, 2014). Their presence on both the cell surface membrane and internal membrane-bound vesicles allows detection of external and internal PAMPs; their ligands include those associated with viruses, bacteria and fungi (Arpaia and Barton, 2011). Downstream mediators include inflammatory cytokines and interferons which have been assessed in cats with FIP, with sometimes conflicting results (Dean *et al.*, 2003; Kipar *et al.*, 2006b). Interferons and the inflammatory cytokine interleukin (IL)-6 can activate members of the signal transducer and regulator of transcription (STAT) family with downstream effects on replication, differentiation or inflammatory potential (Aaronson and Horvath, 2002). Cats with a compromised immune system appear to be more susceptible to FIP, while, paradoxically, the lesions are caused by an excessive immune response (Pedersen, 1987, 2014; Kipar and Meli, 2014). This has been attributed, at least in part, to increased viral replication in immunosuppressed animals and, therefore, an increased likelihood of viral mutations occurring and accumulating (Poland *et al.*, 1996).

TLRs have been associated with susceptibility to many diseases, including chronic inflammatory, viral and more specifically coronaviral diseases (e.g. severe acute respiratory syndrome, SARS) (Dosch *et al.*, 2009). Intriguingly though, both TLR stimulation and antagonism/knock-outs have contributed to exacerbation of disease in different contexts and there exists considerable crossover between receptors and their potential ligands (Arpaia and Barton, 2011).

When FCoV is able to leave the intestine, the mesenteric lymph nodes (MLNs) are the presumed first site of viral spread, potentially representing the interface between local and systemic immune response; support for this assumption are FIP cases that present only with MLN lesions (Kipar *et al.*, 1999). We therefore chose the MLN as our organ of interest, with the aim of comparing key mediators of the innate immune system between uninfected cats and FCoV-infected cats with and without FIP. We hypothesized that in addition to an excessive pro-inflammatory cytokine response, there would be a deficient interferon response, and aimed to gain an insight into which TLR pathways are involved in triggering this response. We also wished to further evaluate the presence and significance of previously published viral S gene variations and determine whether a connection with the host immune response could be detected.

## **Materials and Methods**

### *Case Selection*

The study was undertaken on cats that had all been seen initially as patients at the university small animal clinics and local veterinary practices of Bristol, UK, or Zurich, Switzerland, and humanely destroyed with or without FIP for clinical reasons unrelated to this study (Table 1). A post-mortem examination was performed on each cat with owner consent and samples of MLN were collected into RNeasy<sup>®</sup> (Qiagen, Hombrechtikon, Switzerland) within 2 h of euthanasia and stored at  $-80^{\circ}\text{C}$  until use. The Bristol cases form part of the University of Bristol FIP Biobank built up as a resource for multiple studies; many of these cases were utilized previously (Porter *et al.*, 2014; Barker *et al.*, 2017).

Group 1 (G1) comprised of 40 control cats confirmed to not have FIP and with an alternate confirmed diagnosis (Tables 1A and 1B), and group 2 (G2) consisted of 30 cats

confirmed to have FIP (Table 1C). A diagnosis of FIP was based on relevant clinical findings and compatible gross and/or histological lesions together with immunohistological demonstration of FCoV antigen-positive macrophages within typical lesions (Kipar *et al.*, 1998). The immunohistochemistry was carried out as previously described (Kipar *et al.*, 1998), using a mouse monoclonal primary antibody (clone FIPV3-70 SC 65653, Santa Cruz, Heidelberg, Germany). Based on the results of the reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) for FCoV undertaken on the MLNs, group G1 was then subdivided into G1+ (FCoV-positive) and G1- (FCoV-negative).

#### *RNA Extraction and cDNA Synthesis*

RNA extraction was carried out using the RNeasy Plus Minikit<sup>®</sup> (Qiagen) according to the manufacturer's protocol. Briefly, 30 mg of MLN tissue were disrupted in extraction buffer using a tissue homogenizer (Mixer-Mill 300, Retsch, Haan, Germany) for 40 sec at 30 Hz before on-column extraction and elution of RNA. As pilot tests revealed that significant genomic DNA contamination remained, an optional DNase step was included prior to use of the Superscript IV VILO<sup>®</sup> kit (ThermoFisher Scientific, Waltham, Massachusetts, USA) for cDNA synthesis, following the manufacturer's instructions, in order to avoid possible interference with the RT-qPCR results. Starting RNA levels were equilibrated between samples to 400 ng/ $\mu$ l, using a NanoDrop 2000<sup>®</sup> (ThermoFisher Scientific). Samples were further diluted 1 in 20 prior to RT-qPCR.

#### *Reverse Transcriptase Quantitative Polymerase Chain Reaction*

TaqMan RT-qPCR was performed on an Applied Biosystems 7500 Fast PCR System<sup>®</sup> (ThermoFisher Scientific) using newly developed, or previously published, primer and probe

protocols for: FCoV; feline TLR 1 to 9; STAT 1 to 3; interferon (IFN)- $\alpha$ , - $\beta$  and - $\gamma$ ; IL-1 $\beta$ , -6, -10, -15, and -17; tumour necrosis factor (TNF)- $\alpha$ ; CXC motif chemokine 10 (CXCL10); CC motif chemokine ligand 8 (CCL8); transforming growth factor (TGF)- $\beta$ 1; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as the reference gene (Table 2) (Leutenegger *et al.*, 1999). This gene was chosen based on previous experience in our laboratory and following reference gene comparisons during optimization. All primers and probes were manufactured by Microsynth (Balgach, Switzerland). The hydrolysis probes were labelled with a 5' reporter dye FAM (6-carboxyfluorescein) and a 3' quencher TAMRA (6-carboxy-tetramethylrhodamine).

Those primers and probes that were newly developed were designed using Primer Express<sup>®</sup> software (v3.0.1, Thermo Fisher Scientific) to span an exon–exon junction. These were tested for specificity by conventional PCR of a test sample, gel electrophoresis, sequencing of the resulting extracted band (Microsynth) and evaluation using NCBI BLAST. Conditions were as for RT-qPCR except for omission of the probe. Primer concentrations for this step were 900 nM. Varying primer/probe concentrations were then tested to determine the optimal efficiency and dynamic range as well as replicability using a sample dilution series. All final protocols (Table 2) had an efficiency >95%. Those previously published were tested again in our system, omitting the conventional RT-PCR step. Each reaction comprised 12.5  $\mu$ l TaqMan Fast Universal Master Mix<sup>®</sup> (ThermoFisher Scientific), with 2.5  $\mu$ l cDNA, primer and probe volumes as per Table 2, made up to 25  $\mu$ l with RNase-free water. The thermal profile for all RT-qPCRs was: 50°C for 2 min, 95°C for 10 min, and 45 cycles of 95°C for 10 sec, and 60°C for 1 min. All samples were run in duplicate and any samples with discordant results were repeated. Data collection occurred during the extension phase at 60°C. Appropriate controls were included in each run.



The Applied Biosystems 7500 Software<sup>®</sup> v2.0.6 was used to visualize results and allocate a quantification cycle ( $C_q$ ) to each sample, and the threshold was equilibrated between runs for each target.

### *Viral Sequencing*

The particular codons of interest within the FCoV S gene were 1,058 and 1,060 (Chang *et al.*, 2012). With reference to the sequence used in the original paper, the mutations in question appear to be at positions 1,048 and 1,050 rather than 1,058 and 1,060 as previously described, and will be referred to subsequently by the former numbers.

Following initial FCoV RT-qPCR, all positive samples not analysed for previous studies by Porter *et al.* (2014) or Barker *et al.* (2017) underwent additional conventional RT-PCR and Sanger sequencing targeting the S gene region of interest. PCR was performed using the previously published degenerate primers (Porter *et al.*, 2014). Each reaction comprised 10  $\mu$ l Phusion Flash Master Mix<sup>®</sup> (ThermoFisher Scientific), with 2  $\mu$ l cDNA, 0.5  $\mu$ M each of forward and reverse primers, made up to 20  $\mu$ l with RNase-free water. Reactions were run on a T Professional<sup>®</sup> thermocycler (Biometra GmbH, Göttingen, Germany) with the following thermal profile: 98°C for 10 sec, 40 cycles of 98°C for 1 sec, 52°C for 5 sec, 72°C for 3 sec, followed by 72°C for 1 min. Appropriate controls were included in each run.

The reaction product then underwent gel electrophoresis. Bands of appropriate size were extracted using the GeneJET Gel Extraction Kit<sup>®</sup> (ThermoFisher Scientific) and submitted for Sanger sequencing at a commercial laboratory (Microsynth). When no band was visible, the reaction was repeated using 50 cycles and the product was subjected to gel electrophoresis. Samples still appearing negative were cut out in the region of the expected band, purified and re-subjected to PCR. The bioinformatics software Geneious 9.1.7<sup>®</sup>,

(Biomatters Limited, Silkeborg, Denmark) was used to map the resulting sequences to the reference gene FCoV C1Je (Accession number DQ848678) (Chang *et al.*, 2012).

