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Pantropic canine coronavirus induces canine M1 macrophage polarization *in vitro*

Flavia Volpato Vieira[1,2], Rebeca Figueiredo Nalesso[2], Letícia Colin Panegossi[2], Icaro Alex'Sanderson Pereira de Godoy[2], Jamila Cristina Baptistella[4], Tereza Cristina Cardoso[2,3*]

[1]*Federal Institute of Amazonas, College of Veterinary Medicine, Manaus, Amazonas, Brazil*
[2]*São Paulo State University, College of Veterinary Medicine, Laboratory of Animal Virology and Cell Culture Araçatuba, São Paulo, Brazil*
[3]*São Paulo State University, College of Veterinary Medicine, Laboratory of Veterinary Anatomy and Histology, Araçatuba, São Paulo, Brazil*
[4]*UniSalesiano Araçatuba, College of Veterinary Medicine, São Paulo State, Brazil*
*****Correspondence: tereza.cardoso@unesp.br*

Abstract. Emerging coronavirus infections are a major threat to global public health. In this respect, a novel recombination of canine coronavirus (CCoV) and feline coronavirus (FCoV) was described among human biological samples, giving rise to a potential zoonosis. Despite all efforts, the host–virus immune response related to CCoV is still unknown. In this study, pantropic CCoV infection of canine macrophages, derived from peripheral blood monocytes, was performed. After infection, macrophages were first polarized to the M1 and/or M2 phenotype. Moreover, infection kinetics, cell viability, apoptosis, mitochondrial dysfunction associated with reactive oxygen species and oxide nitric production were measured. Our results demonstrated that virus infection mainly polarized host macrophages to the classically activated (M1) phenotype, as demonstrated by amoeboid morphology with numerous fibrillary cytoplasmic processes followed by classical phenotypes. Viral infection released new particles 18 h post-infection associated with a decrease in viable cells. Furthermore, upon CCoV infection, M1 cells exhibited reduced phagocytosis properties, as evidenced by a neutral red uptake assay. This *in vitro* method opens an avenue for further studies on host-virus interaction.

Keywords: Apoptosis, CCoV, macrophages, mitochondria metabolism, viral enteritis

Coronavírus canino pantrópico induz polarização de macrófagos M1 caninos in vitro

Resumo. As infecções emergentes por coronavírus constituem uma grande ameaça para a saúde pública mundial. A este respeito, foi descrita uma nova recombinação do coronavírus canino (CCoV) e do coronavírus felino (FCoV) em amostras biológicas humanas, dando origem à uma potencial zoonose. Apesar de todos os esforços, a resposta imunitária vírus-hospedeiro relacionada com o CCoV ainda não é conhecida. Neste estudo, foi efetuada uma infecção pantrópica por CCoV em macrófagos caninos derivados de monócitos do sangue periférico. Após a infecção, os macrófagos foram primeiramente polarizados para o fenótipo M1 e/ou M2. Além disso, foram medidas a cinética da infecção, a viabilidade celular, a apoptose, a disfunção mitocondrial associada a espécies reativas de oxigênio e a produção de óxido nítrico. Os nossos resultados demonstraram que a infecção pelo vírus polarizou principalmente os macrófagos do hospedeiro para o fenótipo classicamente ativado (M1), como demonstrado pela morfologia ameboide com numerosos processos citoplasmáticos fibrilares seguidos de fenótipos clássicos. A infecção viral produziu novas partículas 18 horas pós-infecção, associadas a uma diminuição das células viáveis. Além disso, após a infecção por CCoV, as células M1 exibiram propriedades de fagocitose

reduzidas, conforme evidenciado por um ensaio de absorção de vermelho neutro. Este método *in vitro* representa uma via para novos estudos sobre a interação vírus-hospedeiro.

Palavras chave: Apoptose, CCoV, macrófagos, metabolismo mitocondrial, enterite viral

Introduction

Canine coronaviruses (CCoVs) are member of the subfamily *Orthocoronavirinae* that belongs to the family *Coronaviridae*, suborder *Coronavirineae*, order *Nidovirales* genus *Alphacoronavirus* (Belouzard et al., 2012; Groot et al., 2011). The genome consists of genes encoding the structural proteins spike (S), envelope (E), membrane (M) and nucleocapsid (N) and ORFs translated into the nonstructural proteins replicase polyprotein (ORF1), 3a, 3b, 3c, 7a and 7b. Emerging viral infections continue to pose a major threat to global public health (Alfano et al., 2020; Baldanta et al., 2017; Dalskov et al., 2020; Liu & Saif, 2020; Ntafis et al., 2012). The recent emergence of a novel coronavirus (SARS-CoV II), which caused an outbreak of unusual viral pneumonia in tens of people in Wuhan, a central city of China, restated the risk of coronaviruses posed to public health (Liu & Saif, 2020).

To date, two CCoV genotypes are known, designated types I and II, and canine/porcine recombinant viruses have been identified (Chen et al., 2019; Decaro et al., 2008, 2010; Decaro & Buonavoglia, 2008; Decaro & Lorusso, 2020; Enjuanes et al., 2006). Moreover, CCoV-II was subdivided into CCoV-IIa (which derives from recombination with feline coronavirus; FCoV-II) and CCoV-IIb (which derives from recombination with porcine transmissible gastroenteritis virus; TGEV) with no association with clinical disease in dogs (Decaro et al., 2007). However, a few years ago, a highly virulent strain (pantropic CCoV-IIa) was isolated during an outbreak of fatal, systemic disease in puppies in Italy (Decaro et al., 2007).

