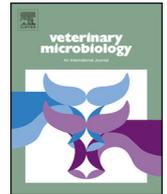




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Altered expression of adhesion molecules on peripheral blood leukocytes in feline infectious peritonitis



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ABSTRACT

Feline infectious peritonitis (FIP) is a fatal, coronavirus-induced systemic disease in domestic and wild felids. The pathology associated with FIP (multifocal granulomatous vasculitis) is considered to be elicited by exaggerated activation and subsequent extravasation of leukocytes. As changes in the expression of adhesion molecules on circulating leukocytes precede their margination and emigration, we reasoned that the expression of leukocyte adhesion molecules may be altered in FIP. In present study, the expression of principal adhesion molecules involved in leukocyte transmigration (CD15s, CD11a, CD11b, CD18, CD49d, and CD54) on peripheral blood leukocytes from cats with naturally occurring FIP ($n = 15$) and controls ($n = 12$) was quantified by flow cytometry using a formaldehyde-based rapid leukocyte preparation technique. T- and B-lymphocytes from FIP patients exhibit higher expression of both subunits (CD11a and CD18) composing the β_2 integrin lymphocyte function-associated antigen (LFA)-1. In addition, the expression of the α_4 subunit (CD49d) of the β_1 integrin very late antigen (VLA)-4 was elevated on B-lymphocytes from FIP patients. The expression of CD11b and CD18, that combine to form the β_2 integrin macrophage-1 antigen (Mac-1), was elevated on monocytes, whereas the density of CD49d was reduced on this population in FIP. Granulocytes of FIP cats displayed an increased expression of the α chain of Mac-1 (CD11b). These observations suggest that leukocytes from FIP patients show signs of systemic activation causing them to extravasate into surrounding tissues and ultimately contribute to pyogranuloma formation seen in FIP.

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1. Introduction

Feline infectious peritonitis, first described in 1963, is a highly fatal, progressive, and immunopathological disease of domestic and wild *Felidae* (Holzworth, 1963). FIP occurs worldwide and is currently one of the leading infectious causes of death in cats. The causative agent of FIP, feline coronavirus (FCoV), is ubiquitous in virtually all cat populations, with seropositivity of up to 90% depending

on environment and geographical area. From the seropositive cats, 5–12% eventually develop FIP (Addie and Jarrett, 1992). FCoV is an enveloped, single-stranded, positive-sense RNA virus belonging to the *Coronaviridae* family within the order of the *Nidovirales* (Gorbalenya et al., 2006). FCoVs are classified into two serotypes (I and II) according to the amino acid sequence of the spike protein (Herrewegh et al., 1998). In addition, each serotype can be further divided into two distinctly different pathotypes, based on their pathogenicity in cats. The most common pathotype in the field, feline enteric coronavirus (FECV), causes a mild, often unapparent enteric infection. In sharp contrast, infection with the virulent pathotype, designated

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feline infectious peritonitis virus (FIPV), manifests as a devastating, highly lethal systemic disease called FIP (Pedersen, 1987). FIP is characterized by fibrinous to granulomatous serositis, often with protein-rich effusions in body cavities, and (pyo)granulomatous inflammatory lesions in various organs (Weiss and Scott, 1981a,b). In the heterogeneous and sometimes confusing histopathological picture of FIP one distinct feature stands out: the multifocal granulomatous vasculitis, restricted to small and medium-sized veins. Morphologically, the vasculitis is typified by a venous and perivenous circular cell infiltrate dominated by monocytes/macrophages intermingled with a few neutrophils and lymphocytes (Kipar et al., 1998). The vasculitis has previously been regarded to be induced by a type III hypersensitivity reaction, involving the deposition of immune complexes within venules leading to complement activation (Jacobsegeels et al., 1982; Pedersen and Boyle, 1980). However, Kipar and co-workers demonstrated that the morphology, cellular composition, and distribution of the vascular lesions in FIP differs significantly from immune-complex-mediated vascular inflammatory processes. Furthermore, the demonstration of FCoV antigen within intravascular leukocytes and among cells in the FIP granulomas indicates that the phlebitis is initiated by activated and FCoV-infected circulating monocytes. Excessive numbers of activated monocytes will emigrate out of the blood circulation and accumulate perivenously. The exaggerated extravasation is associated with enhanced local release of matrix metalloproteinases B (MMP 9) leading to endothelial barrier dysfunction (Kipar et al., 2005). Considering the importance of leukocyte extravasation in the pathogenesis of FIP, comparatively few studies have been aimed at investigating this key pathogenic event. A crucial step in the process of leukocyte recruitment into the parenchyma is the adherence of circulating leukocytes to vascular endothelial cells (EC), which is facilitated by adhesion molecules expressed on the surface of participating cells. Based on their biochemical properties and molecular structure, these adhesion molecules have been grouped into three gene families: the selectins, the integrins, and the immunoglobulin (Ig) supergene family (Carlos and Harlan, 1994). The initial adhesive interaction involves the participation of endothelial selectins that bind with sialyl lewis X (sLex, CD15s) carbohydrate moieties, which can be found on the terminal domains of glycoproteins expressed on the leukocyte surface. Because of its relative low affinity nature, this binding results under hydrodynamic shear flow in leukocyte rolling along the vessel wall. The transiently bound leukocytes are subsequently activated on encountering immobilized chemokines at the endothelial surface. This activation step enables the strengthening of the adhesive forces and leukocytes become firmly attached to the endothelium. This firm adhesion is achieved by the interplay between integrins and Ig supergene family receptors (Ebnet and Vestweber, 1999). The integrins are heterodimer glycoproteins consisting of two non-covalently associated dissimilar proteins designated α and β chain. The integrins are divided into different groups dependent on the common β chain. The β_2 (CD18) subunit combines with α_L (CD11a) subunit to build lymphocyte

