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## Prolonged depletion of circulating CD4<sup>+</sup> T lymphocytes and acute monocytosis after pantropic canine coronavirus infection in dogs

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### ABSTRACT

A hypervirulent strain (CB/05) of canine coronavirus was employed to infect oronasally 11-week-old pups. Peripheral blood monocytes (CD14<sup>+</sup>), T lymphocytes (CD4<sup>+</sup> and CD8<sup>+</sup>) and B lymphocytes (CD21<sup>+</sup>) were studied by flow cytometry within 5 days post-infection (p.i.) and at later time points. Infection with CB/05 resulted in a profound depletion of T cells and a slight loss of B cells in the first week p.i. In particular, while the CD8<sup>+</sup> and the B lymphocytes returned to baseline levels by day 7 p.i., the CD4<sup>+</sup> T cells remained significantly low until day 30 p.i. and recovered completely only at day 60 p.i. Monocytosis was also observed after CB/05 infection with a peak at day 5 p.i. The prolonged depletion of peripheral CD4<sup>+</sup> T cells did not alter the levels of serum IgG or IgM. The impact of CB/05 infection on the immune performance of infected pups is discussed.

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### 1. Introduction

Canine coronavirus (CCoV) is an enveloped, single-stranded RNA virus responsible for mild to severe enteritis in dogs (Tennant et al., 1991). Currently, two CCoV genotypes are known, namely CCoV type I (CCoV-I) and CCoV type II (CCoV-II), the latter being classified into two subtypes, CCoV-IIa and CCoV-IIb, including classical and transmissible gastroenteritis virus-like CCoVs (Decaro et al., 2009, 2010b). A novel hypervirulent CCoV-IIa biotype has been recently identified (strain CB/05) which is responsible for a severe, often lethal, systemic disease characterized by an unusual spreading to several internal organs both under natural and experimental conditions (Buonavoglia et al., 2006; Decaro et al., 2007, 2008; Decaro and Buonavoglia, 2008). An additional finding observed in dogs infected with this pantropic biotype is the lymphopenia associated with the detection of virus in several lymphoid organs (Decaro et al., 2008). Milder clinical signs, including moderate lymphopenia, were also observed in pups recovered from a recent infection caused by an enterotropic strain on CCoV and subsequently infected with strain CB/05, thus showing the absence of a full cross-protection of enteric CCoV against the pantropic variant (Decaro et al., 2010a). These preliminary studies were aimed at evaluating the clinical

signs and pathology induced by the pantropic variant, but did not investigate the specific lymphocyte subsets altered during the lymphopenia. The study of the effect of this hypervirulent CCoV biotype on immune cells would be crucial to understand the pathogenesis of the disease. Thus, in the present study, circulating monocytes and lymphocyte populations (CD4<sup>+</sup>, CD8<sup>+</sup> and CD21<sup>+</sup>) were analyzed by flow cytometry in dogs infected experimentally with strain CB/05. In particular, T lymphocyte subsets, B lymphocytes and monocytes were monitored early after the infection (3–5 days) and at later time points (until day 60).

### 2. Materials and methods

#### 2.1. Origin of the challenge virus

Strain CB/05 was isolated from the lungs of a dead pup (117/05-C) and adapted to growth on A-72 canine cells (Decaro et al., 2007). The challenge virus was titrated on cell cultures (10<sup>5</sup> TCID<sub>50</sub>/ml) and stored at –70 °C.

