Upregulation of endothelial cell adhesion molecules characterizes veins close to granulomatous infiltrates in the renal cortex of cats with feline infectious peritonitis and is indirectly triggered by feline infectious peritonitis virus-infected monocytes in vitro


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One of the most characteristic pathological changes in cats that have succumbed to feline infectious peritonitis (FIP) is a multifocal granulomatous phlebitis. Although it is now well established that leukocyte extravasation elicits the inflammation typically associated with FIP lesions, relatively few studies have aimed at elucidating this key pathogenic event. The upregulation of adhesion molecules on the endothelium is a prerequisite for stable leukocyte–endothelial cell (EC) adhesion that necessarily precedes leukocyte diapedesis. Therefore, the present work focused on the expression of the EC adhesion molecules and possible triggers of EC activation during the development of FIP. Immunofluorescence analysis revealed that the endothelial expression of P-selectin, E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) was elevated in veins close to granulomatous infiltrates in the renal cortex of FIP patients compared to non-infiltrated regions and specimens from healthy cats. Next, we showed that feline venous ECs become activated when exposed to supernatant from feline infectious peritonitis virus (FIPV)-infected monocytes, as indicated by increased adhesion molecule expression. Active viral replication seemed to be required to induce the EC-stimulating activity in monocytes. Finally, adhesion assays revealed an increased adhesion of naive monocytes to ECs treated with supernatant from FIPV-infected monocytes. Taken together, our results strongly indicate that FIPV activates ECs to increase monocyte adhesion by an indirect route, in which proinflammatory factors released from virus-infected monocytes act as key intermediates.

INTRODUCTION

Feline infectious peritonitis (FIP) is a progressive and mostly fatal, immunopathological disease in cats caused by the virulent variant of feline coronaviruses (FCoVs), feline infectious peritonitis virus (FIPV) (Pedersen, 2009). FIP is accompanied by a fibrinous to granulomatous inflammation of serosal membranes and multifocal granulomatous vascular lesions in several vital organs, often leading to effusions in body cavities.

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Abbreviations: FIP, feline infectious peritonitis; EC, endothelial cell; ICAM, intercellular adhesion molecule; VCAM, vascular adhesion molecule; FCoV, feline coronavirus; FIPV, feline infectious peritonitis virus; Ig, immunoglobulin; FHV, feline herpesvirus; PF, paraformaldehyde; PBS, phosphate buffered saline; NGS, normal goat serum; RT, room temperature; N, nucleocapsid; vWF, von Willebrand factor; MFI, mean fluorescent intensity; PBMC, peripheral blood mononuclear cell; MACS, magnetic activated cell sorting; CRFK, Crandell Rees feline kidney; ELISA, enzyme-linked immunosorbent assay; TNF, tumour necrosis factor; IL, interleukin; SD, standard deviation.