function-associated antigen (LFA)-1 or with α_M (CD11b) to form macrophage-1 antigen (Mac-1). They are responsible for interactions with intercellular adhesion molecule (ICAM)-1 (CD54), present constitutively on EC and markedly induced during inflammation. The β_1 integrins named very late antigen (VLA) share a common β_1 (CD29) chain that can be linked with a number of α chains. The VLA-4 receptor contains α_4 subunit (CD49d) and is one of the β_1 integrins responsible for leukocyte extravasation due to its interaction with vascular cell adhesion molecule (VCAM)-1 present on activated endothelium. Both ICAM-1 and VCAM-1 belong to the Ig supergene family and are built of several immunoglobulin domains (Barreiro and Sanchez-Madrid, 2009; Carlos and Harlan, 1994).

Given the large number of leukocytes in FIP lesions, we hypothesized that FIPV infection could alter the expression of adhesion molecules on leukocytes. This might favor leukocyte–endothelial adherence and subsequent migration, thereby inducing endothelial cell damage and contributing to the pathogenesis of FIP. Despite their critical role in the pathological outcome, the expression of adhesion molecules on peripheral leukocytes during FIPV infection remains mainly undescribed. Identification of adhesion molecules with altered expression in FIP will not only provide invaluable insights to further elucidate the pathogenesis but this essential data can also assist in the development of more accurate diagnostic methods.

Therefore, the principal aim of present study was to quantify the expression of adhesion molecules on the main peripheral leukocyte populations in FIP patients and healthy controls by flow cytometry using the formaldehyde-based rapid leukocyte preparation technique (Hamblin et al., 1992). With a panel of thoroughly validated monoclonal antibodies, the main adhesion molecules associated with leukocyte transendothelial migration were studied: CD15s, CD11a, CD11b, CD18, CD49d, and CD54.

2. Materials and methods

2.1. Patients and controls

Fifteen cats, naturally infected with FCoV and clinically strongly suspected of FIP (based on cat profile, clinical signs and blood and/or exudate examination) were included in this study. The characteristics and pathological findings of these cats are listed in Table 1. In all cases, the presumptive diagnosis of FIP was confirmed by post-mortem examinations that comprised necroscopy, histology and immunohistochemistry for FCoV-antigens. Patients were not receiving corticosteroids or other immunosuppressive therapy. Serology for feline immunodeficiency virus (FIV) and for feline leukaemia virus (FeLV) was performed using a commercially available ELISA kit (Witness[®] FeLV-FIV, Synbiotics Corporation, San Diego, CA, USA) according to the procedure advised by the manufacturer. All cats were confirmed not to be infected with FIV and FeLV.

A control group of twelve specific pathogen free (SPF) cats were permanently kept at the animal facility of the Faculty of Veterinary Medicine of Ghent University. At the time of sampling, these cats were clinically healthy and did

Table 1
Characteristics and pathological findings of FIPV-infected cats enclosed in this study.

	Breed	Sex	Age	FCoV antibody titer ^a		Pathological form	Lesions
				Serum	Exudate		
1	Scottish fold	M	5 mo	≥12,800	6400	Effusive	M, I
2	Domestic shorthair	F	3 mo	3200	1600	Effusive	I, Li, S, U
3	Domestic shorthair	F	2 y	≥12,800	≥12,800	Effusive	I
4	Burmese	M	2.5 mo	≥12,800	–	Non-effusive	I, K, P
5	British shorthair	M	6 y	≥12,800	≥12,800	Effusive	M, I, U
6	Domestic shorthair	F	6 mo	≥12,800	≥12,800	Effusive	M, I
7	Domestic shorthair	M	4 mo	1600	1600	Effusive	M, I
8	British shorthair	F	8 mo	≥12,800	≥12,800	Effusive	I, K, Pe
9	British shorthair	M	6 mo	≥12,800	≥12,800	Effusive	M, I
10	Norwegian forest cat	M	3 y	≥12,800	≥12,800	Effusive	M, I, K, U, Lu, P
11	Domestic shorthair	F	1 y	6400	3200	Effusive	I, Pe, D
12	Sacred birman	M	1 y	≥12,800	–	Non-effusive	K, Li, Lu
13	Domestic shorthair	M	5 mo	≥12,800	≥12,800	Effusive	I, S, M, Li, St
14	Domestic shorthair	M	2 y	≥12,800	≥12,800	Effusive	Lu, S, Li, U, D, K, I
15	Domestic shorthair	F	6 mo	6400	6400	Effusive	S, I, M, Li, D

M: male; F: female; mo: month; y: year; M: mesentery; I: intestines; Li: liver; S: spleen; U: urinary bladder; K: kidney; P: pleura; Pe: peritoneum; Lu: lungs; D: diaphragm; St: stomach; –, exudate not present.