### *Statistical Analysis*

Relative mRNA transcription levels were calculated using the comparative  $C_q$  method (Pfaffl, 2001). The  $C_q$  of each target was first normalized to GAPDH as the endogenous reference ( $\Delta C_q$ ) and then expressed relative to the G1  $\Delta C_q$  mean as the calibrator ( $2^{-\Delta\Delta C_q}$ ). For FCoV RT-qPCR results, the mean of G1+ was instead used as the calibrator (to allow for visualization graphically).

The statistical programme SPSS Statistics v.25<sup>®</sup> (IBM, Armonk, New York, USA) was used for all analyses and graphical data presentation. Data were first assessed for normality using a Shapiro-Wilk test. As almost all data failed the test, so non-parametric measures were applied. A two-tailed Mann-Whitney test with a significance level of  $P \leq 0.05$  was used to compare results between groups for each target molecule. Firstly, cats with and without FIP (G1 versus G2) were compared, followed by comparisons between each of the three groups (G1-, G1+ and G2) in turn. Within G2, comparisons were made between cats with and without cavitory effusions and with and without histologically observed FIP lesions in the MLNs. Correlation between relative FCoV levels and inflammatory mediator gene expression levels, and also between individual inflammatory mediator gene expression levels, was analysed within G2 using a one-tailed Spearman's rank test. Here a cut off of  $P \leq 0.01$  was used, with  $P \leq 0.05$  indicating weak correlation.

## **Results**

### *Feline Coronavirus Status within the Study Population*

Signalments of the cats are shown in Tables 1A–1C. All MLN samples from cats with FIP (G2) were positive for FCoV ( $n = 30$ ). Of the 40 cats without FIP (G1), 10 (25%) also had a positive FCoV RT-qPCR result, these were assigned to a new sub-group (G1+). However, the relative FCoV load was clearly, and significantly, lower in G1+ than in G2 (Fig. 1).

None of the G1 cats exhibited histological changes suggestive of FIP in any tissue examined, including the MLNs when available for histology (25 of 30 from G1- and seven of 10 from G1+). Inflammation of other aetiologies was observed in the MLNs of two of the 30 G1- cats and none of the G1+ animals. All G1 samples were also negative for FCoV antigen by immunohistochemistry.

#### *Association between Key Pathological Findings and Relative Viral Load in Mesenteric Lymph Nodes of Cats with Feline Infectious Peritonitis*

The MLNs were available for histological examination in 28 of the 30 cats with FIP. In 21 cases (75%), these exhibited the typical pyogranulomatous lesions, with or without associated serosal lesions on the lymph node capsule (e.g. serofibrinous to granulomatous serositis). All samples with typical pyogranulomatous lesions also showed FCoV antigen in lesional macrophages (Figs. 2a, 2b). Seven MLNs had no typical lesions; among these was only one case (G2.19) in which FCoV antigen was detected, in low numbers of macrophages within the marginal sinus, suggesting an early lesion (Figs. 2c, 2d). There was no significant difference in FCoV load found between MLNs with and without lesions, although those with lesions had a tendency to higher FCoV levels (Fig. 1).

Of the 30 cats with FIP, 22 exhibited effusions (Table 1C). These were not associated with a higher relative FCoV load in the MLNs in comparison with the cats without effusion ( $n = 6$ ; data not available for two cats).

*Association between Feline Infectious Peritonitis and Feline Coronavirus Status, Disease Features, Viral Load and Gene Expression of Immune Mediators*

In order to evaluate the effect of FCoV infection and FIP on target gene transcription, G1 and G2 were first compared with each other before comparisons between all three groups (G1+, G1- and G2). The assessed target genes are described below according to their positions in immune signalling pathways as first line receptors, inflammatory mediators or signal transducers. Detailed results are provided in Table 3.

*Toll-like Receptors:* Relative TLR2, 4, and 8 gene transcription levels were significantly higher in G2 than G1. Within G1 there was no difference between virus-positive and virus-negative MLNs for these TLRs; however, TLR9 gene expression, although not elevated in G2, was significantly higher in G1+ than in G1- (Fig. 3).

In G2 cats, gene transcription levels were compared between MLNs with and without FIP lesions, and in relation to the presence of effusions. A significant difference was found only for TLR2 (higher expression in MLNs with lesions) (Fig. 3, Table 3); in contrast, TLR2 expression appeared slightly lower in cats with effusions (Fig. 3). A possible trend not reaching significance was for a slightly higher TLR4 expression level in MLNs with lesions, while TLR3 and 9 gene expression levels were slightly lower (Fig. 3). Investigating this further, we found that TLR3 gene expression levels in G2 MLNs without lesions were also slightly higher than levels in G1 (which were similar to those in G2 MLNs with lesions), suggesting a potential negative regulation of TLR3 by FCoV (Fig. 3).

*Cytokines and Chemokines:* Relative IL-1 $\beta$ , IL-6, IL-15, TNF- $\alpha$ , IFN- $\alpha$ , - $\beta$ , - $\gamma$ , CCL8 and CXCL10 gene transcription levels were all significantly higher in G2 compared with G1 (Fig. 4). None of these showed any significant difference between G1+ and G1-. For most cytokines, G1+ and G1- clustered together; however, for IL-6, TNF- $\alpha$  and IFN- $\gamma$ , G1+ appeared to cluster slightly between the other two groups (G1- and G2), suggesting a possible intermediate stage (Fig. 4). Between groups, the fold differences in the chemokine gene expression levels (CXCL10 and CCL8) were mainly in the range of 10–100 $\times$ , while those for the pyrogenic cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) rarely exceeded 10 $\times$ . IL-10, IL-17 and TGF- $\beta$  gene transcription levels showed no intergroup differences (Fig. 4, Table 3).

For IL-1 $\beta$ , IL-6, IFN- $\gamma$  and CCL8, a possible trend towards increased transcription (not reaching significance) was observed in G2 MLNs with lesions compared with those without (Fig. 4).

*Signal Transducers and Activators of Transcription:* STAT1 and 2 gene expression levels were significantly higher in G2 than G1. For both transcription factors, gene expression levels were also higher in G1+ than in G1-; significantly so for STAT2. STAT3 gene expression levels were similar across all groups (Fig. 5).

In G2, STAT2 gene expression levels were significantly higher in cats with effusions (Fig. 5, Table 3). For STAT1, there was an insignificant trend to be higher with effusions (Fig. 5).

*Correlation of Target Immune Mediators and Feline Coronavirus Load in Cats with Feline Infectious Peritonitis:* The majority of gene targets elevated in G2 also showed significant positive correlation with relative viral load (Supplementary Table 1). These included TLR 2

and 4, the cytokines IL-1 $\beta$ , IL-6, together with STAT2, CXCL10, CCL8, IFN- $\beta$  and IFN- $\gamma$  ( $P \leq 0.01$ ). TLR8 and IFN- $\alpha$  gene expression showed weaker correlation ( $P \leq 0.05$ ), while STAT1, TGF- $\beta$ , and TNF- $\alpha$  gene expression showed no correlation, and TLR9 a weak, although significant, negative correlation (Table 4).

Expression of genes encoding IL-6, IL-17 and STAT3, a ‘holy trinity’ of autoimmunity (Camporeale and Poli, 2012), was significantly correlated despite the latter two not showing any correlation with FCoV.

### *Partial S Gene Sequencing*

Of the 40 cats shown by RT-qPCR to carry FCoV in their MLNs, 38 had analyzable S gene sequences following conventional PCR. From the remaining two cats (G1+ cats 1 and 6) it was not possible to obtain samples of sufficient quality even after repeated attempts (Table 1B).

Of the 30 cats with FIP, one was infected with FCoV serotype 2 for which the previously described S gene sequence characterization is not applicable (Herrewegh *et al.*, 1998; Barker *et al.*, 2017). Twenty-six MLN samples contained virus that encoded leucine (M1048L) (cDNA sequence TTG, CTG or TTA). The remaining three cats encoded methionine at codon 1,048 (cDNA sequence ATG). The results are shown in Table 1C. Of the eight sequences obtained from the G1+ cats, all encoded leucine (M1048L). Of the three cases encoding methionine at codon 1,048, two encoded alanine at the codon 1,050 (S1050A), whilst the third encoded serine (Table 1B).

The small methionine group size ( $n = 3$ ) including only one cat that carried the ‘enteric’ virus (M1048, S1050) was not considered valid for statistical comparison with the leucine group, ‘systemic’ virus. Instead, individual cases were plotted, revealing the

methionine group to fall within the range of the leucine group for every target, including FCoV load (Fig. 1).