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The histologic picture of FIP lesions is characterized by an extensive focal infiltration of macrophages, fewer neutrophils and T-cells, and a variable amount of B-cells and plasma cells into the parenchyma (Berg et al., 2005; Kipar et al., 1998, 2005). The vascular inflammation is restricted to small- and medium-sized veins and is mainly observed in the renal cortex (stellate veins), leptomeninges and eyes (Kipar et al., 2005). Macrophages are the predominant inflammatory cells in FIP lesions, and it is believed that the infection of these cells and their progenitors, the monocytes, is one of the key pathogenic events in the development of FIP (Rottier et al., 2005; Stoddart & Scott, 1989). These in vivo target cells of FIPV are part of the mononuclear phagocyte system, involved in the inflammatory response. A prerequisite for circulating leukocytes to cross the endothelium and gain access to the extravascular tissue is the adhesion of these cells to vascular endothelial cells (ECs) (Chavakis et al., 2009). Studies have uncovered this process as a well-regulated cascade of events, with each step controlled by both constitutive and dynamically regulated complementary adhesion molecules expressed on the surface of both participating cell types (Barreiro & Sánchez-Madrid, 2009). Based on their biochemical properties and molecular structures, the adhesion molecules have been grouped into at least three families: the selectins, the integrins and the immunoglobulin (Ig) supergene family (Carlos & Harlan, 1994). Members of the selectin family, P-selectin (CD62P) and E-selectin (CD62E), are selectively expressed on activated ECs. Their leucin domain interacts with leukocyte ligands bearing sialyl Lewis X (sLex; CD15s)-related determinants, initiating reversible leukocyte tethering and rolling under conditions of flow (Sperandio, 2006). Subsequently, the firm cell arrest and final transmigration are mediated by proteins of the Ig supergene family, expressed on the endothelium, and their counter-structures on leukocytes, the integrins. Intercellular adhesion molecule 1 (ICAM-1; CD54), ligand for the β2 integrins lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18) and macrophage 1 antigen (Mac-1; CD11b/CD18) are constitutively expressed on EC at low levels, but are upregulated following inflammatory stimulation. Vascular cell adhesion molecule 1 (VCAM-1; CD106), the ligand for the β1 integrin very late antigen 4 (VLA-4; CD49d/CD29), is only expressed on activated ECs (Kelly et al., 2007). A previous study conducted by our laboratory demonstrated significant alterations in adhesion molecule expression on peripheral blood leukocytes from FIP patients, reflecting their activated state that predisposes them to extravasate (Olyslaegers et al., 2013a). Yet, there are no studies examining the reciprocal expression of their counter receptors on the vascular endothelium in FIP. We hypothesized that FIPV-infected monocytes/macrophages act as central regulators of inflammation by releasing proinflammatory mediators that may activate the ECs in a paracrine fashion. In this study, the in situ expression of P-selectin, E-selectin, ICAM-1 and VCAM-1 on ECs was quantified in kidney samples from FIP cats and compared with the expression in healthy subjects, using immunofluorescence. It was further examined whether EC activation in the renal cortex of FIP patients occurs locally or systemically by comparing regions with and without lesions. Next, an in vitro model of feline venous ECs was used to evaluate the ability of FCoV-infected monocytes to generate soluble mediators, which activate ECs by inducing enhanced surface expression of adhesion molecules. Finally, it was analysed whether the altered adhesion molecule expression also facilitated endothelial binding of uninfected monocytes.

RESULTS

In situ expression of endothelial cell adhesion molecules in FIP patients compared with healthy controls

The endothelial expression of P-selectin, E-selectin, ICAM-1 and VCAM-1 in FIP cats was compared with healthy controls, using immunofluorescence (Fig. 1). In the outer renal cortex of healthy cats, minimal endothelial expression of P-selectin, E-selectin, ICAM-1 and VCAM-1 was detected. However, in FIP cats, the surface expression of these molecules was upregulated on ECs in stellate veins close to a granulomatous infiltrate, although the difference did not reach a significant level for VCAM-1 (P=0.08) (Fig. 1). It was further examined whether EC activation occurs only locally or also systemically in FIP cats, by comparing the expression of the adhesion molecules in veins close to granulomatous infiltrates and veins remote from inflammatory lesions. For all adhesion molecules, the upregulation was limited to the veins that were located close to the granulomatous infiltrates (Fig. 1).

Induction of endothelial cell adhesion molecule expression by culture fluids from peripheral blood monocytes infected with FIPV 79-1146, FCoV 79-1683 or FHV-1