^a Immunoperoxidase monolayer assay (IPMA) antibody titer.

not show any hematological or biochemical abnormalities attributed to FIP or any other infectious disease. Because client-owned FIP cats were used on the basis of availability, and SPF cats, currently present in the animal facility, were enclosed as controls, it was not possible to match controls one-to-one to cases based on age or other factors.

The study and its methodology were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (application EC2012/043) and informed consent was obtained from the owners of all patients.

2.2. Preparation of leukocytes

Peripheral blood leukocytes from patients and controls were prepared for labeling and flow cytometry by the formaldehyde-based rapid leukocyte preparation technique. Peripheral blood was sampled from the external jugular vein into heparin (Leo, Zaventem, Belgium) at a final concentration of 10 U/ml. The fresh blood was mixed, within 5 min, with an equal volume of prewarmed (37 °C) 0.4% paraformaldehyde (PF) in phosphate buffered saline (PBS) (Vel chemicals, UCB, Brussels, Belgium) and incubated for 4 min at 37 °C. An excess (20 ml) of warmed (37 °C) lysing buffer pH 7.4 (0.83% ammonium chloride/0.01 M Tris) was then added and incubated at 37 °C until the erythrocytes were lysed (5 min). Cells were sedimented by centrifugation at 200 × g for 5 min at room temperature and the supernatant was discarded. The leukocyte-rich pellet was washed twice with PBS and finally the leukocytes were resuspended in 2 ml PBS, and stored at 4 °C until labeling the next day.

2.3. Cell labeling

To prevent non-specific binding, 2.5×10^7 leukocytes were first pre-incubated with PBS containing 1% bovine serum albumin (BSA) (Sigma–Aldrich GmbH, Steinheim, Germany) and 10% normal goat serum (NGS) for 30 min at 37 °C. Thereafter, cells were washed with PBS and

incubated with saturating amounts of monoclonal antibodies (mAbs) against the different adhesion molecules (CD15s, CD11a, CD11b, CD18, CD49d, or CD54) in separate tubes for 1 h at 37 °C. After a washing step, Alexa fluor 647-conjugated goat anti-mouse IgG₁ or IgM Abs (Molecular Probes, Eugene, Oregon, USA) were added and incubation was carried out for 1 h at 37 °C. Next, the residual free binding sites of the secondary Abs were saturated by adding an excess of non-specific subclass matched Abs raised in the same species as the primary Abs against the adhesion molecules (mouse IgG₁ or IgM) for 30 min at 37 °C. In the second stage of the staining procedure, the samples were divided over three additional tubes to define the different leukocyte subsets within each adhesion molecule. T-cells were identified by anti-CD3 mAbs (Nishimura et al., 2004), visualized with the fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG₃ Abs (Molecular probes). Anti-CD21 mAbs pre-labeled with Alexa fluor 488-conjugated Fab fragments (Zenon mouse IgG₁, labeling kit, Invitrogen, Carlsbad, USA) was used as marker for B-cells. A double staining with anti-CD13 and anti-granulocytes (VMRD Inc., Pullman, WA, USA), pre-conjugated with Zenon Alexa Fluor 488 or R-phycoerythrin (PE) Mouse IgG₁ labeling kits (Invitrogen) respectively, was performed to distinguish the monocytes (only CD13 positive) and the granulocytes (double positive). Control samples for each antibody, prepared with subclass matched irrelevant mAbs, were run in parallel to identify non-specific background fluorescence. The mAbs with human or dog as target species either reacted with cat according to the manufacturer (CD11b, CD18, and CD21) or had previously been shown to cross-react with feline leukocytes (CD11a and CD49d) (Kaname et al., 2002). For mAbs raised against human antigens with unknown feline cross-reactivity (CD15s and CD54), the tissue distribution and fluorescence patterns of the antigens recognized by these mAbs were determined in cat and were almost identical to those of humans indicating that the mAbs recognized the cat homologues of the human antigens. The

Table 2
Monoclonal antibodies used for surface molecule labeling.