#### 2.2. Experimental infection

Thirteen antibody profile defined, 11-week-old beagle pups negative for faecal CCoV RNA and for serum antibodies to CCoV were included in the study. The infection was performed at the isolation unit of the Animal Hospital, Faculty of Veterinary Medicine

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of Bari, according to the animal health and well-being regulations. The study was authorized by the Italian Ministry of Health (no. 159/2006-C) and was approved by the Ethical Committee of the University of Bari. Dogs were provided water *ad libitum* and fed twice daily throughout the study. For the experimental infection, 10 dogs received 3 ml of stock viral suspension of CB/05 virus (2 ml orally and 1 ml intranasally) and were observed for a period of 5 ( $n=10$ ), 24 ( $n=4$ ) or 60 ( $n=2$ ) days. To assess the presence and the distribution in internal organs of strain CB/05 at different time points, 6 infected dogs were randomly selected and euthanized at day 5 p.i. and other 2 dogs at day 24 p.i. Euthanasia was achieved by intravenous administration of 10 mg/Kg body weight of Zoletil 100 (Virbac S.r.l., Italy) followed by 0.5 ml/Kg body weight of Tanax (Intervet Italia, Italy). The two remaining pups were kept alive until day 60 p.i. Clinical signs of CCoV infection, serum antibodies against CCoV and viral shedding were monitored as previously described (Decaro et al., 2008, 2010a; Decaro and Buonavoglia, 2008). Three control dogs were not infected and received oronasally (2 ml orally and 1 ml intranasally) 3 ml of supernatants from uninfected A-72 cells (same number of passages as the A-72 cells used to propagate the stock virus). The control dogs were euthanized at day 5 p.i. ( $n=1$ ) or at day 24 p.i. ( $n=1$ ) or monitored until day 60 p.i. ( $n=1$ ).

### 2.3. Analysis of peripheral blood cells by flowcytometry

EDTA-treated blood samples were collected before the infection (day 0) and at 3 and 5 days p.i. For kinetics studies, blood samples were also collected from dogs not subjected to euthanasia at later time points as indicated in the figures. Blood samples were labelled with the following monoclonal antibodies from AbD Serotec (Oxford, UK): anti-dog CD3-PE (clone CA 17.2A12 for T lymphocytes), anti-dog CD4-FITC (clone YKIX302.9 for T-helper and neutrophils), anti-dog CD8-FITC (clone YCATE55.9 for T cytotoxic/suppressor), anti-dog CD21-PE (clone CA2.1D6, B cells), anti-human CD-14-PE (clone TUK4, monocytes). Since the CD4 molecule is expressed also on neutrophils in dogs, the CD4<sup>+</sup> T cells were studied by double labelling the blood cells with the anti-CD3 and the anti-CD4 monoclonal antibodies (i.e., CD3<sup>+</sup> CD4<sup>+</sup> cells). To remove contaminating erythrocytes a lysing solution was employed (Versa-Lyse, Beckman Coulter, Miami, FL, USA). The cell suspensions were then analyzed using the Epics XL flowcytometer (Beckman Coulter). Before the acquisition, the optical path was adjusted by testing with the optics calibrator Flow Check (Beckman Coulter). Data acquisition and analysis was performed with the Elite workstation. A count cycle contained 10,000 cells. The three major cell populations (i.e., granulocytes, lymphocytes and monocytes) were identified by means of proper gating and compensation in a forward-versus side-scatter dot plot and then, by using fluorescence signals. To determine the absolute numbers of cell populations (i.e., number of cells/ $\mu$ l), the internal standard fluorescence beads (Flow-Count, Beckman Coulter) were used.

### 2.4. Measurement of total IgG and IgM in sera

Total IgG and IgM were measured in the serum samples by using ELISA quantification kits (Bethyl, Montgomery, USA) following the manufacturer's instructions.

### 2.5. Histology

Formalin-embedded samples from duodenum and mesenteric lymph nodes were cut in 3  $\mu$ m sections and stained with H&E for histological examination.

### 2.6. Statistical analysis

The differences between the experimental group and the control group and those within the same group were analyzed by the paired Student's *t* test. A *p* value <0.05 was considered significant (confidence level 95%). Data are expressed as the arithmetic mean  $\pm$  S.D.