Recently, an isolated HLJ-073 strain causing gross multiple organ lesions and diarrhoea was isolated and identified as a pantropic strain in China. Cell tropism experiments suggested that HLJ073 could effectively replicate in canine macrophages/monocytes and human THP-1 cells (Chen et al., 2019). Moreover, a novel canine-feline recombinant *alphacoronavirus* was isolated from a human patient in 2021, bringing a new potential of zoonosis (Tian et al., 2021; Vlasovaet al., 2022a; Vlasova et al., 2022b). The emergence of pantropic CCoV strains worldwide is considered a neglected pathogen; studies on its pathogenesis and host immune response are missing.

Macrophages are specialized cells involved in the detection, phagocytosis and destruction of bacteria and other harmful organisms. In addition, they can also present antigens to T cells and initiate inflammation by releasing molecules (known as cytokines) that activate other cells (Knoll et al., 2021). For example, pulmonary macrophages in COVID-19 derived from infiltrating inflammatory monocytes are in a hyperactive activated state, resulting in a detrimental loop of proinflammatory cytokine release and recruitment of cytotoxic effector cells, thereby exacerbating tissue damage at the site of infection (Heinrich et al., 2017). By simplified classification, canine macrophages were divided into classically activated (M1) and activated (M2) macrophages based on their activation states. Some viruses use these cells to hide themselves from the immune system to initially propagate and generate new progeny virus particles and even to spread new viruses in the host body (Heinrich et al., 2017). One of the most modern mechanisms applied by viruses to succeed in this process is to consume cell energy.

Mitochondria are implicated directly in several immune-host and viral response (Scott, 2010). Moreover, viral proteins inserted in mitochondrial membranes present anti- and/or proapoptotic effects, affecting cell survival/death pathways (El-Bacha & Da Poian, 2013; Ohta & Nishiyama, 2011; Perlman & Netland, 2009). However, a dysregulated macrophage response can be damaging to the host, as is seen in the macrophage activation syndrome induced by severe infections, including infections with the related virus SARS-CoV (Perlman & Netland, 2009). Interestingly, death due to COVID-19 revealed a high infiltration of macrophages within the area of bronchopneumonia (Beniac et al., 2006).

This study was realized to investigate whether pantropic CCoV strain-infected canine monocytes induce differentiation into primed M1 macrophages. In addition, mitochondrial dysfunction at 6, 12 and 18 h post-infection was addressed. For this purpose, viral replication, phagocytosis, mitochondrial membrane depolarization and respiratory chain complexes were detected. Other parameters were

measured as cell viability, nitric oxide production, and reactive oxygen species associated with apoptosis events.

Materials and methods

Cell culture, blood cell isolation and virus strain

AF-72 cells (ATCC, CRL 1542) were cultured in MEM (Sigma–Aldrich®) supplemented with antimycotic/antibiotic 1X solution, 10% foetal calf serum (Sigma–Aldrich®), 2 mM L-glutamine (Sigma–Aldrich®), and nonessential amino acids (100x, Invitrogen®, Life Technologies, Carlsbad, CA, USA). Cultures were incubated at 38.5 °C in 5% CO_2 with 95% humidity. Canine macrophages were derived from peripheral blood monocytes (PBMCs) obtained from healthy dogs maintained in veterinary school under sanitary health conditions and followed protocols of animal experimentation (CEEA 2016/09854-2). In order to confirm absence of CCoV previously infection, these animals were searching weekly using RT-PCR and serology analysis. Briefly, PBMCs were obtained using lymphocyte separation medium (ACCUSPIN™ System-Histopaque® -1077; Sigma–Aldrich, St Louis, MO) and plated on collagen-coated plates in 24-well polystyrene cell culture plates (SigmaScreen™) by the addition of Roswell Park Memorial Institute (RPMI)-medium (Sigma–Aldrich). Nonadherent cells were removed after 16 h in culture, and adherent monocytes were ready to use. Pantropic CCoV strain, a field isolate catalogue as 06/2015 Brazil (GenBank access number KR105604), was used, maintained after infection of AF-72 cell monolayers and stored at -196 °C.

Study design

Briefly, this study was divided into two distinct experiments: (1) M1-primed canine macrophages were induced for six days in 10 ng/mL macrophage colony-stimulating factor from dogs (GM-CSF; R&D Systems, Minneapolis MN, USA) and cultured in RPMI medium (Sigma–Aldrich) supplemented with 10% Xeno-Free® foetal bovine serum (Sigma–Aldrich); (2) CCoV infection in canine blood-derived monocytes performed at an MOI of 1 and viral absorption at 30 min at 38.5 °C/5% CO_2 at 6, 12 and 18 h post-infection (p.i.) in order to evaluate early events after virus infection.