To examine the hypothesis that FCoV-infected monocytes secrete factor(s) that activate ECs, supernatant derived from FIPV 79-1146-, FCoV 79-1683-, UV-inactivated FIPV 79-1146- or mock-inoculated monocytes, cultured for 48 h, was added to feline venous EC cultures. An additional cat virus, feline herpesvirus 1 (FHV-1), was also included to determine if the obtained results were FCoV specific. The observed numbers of infected monocytes (Table 1), assessed by immunofluorescence 24 and 48 hours post-inoculation (h.p.i.), strongly varied between the three blood donors, which has also been demonstrated by Dewerchin et al. (2005) for FIPV 79-1146 and FCoV 79-1683. This cat-dependent variation, however, was barely reflected in the intracellular and extracellular virus titres (Fig. 2). Especially for cat 3, a very limited percentage of FIPV 79-1146- and FCoV 79-1683-infected cells was observed, although virus titres were comparable to those observed for cat 1 and cat 2. This may suggest that, although the number of infected monocytes was lower, more virus per infected cell was produced. Furthermore, FCoV 79-1683 inoculation of monocytes from all cats led to very low infection percentages and virus titres in comparison to FIPV 79-1146 infection, confirming previous findings with these strains (Dewerchin et al., 2005; Rottier et al., 2005; Stoddart & Scott, 1989).
After incubating ECs with the supernatant of inoculated monocytes for 6 h (E-selectin) or 12 h (VCAM-1), the surface expression of the two EC adhesion molecules was determined by a cell-based ELISA. Parallel wells were treated with TNF-α and IL-1β as positive controls. The effect of infection on adhesion molecule expression strongly varied between cats, as depicted in Fig. 3. ECs treated with supernatant of both FIPV- and FHV-1-infected monocytes from cat 2 showed a marked increase in both E-selectin (4.2-fold and 4.6-fold, respectively) and VCAM-1 (3.7-fold and 4-fold, respectively).

**Fig. 1.** Expression of endothelial adhesion molecules in renal veins. Cryosections from the renal cortex of healthy cats (HC), FIP cats at sites and remote from inflammatory lesions (R) and FIP cats at sites close to granulomatous infiltrates (L) were stained with mAbs against P-selectin, E-selectin, ICAM-1 or VCAM-1 (red) together with the endothelial marker von Willebrand factor (green) and FCoV (blue). Expression levels are presented in mean fluorescent intensity (MFI). Significant differences from the healthy controls are indicated by an asterisk (P<0.05).
expression in comparison to supernatant from mock-inoculated monocytes. Similar results were obtained for cat 1, although the upregulation of E-selectin and VCAM-1 was less pronounced. Culture fluids from infected monocytes from cat 3 did not seem to cause a distinct increase in adhesion molecule expression. Despite the large variation between cats, cat 3 did not seem to cause a distinct increase in adhesion molecule expression. Despite the large variation between cats, cat 3 did not seem to cause a distinct increase in adhesion molecule expression. Despite the large variation between cats, cat 3 did not seem to cause a distinct increase in adhesion molecule expression. Despite the large variation between cats, cat 3 did not seem to cause a distinct increase in adhesion molecule expression. Despite the large variation between cats, cat 3 did not seem to cause a distinct increase in adhesion molecule expression. Despite the large variation between cats, cat 3 did not seem to cause a distinct increase in adhesion molecule expression. Despite the large variation between cats, cat 3 did not seem to cause a distinct increase in adhesion molecule expression. Despite the large variation between cats, cat 3 did not seem to cause a distinct increase in adhesion molecule expression.

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<td>Mean±sd</td>
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**Table 1. Percentage of viral antigen-positive monocytes**

**Increased adhesion of naive monocytes to endothelial cell cultures that are pretreated with culture fluids from FIPV 79-1146-infected peripheral blood monocytes**

We evaluated whether FIPV 79-1146 induced alteration in adhesion molecule expression also permits an enhanced binding of uninfected monocytes to the activated endothelium. Magnetic-activated cell sorting (MACS) purified monocytes were layered for 30 min onto EC monolayers that were pretreated with UV-irradiated supernatant of mock- or FIPV 79-1146-infected monocytes, TNF-α, or IL-1β for 12 h. Non-adherent cells were removed and the adherent monocytes were counted in 10 random microscopic fields for each well, and each experiment was performed in triplicate. The results are shown in Fig. 4. Only small numbers of monocytes adhered to the negative control EC monolayers that were pretreated with supernatant from mock-inoculated monocytes. In contrast, an increased adhesion of monocytes to ECs that were exposed to culture fluids of FIPV-infected monocytes was observed. This was more pronounced after pretreatment for 12 h (4-fold; P=0.05) than 6 h (1.7-fold; P=0.05, data not shown). An even more pronounced increase in monocyte adherence was observed in the positive control wells, where ECs were pretreated with TNF-α (5-fold; P=0.05) or IL-1β (8-fold; P=0.05).