## 3. Results

### 3.1. Absolute counts of peripheral blood CD14<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD21<sup>+</sup> cells early after CB/05 infection

Acute lymphopenia has been described to occur after CB/05 infection. In order to determine if monocytes were also altered during the course of the infection, peripheral blood from ten dogs infected with CB/05 was subjected to flow cytometry analysis and absolute counts of CD14<sup>+</sup> cells (i.e., monocytes) were measured 3 and 5 days p.i. As shown in Fig. 1A, CD14<sup>+</sup> cells increased significantly by day 3 and augmented even further (although with a greater level of variability) by day 5 p.i. As expected, lymphocytes were significantly reduced by day 3 (average reduction 44%, range 11–60%) and remained at this level by day 5 p.i. (average reduction 45%, range 20–71%). The CD4<sup>+</sup> and CD8<sup>+</sup> T cell absolute counts were also quantified in the infected dogs and Fig. 1B shows that the CD4<sup>+</sup> T cell population was significantly reduced by day 3 (average reduction 63%, range 52–73%) and remained low at day 5. A similar trend was observed for the CD8<sup>+</sup> T cells; in particular, they dropped to approximately 50% of their initial counts on day 3 (range 12–61%) and remained low at day 5 p.i. (Fig. 1C). The B lymphocytes (i.e., CD21<sup>+</sup>) showed a 36% reduction by day 3 p.i. (range 32–62%) followed by a rapid recovery by day 5 (Fig. 1D).

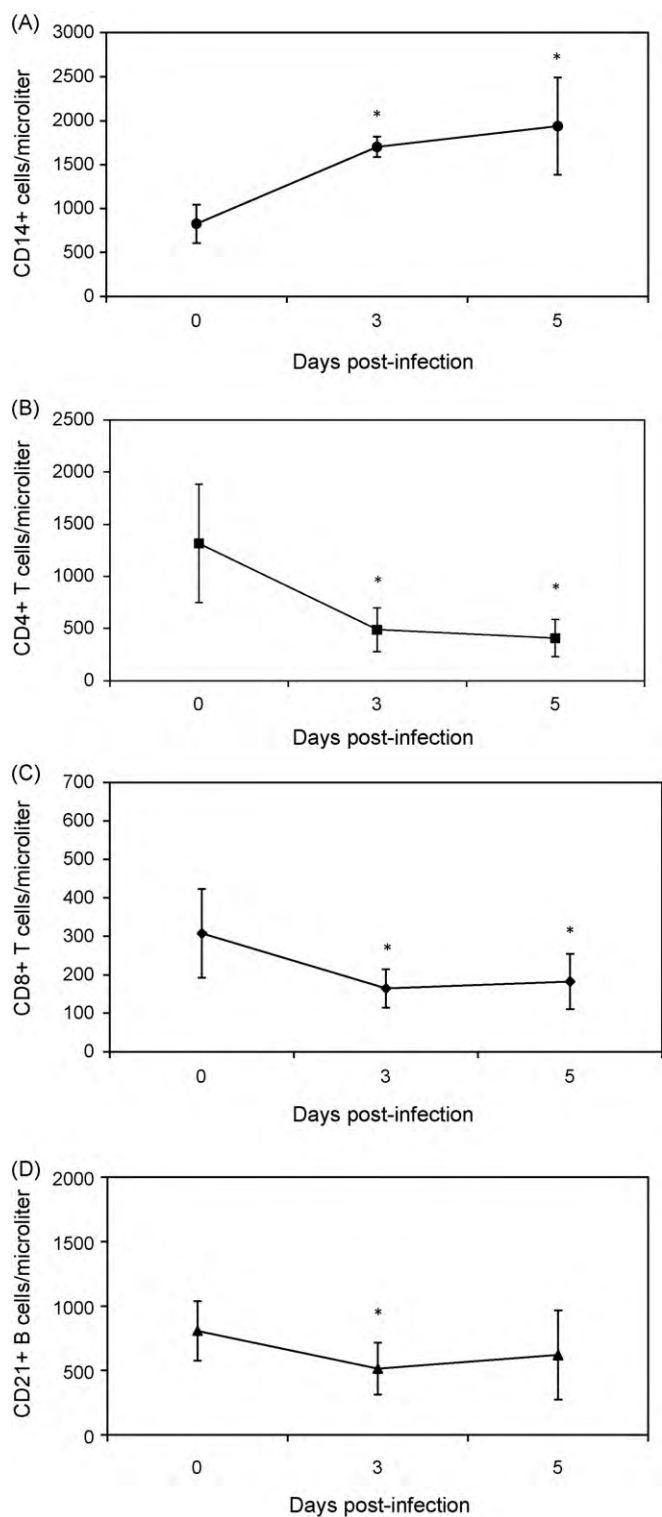
The three control dogs (not infected) did not show any significant difference in any cell population during the study period (data not shown).