Macrophage phenotyping

To confirm the M1 macrophage-derived PBMC phenotype among the studied groups, flow cytometry and immunofluorescence analysis were performed. The antibodies used were the same as described previously with some modifications (Heinrich et al. 2017). Rat anti-dog MHCII labelled with FITC (cat # MCA 1044F, clone YKIX334.2) and rat anti-dog CD32 labelled with Cy3 (cat # MCA1075F, clone YKIX753.22.2) were all purchased from Bio-Rad™ antibodies. After CCoV suspension adsorption, cell monolayers were washed with phosphate-buffered saline (PBS, 7.2), and fresh medium was added. CCoV-infected and uninfected cells were monitored under phase contrast using an Olympus IX-70 microscope (Olympus®, Tokyo, Japan) at 6, 12 and 18 h post-infection (p.i.). AF-72 cells (1 x 10^4 per well) were seeded in 96-well plates to allow virus recovery from CCoV-infected and uninfected cells.

Virus titration, recovery and detection

Susceptibility of macrophages to CCoV infection was verified by morphological changes (cytopathic effect, virus recovery after AF-72 infection and indirect immunofluorescence assay (IFA) at 6, 12 and 18 h post-infection (p.i).

For CCoV infection, AF-72 cells were used to virus recovery using supernatant of canine macrophages infected monolayers. This procedure was performed at a multiplicity of infection (MOI) of 1.0 in cell culture incubated at 39.5 °C, 5% CO_2, and 95% humidity with density of 1 x 10^5 cells/cm^2. Infected and control macrophages cells were visualized under phase-contrast using Olympus IX 70 microscope (Olympus, Tokyo, Japan) and at least 10 fields were analyzed for each condition. The photographs were taken at 40 x magnification using cell Sens software (Olympus).

The $TCID_{50}$ were applied to infected and control (mock-infected cells supplemented with culture medium) AF-72 cells to measure viral infective particles recovery and the data were transformed in Log $TCID_{50}/_{50\mu L}$. AF-72 CCoV-infected cells were used as a positive control. DAPI (1 mg/ml) was used as a

counterstain for each slide. Virus suspension harvested from each p.i. was serially diluted 10-fold and added to each well with 100 µl in six replicates to perform virus titration on AF-72 cell monolayers. The titer was calculated by the 50% tissue culture infection dose ($TCID_{50}$) according to the Reed and Muench method. The purification and electron microscopy protocol for negative-stain and thin-section examination was performed (Beniac et al., 2006; Vlasova, et al., 2022).

Phagocytic activity assay and cell viability

The phagocytic capacity of M1 cells was detected by a neutral red uptake assay following the manufacturer's instructions (TOXI-4 Kit, Sigma–Aldrich) (Khatua et al., 2022). Cells at 1.0×10^3/50 µl were seeded in 96-well plates and incubated overnight at 5% CO_2 and 39.5 °C. The cells were infected with CCoV at an MOI of 3 for 6, 12 and 18 h. Next, 50 µl of 0.1% neutral red solution was added to each well and incubated for 1 h. Then, 100 µl of cell lysis solution (ethanol: ice acetic acid = 1:1, v:v) was added to each well, and the wells were incubated at room temperature for 10 min. A microplate reader (ThermoFisher, CA, USA) was used at an absorption wavelength of 540 nm. The uptake efficiency was calculated by normalizing the sample absorbance compared to that of the control represented by noninfected M1 cells as 100%. Cell viability was determined using the MTT assay following the manufacturer´s instructions (Sigma–Aldrich). The optical density (OD) was determined at 570-600 nm using BioPhotometer™ (Eppendorf). The survival ratio of infected cells was expressed as a percentage of the vehicle control (DMSO).

Statistical analysis

The data were computed from infected and noninfected cells at all times p.i. All experiments were performed at least in triplicate. Descriptive statistics include the mean ± standard deviation (s.d.). A p value < 0.005 was considered significant. All statistical analyses were performed using Prism software (GraphPad® v 9.1, 274 CA, USA) by One-way ANOVA data analysis.

Results

Macrophage phenotyping and CCoV replication

Morphologically, differences between uninfected canine monocytes and M1-polarized cells were similar to those reported in a previous study. Herein, CCoV infection induced only M1-primed cells. The majority of M1 macrophages were amoeboid and had numerous fibrillary cytoplasmic processes on the cellular surface and intense cytoplasm vacuolization in comparison to uninfected M1 cells (Fig. 1).