## Discussion

As predicted from previous studies, the results of the present investigation confirm the complex effect of FCoV on the immune system in association with FIP. The disease is caused by an exaggerated immune response to FCoV, but it is well known that cats can also carry FCoV systemically without developing FIP (Meli *et al.*, 2004). Here we have assessed some of the key mediators of the innate immune response, focussing on the MLN, the most likely first site of infection beyond the intestine and one of the main sites of viral persistence in experimentally infected healthy animals (Kipar *et al.*, 2010). By comparing FCoV-positive, lesion-free MLNs from cats affected by diseases other than FIP, with both FCoV-negative cats without FIP and cats with FIP, we aimed to separate the direct viral effects from the host effects contributing to FIP in a natural setting.

FIP presents as a spectrum of disease with variable duration rather than as a discrete clinical picture; as such the pathological features also vary. This was reflected by variation in organ involvement and presence of effusions in our case cohort. We therefore also wanted to assess whether the inflammatory mediator production in the MLNs showed any correlation with the form of disease. Although vascular permeability is, to a large extent, cytokine mediated (Takano *et al.*, 2011), and we found upregulation of cytokine genes with a role in vascular permeability in the FIP cases, the inflammatory mediator gene expression profile of the MLNs differed only minimally between cats with and without effusions. This suggests that the MLN is unlikely to make a large systemic contribution to vascular permeability. Similarly, Safi *et al.* (2017) evaluated inflammatory mediators within peripheral blood mononuclear cells of FIP cats with and without effusions and found little consistent pattern to

distinguish those with effusions from those without. Both studies therefore provide further, although indirect, support that vascular endothelial growth factor, which was previously shown to correlate with the degree of effusion in FIP, is key to this phenomenon (Takano *et al.*, 2011). Interestingly, the presence or absence of histological FIP lesions was not correlated with many significant differences between mediator gene expression. Alongside this, and surprisingly, although FCoV levels appeared higher in association with lesions, they were not significantly so. This may partly explain the lack of significant differences in mediator gene expression. Additionally, it cannot be entirely excluded that the area of the MLN sampled for RNA extraction failed to correlate with the histological findings in some cases. An alternative explanation for the lack of significant differences between gene expression for most mediators in MLNs, while many exhibit higher overall levels in FIP, would be that systemic stimulation to upregulate inflammatory mediators is more relevant than local or lesion-specific stimulation. Finally, as trends were occasionally observed when cases with and without effusions and MLN lesions were compared, the lack of significance may also be due to the small group sizes once subgroups were created, which was a limitation of this study.

The MLNs of cats without FIP had significantly lower viral loads than their counterparts from cats with FIP. This confirms previous findings in natural infection, where cats with FIP were reported to carry higher viral loads in haemopoietic and lymphoid tissues, including MLNs, than asymptomatic FCoV-infected cats (Kipar *et al.*, 2006a). Without the disease, however, the presence or absence of FCoV in the MLN seems not to influence the transcription level of most of our target immune mediators. This would indicate that, in the main, the host response has a greater influence than any direct viral effect. Still, there were exceptions. Even among those mediators not attaining significance, IFN- $\gamma$ , IL-6 and TNF- $\alpha$  showed a trend towards higher gene expression levels in FCoV-positive MLNs. This suggests at least a modest direct viral effect; it may have been masked by low group numbers, requiring a larger sample size to confirm or refute. Another study limitation was the



composition of the groups. As all were field cases it was not possible to control for confounding factors, e.g. ensuring control cases were free of any inflammatory processes, that FIP cases were at similar disease stages, and that cats were initially subject to the same FCoV infection pressures.

Inflammatory cytokines have been previously studied in FIP, with conflicting results, possibly dependent on variations in disease form between animals included in the different studies, and/or the type of sample/organ evaluated. TNF- $\alpha$  gene transcription, for example, was found to be decreased in the MLNs of cats with FIP compared with FCoV-free specific pathogen-free cats, while IL-1 $\beta$  gene expression was elevated (Kipar *et al.*, 2006b). In the present study, gene expression for all three pyrogenic cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) was upregulated in the MLNs in FIP, as well as that for IL-15 (a stimulator of lymphocyte proliferation). We also found significantly higher transcription levels for the monocyte-recruiting chemokines CXCL10 and CCL8, which have both been found to be upregulated in Crandell–Rees feline kidney cells after in-vitro FCoV infection (Harun *et al.*, 2013), indicating a mechanism of monocyte recruitment as a direct viral effect. Our results confirm their relevance *in vivo* with recruitment of monocytes as the infected cell type being a potential amplifying step that is worthy of further investigation. The increase in inflammatory cytokine gene transcription supports the observation that an overexuberant inflammatory response is a key factor in the development and progression of FIP. Expression of the gene encoding the anti-inflammatory cytokine IL-10 was, in contrast, not upregulated in FIP, implying there was no local brake on the inflammatory process. This is in line with previous findings, where IL-10 expression was higher in the spleen of healthy FCoV-infected cats, but not in the MLN (Kipar *et al.*, 2006b). Gene expression for the interferons was also higher in FIP, IFN- $\gamma$  being one of the cytokines to show an intermediate level in infected asymptomatic cats in our study. These type I and II interferons have major antiviral roles in the innate immune system. IFN- $\gamma$  in particular has been of interest in FIP as levels of this potentially

protective cytokine tend to be low in the peripheral blood of diseased animals and host gene polymorphisms have been identified which may contribute to resistance against the disease (Gelain *et al.*, 2006; Hsieh and Chueh, 2014). Similarly to the apparent lack of impact of mediator levels on the presence of lesions or effusions in FIP, this suggests that MLN IFN production has a more local effect.

TLRs have been used for targeted therapy against a number of diseases in human medicine, both with adjuvants and inhibitors; however, veterinary medicine lags behind in this respect (Hennessy *et al.*, 2010; Klingemann, 2018). Here we identified increased gene expression levels of TLRs 2, 4, 8 and 9 with FIP and FCoV infection, respectively, indicating a possible role for these molecules in FIP and hence identifying them as potential targets for FIP control. Assessment at the protein level would be a useful avenue for further investigations; however, this is particularly challenging in feline studies owing to the lack of availability of appropriate antibodies. In most mammals, TLRs 2 and 4 are located on the cell membranes, while TLRs 8 and 9 are found in intracytoplasmic vesicles, most commonly in professional antigen presenting cells (Lester and Li, 2014). TLR2, together with TLRs 1, 6 and 10, comprise the TLR1 family (Roach *et al.*, 2005). These latter three receptors arose through evolutionary gene duplication (Hughes and Piontkivska, 2008, Hennessy *et al.*, 2010). TLR2 is able to signal as a heterodimer with any of its co-family members in order to allow a wider range of antigen recognition. It is typically responsible for detecting bacterial and fungal components (Beutler, 2009). TLR2 has been linked to detection of the SARS-CoV S protein *in vitro* (Dosch *et al.*, 2009); its upregulation in FIP could indicate that the FCoV S protein is also able to act as a ligand.

TLR4 classically detects lipopolysaccharide; however, one study linked it to protection against murine coronavirus, as TLR4-deficient mice were found to exhibit greater susceptibility to murine hepatitis virus infection. The precise mechanism was not established

in that case, but it involved inflammatory cell influx in the TLR4-deficient mice (Khanolkar *et al.*, 2009). No such protective effect was observed in our study, despite upregulation of TLR4 gene expression in the MLNs in association with FIP, although its individual effect in this case cannot be separated from the mediator milieu.

TLR9 gene expression was not elevated in the MLNs of cats with FIP, but was instead increased in the FCoV-positive MLNs of cats without FIP. Considering that a previous *in vitro* study found reduced viral replication when TLR9 was stimulated with a synthetic CpG ligand prior to FCoV infection (Robert-Tissot *et al.*, 2012), the increased gene expression in FCoV-infected cats without FIP could indicate that TLR9 has a protective effect, which may even have helped prevent the development of disease. Stimulation by co-infectious agents could therefore also be hypothesized to be protective against FIP. Along these lines, co-infection must also be considered a possible alternative explanation for the raised TLR 2 and 4 gene expression levels, as these TLRs are more typically associated with bacterial infections. Enteric coronavirus infection or the generalized inflammatory state induced by FIP may have increased the permeability of the intestinal barrier to microorganisms. The resulting TLR stimulation would therefore not be virus induced. A third alternative is the upregulation of TLR 2 and 4 by endogenous ligand stimulation, reported as a response to alarmin release from damaged cells (van Beijnum *et al.*, 2008). This alternative also fits with upregulation of TLR2 gene expression in lesional MLNs compared with non-lesional MLNs in FIP.