**DISCUSSION**

A pathological characteristic of FIP is the infiltration of large numbers of leukocytes into the parenchyma, causing functional impairment of the affected organs (Berg et al., 2005; Kipar et al., 1998, 2005). Decisive for the invasion of tissues by inflammatory cells is their transmigration across the endothelium. This leukocyte recruitment is known to be a multistep process that is largely staged at the vascular level. Leukocytes first roll on and subsequently firmly adhere to the endothelium before they ultimately emigrate from the vascular lumen. These events are orchestrated by the engagement of increasingly well-defined ligand pairs (Carlos & Harlan, 1994). In the present study, immunofluorescent staining revealed an elevated expression of P-selectin, E-selectin, ICAM-1 and VCAM-1 on ECs in the renal cortex close to granulomatous infiltrates compared to healthy controls. As the analysed molecules mediate the arrest of circulating leukocytes on the endothelium and thereby facilitate their influx in tissues, our results provide further support for a prominent role of leukocyte extravasation in the pathogenesis of FIP. Interestingly, further comparison between the expression of the adhesion molecules in veins close to a granulomatous infiltrate and veins remote from inflammatory lesions showed that this upregulation was limited to the veins that were located close to the granulomatous infiltrates in the renal cortex. This suggests that local production and release of EC-activating factors, most likely by infected macrophages in the granulomatous infiltrates, is responsible for the elevated expression of adhesion molecules on ECs and subsequent local influx of activated leukocytes that induce phlebitis. Interestingly, in contrast to these endothelial adhesion molecules, Kipar et al. (2005) reported a systemical upregulation of the MHC II antigen, another marker for EC activation, on ECs of both veins and arteries in cats with FIP phlebitis and intense monocyte-associated viraemia, regardless of the presence of inflammatory infiltrates.
It is well recognized that the expression of endothelial adhesion molecules is dynamically regulated by inflammatory mediators (Carlos & Harlan, 1994). The local upregulation of P-selectin, E-selectin, ICAM-1 and VCAM-1 in veins close to granulomatous infiltrates indicates prior exposure of the endothelium to proinflammatory chemokines or cytokines. It has been shown that FIPV-infected monocytes release TNF-α and IL-1β, two key cytokines involved in EC activation (Kipar et al., 2005; Regan et al., 2009). To demonstrate that transferable factors derived from FCoV-infected monocytes initiate EC activation, the expression of adhesion molecules on a feline venous EC line was measured after treatment with supernatant from inoculated monocytes. The surface expression of endothelial E-selectin was significantly upregulated after 6 h of exposure to culture fluids of FIPV-infected monocytes. As the selectins are known to be important for the initial interaction between leukocytes and the endothelium (Vestweber & Blanks, 1999), we hypothesize that elevated E-selectin expression mediates leukocyte rolling, and thereby initiates their extravasation. VCAM-1 is a member of the Ig supergene family and is involved in firm adherence of leukocytes to the endothelium (Carlos & Harlan, 1994). VCAM-1 is not present on resting ECs, but cytokines can elicit upregulation with peak expression after 12 h (Kelly et al., 2007). In the present study, there was a baseline expression of VCAM-1 and marked, although not statistically significant, upregulation of surface expression after exposure for 12 h to the supernatant of FIPV-infected monocytes. The enhanced surface expression of