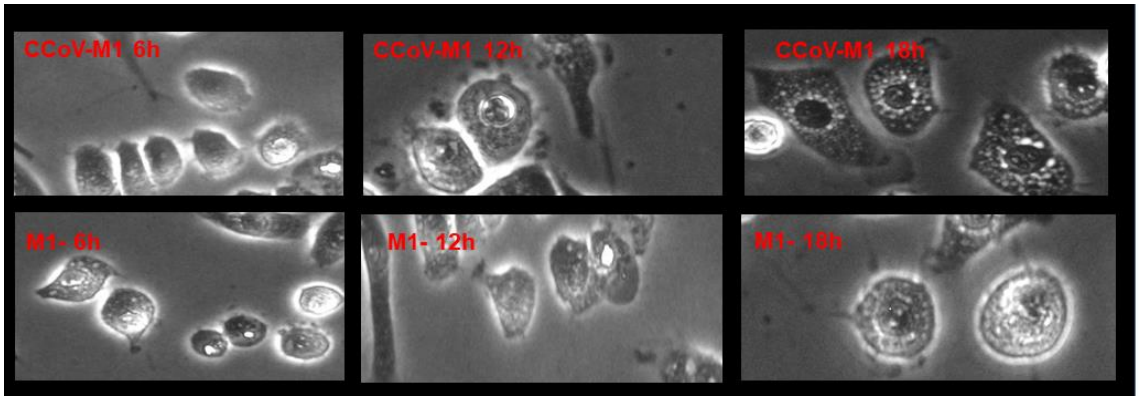

Figure 1. Characterization of canine macrophage culture. A) Phase contrast microscopy of CCoV-infected and uninfected polarized M1 canine macrophages (magnification 100X). CCoV-M1 did not differ from M1 uninfected cells, showing small and roundish morphology and lacking cytoplasmic extensions at 6 h. CCoV-M1 at 18 h p.i. demonstrated an enlarged amoeboid cell shape with roundish cell bodies and numerous delicate cytoplasmic extensions and vacuolization.

The canine macrophage phenotype was analysed after CCoV infection and no infection but stimulation of M1 polarization (Fig. 2A and B). In addition, infection of AF-72 cells, known to be permissive to CCoV infection, with suspension prepared from infected M1, confirmed CCoV antigens (Fig. 3A and B). In this study, no positive results were obtained of M2 canine-primed macrophages after CCoV infection (data not shown). Conversely, viral titers increased continuously, with a significant

difference between the four evaluated moments (Fig. 3C). Finally, CCoV particles from AF72 cells were purified, and electron microscopy revealed coronavirus-like images (Fig. 3D).

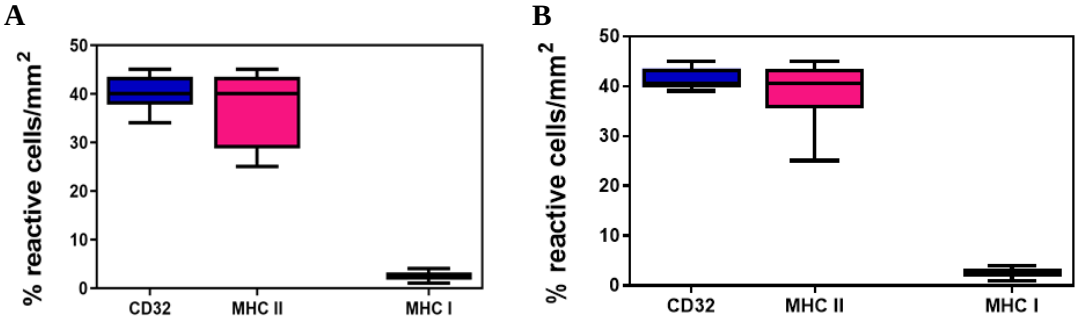

Figure 2. Characterization of canine macrophage culture. **A**) Flow cytometry of canine M1 MHC I/II revealed that 44.58% of isolated cells were positively marked by the MHCII phenotype. B) A total of 41.57% of isolated cells were positive for the CD32 phenotype. **B**) CCoV-M1 phenotyping showing 42.12% MHCII, 39.8% CD32 and low MHCI cell markers.

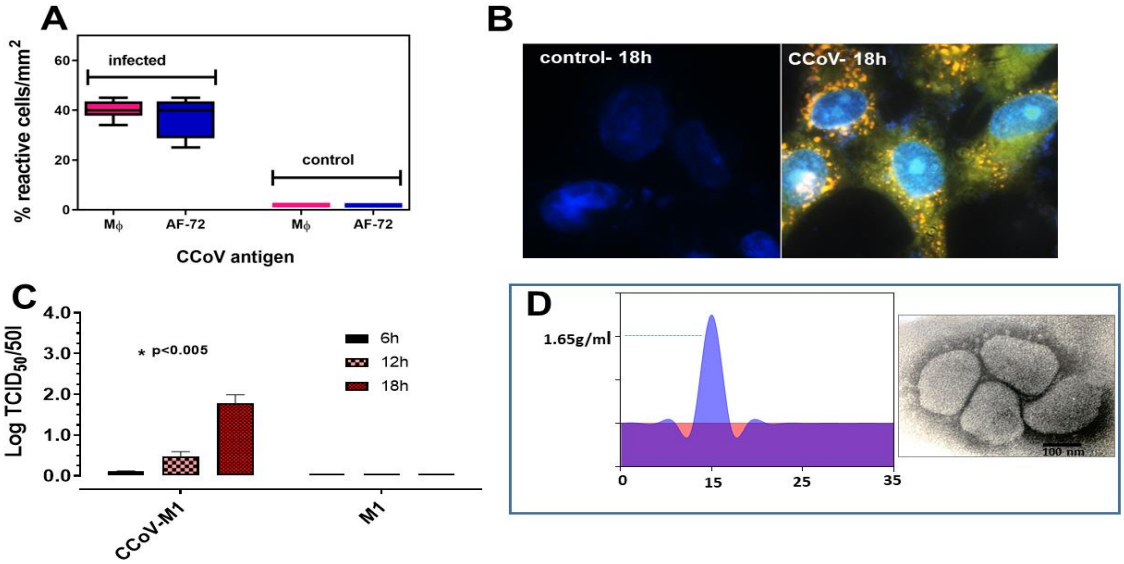

Figure 3. A) After infection, almost > 40% of M1 cells showed positivity for viral antigens different from the control cells; **B**) AF-72-infected cells by macrophage-CCoV-infected culture suspensions, showing viral antigens (fluorescence inside cytoplasm), counterstained with DAPI (blue-nucleus). **C** (magnification 100X); CCoV titration demonstrated an increase in virus production according to post infection points; **D**) Chromatography graph demonstrating a peak of probable CCoV particles followed by characteristic images of coronavirus-like particles taken under electron microscopy (EM).