From their known ligands, TLRs 3, 7 and 8 would be predicted to be triggered in infection by FCoV as it is a ssRNA virus (triggering TLRs 7 and 8), possessing a double-stranded RNA intermediate replicating phase (triggering TLR3) (Arpaia and Barton, 2011). That no upregulation occurs for TLR3 and TLR7 with FIP suggests either the lack of an appropriate trigger (TLR 7 and 8 are known to show differing, if overlapping, specificity, and dsRNA intermediate replicates are a minority of the viral RNA present; Jensen and Thomsen,

2012), or that the virus is able to inhibit TLR transcription. SARS-CoV is known to inhibit both TLR 3 and 7 signalling via papain-like protease activity (PLpro) (Li *et al.*, 2016). This mechanism may also contribute in FCoV infection, but would be expected to affect the signalling pathways rather than the TLR mRNA levels directly. In cats with FIP, we observed slightly lower TLR3 gene transcription in MLNs with typical FIP lesions, as compared with MLNs without lesions, down to the levels seen in MLNs from cats without FIP. This could indicate a general systemic stimulus to upregulate TLR3 in FIP, which is counteracted locally by viral inhibition of TLR3. Prior stimulation of TLR3 has also been shown *in vitro* to contribute to defence against murine coronavirus via type I interferon induction (Mazaleuskaya *et al.*, 2012), so is another potential avenue for future FIP research. A larger sample population, in particular with larger numbers of systemically infected cats without FIP, might have revealed significant intergroup differences for TLR3.

The STAT transcription factors are a key part of the antiviral pathways, mediating many downstream IFN effects (Aaronson and Horvath, 2002). They have also been linked to other coronavirus infections (e.g. STAT1 knock-out mice show a markedly increased susceptibility to SARS-CoV, while avian infectious bronchitis coronavirus uses STAT1 inhibition of IFN responses) (Frieman *et al.*, 2010; Kint *et al.*, 2015). STAT 1 and 2 gene transcription levels correlated with type II and I interferon transcription levels, respectively, in our study, while in virus-positive MLNs of cats without FIP, STAT 1 and 2 levels (the latter significantly so), as well as IFN- $\gamma$  levels, lay between the other two groups. This shows that the levels of IFNs and their downstream transcription factors are closely linked. Interestingly, STAT2 gene expression levels were significantly higher in the MLNs of cats with FIP and with effusions, a finding that cannot be readily explained. STAT2 has been linked to IL-6 upregulation, which itself has been linked to increased vascular permeability (Maruo *et al.*, 1992; Nan *et al.*, 2018); however, the IL-6 gene was not upregulated in our cohort, suggesting that responsibility lies with another pathway.

The results of our S gene codon mutation analysis add weight to recent findings that the M1058L mutation (referred to as M1048L in the present study due to re-evaluation of the reference sequence) is likely to contribute to systemic spread, but does not itself confer pathogenicity (Chang *et al.*, 2012; Porter *et al.*, 2014). This indicates that further host and/or viral factors are required for the development of FIP or, more precisely, the activation of virus-infected monocytes as a prerequisite to set off FIP vasculitis (Kipar and Meli, 2014). Most likely owing to the low viral RNA levels within the MLNs of cats without FIP, obtaining an adequate sequence from this group proved problematic. Other researchers experienced similar problems, often finding that FCoV RT-PCR-positive samples from cats without FIP were not amenable to sequencing (Felten *et al.*, 2017b). The lack of FCoV antigen expression in these cats was not unexpected and reflects the rarity of infected cells and/or the low virus load in infected cells; this is in line with the results of a previous study that found only rare positive macrophages in the MLNs of experimentally persistently-infected cats (Kipar *et al.*, 2010).

It was not possible to compare statistically the induced immune response of viruses showing S protein amino acid variations (codons 1,048 and 1,050) as only one cat had the 'enteric' form.

The future outcome of our FCoV infected cats without FIP, had they not succumbed to other diseases, is unknown, as is the contribution of yet to be defined viral factors. These cats may have remained carriers or have been demonstrating a transitional phase to later development of disease. However, based on our observations, activation of genes encoding TLRs 2, 4 and 8 in MLNs is associated with a negative outcome (i.e. FIP), while carrier animals upregulated the gene encoding TLR9. IFN- $\gamma$ , and particularly STAT2 with its myriad opportunities to direct cell fate, displayed intermediate levels of upregulation in the MLNs of

the carrier/transitional group, not associated with widespread increase in mediators of inflammation.

This study is only the start of determining the extent of involvement of PRRs in FIP; the downstream effects of these transcriptional alterations must be further investigated. However, our results reinforce the need for a balanced immune response against the virus, with the hypothesis that the moderate response in cats without FIP is part of the key to controlling the virus; when this balance is lost the animal may be at risk of succumbing to disease.

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### **Conflict of Interest Statement**

The authors declare no conflicts of interest with respect to the publication of this manuscript.

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## Figure Legends

Fig. 1. Boxplots demonstrating relative levels of FCoV transcription in G1+ and G2. The amount of FCoV was calculated by  $2^{-\Delta\Delta Cq}$ , using fGAPDH as the internal reference gene and expressed as an n fold difference relative to the G1+ mean as a calibrator. The boxes depict the median and interquartile (IQ) range with whiskers extending to the highest and lowest values which are within  $1.5 \times$  the IQ range. Outliers beyond this are individually marked. The three columns of individual crosses within G2 depict the three variations in the viral S protein at codons 1,048 and 1,050 respectively. From left to right: L, leucine at 1,048 ('systemic' virus); M&A, methionine and alanine ('systemic' virus); M&S, methionine and serine ('enteric' virus). 2E+/- and 2L+/- represent relative FCoV levels amongst MLN of cats with and without effusions/lesions.

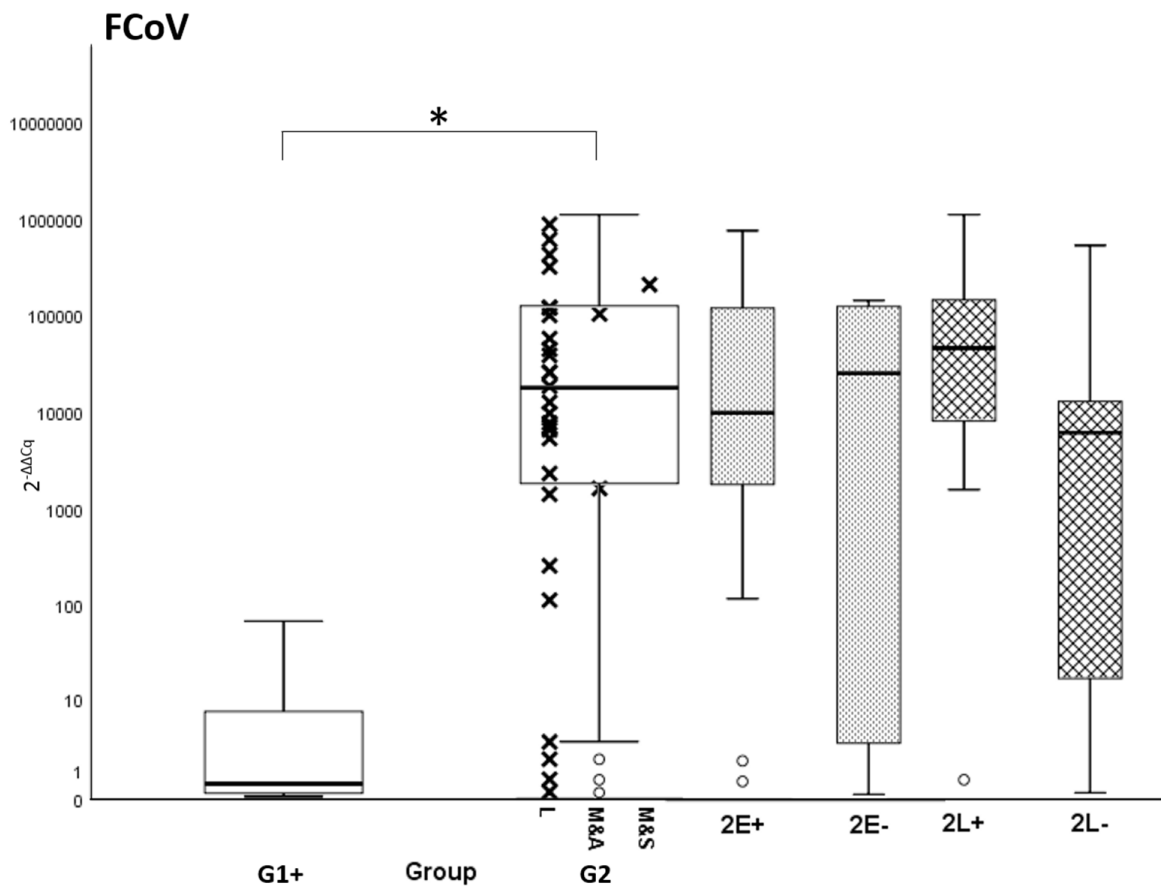


Fig. 2. Examples of MLNs with and without lesions from cats with FIP. (a, b) Case G2.5. (a) Focal pyogranulomatous inflammation with central necrosis (\*). HE. (b) Viral antigen expression is seen in abundant intact lesional macrophages. IHC. (c, d) Case G2.19. (c) Reactive hyperplasia with expansion of the marginal sinus by macrophages (\*). HE. (d) Some of the latter are FCoV antigen-positive. IHC.