Fig. 2. FCoV 79-1683, FIPV 79-1146 and FHV-1 replication in feline monocytes from three different blood donors. Cells were inoculated with FCoV 79-1683, FIPV 79-1146 and FHV-1 at an m.o.i. of 1. At 1, 24 and 48 h.p.i., the intracellular and extracellular virus titres were determined.
adhesion molecules was directly related to enhanced binding of uninfected monocytes to feline venous ECs exposed to supernatant of FIPV-infected monocytes. The increase in monocyte adhesion was more pronounced after pretreatment
for 12 h than 6 h and paralleled the time required for maximum expression of VCAM-1. The concurrence of the increased adhesion of naive monocytes to endothelium with the increased surface expression of adhesion molecules indicates the biological significance of adhesion molecule expression. These data collectively demonstrate that transferrable factors derived from FIPV 79-1146-infected monocytes are capable of activating ECs to almost the same extent as known proinflammatory stimuli, such as TNF-α and IL-1β. In contrast to FIPV 79-1146, supernatant from monocytes inoculated with FCoV 79-1683 was not able to significantly increase the expression of E-selectin and VCAM-1, which was not very surprising considering the low number of infected monocytes and virus titres after inoculation, and hence the low number of cytokine-producing monocytes. Furthermore, as FCoV 79-1683 is considered to be an avirulent FIPV strain (Pedersen, 2009), this virus strain might not only be less capable of recruiting in monocytes but also less capable of activating them. Supernatant from FHV-1-infected monocytes was also included, showing that the enhanced surface expression of adhesion molecules is not FIPV-specific. Another member of the Ig supergene family, ICAM-1, is also important in leukocyte adherence and is constitutively expressed at low levels on ECs, but is significantly upregulated following inflammatory stimulation (Kelly et al., 2007). However, we have previously observed a complete loss of both constitutive as well as inducible expression of ICAM-1 on the feline venous EC line (Olyslaegers et al., 2013b). In addition, the expression of P-selectin on the feline venous EC line was not or only marginally induced by TNF-α and IL-1β (data not shown), which has also been described for human umbilical vein ECs (Yao et al., 1996) and certain mouse endothelium cell cultures (Hahne et al., 1993). Therefore, we were not able to assess the expression of P-selectin and ICAM-1 in our EC model system. Since ICAM-1 and P-selectin are known to mediate adherence (Beekhuizen & Van Furth, 1993) and because our immunofluorescence analysis demonstrated elevated expression of ICAM-1 and P-selectin on ECs of adjacent vessels, we expect that besides E-selectin and VCAM-1, ICAM-1 and P-selectin also play essential roles in leukocyte extravasation during FIPV infection.

The development of FIP may not only depend on the virus (virulent versus avirulent) but also on genetic (Foley & Pedersen, 1996) and other host (Poland et al., 1996) factors. Here, a clear cat-dependent variation was observed when supernatant of infected monocytes was tested for its ability to induce adhesion molecule expression on ECs. This could be explained by differences in the number of infected monocytes, as suggested by the results obtained for ECs that were treated with supernatant from FIPV 79-1146-infected monocytes from cat 3, for which low infection percentages were obtained. However, even though similar infection percentages were obtained for cat 1 and cat 2, the upregulation of both E-selectin and VCAM-1 was clearly more pronounced on ECs that were treated with supernatant from FCoV 79-1683-infected monocytes from cat 2. This may suggest that monocytes from certain cats are more potent to produce the crucial cytokines that activate ECs and therefore might predispose these cats to develop FIP. This is supported by the results obtained for FHV-1, for which the infection percentages from cat 3 were quite similar to those of cat 1 and cat 2, but only in cats 1 and 2 was an increased expression observed.

In conclusion, a contained upregulation of vascular adhesion molecules is expected to play an important role in the localized recruitment and retention of inflammatory cells, which typify FIP lesions. In addition, our data strongly suggest that a major mechanism of monocyte extravasation in FIP is the ability of infected monocytes to induce adhesion molecule expression on ECs, which allows adherence and subsequent transmigration of monocytes through the EC layer. This pattern of adhesion molecule induction might not only serve as a strategy for viral dissemination throughout the body but can also facilitate the recruitment of naive monocytes into the inflammatory process, providing a continuous inflow of susceptible cells for viral replication. Understanding the interactions between monocytes and the endothelium not only gives key insights into the pathogenesis of FIPV-associated tissue injury but may also provide future strategies for therapeutic interventions.