CCoV infection induces lower cell proliferation, neutral red uptake and early events of apoptosis

With regard to cell proliferation, there was a significant decrease between 6 and 18 h pi. ($p < 0.005$) (Fig. 3A). The results showed that CCoV infection significantly inhibited M1 phagocytosis in the infected group compared with the control group by analysis of neutral red uptake (Fig. 3B).

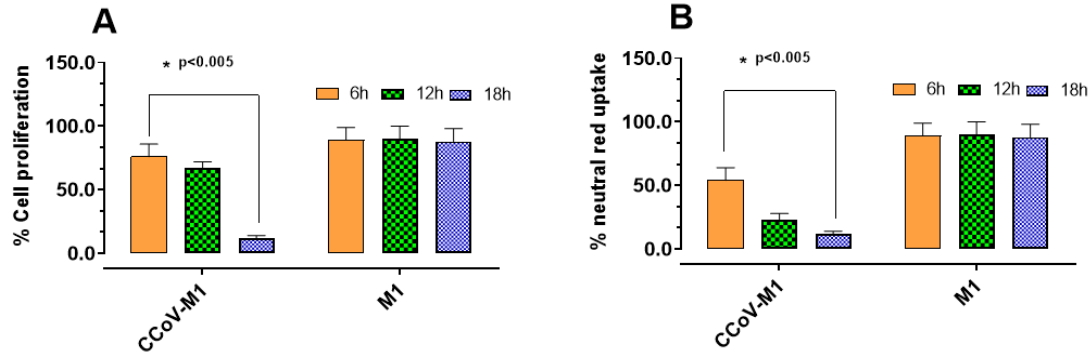

Figure 4. A) Cell proliferation after CCoV infection decreased significantly at 18 h p.i. B) Neutral red uptake test showing a continuous drop at 6, 12, and 18 h p.i.

Discussion

Coronaviruses are pathogens with a serious impact on human and animal health. They mostly cause enteric or respiratory disease, which can be severe and life threatening (Khatua et al., 2022; Saleh et al., 2020). Moreover, the outcome of SARS -like outbreaks, the importance of advancing our knowledge on the replication of these viruses and their interactions with the host, need further research (Ohta & Nishiyama, 2011; Poon et al., 2010). Next, a role in basic virus replication or virus assembly interactions with the host cell creates an optimal environment for coronavirus replication (Merad & Martin, 2020). Due to the complexity of the coronavirus replication cycle and host specificity, our knowledge of host factors involved in coronavirus replication is still in an early stage compared to what is known for some other +RNA viruses (Belouzard et al., 2012).

In the present study, pantropic CCoV was shown to be effective in promoting canine macrophages into M1 polarization culture infection, in view of the positive immunostaining in the cells of the experiment, in association with increased viral titers during all p.i. This demonstrates the ability of the pantropic variant to cause infection and to generate new infectious particles, a fact proven by the subsequent infection of AF-72 cells with the culture of infected macrophages. AF-72 cells are known to be permissive to CCoV infection and were therefore used as a positive control in the experiment. The successful phenotyping of M1 was based on a previous study that standardized the most important cell domains for canine studies (Heinrich et al., 2017).

For instance, following classical stimulation with pro-inflammatory cytokines, murine macrophages produce NO, whereas human macrophages nearly lack synthesis of NO in response to classically activating stimuli (Vieira et al., 2018). Herein, NO production did not interfere with antiviral activity since new CCoV particles were produced. However, the literature indicates MHC class II as a panmacrophage marker that is expressed on both M1 and M2 macrophages in mice (Heinrich et al., 2017). A recent work in China also found the ability of a new variant of CCoV, isolated from a fatal case, to cause infection and replicate in culture in macrophages (Hossain et al., 2021). In this sense, the ability to alter cell tropism and cause infection in macrophages seems to be a key factor in understanding the disease-related pathogenesis caused by CCoV variants.

The frequent appearance of new variants of coronavirus occurs due to several factors, such as errors committed by RNA polymerase and the high frequency of genetic recombination between different coronaviruses (Pratelli, 2011; Uchida et al., 2019). These changes lead to changes in the S protein, which is responsible for coronavirus tropism (Vieira et al., 2018). In contrast to typical CCoV infection, the panthropic variant demonstrates the ability to spread through different organs, causing severe and often fatal systemic disease (Marfè et al., 2011). It is suspected that the mutation observed in CCoV probably changes the cellular tropism, making it able to infect canine macrophages efficiently, as demonstrated in this study (Elmore, 2007; Licitra et al., 2014; Rottier et al., 2005).