Antibody	Ligand	Comment	Isotype	Source
CSLEX1	CD15s	Specific for the α 2-3 sialosylated form of lacto-N-fucopentaose III	IgM, κ	BD Biosciences ^a
25.3	CD11a	Recognizes structural epitope on α _L -chain of LFA-1	IgG1	Beckman Coulter ^b
CA16.3E10	CD11b	Recognizes structural epitope on α _M -chain of Mac-1	IgG1	Serotec ^c
CA1.4E9	CD18	Recognizes structural epitope on common β ₂ -chain of integrins	IgG1	Serotec
HP2/1	CD49d	Recognizes structural epitope on α ₄ chain of VLA-4	IgG1	Serotec
RR1/1	CD54	Recognizes ICAM-1	IgG1	Santa Cruz Biotechnology, Inc. ^d
NZM1	CD3	Recognizes the epsilon chain of feline CD3	IgG1	Y. Nishimura ^e
CA2.1D6	CD21	Recognizes complement receptor 2 expressed by B-cells	IgG1	Serotec
25-2B	CD13	Recognizes aminopeptidase N present on cells of the myeloid lineage	IgG1	VMRD ^f
CL35A	Undetermined	Granulocyte marker	IgG1	VMRD

^a BD Biosciences, CA, USA.

^b Beckman Coulter, Roissy, France.

^c Serotec, Oxford, UK.

^d Santa Cruz Biotechnology Inc., CA, USA.

^e Yorihiro Nishimura, University of Tokyo, Japan.

^f VMRD, Pulman, USA.

optimal dilution of each Ab was determined prior to experiments and the same batches of Ab were used throughout the study. For details on antibodies see [Table 2](#).

Labeled leukocytes were resuspended in 500 μ l of 1% PF in PBS and left overnight in the dark at 4 °C before flow cytometric analysis.

2.4. Flow cytometry

Flow cytometry was performed using a FACSCanto flow cytometer equipped with FACSDiva software (BD Biosciences, Mountain View, CA, USA). The instrument was calibrated before each experiment with the same lot of fluorescence reference beads (rainbow calibration particles (8 peaks), BD Biosciences) to ensure that relative fluorescence values were comparable between runs. For each sample, a minimum of 10,000 events was acquired within the leukocyte gate. Forward and side scatter (FSC and SSC) were collected on a linear scale, while fluorescence was measured using a five decade logarithmic scale. Gates were set to isolate singlet events from debris and aggregates. Next, leukocyte subpopulations were carefully delineated according to their light scatter characteristics and cell marker expression ([Fig. 2](#)). The mean fluorescence intensity (MFI) in arbitrary units (linear conversion of \log_{10} fluorescence) was used as a relative indicator of the surface density of each adhesion molecule on the respective leukocyte population. These values were corrected by subtracting control fluorescence of the same subpopulation in which primary mAbs were substituted by irrelevant isotype matched mAbs. For unimodally distributed markers, results were expressed as MFI of the whole leukocyte subpopulation. For bimodally distributed markers, the results were described in two ways: the proportion (%) of brightly positive cells within each subpopulation, and next the MFI of the brightly (+) subset. The spectral overlap of Alexa Fluor 488- and PE-labeled antibodies was corrected using electronic compensation.

To examine whether differences in expression was attributed to differences in cell size the FSC measurements on the different cell populations of the patients were compared with those of the controls.

2.5. Statistic analysis

The results were summarized and analyzed using non-parametric methods, since the distribution of data was non-Gaussian. The receptor density, expressed as MFI, was presented as median value, 25–75th percentile, and range. The significance of differences between FIP patients and controls was calculated with the Mann–Whitney *U*-test (two-tailed). Correlations were calculated using Spearman's rank test. *P* values equal or lower than 0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Leukocyte preparation technique and flow cytometric analysis

Peripheral blood leukocytes from patients and controls were prepared for antibody labeling and flow cytometry by the formaldehyde-based leukocyte preparation technique. Representative light scattergrams of peripheral blood leukocytes from healthy subjects and FIP cats obtained with this technique are shown ([Fig. 1](#)). Striking differences in FSC/SSC profiles of leukocytes from healthy and FIP cats were noted. The technique applied on blood of healthy subjects permitted to clearly define lymphocytes, monocytes and granulocytes based on their intrinsic forward and sideward light scattering characteristics. In contrast, these main leukocyte populations could not be identified simply by their FSC and SSC properties in FIP samples because of a considerable overlap between granulocytes and monocytes. Furthermore, a substantial diminution of the lymphocyte count in FIP cats made it difficult to separate this population from cell debris. Since gating of leukocyte populations based on physical characteristics could not be applied on FIP samples and to discriminate between B- and T-lymphocytes within the lymphocyte gate, specific cell markers were used to identify the different subpopulations in leukocytes from controls and patients. One color staining with anti-CD3 (T-lymphocytes) and anti-CD21 (B-lymphocytes) plotted