### 3.2. Absolute counts of peripheral blood CD14<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD21<sup>+</sup> cells at later time points after CB/05 infection

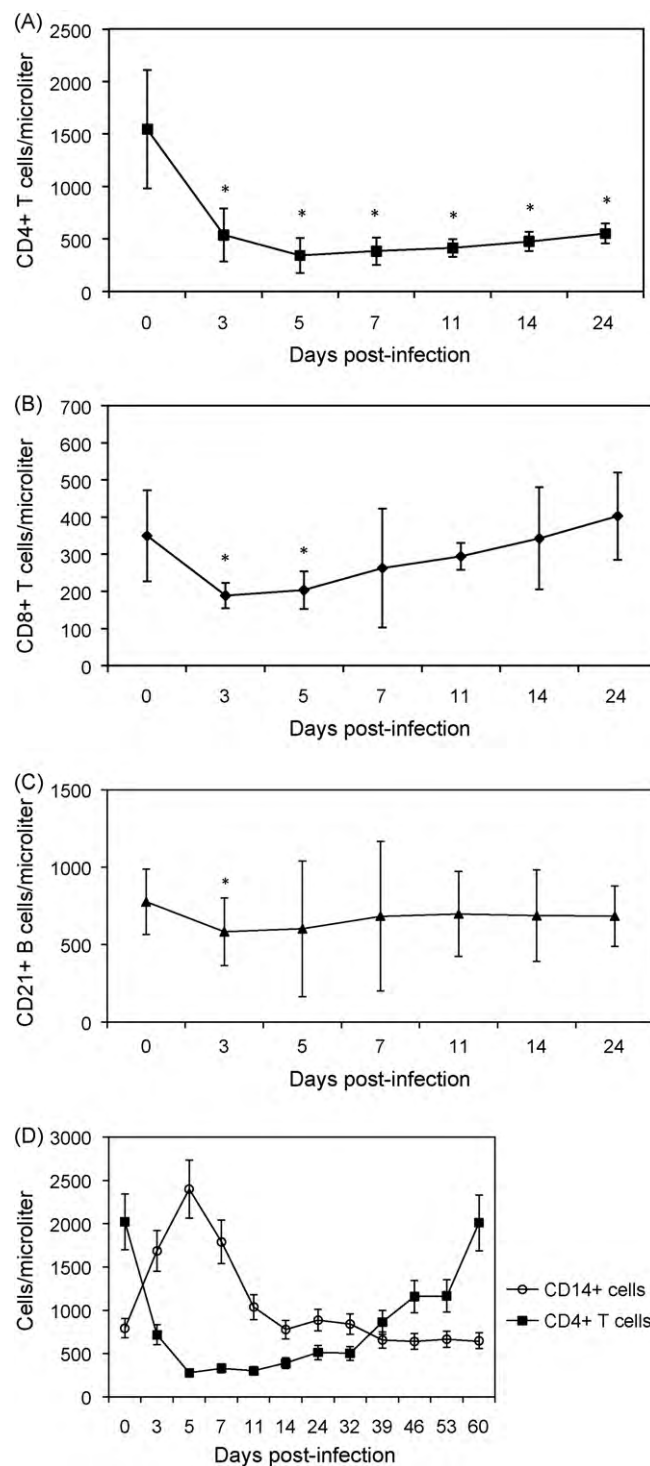
To investigate the dynamics of both the lymphocyte depletion and the monocyte increment, dogs were monitored for a longer period of time and the absolute counts of CD4<sup>+</sup>, CD8<sup>+</sup>, CD21<sup>+</sup> and CD14<sup>+</sup> cells were measured until day 24 p.i. (four dogs) or until day 60 p.i. (two dogs). While the CD8<sup>+</sup> subset returned to basal level at day 7 and did not undergo any further change (Fig. 2B), the CD4<sup>+</sup> subset remained significantly depleted until day 24 (Fig. 2A). In addition, the CD21<sup>+</sup> B cell population which returned to basal level on day 5–7 did not change at later time points p.i. (Fig. 2C). Finally, two dogs were studied until day 60 p.i. to monitor in particular the CD4<sup>+</sup> and CD14<sup>+</sup> cell populations (Fig. 2D). The CD14<sup>+</sup> monocytes and the CD4<sup>+</sup> T cells returned to basal levels at day 14 p.i. and 60 days p.i., respectively.