Although studies involving macrophage infection by CCoV have been performed *in vitro*, other serious systemic diseases caused by coronavirus, such as severe respiratory distress syndrome (SARS-CoV) in humans and feline infectious peritonitis (FIP), show that the involvement of macrophages is determinant for the course of viral infection (Elmore, 2007; Licitra et al., 2014; Rottier et al., 2005). Infection of macrophages and dendritic cells by feline infectious peritonitis virus (VFIP) leads to an aberrant pattern of cytokine and chemokine expression, leading to lymphoid depletion and high viral titers (Timurkan et al., 2021).

The association between CCoV and lymphopenia is already well described in dogs, and it is believed that in a similar way to what occurs in FIP, macrophages may be indirectly related to the development of marked lymphopenia similar to that observed in CCoV infections (Rottier et al., 2005). The TNF-α (tumour necrosis factor) produced by activated macrophages and lymphocytes is one of the molecules that induces apoptosis in uninfected thymus lymphocytes (Marinaro et al., 2010). Although it is believed that lymphopenia can occur in CCoV infection for similar reasons, this association has not yet been determined (Dean et al., 2003).

Additionally, when evaluating the cell proliferation of the cells of the culture, it was observed that there was a significant decrease 18 h p.i. when compared to the control cells, showing that CCoV directly

affected cell viability. Another important factor was the continuous increase in mitochondrial depolarization, evaluated by the JC-1 probe. The combination of these parameters shows that mitochondrial (intrinsic) pathways of apoptosis are probably activated by viral infection, as the change in mitochondrial permeability is determinant for this activation to take place. Moreover, CCoV infection resulted in truncated Bid translocation from the cytosol to the mitochondria, indicating that both intrinsic and extrinsic apoptosis pathways are involved in viral infection(Cron et al., 2023; Gioti et al., 2021; Lemke, 2019; Ruggieri et al., 2007; Sanz et al., 2014).

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Conflicts of interest

The authors declare no conflicts of interest.

References

Alfano, F., Fusco, G., Mari, V., Occhiogrosso, L., Miletti, G., Brunetti, R., Galiero, G., Desario, C., Cirilli, M., & Decaro, N. (2020). Circulation of pantropic canine coronavirus in autochthonous and imported dogs, Italy. *Transboundary and Emerging Diseases*, *67*(5), 1991–1999. https://doi.org/10.1111/tbed.13542.

Baldanta, S., Fernandez-Escobar, M., Acin-Perez, R., Albert, M., Camafeita, E., Jorge, I., Vazquez, J., Enríquez, J. A., & Guerra, S. (2017). ISG15 governs mitochondrial function in macrophages following vaccinia virus infection. *PLoS Pathogens*, *13*(10), e1006651. https://doi.org/10.1371/journal.ppat.1006651.

Belouzard, S., Millet, J. K., Licitra, B. N., & Whittaker, G. R. (2012). Mechanisms of coronavirus cell entry mediated by the viral spike protein. *Viruses*, *64*(6), 1011–1033. https://doi.org/10.3390/v4061011.

Beniac, D. R., Andonov, A., Grudeski, E., & Booth, T. F. (2006). Architecture of the SARS coronavirus prefusion spike. *Nature Structural & Molecular Biology*, *13*(8), 751–752. https://doi.org/10.1038/nsmb1123.

Chen, S., Liu, D., Tian, J., Kang, H., Guo, D., Jiang, Q., Liu, J., Li, Z., Hu, X., & Qu, L. (2019). Molecular characterization of HLJ-073, a recombinant canine coronavirus strain from China with an ORF3abc deletion. *Archives of Virology*, *164*(8), 2159–2164. https://doi.org/10.1007/s00705-019-04296-9.

Cron, R. Q., Goyal, G., & Chatham, W. W. (2023). Cytokine storm syndrome. *Annual Review of Medicine*, *74*, 321–337. https://doi.org/10.1146/annurev-med-042921-112837.

Dalskov, L., Møhlenberg, M., Thyrsted, J., Blay-Cadanet, J., Poulsen, E. T., Folkersen, B. H., Skaarup, S. H., Olagnier, D., Reinert, L., & Enghild, J. J. (2020). SARS-CoV-2 evades immune detection in alveolar macrophages. *EMBO Reports*, *21*(12), e51252. https://doi.org/10.15252/embr.202051252.

Dean, G. A., Olivry, T., Stanton, C., & Pedersen, N. C. (2003). In vivo cytokine response to experimental feline infectious peritonitis virus infection. *Veterinary Microbiology*, *97*(1–2), 1–12. https://doi.org/10.1016/j.vetmic.2003.08.010.

Decaro, N. & Buonavoglia, C. (2008). An update on canine coronaviruses: viral evolution and pathobiology. *Veterinary Microbiology*, *132*(3–4), 221–234. https://doi.org/10.1016/j.vetmic.2008.06.007.

Decaro, N., Campolo, M., Lorusso, A., Desario, C., Mari, V., Colaianni, M. L., Elia, G., Martella, V. & Buonavoglia, C. (2008). Experimental infection of dogs with a novel strain of canine coronavirus causing systemic disease and lymphopenia. *Veterinary Microbiology*, *128*(3–4), 253–260. https://doi.org/10.1016/j.vetmic.2007.10.008.