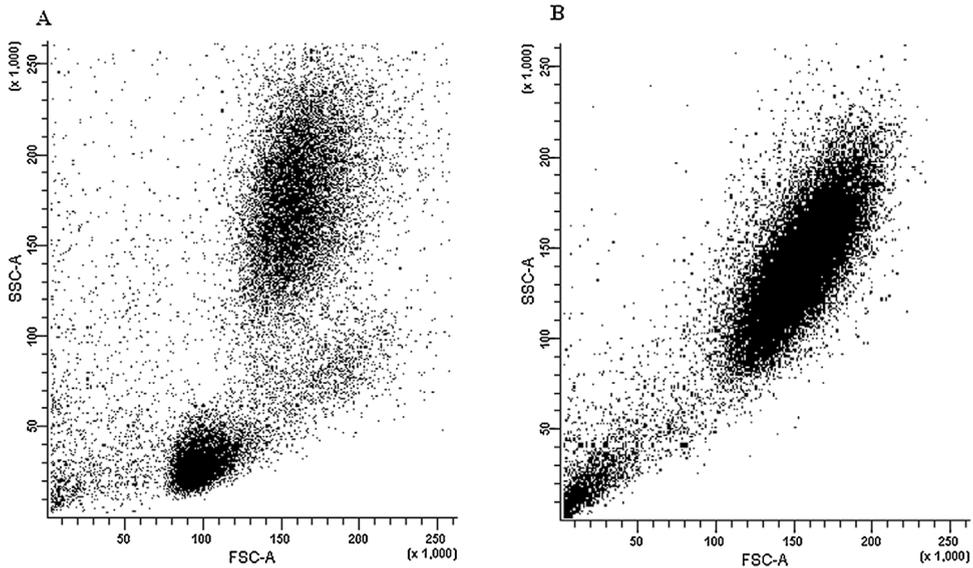


Fig. 1. Light scattergrams of peripheral blood leukocytes. Peripheral blood leukocytes from controls (A) and FIP patients (B) were prepared for flow cytometry by the formaldehyde-based rapid leukocyte preparation technique. The FSC/SSC profiles acquired with this technique differ considerably between the two groups.

against SSC and two color dot plots with anti-CD13 and anti-granulocytes (monocytes and granulocytes) are shown in Fig. 2.

Finally, it was ascertained that the leukocyte preparation technique only rendered surface antigens and not intracellular antigens accessible for antibodies. The processing did not permeabilise leukocyte membranes, as evidenced by our failure to stain cells with antibodies to the cytoplasmic antigen vimentin (data not shown).

3.2. Expression patterns of adhesion molecules

Following distribution patterns of adhesion molecules were observed in healthy cats as well as in FIP cats

by histogram analysis: Within the CD21⁺ leukocyte subpopulation (B-cells), each adhesion molecule showed a unimodal fluorescence distribution, indicating that adhesion molecules were expressed by all (CD11a, CD18, and CD49d) or none (CD15s, CD11b, and CD54) of the B-lymphocytes. On T-lymphocytes the same distribution of adhesion molecules was seen as on B-lymphocytes, except the VLA-4 expression (CD49d) on T-lymphocytes was characterized by a clear bimodal distribution containing a negative and a positive subset. The expression profiles of adhesion molecules on granulocytes and monocytes were similar to each other. The entire granulocyte and monocyte population was brightly positive for CD11a, CD11b, and CD18 and dimly

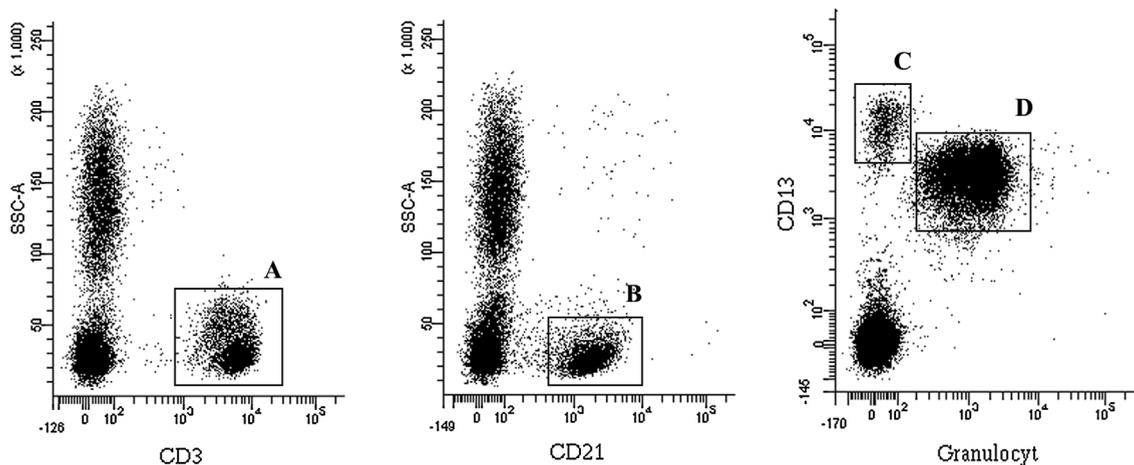


Fig. 2. Identification of the leukocyte subpopulations. T-lymphocytes (A), B-lymphocytes (B), monocytes (C), and granulocytes (D) were carefully gated according to their intrinsic light scatter characteristics and expression of specific cell markers.

positive for CD54, presenting as an unimodal distribution of fluorescence intensities. By contrast, the histogram of CD15s on both populations showed a bimodal distribution. CD49d expression on both monocytes and granulocytes revealed an unimodal peak. However,

monocytes expressed a moderate to high level of this molecule whereas granulocytes express only little surface VLA-4. Representative histograms of adhesion molecules with an unimodal or bimodal expression pattern are shown in Fig. 3.