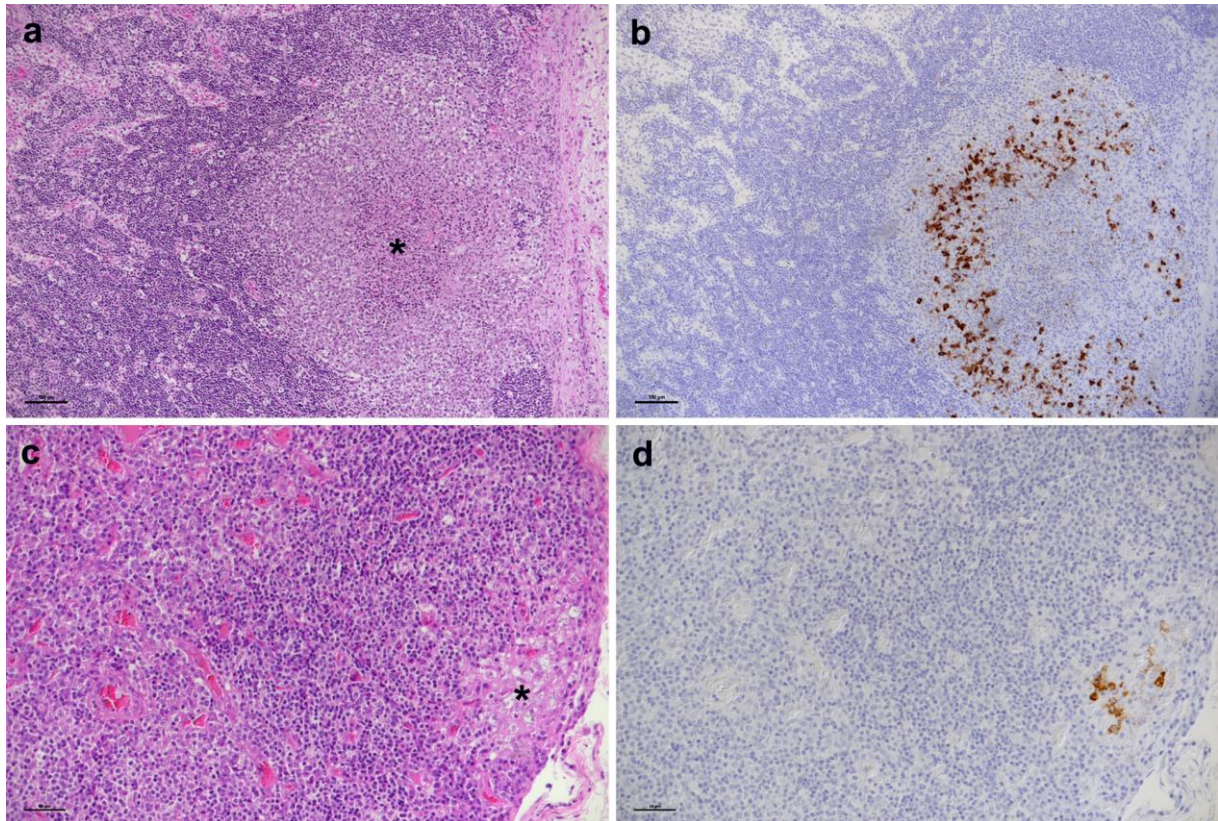


Fig. 3. Boxplots of relative levels of TLR gene expression in each group. The amount of target was calculated by  $2^{-\Delta\Delta Cq}$ , using fGAPDH as the internal reference gene and expressed as an n fold difference relative to the G1 mean as a calibrator. The boxes depict the median and interquartile (IQ) range with whiskers extending to the highest and lowest values, which are within  $1.5 \times$  the IQ range. Outliers beyond this are individually marked. \* marks significant differences between individual groups ( $P \leq 0.05$ ) or, where joined by a bar, between G1 as a whole and G2. 2E+/- and 2L+/- represent relative gene expression levels among MLNs of cats with and without effusions/lesions.

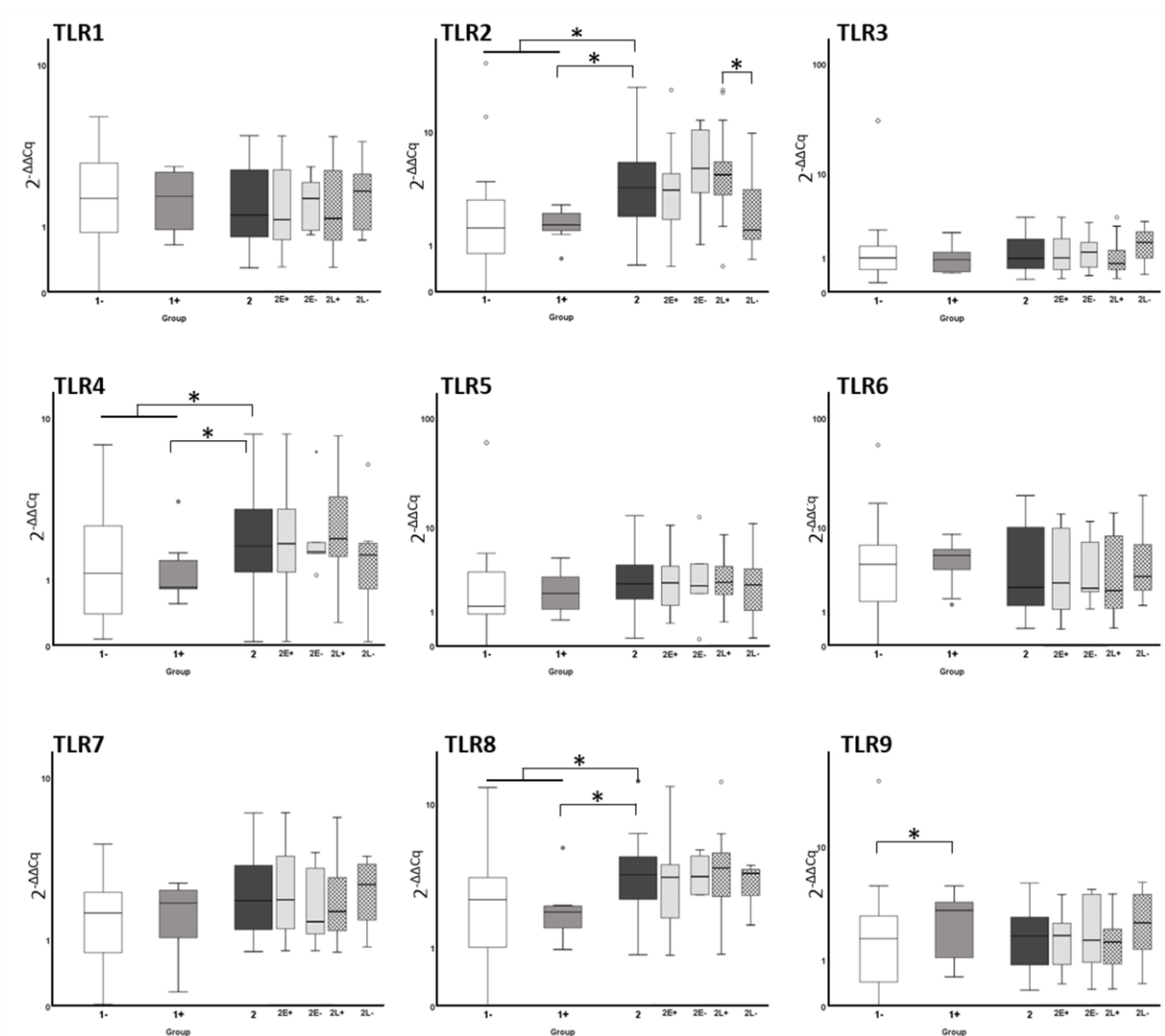




Fig. 4. Boxplots of relative levels of cytokine and chemokine gene expression in each group. The amount of target was calculated by  $2^{-\Delta\Delta Cq}$ , using fGAPDH as the internal reference gene and expressed as an n fold difference relative to the G1 mean as a calibrator. The boxes depict the median and interquartile (IQ) range with whiskers extending to the highest and lowest values, which are within  $1.5 \times$  the IQ range. Outliers beyond this are individually marked. \* marks significant differences between individual groups ( $P \leq 0.05$ ) or, where joined by a bar, between G1 as a whole and G2. 2E+/- and 2L+/- represent relative gene expression levels amongst MLNs of cats with and without effusions/lesions.