Decaro, N., Elia, G., Martella, V., Campolo, M., Mari, V., Desario, C., Lucente, M. S., Lorusso, E., Kanellos, T., & Gibbons, R. H. (2010). Immunity after natural exposure to enteric canine coronavirus

does not provide complete protection against infection with the new pantropic CB/05 strain. *Vaccine*, *28*(3), 724–729. https://doi.org/10.1016/j.vaccine.2009.10.077.

Decaro, N., & Lorusso, A. (2020). Novel human coronavirus (SARS-CoV-2): A lesson from animal coronaviruses. *Veterinary Microbiology*, *244*, 108693. https://doi.org/10.1016/j.vetmic.2020.108693.

Decaro, N., Martella, V., Elia, G., Campolo, M., Desario, C., Cirone, F., Tempesta, M., & Buonavoglia, C. (2007). Molecular characterisation of the virulent canine coronavirus CB/05 strain. *Virus Research*, *125*(1), 54–60. https://doi.org/10.1016/j.virusres.2006.12.006.

El-Bacha, T., & Da Poian, A. T. (2013). Virus-induced changes in mitochondrial bioenergetics as potential targets for therapy. *The International Journal of Biochemistry & Cell Biology*, *45*(1), 41–46. https://doi.org/10.1016/j.biocel.2012.09.021.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic Pathology*, *35*(4), 495–516. https://doi.org/10.1080/01926230701320337.

Enjuanes, L., Almazán, F., Sola, I., & Zuniga, S. (2006). Biochemical aspects of coronavirus replication and virus-host interaction. *Annual Review of Microbiology*, *60*, 211–230. https://doi.org/10.1146/annurev.micro.60.080805.142157.

Gioti, K., Kottaridi, C., Voyatzaki, C., Chaniotis, D., Rampias, T., & Beloukas, A. (2021). Animal coronaviruses induced apoptosis. *Life*, *11*, 185. https://doi.org/10.3390/life11030185.

Groot, R. J., Baker, S. C., Baric, R., Enjuanes, L., Gorbalenya, A. E., Holmes, K. V, Perlman, S., Poon, L., Rottier, P. J. M., & Talbot, P. J. (2011). *Family coronaviridae* (pp. 1–23). Elsevier.

Heinrich, F., Lehmbecker, A., Raddatz, B. B., Kegler, K., Tipold, A., Stein, V. M., Kalkuhl, A., Deschl, U., Baumgärtner, W., & Ulrich, R. (2017). Morphologic, phenotypic, and transcriptomic characterization of classically and alternatively activated canine blood-derived macrophages in vitro. *PloS One*, *12*(8), e0183572. https://doi.org/10.1371/journal.pone.0183572.

Hossain, M. E., Islam, A., Islam, S., Rahman, M. K., Miah, M., Alam, M. S., & Rahman, M. Z. (2021). Detection and Molecular Characterization of Canine Alphacoronavirus in Free-Roaming Dogs, Bangladesh. *Viruses*, *14*(1), 67. https://doi.org/10.3390/v14010067.

Khatua, S., Simal-Gandara, J., & Acharya, K. (2022). Understanding immune-modulatory efficacy in vitro. *Chemico-Biological Interactions*, *352*, 109776. https://doi.org/10.1016/j.cbi.2021.109776.

Knoll, R., Schultze, J. L., & Schulte-Schrepping, J. (2021). Monocytes and Macrophages in COVID-19. *Frontiers in Immunology*, *12*, 720109. https://doi.org/10.3389/fimmu.2021.720109.

Lemke, G. (2019). How macrophages deal with death. *Nature Reviews Immunology*, *19*, 539–549. https://doi.org/10.1038/s41577-019-0167-y.

Licitra, B. N., Whittaker, G. R., Dubovi, E. J., & Duhamel, G. E. (2014). Genotypic characterization of canine coronaviruses associated with fatal canine neonatal enteritis in the United States. *Journal of Clinical Microbiology*, *52*(12), 4230–4238. https://doi.org/10.1128/JCM.02158-14.

Liu, S.-L., & Saif, L. (2020). Emerging viruses without borders: the Wuhan coronavirus. In *Viruses* (Vol. 12, Issue 2, p. 130). MDPI. https://doi.org/10.3390/v12020130.

Marfè, G., Tafani, M., Fiorito, F., Pagnini, U., Iovane, G., & De Martino, L. (2011). Involvement of FOXO transcription factors, TRAIL-FasL/Fas, and sirtuin proteins family in canine coronavirus type II-induced apoptosis. *PLoS One*, *6*(11), e27313. https://doi.org/10.1371/journal.pone.0027313.

Marinaro, M., Mari, V., Bellacicco, A. L., Tarsitano, E., Elia, G., Losurdo, M., Rezza, G., Buonavoglia, C., & Decaro, N. (2010). Prolonged depletion of circulating CD4+ T lymphocytes and acute monocytosis after pantropic canine coronavirus infection in dogs. *Virus Research*, *152*(1–2), 73–78. https://doi.org/10.1016/j.viruses.2010.06.006.