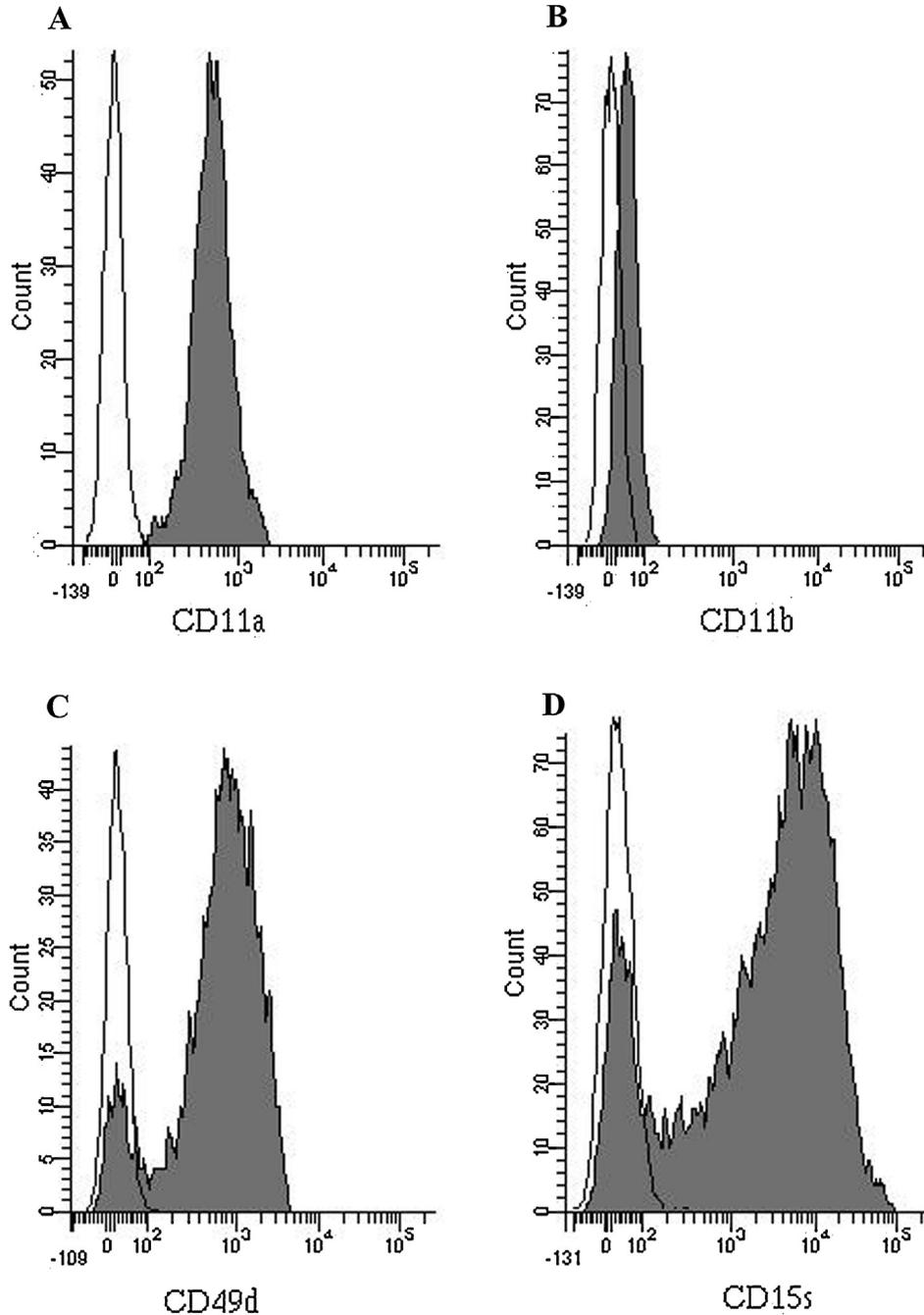


Fig. 3. Expression patterns of adhesion molecules. Representative histograms of adhesion molecules plotting logarithm of fluorescence intensity per cell against cell number results in either unimodal (A and B) or bimodal distribution (C and D) patterns. On B-lymphocytes, CD11a (A) and CD11b (B) expression, display an unimodal fluorescence distribution, but CD11a is expressed by all the cells whereas CD11b expression is absent on the entire population. CD49d (C) and CD15s (D) are only expressed by a proportion of the T-lymphocyte and granulocyte population, respectively, and histograms demonstrate a typical bimodal fluorescence distribution. Open histograms illustrate the binding of the irrelevant, isotype-matched mAbs. Full histograms represent the binding of the relevant mAbs.