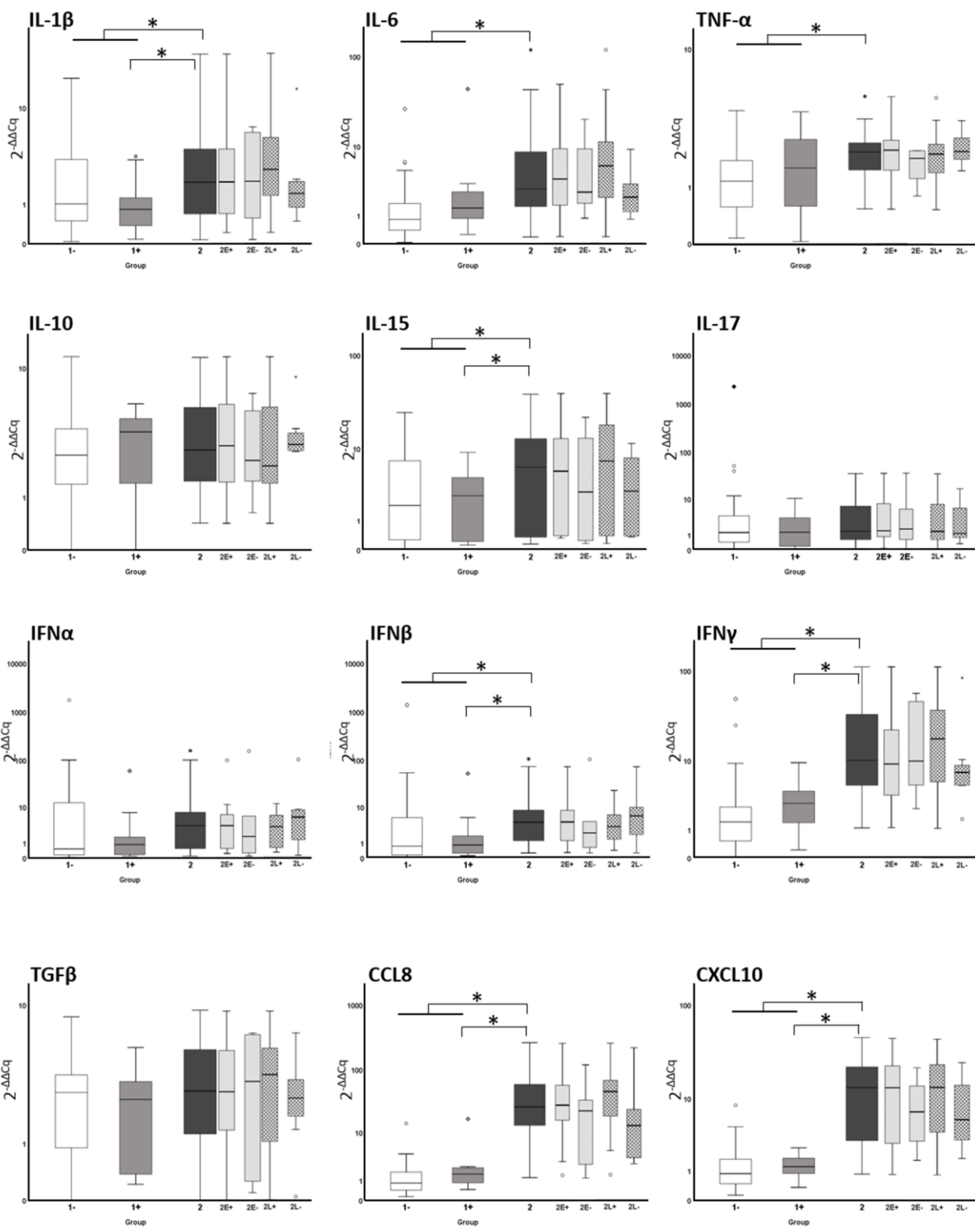
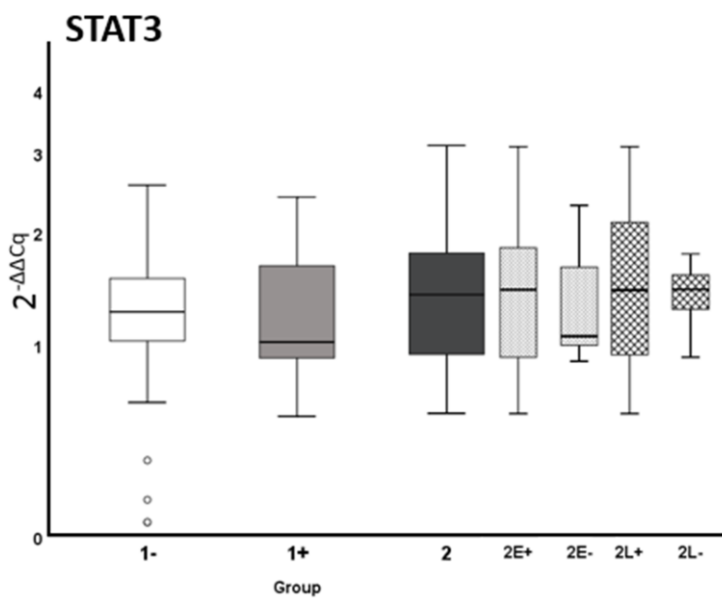
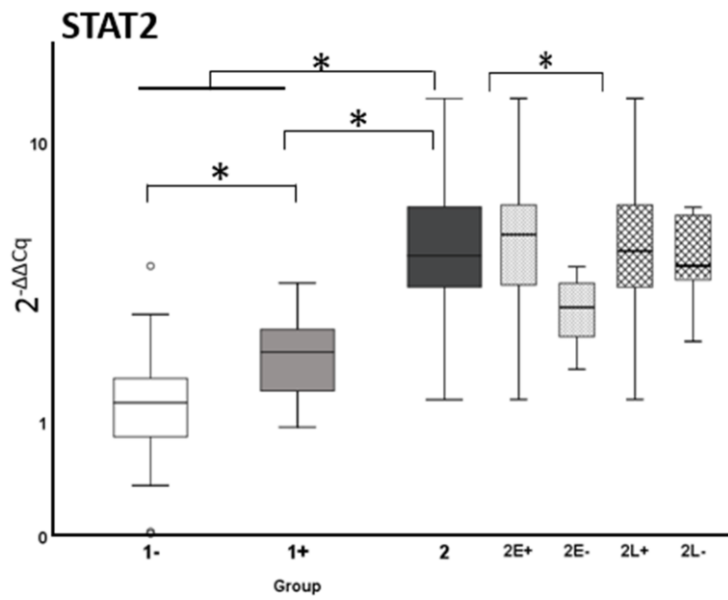
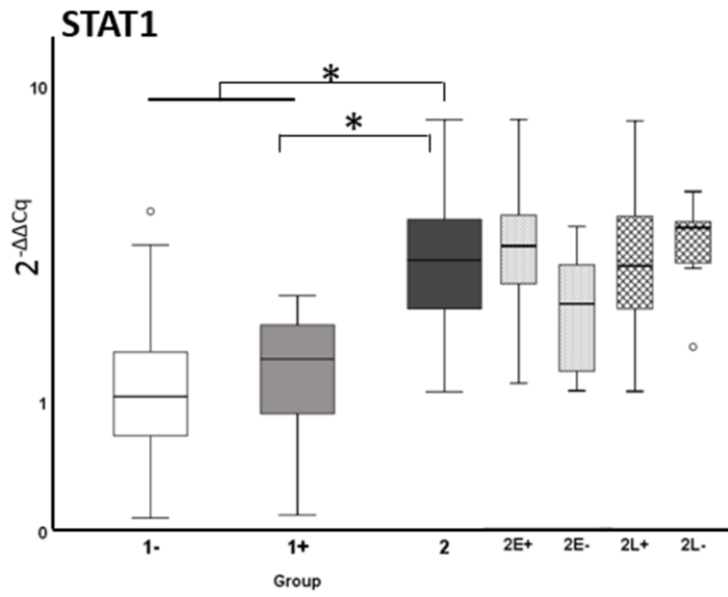


Fig. 5. Boxplots of relative levels of STAT gene expression in each group. The amount of target was calculated by  $2^{-\Delta\Delta Cq}$ , using fGAPDH as the internal reference gene and expressed as an n fold difference relative to the G1 mean as a calibrator. The boxes depict the median and interquartile (IQ) range with whiskers extending to the highest and lowest values, which are within  $1.5 \times$  the IQ range. Outliers beyond this are individually marked. \* marks significant differences between individual groups ( $P \leq 0.05$ ) or, where joined by a bar, between G1 as a whole and G2. 2E+/- and 2L+/- represent relative gene expression levels amongst MLNs of cats with and without effusions/lesions.



**Table 1:** Signalment, pertinent histological and immunohistological findings, and Sanger sequencing results of all cases.

**A. Group 1-:** Cats without FIP and without evidence of systemic FCoV infection as determined by RT-qPCR on the mesenteric lymph node.