Merad, M., & Martin, J. C. (2020). Pathological inflammation in patients with COVID-19: a key role for monocytes and macrophages. *Nature Reviews Immunology*, *20*(6), 355–362. https://doi.org/10.1038/s41577-020-0331-4.

Ntafis, V., Xylouri, E., Mari, V., Papanastassopoulou, M., Papaioannou, N., Thomas, A., Buonavoglia, C., & Decaro, N. (2012). Molecular characterization of a canine coronavirus NA/09 strain detected in a dog's organs. *Archives of Virology*, *157*, 171–175. https://doi.org/10.1007/s00705-011-1141-6.

Ohta, A., & Nishiyama, Y. (2011). Mitochondria and viruses. *Mitochondrion*, *11*(1), 1–12. https://doi.org/10.1016/j.mito.2010.08.006.

Perlman, S., & Netland, J. (2009). Coronaviruses post-SARS: update on Coronaviruses post-SARS: update on. *Nature Reviews Microbiology*, *7*(6), 439–450. https://doi.org/10.1038/nrmicro2147.

Poon, I. K. H., Hulett, M. D., & Parish, C. R. (2010). Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death & Differentiation*, *17*(3), 381–397. https://doi.org/10.1038/cdd.2009.195.

Pratelli, A. (2011). Basic science track entry and release of canine coronavirus from polarized epithelial cells. *Quarterly Journal of Microbiological Sciences*, *34*(1), 25–32.

Rottier, P. J. M., Nakamura, K., Schellen, P., Volders, H., & Haijema, B. J. (2005). Acquisition of macrophage tropism during the pathogenesis of feline infectious peritonitis is determined by mutations in the feline coronavirus spike protein. *Journal of Virology*, *79*(22), 14122–14130. https://doi.org/10.1128/JVI.79.22.14122-14130.2005.

Ruggieri, A., Di Trani, L., Gatto, I., Franco, M., Vignolo, E., Bedini, B., Elia, G., & Buonavoglia, C. (2007). Canine coronavirus induces apoptosis in cultured cells. *Veterinary Microbiology*, *121*, 64–72. https://doi.org/10.1016/j.vetmic.2006.12.016.

Saleh, J., Peyssonnaux, C., Singh, K. K., & Edeas, M. (2020). Mitochondria and microbiota dysfunction in COVID-19 pathogenesis. *Mitochondrion*, *54*, 1–7. https://doi.org/10.1016/j.mito.2020.06.008.

Sanz, A. B., Sanchez-Niño, M. D., Izquierdo, M. C., Gonzalez-Espinoza, L., Ucero, A. C., Poveda, J., Ruiz-Andres, O., Ruiz-Ortega, M., Selgas, R., Egido, J., & Ortiz, A. (2014). Macrophages and recently identified forms of cell death. *International Reviews of Immunology*, *33*, 9–22. https://doi.org/10.3109/08830185.2013.771183.

Scott, I. (2010). The role of mitochondria in the mammalian antiviral defense system. *Mitochondrion*, *10*, 316–320. https://doi.org/10.1016/j.mito.2010.02.005.

Tian, Z., Pan, Q., Zheng, M., Deng, Y., Guo, P., Cong, F., & Hu, X. (2021). Molecular characterization of the FCoV-like canine coronavirus HLJ-071 in China. *BMC Veterinary Research*, *17*, 1–9. https://doi.org/10.1186/s12917-021-03073-8.

Timurkan, M. O., Aydin, H., Dincer, E., & Coskun, N. (2021). Molecular characterization of canine coronaviruses: an enteric and pantropic approach. *Archives of Virology*, *166*, 35–42. https://doi.org/10.1007/s00705-020-04826-w.

Uchida, M., Uchida, K., Maeda, S., & Yonezawa, T. (2019). Expression of apoptosis inhibitor of macrophages in tissue macrophages, leukocytes and vascular endothelial cells of dogs. *Tissue and Cell*, *58*, 112–120. https://doi.org/10.1016/j.tice.2019.05.002.

Vieira, F. V, Vieira, D. S., Gameiro, R., Ferreira, H. L., & Cardoso, T. C. (2018). Digital PCR Platform as a Tool to Determine the Canine Coronavirus (CCoV) Genome in Clinical Samples. *Archives on Veterinary Science and Technology*, *4*(2), 1–5. https://doi.org/10.29011/2637-9988/100042.

Vlasova, A. N., Diaz, A., Damtie, D., Xiu, L., Toh, T.-H., Lee, J. S.-Y., Saif, L. J., & Gray, G. C. (2022). Novel canine coronavirus isolated from a hospitalized patient with pneumonia in East Malaysia. *Clinical Infectious Diseases*, *74*(3), 446–454. https://doi.org/10.1093/cid/ciab456.

Vlasova, A. N., Toh, T.-H., Lee, J. S.-Y., Poovorawan, Y., Davis, P., Azevedo, M. S. P., Lednicky, J. A., Saif, L. J., & Gray, G. C. (2022). Animal alphacoronaviruses found in human patients with acute respiratory illness in different countries. *Emerging Microbes & Infections*, *11*(1), 699–702. https://doi.org/10.1080/22221751.2022.2040341.

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