### 3.3. Measurements of total IgM and IgG in the serum samples of dogs infected with the CB/05 CCoV

To determine whether the prolonged depletion of CD4<sup>+</sup> T cells affected the production of immunoglobulins in the serum, IgG and IgM were measured in four dogs at weekly intervals following CB/05 infection. Sera were taken before the infection (day 0; baseline level) and once a week for 3 weeks p.i. (days 7, 14, 21) and were subjected to ELISAs assays. When compared to baseline levels, the amount of serum IgG or IgM did not change, in any dog, at any time point after the infection (data not shown).



**Fig. 1.** Kinetics of CD14<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD21<sup>+</sup> peripheral blood cells in dogs ( $n = 10$ ) infected with the pantropic CB/05 CCoV. Blood was taken 3 and 5 days following the infection and the absolute numbers of cells were measured by flow cytometry. Data are expressed as the arithmetic mean  $\pm$  S.D. Asterisks indicate statistical significance ( $p < 0.05$ ) between the indicated absolute count value and the baseline value (i.e., day 0).

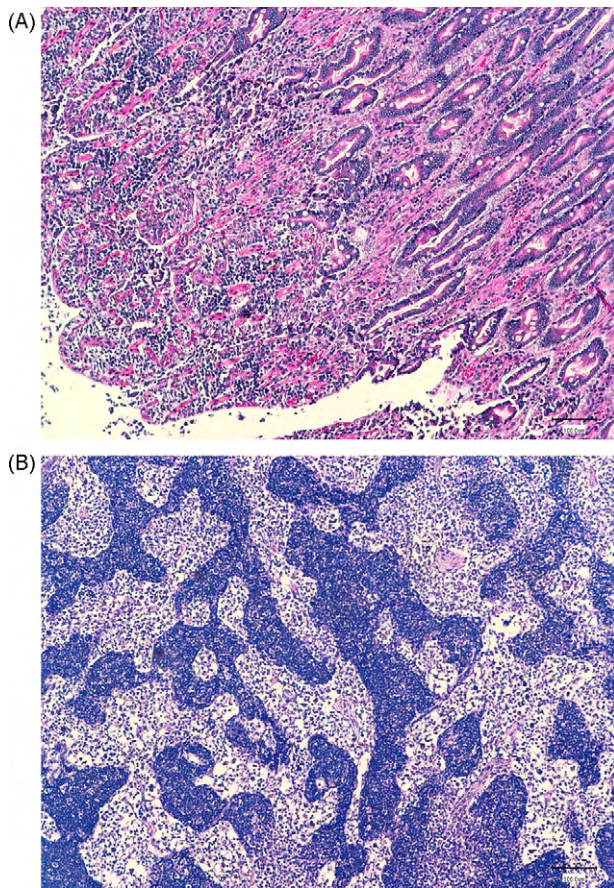


**Fig. 2.** Kinetics of CD14<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD21<sup>+</sup> peripheral blood cells in dogs infected with the pantropic CB/05 CCoV. Blood was taken until day 24 p.i. ( $n = 4$ ) or day 60 p.i. ( $n = 2$ ) and the absolute numbers of cells were measured by flow cytometry. Data are expressed as the arithmetic mean  $\pm$  S.D. Asterisks indicate statistical significance ( $p < 0.05$ ) between the indicated absolute count value and the baseline value (i.e., day 0).

### 3.4. Clinical signs, post-mortem findings, viral shedding and serology in dogs after experimental infection with strain CB/05

Starting from day 3 post-infection (p.i.) all infected pups displayed clinical signs of depression, diarrhoea and fever (39.5–39.9°C), that were slightly milder than those described in