	Breed	Age	Sex	Diagnosis	MLN	
					Histology	IH (FCoV Ag)
1	Ragdoll	4 y	MN	Congestive heart failure	Normal	-
2	Bengal	11 y	MN	Colonic adenocarcinoma	Normal	ND
3	DSH	adult	FN	DCM, chronic kidney disease	Follicular hyalinosis	-
4	DSH	adult	MN	Acute myeloid leukaemia	Leukaemia	-
5	Birma	1 y	MN	Hippocampal necrosis	Normal	-
6	House cat	14 y	MN	Haemorrhage in brain	Follicular hyalinosis	ND
7	DSH	8 y	MN	Chemodectoma	Normal	-
8	Birman	13 y		Pyothorax and pneumonia	Neutrophilic and histiocytic inflammation	-
9	DSH	6 y	MN	Astrocytoma	Normal to reactive hyperplasia	-
10		10 y	MN	Diabetes mellitus	Reactive hyperplasia and amyloidosis	-
11	DSH	12 y		Aplastic anaemia	Neutrophilic inflammation	-
12	DSH	6 y		Diarrhoea, suspected torovirus	ND	ND
13	DLH	8 y		Gastric lymphoma	Normal	-
14	DSH	5 y	MN	Suppurative meningitis	Mild depletion	-
15	DSH	3 y	MN	Lymphocytic cholangiohepatitis	Normal to reactive hyperplasia	ND
16	DSH	2 y	MN	Hepatitis and pyelonephritis	Reactive hyperplasia and sinus histiocytosis	-
17	DSH	4 y	FN	Granulomatous rhinitis & encephalitis	ND	ND
18	DSH	8 y	FN	Chronic enteropathy	ND	ND
19	DSH	1 y	FN	Poxviral pneumonia	ND	ND
20	DSH	4 y	FN	Hepatic encephalopathy	ND	ND
21	Ragdoll	3 y	MN	Hypertrophic cardiomyopathy	ND	ND
22	DSH	13 y	FN	Focal intestinal necrosis	Normal	ND
23	DSH		FE	Behavioural	Normal to reactive hyperplasia	-
24	DSH	3 y	FN	Invasive meningioma	Normal to reactive hyperplasia	-
25	Maine Coon	9 y		Meningioencephalitis	Normal	-
26	DSH	5 y	FN	Pulmonary adenocarcinoma	Tumour emboli	ND
27	Devon Rex	8 y		Inflammatory bowel disease	Normal	-
28	DSH	9 y	MN	Multicentric lymphoma	Reactive hyperplasia	-
29	DSH	10 m	MN	Hypertrophic cardiomyopathy	Reactive hyperplasia and sinus histiocytosis	-
30	Bengal	7 y	FN	Jejunal constriction	Follicular depletion	-

MLN, mesenteric lymph node; IH, immunohistology; Ag, antigen; DSH, domestic short hair; DLH, domestic long hair; blank, data not available; F/M(E/N), female/male (entire/neutered); DCM, dilated cardiomyopathy; ND, not done; -, negative.

**B. Group 1+:** Cats without FIP but with evidence of systemic FCoV infection as determined by FCoV RT-qPCR on the mesenteric lymph node.

	Breed	Age	Sex	Diagnosis	MLN					
					Histology	IH (FCoV Ag)	Sequencing			
							codon 1048		codon 1050	
1	Maine Coon	1 y		Pleural effusion (FCoV RT-qPCR -ve)	ND	ND	Not possible			
2	DSH	3y	MN	Lethargy, weight loss, anaemia	ND	ND	TTG	Leu	ND	
3	DSH	10 y	MN	Diabetes mellitus	Reactive hyperplasia with collagen scars	-	CTG	Leu	ND	
4	Ragdoll	4 m	ME	Severe interstitial pneumonia	Normal to reactive hyperplasia	-	CTG	Leu	TCC	Ser
5	Havana	4 y	FN	Nasal lymphoma	ND	ND	TTG	Leu	TCT	Ser
6	DSH	10 y	FN	Round cell neoplasia	Sinus histiocytosis	-	not possible			
7	DSH	8 y	MN	Pleural effusion (FCoV RT-qPCR -ve)	Normal	-	TTG	Leu	ND	
8	DSH	18 y	FN	Chronic kidney disease	Sinus histiocytosis	ND	TTG	Leu	TCT	Ser
9	DSH	10 y	MN	Lymphoma	Normal	-	CTG	Leu	ND	
10	DSH	-	FE	Anaesthetic death	Normal to reactive hyperplasia	-	CTG	Leu	ND	

MLN, mesenteric lymph node; IH, immunohistology; Ag, antigen; DSH, domestic short hair; blank, data not available; F/M(E/N), female/male (entire/neutered); -, negative; ND, not done.

C. Group 2: Cats with FIP.

	Breed	Age	Sex	Effusion	MLN					
					FIP lesions	IH (FCoV Ag)	Sequencing			
							Codon 1048		Codon 1050	
1	DSH	10 y	FN	+ (A)	Necrotising and pyogranulomatous	+	TTG	Leu	TCT	Ser
2	Norwegian Forest	8 m	MN	-	Necrotising and pyogranulomatous and lymphoplasmacytic	+	CTG	Leu	TCT	Ser
3		4 m	ME	+ (A)	Granulomatous	+	ATG	Met	GCT	Ala
4		1.5 y	MN	+ (A)	Pyogranulomatous	+	TTG	Leu	TCA	Ser
5	Maine Coon	1 y	MN	+ (A)	Pyogranulomatous and lymphoplasmacytic	+	TTG	Leu	TCC	Ser
6	DSH	6 m	MN	+ (A, P)	Granulomatous	+	TTG	Leu	TCT	Ser
7	DSH	4 m	FE	+ (M)	Granulomatous	+	TTG	Leu	TCT	Ser
8	BSH	6 y	MN	+ (A)	Pyogranulomatous	+	TTG	Leu	TCC	Ser
9	Persian	5 m	FE	+ (A)	Pyogranulomatous	+	TTG	Leu	TCT	Ser
10		3 y		+ (A)	Granulomatous	+	TTG	Leu	ND	
11	Burmese	3 m	ME	+ (T)	Necrotising and granulomatous	+	TTG	Leu	ND	
12	Abyssinian	4 m	FE	+ (A)	Pyogranulomatous	+	TTG	Leu	ND	
13	DSH	-		+ (T)	ND	ND	TTG	Leu	ND	
14	DSH	5 m		+ (A, T)	Necrotising and pyogranulomatous	+	TTG	Leu	ND	
15	Siamese	1 y		+	Pyogranulomatous	+	CTG	Leu	TCC	Ser
16	BSH	10 m	MN	+	Sinus histiocytosis	-	TTG	Leu	TCC	Ser
17	DSH	2 y	MN	+	Reactive hyperplasia	-	TTG	Leu	TCT	Ser
18	Siamese	3 y	MN	+ (A, T)	Normal	-	c/tTG	Leu	TCT	Ser
19	Birman	12 y	MN	+ (M)	Reactive hyperplasia	+	TTG	Leu	TCC	Ser
20	BSH	1 y	FN	+ (A, T)	Pyogranulomatous	+	ATG	Met	TCC	Ser
21	DSH	2 y	MN	-	Granulomatous	+	ATG	Met	GCC	Ala
22	Oriental	3 y	ME	-	Granulomatous	+	TTG	Leu	TCC	Ser
23	Birman	8 m	ME	-	ND	ND	TTA	Leu	TCA	Ser
24	Ragdoll	10 m	FN		Necrotising and granulomatous	+	TTG	Leu	TCC	Ser
25	BSH	2 y	MN	+ (A)	Necrotising and pyogranulomatous	+	TTG	Leu	TCC	Ser
26	DSH	6 m	FE	-	Normal	-	CTG	Leu	TCT	Ser
27	DSH	1 y		+ (A)	Reactive hyperplasia	-	FCoV Type II			
28	DSH	4 m			Reactive hyperplasia	-	TTG	Leu	TCC	Ser
29	DSH	7 m		-	Pyogranulomatous	+	TTG	Leu	ND	
30	DSH		ME	+ (A)	Pyogranulomatous	+	TTG	leu	ND	

MLN, mesenteric lymph node; IH, immunohistology; Ag, antigen; DSH, domestic short hair; blank, data not available; BSH, British long hair; F/M (E/N), female/male (entire/neutered); +, positive/present; -, negative/absent; A, abdominal; P, pericardial; M, multicavitary; T, thoracic; ND, not done; Leu, leucine; Ala, alanine; Met, methionine; Ser, serine. Nucleotide bases in lower case indicate a mixed infection.