**METHODS**

**Animals and tissues.** Nine cats, between 5 months and 5 years old, presenting clinical signs consistent with FIP were referred from private clinicians. In all cases, the presumptive diagnosis of FIP was confirmed.
by post-mortem examinations including the demonstration of viral antigen in FIP lesions using immunofluorescence. Immunofluorescence analysis was performed on cryosections, or, if present in sufficient quantities, asctes. The viral antigens were visualized with polyclonal FITC-labelled anti-FCoV antibodies (VMRD) and the technique is similar to the immunofluorescent staining described below. Eight healthy, conventionally housed cats, between 5 months and 5 years old, that had to be euthanized for health-unrelated problems, were selected as control animals. All cats included in the study were confirmed not to be infected with feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) and were not receiving any form of immunoregulatory drugs to avoid potential confounding factors on the adhesion molecule expression.

In kidneys, FIP lesions are generally presented as a focal phlebitis and periphlebitis of the stellate veins in the outer renal cortex (Kipar et al., 2005). This tissue was selected for sampling, as these vascular lesions are unaltered tissue. If no macroscopic signs of phlebitis were observed, stellate veins directly next to a granulomatous infiltrate were sampled. Regardless of the presence of phlebitis, these samples will further be referred to as veins close to granulomatous infiltrates, as every sample contained FCoV antigen-positive cells in the adjacent tissue. Control specimens were taken from the kidneys of FIP cats at sites remote from contained FCoV antigen-positive cells in the adjacent tissue. Control samples will further be referred to as veins close to granulomatous infiltrates, as every sample contained FCoV antigen-positive cells in the adjacent tissue. Control samples were taken from the kidneys of FIP cats at sites remote from the renal cortex, where the stellate veins are situated. Quantitative analysis of fluorescent signals associated with adhesion molecule expression was carried out using the NIH ImageJ software program (National Institutes of Health). In brief, the original multicolour image (RGB) was split into single colour channels and the endothelium (vWF positive) was outlined on the green digital image, using a freehand selection tool. Thereafter, this defined area was copied to the red digital image (adhesion molecule expression) and the mean brightness value of this selection, expressed in arbitrary units, was used as an indicator of red fluorescence intensity. The measurements were only performed on the veins that were cut in crossection to reduce the impact of apparent lack of immunostaining due to tangential or oblique planes of section across the endothelium. The average staining intensity, mean fluorescent intensity (MFI), was calculated from the three images acquired from one cryosection and normalized with the average of three independent background values on the same slide.

Isolation of blood monocytes. Purpose-bred FCoV-, FeLV- and FIV-negative cats, permanently kept at the animal facility of the Faculty of Veterinary Medicine of Ghent University, were used as blood donors. Mononuclear cells were separated by density gradient centrifugation on Ficoll-Paque, as previously described (Dewerchin et al., 2005). Briefly, heparinized (15 U ml⁻¹; Leo) blood collected from the jugular vein was mixed 1:1 with cold PBS, layered onto Ficoll-Paque (GE Healthcare) and centrifuged at RT. The cell layer at the interface, consisting mainly of peripheral blood mononuclear cells (PBMCs), was collected and a lysis step was performed to remove residual red blood cells.

For virus inoculation, monocytes from three different blood donors were seeded in 24-well tissue culture plates at a concentration of 4×10⁵ cells ml⁻¹ in RPMI 1640 medium (Invitrogen) containing 10% FCS (Greiner Bio-One), 100 U ml⁻¹ penicillin, 0.1 mg ml⁻¹ streptomycin, 0.1 mg ml⁻¹ gentamycin, 10 U ml⁻¹ heparin, 1 mM sodium pyruvate and 1% non-essential amino acids (100×) (Invitrogen).

For adhesion assays, monocytes from one donor, which was different from the donors for viral infection, were immediately separated from the Ficoll-purified PBMC fraction by positive MACS (Miltenyi-Biotec) using the monocyte marker DH59B (VMRD) and anti-mouse IgG microbeads, together with LS separation columns (Miltenyi Biotec). After this procedure, the resulting cell population was represented by >95% monocytes, as assessed by flow cytometry.

Virus and mode of infection. The serotype II FCoV strain 79-1146, obtained from the American Type Culture Collection (ATCC), and the serotype II FIPV strain 79-1146, kindly provided by Dr Egberink (Utrecht University, The Netherlands), both passaged on Crandell Rees feline kidney (CRFK) cells, were used. The FHV-1 strain was obtained from a positive diagnostic sample in our laboratory and passaged on CRFK cells.