3.3. Expression level and distribution of adhesion molecules in FIP compared with controls

The surface expression of adhesion molecules was compared between FIP cats ($n = 15$) and healthy controls ($n = 12$) using the logarithmic MFI parameter of the entire leukocyte subpopulation (unimodally distributed molecules) or of only the brightly positive subset (bimodally distributed molecules). In addition, the proportion of brightly positive cells within each subpopulation was compared between patients and controls for adhesion molecules with a bimodal distribution.

3.4. sLex expression

No significant differences were observed in the level of sLex (CD15s) expression on any leukocyte population from patients compared with controls (Fig. 4). However, the high degree of intersubject variability in expression of sLex on all the leukocyte populations in both FIPV-infected and uninfected groups was noteworthy. The proportion of the monocyte and granulocyte population brightly expressing sLex also varied greatly among individuals and there were no significant differences in the percentages of cells expressing the molecule when patients and controls were compared.

3.5. Integrin expression

Fig. 5 shows the expression of the β_1 and β_2 integrins on the peripheral blood leukocytes from healthy controls compared with FIP cats.

Granulocytes expressed low levels of the β_1 integrin VLA-4 (CD49d/CD29) and these levels were not significantly different between patients and controls. On the

contrary, lymphocytes and monocytes displayed moderate to high expression levels of CD49d. Significant differences in MFI associated with CD49d-expression on B-lymphocytes and monocytes were detected in the FIP cats compared with the control cats. The relative number (%) of CD49d-positive T-lymphocytes appeared comparable between FIP cats ($75.7 \pm 11.6\%$) and healthy controls ($83.3 \pm 4.8\%$).

The expression of the β_2 integrins LFA-1 and Mac-1 was increased on leukocytes from FIP patients. More specific, the CD11a (α_L -chain of LFA-1) expression on FIP lymphocytes (B- and T-cells) was significantly higher than that of the controls. Granulocytes and monocytes from FIP patients expressed significantly higher density of the CD11b (α_M -chain of Mac-1) molecule. The expression of CD18 (common β_2 -chain) was up regulated on all leukocyte populations in the patient group although the difference did not achieve significance for the granulocyte population.

The possibility that the observed differences in fluorescence were due to a difference in size between the cells of the patient and control group was explored by comparing the mean forward scatters. There were no substantial differences in the mean forward scatters of B-lymphocytes, T-lymphocytes, granulocytes and monocytes showing that the differences in the MFIs were not attributable to differences in size between both groups.

3.6. ICAM-1 expression

MFI values for ICAM-1 (CD54) expression were not significantly different between patients and controls for any leukocyte population (Fig. 6). The expression of ICAM-1 was found to be extremely variable on monocytes and granulocytes from FIP cats.

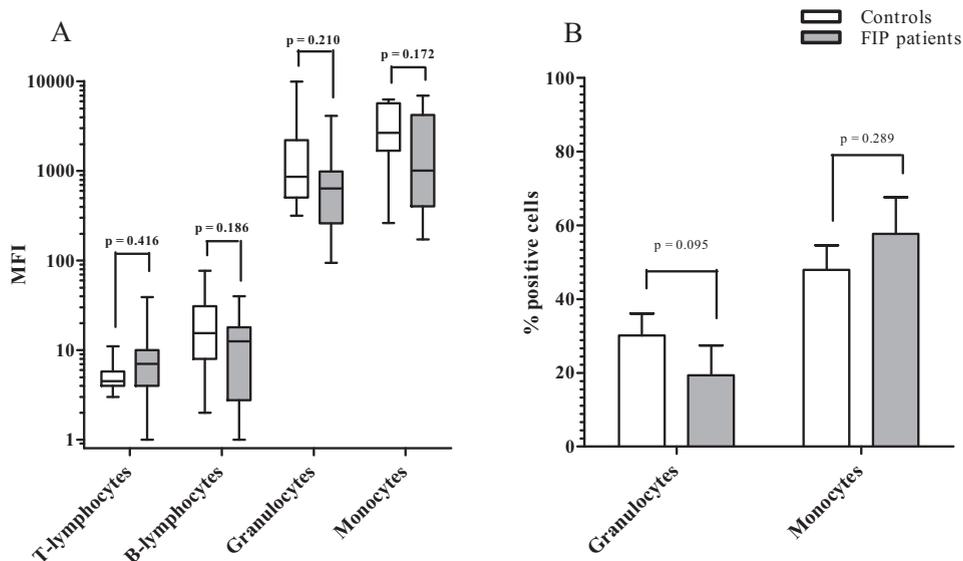


Fig. 4. Expression level and distribution of sLex on leukocyte subpopulations from controls and FIP patients. sLex (CD15s) expression shows a unimodal distribution on B- and T-lymphocytes and a bimodal distribution on granulocytes and monocytes by histogram analysis. Expression level (A) was expressed as MFI of the whole leukocyte population (unimodal) or of the brightly positive subset (bimodal). Distribution (B) of sLex on granulocytes and monocytes was assessed by percentage CD15s-positive cells within the respective subpopulation.