**Fig. 3.** Paraffin-embedded histological sections stained with H&E, 10 $\times$ ; (A) damage of the epithelium and infiltration of the lamina propria by lymphocytes and plasma cells in the duodenum; (B) severe lymphoid depletion in mesenteric lymph nodes.

previous experimental infections (Decaro et al., 2008). Six randomly selected dogs were euthanized at day 5 p.i. and necropsy revealed the presence of enteritis (6 dogs out of 6 euthanized), enlargement of spleen (5/6), and mesenteric lymph nodes (6/6), infarctious areas in the renal cortex (3/6) and patchy areas of consolidation in the apical lobes of lungs (2/6). Histological examination of duodenum at day 5 p.i. revealed a diffuse epithelial damage and several lymphocytes and plasma cells infiltrating the lamina propria (Fig. 3A). In addition, sections of mesenteric lymph nodes showed a severe lymphoid depletion (day 5 p.i.; Fig. 3B). Two infected pups were euthanized at day 24 p.i. while the remaining two infected dogs were monitored until day 60 p.i.; these four dogs displayed similar clinical signs (fever, loss of appetite, depression and diarrhoea) that persisted until days 10–12 p.i. Post-mortem findings in pups euthanized at day 24 p.i. consisted only in enlargement of spleen and lymph nodes. All uninfected pups remained healthy throughout the study and no gross lesions were evident in control dogs euthanized at day 5 p.i. ( $n = 1$ ) or day 24 p.i. ( $n = 1$ ).

All infected pups shed the virus in their feces as long as they were retained in the study (i.e. until day 5 p.i., for dogs euthanized at day 5 or until day 24 p.i. for dogs euthanized at day 24 p.i.). In particular, the two dogs that were monitored for 60 days shed the virus until days 30–32 p.i. As described elsewhere (Decaro et al., 2008, 2010a; Decaro and Buonavoglia, 2008), seroconversion was studied by using ELISA and virusneutralization tests. Seroconversion was observed in the four dogs that were kept until day 24 p.i. and day 60 p.i. (data not shown). Neither viral shedding nor CCoV-specific serum antibodies were detected in control dogs at any time point.