Seventy-two hours post-seeding, non-adherent PBMCs were removed by washing the dishes twice with RPMI 1640. The adherent cell population consisted of 94.3±1.5% monocytes, as assessed by immunofluorescent staining with the monocyte marker DH59B. The monocytes were mock-inoculated or inoculated at an m.o.i. of 1 with UV-inactivated FIPV 79-1146 or replication-competent FHV-1, FCoV 79-1683 or FIPV 79-1146. For UV inactivation, a thin layer of viral suspension was exposed to shortwave UV light for 10 min. Inactivation of virus infectivity was verified by plaque assay on CRFK cells. After 1 h incubation at 37°C, the inocula were removed, and cells were washed twice with RPMI and further incubated with fresh media. Monocyte supernatant was collected 48 h.p.i. and centrifuged to remove any cellular debris. In

Quantitative evaluation. Images were acquired with a confocal microscope (Leica Microsystems) using fixed settings for excitation and detection. In FIP cats, images were recorded at sites containing granulomatous infiltrates (three images) and at non-infiltrated sites remote from inflammatory lesions (three images), based on FCoV antigen expression (Fig. 1). For the healthy controls, three images were recorded in the same anatomical region as the FIP cats, being the outer renal cortex, where the stellate veins are situated. Quantitative analysis of fluorescent signals associated with adhesion molecule expression was carried out using the NIH ImageJ software program (National Institutes of Health). In brief, the original multicolour image (RGB) was split into single colour channels and the endothelium (vWF positive) was outlined on the green digital image, using a freehand selection tool. Thereafter, this defined area was copied to the red digital image (adhesion molecule expression) and the mean brightness value of this selection, expressed in arbitrary units, was used as an indicator of red fluorescence intensity. The measurements were only performed on the veins that were cut in crossection to reduce the impact of apparent lack of immunostaining due to tangential or oblique planes of section across the endothelium. The average staining intensity, mean fluorescent intensity (MFI), was calculated from the three images acquired from one cryosection and normalized with the average of three independent background values on the same slide.

Immunofluorescence. Cryosections (10 μm) were prepared with a cryostat (Leica CM 1950; Leica Microsystems), air-dried and fixed for 10 min in 4 % paraformaldehyde (PF). All incubation steps were performed in a moist chamber at 37°C for 1 h (unless mentioned otherwise), and each step was followed by two thorough washings in PBS (pH 7.4) in the dark. As monoclonal antibodies were used for the immunofluorescence staining, the primary antibodies were visualized with secondary antibodies, as described below. For the quantitative analysis of the staining intensity, the images were acquired from one cryosection and the mean brightness value of this selection, expressed in an arbitrary unit (AU), was calculated from the three images acquired from one cryosection and normalized with the average of three independent background values on the same slide.

Virus and mode of infection. The serotype II FCoV strain 79-1683, obtained from the American Type Culture Collection (ATCC), and the serotype II FIPV strain 79-1146, kindly provided by Dr Egberink (Utrecht University, The Netherlands), both passaged on Crandell Rees feline kidney (CRFK) cells, were used. The FHV-1 strain was obtained from a positive diagnostic sample in our laboratory and passaged on CRFK cells.
order to inactivate virus, the supernatant was irradiated with UV prior to storage at −70 °C.

At 24 and 48 h.p.i., the expression of viral antigen in infected cells and the percentage of infected monocytes were assessed by immunofluorescence. Cells were fixed with 4 % PF in PBS for 10 min at RT, followed by permeabilization with 0.1 % Triton X-100 for 2 min at RT. Cells were incubated with primary mAb dilutions containing 10 % normal goat serum against the N protein for FCoV 79-1683 and FIPV 79-1146 (10A12, produced and characterized in our laboratory) and against the gB, gC and gD glycoproteins for FHV-1 for 1 h at 37 °C. After two washes in PBS, FITC-conjugated goat anti-mouse antibodies (Molecular Probes) were added for 1 h at 37 °C to detect the binding of the primary antibodies. Nuclei were stained with Hoechst 33342 (Molecular Probes) for 10 min at RT. The coverslips were washed two times in PBS, mounted and examined for fluorescence.