**Table 2 Primer and probe sequences used for RT-qPCR and conventional RT-PCR**

<i>Gene</i>	<i>Reference or accession number</i>	<i>Primer and probe sequences (5'-3') where not previously published</i>		<i>PCR product length (base pairs)</i>
GAPDH, IL-10	Leutenegger <i>et al.</i> , 1999			
FCoV (RT-qPCR)	Gut <i>et al.</i> , 1999			
FCoV (conventional)	Porter <i>et al.</i> , 2014			
TLR1, 2, 4, 5, 6, 7, 9	Ignacio <i>et al.</i> , 2005			
TLR3, 8, IL-15, IFN- $\alpha$ , - $\beta$	Robert-Tissot <i>et al.</i> , 2011			
IL-1 $\beta$ , IL-6, TNF- $\alpha$	Kipar <i>et al.</i> , 2001			
TGF- $\beta$	Taglinger <i>et al.</i> ,			
IL-17	XM_006931816.1	F-16	ACTTCATCCATGTTCCCATCACT	126
		R-141	CACATGCTGAGGAAAATTCTTGTC	
		P-83	CATTCCCACAAAATCCAGGATGCC	
STAT1	XM_006935443	F-1649	TTGACCTCGAGACGACCTCTCT	135
		R-1783	GCGGGTTCAGGAAGAAGGA	
		P-1686	CTCCAATGTCAGCCAGCTCCCGAGT	
STAT2	XM_003988893	F-1182	GCCCAGGTCACGGAGTTG	122
		R-1303	ACAGTGAAC TTGCTCCCTGTCTT	
		P-1212	CTGCACAGAGCCTTTGTGGTAGAAACCC	
STAT3	XM_006940361.2	F-1626	GCCAGTTGTGGTGATCTCCAA	133
		R-1758	TTGATCCCAGGTTCCAATCG	
		P-1696	CTGACCAACAACCCCAAGAACGTGAACTTT	
CCL8	XM_003996558	F-95	GGCCACCTTCAGCATCCA	82
		R-176	CCCTTTGACCACACTGAAGCA	
		P-121	CTCAGCCAGGTTTCAGTTTCCATCCCA	
CXCL10	XM_003985274.3	F-386	TGCCATCATTCCCTACATTCTT	78
		R-463	CAGTGGTTGGTCCCTTTTAGGA	
		P-411	CAAGCCCTAATTGTCCCTGGATTGCAG	
IFN- $\gamma$	NM_001009873.1	F-214	TGGAAAGAGGAGAGTGATAAAACAA	122
		R-335	TCCTTGATGGTGTCCATGCT	
		P-284	ACCTGAAAGATGATGACCAGCGCATTCAA	

Accession number, NCBI accession number; F, forward primer and start site; R, reverse primer and start site; P, probe and start site. All final reactions contained equivalent F and R concentrations of 900 nM and 250 nM for P, with the exception of FCoV RT-qPCR, 300 and 250 nM; FCoV conventional, 500nM; TGF- $\beta$ , 200 and 50 nM; STAT3, 600 and 250 nM, respectively.



**Table 3:** Results of statistical comparisons between groups of cats, using a two-tailed Mann-Whitney test

	Statistical comparison between			FIP group	
	G1 vs G2	G1- vs G1+	G1+ vs G2	Effusions present vs absent	MLN lesions present vs absent
FCoV	<b>0.000*</b>	<b>0.000*</b>	<b>0.000*</b>	0.764	0.071
TLR1	0.610	0.914	0.794	0.643	0.533
TLR2	<b>0.000*</b>	0.724	<b>0.002*</b>	0.259	<b>0.048*</b>
TLR3	0.569	0.724	0.656	0.682	0.189
TLR4	<b>0.019*</b>	0.794	<b>0.022*</b>	1.000	0.208
TLR5	0.053	0.508	0.396	0.806	0.756
TLR6	0.859	0.286	0.469	0.764	0.228
TLR7	0.059	0.770	0.272	0.427	0.568
TLR8	<b>0.012*</b>	0.246	<b>0.015*</b>	0.566	0.435
TLR9	0.991	<b>0.031*</b>	0.140	0.764	0.189
STAT1	<b>0.000*</b>	0.315	<b>0.000*</b>	0.052	0.466
STAT2	<b>0.000*</b>	<b>0.017*</b>	<b>0.000*</b>	<b>0.017*</b>	0.717
STAT3	0.260	0.569	0.414	0.764	1.000
IFN- $\alpha$	<b>0.041*</b>	1.000	0.077	0.604	0.499
IFN- $\beta$	<b>0.004*</b>	0.770	<b>0.036*</b>	0.566	0.604
IFN- $\gamma$	<b>0.000*</b>	0.131	<b>0.003*</b>	0.806	0.249
IL-1 $\beta$	<b>0.026*</b>	0.432	<b>0.031*</b>	0.849	0.272
IL-6	<b>0.001*</b>	0.209	0.177	1.000	0.208
IL-10	0.296	0.469	0.939	0.604	0.272
IL-15	<b>0.019*</b>	0.794	<b>0.039*</b>	0.53	0.376
IL-17	0.440	0.528	0.286	0.723	1.000
TGF- $\beta$	0.430	0.508	0.396	0.978	0.678
TNF- $\alpha$	<b>0.004*</b>	0.432	0.346	0.309	0.405
CXCL10	<b>0.000*</b>	0.396	<b>0.000*</b>	0.441	0.263
CCL8	<b>0.000*</b>	0.177	<b>0.000*</b>	0.46	0.071

\* indicates significance level of  $p \leq 0.05$ . In the first three columns the second group of the comparison is significantly higher in all cases (e.g. for G1 vs G2, G2 levels are higher). In the FIP columns the value of the 'present' group is in both cases higher than in the 'absent' group.

**Table 4:** Summary of Spearman's rank one-tailed correlation results within the FIP group, showing immune mediators with significant results.

	FCoV	TLR2	TLR4	TLR8	TLR9	STAT1	STAT2	STAT3	IFN- $\alpha$	IFN- $\beta$	IFN- $\gamma$	IL-1 $\beta$	IL-6	IL-15	IL-17	TGF- $\beta$	TNF- $\alpha$	CXCL10	CCL8
FCoV	●	↑↑	↑↑	↑	↑		↑↑		↑	↑↑	↑↑	↑↑	↑↑	↑				↑↑	↑↑
TLR2	↑↑	●	↑↑	↑↑			↑	↑↑		↑↑	↑↑	↑↑	↑↑					↑↑	↑↑
TLR4	↑↑	↑↑	●	↑↑		↑	↑↑	↑↑		↑	↑↑	↑↑	↑↑	↑	↑↑			↑↑	↑↑
TLR8	↑	↑↑	↑↑	●				↑↑				↑↑	↑↑					↑	↑
TLR9	↓				●	↑↑	↑	↑↑								↑↑	↑		
STAT1			↑		↑↑	●	↑↑	↑↑				↑	↑	↑	↑		↑↑	↑↑	↑
STAT2	↑↑	↑	↑↑		↑	↑↑	●	↑↑		↑	↑	↑↑	↑↑	↑↑	↑		↑↑	↑↑	↑↑
STAT3		↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	●		↑		↑↑	↑↑		↑↑	↑	↑↑	↑↑	↑↑
IFN- $\alpha$	↑							↑	●	↑↑	↑↑	↑	↑		↑		↑		↑
IFN- $\beta$	↑↑	↑↑	↑				↑	↑	↑↑	●	↑↑		↑		↑		↑↑	↑	↑↑
IFN- $\gamma$	↑↑	↑↑	↑↑				↑	↑	↑↑	↑↑	●	↑↑	↑↑		↑		↑	↑↑	↑↑
IL-1 $\beta$	↑↑	↑↑	↑↑	↑↑		↑	↑↑	↑↑	↑		↑↑	●	↑↑		↑↑	↑	↑↑	↑↑	↑↑
IL-6	↑↑	↑↑	↑↑	↑↑		↑	↑↑	↑↑	↑	↑	↑↑	↑↑	●		↑		↑↑	↑↑	↑↑
IL-15	↑		↑			↑	↑↑							●				↑↑	↑
IL-17			↑↑			↑	↑	↑↑	↑	↑	↑	↑↑	↑		●		↑		
TGF- $\beta$					↑↑			↑				↑				●	↑		
TNF- $\alpha$					↑	↑↑	↑↑	↑↑	↑	↑↑	↑	↑↑	↑↑		↑↑	↑	●	↑	↑
CXCL10	↑↑	↑↑	↑↑	↑		↑↑	↑↑	↑↑		↑	↑↑	↑↑	↑↑	↑↑			↑	●	↑↑
CCL8	↑↑	↑↑	↑↑	↑		↑	↑↑	↑↑	↑	↑↑	↑↑	↑↑	↑↑	↑			↑	↑↑	●

↑↑, positive correlation at a significance level of  $p \leq 0.01$ ; ↑, positive correlation at a significance level  $p \leq 0.05$ ; ↓, negative correlation at a significance level of  $p \leq 0.05$