#### 4. Discussion

A novel hypervirulent biotype of CCoV (CB/05) shows an unusual ability to spread to several internal organs and causes a systemic disease, often lethal in young dogs. The pathogenesis of the CB/05 CCoV infection is not completely known; upon entry via the oronasal mucosa, a low, when detectable, viremia is established which is probably responsible for the dissemination of the virus to several other anatomical sites (including lymphoid organs such as the thymus, spleen and mesenteric lymph nodes) where it may cause a damage (Decaro et al., 2008, 2010a,b; Decaro and Buonavoglia, 2008). The direct involvement of the immune system in the pathogenesis of CB/05 infection was suggested by the ability to cause lymphopenia (Decaro et al., 2007, 2008, 2010a). In the present study, a transient reduction in the number of all peripheral lymphocyte subsets including CD4<sup>+</sup>, CD8<sup>+</sup>, B cells and an increment of circulating CD14<sup>+</sup> monocytes were observed at the acute stage of the CB/05 infection. Subsequently, it was found that the CD4<sup>+</sup> T cell pool remained depleted for approximately 30 days p.i. and returned to basal levels only 60 days p.i.

The frequencies of lymphocytes and lymphocyte subsets are crucial to maintain immune system function. Usually viral infections, immunodeficiency diseases and other infectious diseases determine abnormal changes in the levels of lymphocytes subsets (Veazey et al., 2003; Dunne et al., 2002; Cui et al., 2003). In particular, viral survival and persistence may require immune suppression and, for this reason, lymphopenia is a common consequence of many viral infections in humans and animals. Coronaviruses, morbilliviruses, immunodeficiency viruses as well as influenzaviruses have all been shown to induce significant lymphopenia which can, in some cases, be lethal. (Wong et al., 2003; Cui et al., 2003; Okada et al., 2000; Tsukahara et al., 1992; Hopper et al., 1989; Auwaerter et al., 1999; McKenzie et al., 1986; Van Campen et al., 1989; Gourgeon et al., 1996).

During viral infection, lymphocytes, either infected or uninfected, can be killed by mechanisms ranging from the direct action of the virus (or viral components) to the cytolytic action of host cells and host soluble mediators (Ahr et al., 2004; Maury and Lähdevirta, 1990; Dean et al., 2003).

The CD13/aminopeptidase N (APN), which is the receptor for CCoV and several group I coronaviruses, is not normally present on mature circulating lymphocytes but it is mostly expressed on epithelial cells and on monocytes/macrophages (Tresnan et al., 1996; Savarino et al., 2003; Hofmann and Pöhlmann, 2004). Studies in humans have also shown that immature B and T cells are CD13 positive but become negative upon maturation; thus, lymphocytes of peripheral blood, spleen or tonsils are CD13 negative (Riemann et al., 1999; Drexler, 1987; Syrjäälä et al., 1994; Spit et al., 1995). Since the distribution of virus receptors determines the tissue tropism of a virus, it could be speculated that a direct cytolytic effect of CB/05 on circulating lymphocytes would not be likely to occur and that other mechanisms are responsible for the extensive loss of lymphocytes observed after CB/05 infection.

A reduced production of mature lymphocytes by a primary lymphoid organ such as the thymus cannot be excluded since high levels of CB/05 RNA copies were found in this lymphoid organ (Decaro et al., 2010a). In this regard, the thymus is a target organ in several infectious diseases (Savino, 2006) and many viruses (including coronaviruses), protozoa, and fungi can invade/infect the thymus interfering with the thymocyte differentiation process. It is clear that both endogenous and infectious agent-derived moieties can determine apoptosis of lymphocytes. Among the former, TNF-alpha is one of the molecules leading to apoptosis of uninfected lymphocytes in the thymus (Savino, 2006); it is remarkable that the excessive production of TNF-alpha by feline infectious peritonitis virus (FIPV)-activated lymphocytes (Dean et al., 2003)

was shown to be responsible for the apoptosis of lymphocytes in cats with FIP and TNF- $\alpha$  positively correlated with lymphopenia in HIV infection (Maury and Lähdevirta, 1990). Along these lines, another coronavirus, the mouse hepatitis virus (MHV)-A59, was shown to induce thymic atrophy not from a generalized lytic infection of T lymphocytes but rather from apoptosis of immature double-positive T cells caused by infection of a small proportion of thymus epithelial cells or from dysregulated secretion of cytokines (Godfraind et al., 1995). It would be interesting to determine whether the CD13/APN receptor is expressed on immature lymphocytes also in dogs thus rendering this thymic cell population susceptible to CB/05 infection and to determine whether the secretion of TNF- $\alpha$  contributes to the lymphopenia observed in dogs infected with CB/05.