At 1, 24 and 48 h.p.i., the culture medium was harvested and centrifuged at 400 g for 10 min. The supernatant was used for the determination of extracellular virus titres. Intracellular virus was obtained by scraping the remaining cells in 1 ml fresh medium. The cells were transferred to the Eppendorf with the pellet of the centrifuged extracellular virus and subjected to two freeze–thaw cycles to release the intracellular virus. The samples were stored at −70 °C until titration. Virus titres were assessed by determining of the TCID50 using CRFK cells. The 50 % endpoint was calculated according to the method of Reed & Muench (1938).

Feline venous endothelial cells. A feline venous EC line generated in our laboratory by simian virus 40 T antigen-induced and human telomerase reverse transcriptase-induced immortalization of primary venous ECs was propagated as previously described (Olyslaegers et al., 2013b).

Adhesion molecule expression assay. Surface expression of the adhesion molecules E-selectin and VCAM-1 on ECs was determined using an in-house developed cell-based ELISA, as previously described (Olyslaegers et al., 2013b). Briefly, ECs were grown to confluence in gelatin-coated 96-well plates (BD Biosciences). EC monolayers were treated with UV-irradiated supernatant of mock-, FHV-1-, FCoV 79-1683-, FIPV 79-1146- or UV-inactivated FIPV 79-1146-inoculated monocytes, recombinant feline TNF-α (1 ng ml−1; R&D Systems) or recombinant feline IL-1β (1 ng ml−1; R&D Systems) for 6 h (E-selectin) or 12 h (VCAM-1). Following this incubation period, ECs were washed once with RPMI and incubated for 1.5 h on ice with mouse mAbs against E-selectin or VCAM-1. After three washings, the EC monolayers were fixed with 1 % PF. Bound primary antibodies were detected by a peroxidase-conjugated goat anti-mouse IgG (Molecular Probes) for 1 h at RT. After a triple wash, a colorimetric reaction was performed using tetramethylbenzidine substrate solution (R&D Systems). The colour reaction was stopped after 15 min using 1 M H2SO4 and the absorbance was measured at 450 nm.

Monocyte–endothelial adhesion assay. ECs were grown to confluence in 16-well Lab-Tek II chamber slides (Thermo Scientific) and treated with UV-irradiated supernatant of mock- or FIPV 79-1146-inoculated monocytes, collected 48 h.p.i., TNF-α (1 ng ml−1) or IL-1β (1 ng ml−1) for 12 h at 37 °C, 5 % CO2. After incubation, EC monolayers were washed once with RPMI and 5×104 MCS-purified monocytes in RPMI were added per well and coincubated for 30 min at 37 °C. Non-adherent monocytes were removed by washing thrice with RPMI. Finally, 4 % PF was added for 10 min to fix the monocytes to the endothelial monolayer. The cell nuclei were stained with Hoechst 33342 (Molecular Probes) and the chamber slides were mounted in glycerol/PBS (0.9:0.1, v/v) with 2.5 % 1,4-diazabicyclo[2.2.2]octane and analysed using fluorescence microscopy (Leica Microsystems). Monocytes were readily distinguishable from ECs by their smaller, denser, bean-shaped nuclei. The number of adherent monocytes was determined for each treatment, taking the mean of adherent cells counted in 10 randomly selected microscopic fields at 400× magnification. Three independent experiments were performed using culture fluids of monocytes from three different blood donors and MACS-purified monocytes from another donor, and the results are plotted as mean±SD of the number of adherent monocytes per microscopic field.

Statistical analysis. All statistical analyses were carried out using the Mann–Whitney U test with the exception of the comparison of the expression of adhesion molecules between unaffected and affected tissues within an FIP patient, where the Wilcoxon matched-pairs signed-rank test was more appropriate. Differences were considered significant if P<0.05. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software).

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