Depletion of circulating CD4<sup>+</sup> T cells, although transient, may affect humoral as well as cell-mediated immunity thus compromising the ability to generate or maintain an effective immune response. Indeed, the CD4<sup>+</sup> T cells play a central role in immune protection (Zhu and Paul, 2008; Bluestone et al., 2009). In humans and mice, several subpopulations of CD4<sup>+</sup>/Th cells (e.g., Th1, Th2, Th9, Th22, Th17) and CD4<sup>+</sup> Treg have been described and they provide support for a plethora of immune responses. Although extensive studies have not been systematically conducted in other animal species, it is clear that subpopulations of CD4<sup>+</sup> T cells supportive of diverse immune responses exist in species other than humans and mice. Thus, it is conceivable that a prolonged depletion of CD4<sup>+</sup> T cells may alter the immune performance of infected pups. For instance, a decline in antibody responses may be indicative of a loss of T cell help. The antibody response (also the serum neutralization activity) against CCoV is detectable 7 days following CB/05 infection in dogs (Decaro et al., 2008, 2010a; Decaro and Buonavoglia, 2008) and reaches a peak 14–21 days p.i. when the CD4<sup>+</sup> T cells are still massively depleted. In addition, when CCoV seropositive dogs were infected with the hypervirulent CB/05 strain, a boost of the serum IgG against CCoV (as well as a boost of seroneutralization titers) was observed (Decaro et al., 2010a). In the present study, dogs infected with CB/05 seroconverted (with antibody titers comparable to those observed in previous experimental infections); in addition, the total amount of serum IgG and IgM from the same dogs infected with CB/05 was not altered through day 21 p.i. Collectively the data suggest that, in dogs surviving the CB/05 infection, the circulating CD4<sup>+</sup> T cell pool although reduced, is sufficient to provide a CCoV-specific T cell-help to support/maintain antibody responses. It would be interesting to determine whether the antibody response to specific antigens (i.e., vaccines) is affected by the depletion of CD4<sup>+</sup> T cells and if cell-mediated immunity (either against CCoV or other infectious agents/vaccines) is altered as it occurs in several other viral infections (Kuroda, 2010; de Groot-Mijnes et al., 2005; McChesney et al., 1988). These are important issues to address since routine vaccinations are usually carried out in pups at the age of 40–60 days, when CCoV infection is frequently observed.

Finally, the consequences of the massive CD4<sup>+</sup> T cell depletion are difficult to predict in the field since dogs included in the study were kept in an animal facility and were therefore under controlled conditions. However, a potential exposure to opportunistic infections in pups infected with strain CB/05 cannot be excluded. It is noteworthy that the lowest numbers of CD4<sup>+</sup> T cells were found at day 3–5 p.i. when the peak of monocytosis was observed. Thus, it could be hypothesized that a certain level of immune functionality could be maintained by a compensatory role played by the innate immune system (i.e. monocyte/macrophages) as it occurs in HIV infection (Kuroda, 2010). Regarding the mechanisms responsible for the monocytosis, it could be suggested that the production of monocyte growth factors may account for the observed monocytosis in CB/05-infected dogs (Hopper et al., 1989; McKenzie et al.,

1986; Maul et al., 1985; Liu et al., 2002; Tsukahara et al., 1992; Auwaerter et al., 1999; Goto et al., 2003).

Unravelling the mechanisms that lead to monocytosis, to lymphopenia, and to selective prolonged loss of CD4<sup>+</sup> T cells may help define the pathogenesis of the pantropic CB/05 infection and may contribute to design new targets for drug and vaccine development.

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