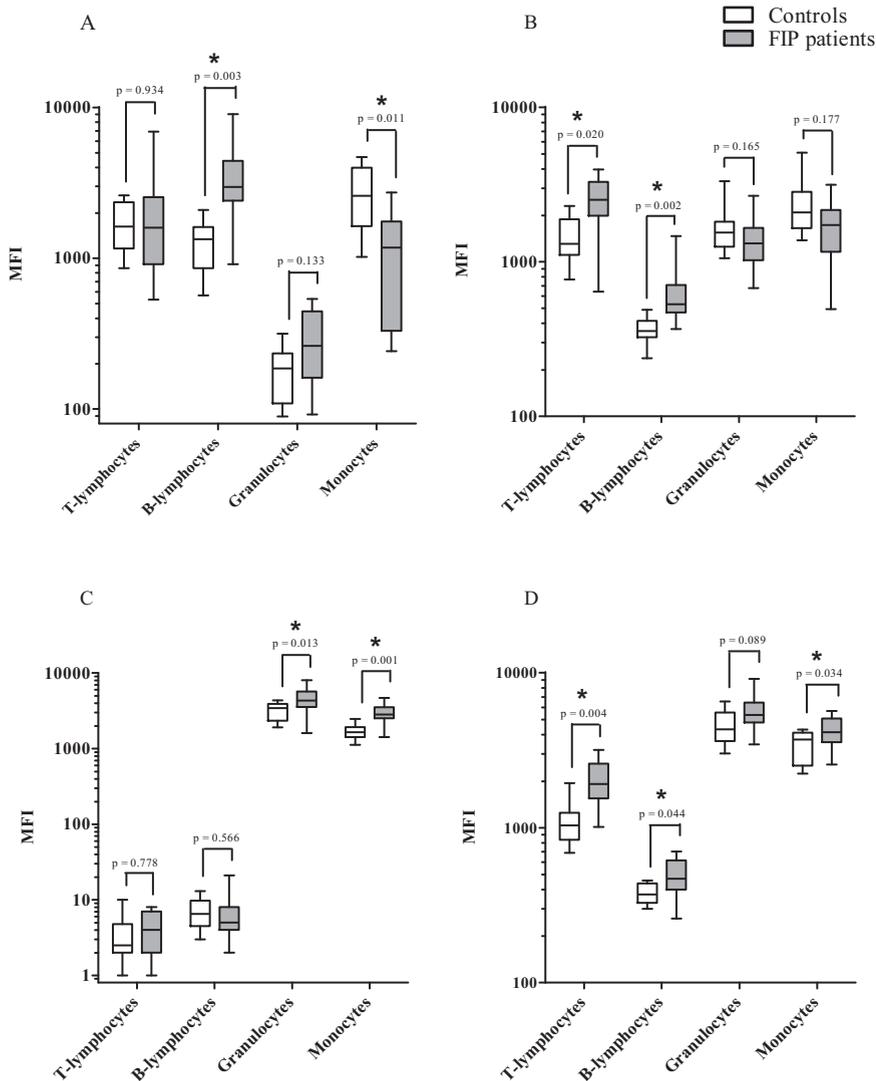


Fig. 5. Expression levels of integrins on leukocyte subpopulations from controls and FIP patients. Expression of integrins is unimodally distributed on all the leukocyte subpopulations. Expression level of CD49d (A), CD11a (B), CD11b (C), and CD18 (D) was compared between controls and FIP patients using the logarithmic MFI parameter of the entire leukocyte subpopulation.

3.7. Clinical correlation

Correlations between adhesion molecule expression on the different leukocyte populations and some aspects of the disease were sought using Spearman's rank coefficient. The immune and disease status of the patients was defined by various laboratory data (lymphocyte count and neutrophil count (relative and absolute numbers) and blood albumin). A significant correlation was found for CD18 expression on B-lymphocytes and albumin level (Spearman coefficient = -0.7273 ; $P = 0.0144$).

4. Discussion

FIP pathology is typically characterized by widespread granulomatous vascular lesions. It is accepted today that the inflammatory reaction in FIP lesions is initiated by

excessive leukocyte migration from the bloodstream into surrounding tissues (Kipar et al., 2005). To date, adhesion molecules, which play a major role in leukocyte extravasation, have not been well characterized in FIPV infection. To the best of our knowledge, this is the first comparative analysis of the quantitative expression of leukocyte adhesion molecules between normal and FIP cats using a whole blood flow cytometric assay. Currently, researchers are well aware that the expression of function-associated antigens, like adhesion molecules, can be rapidly and artefactually modulated if cells are activated *in vitro*. This activation can readily occur during conventional cell preparation and labeling procedures (Forsyth and Levinsky, 1990; Macey et al., 1995). To overcome these problems and to minimize *ex vivo* alterations, we have successfully applied the formaldehyde-based rapid leukocyte preparation technique, previously designed to prepare

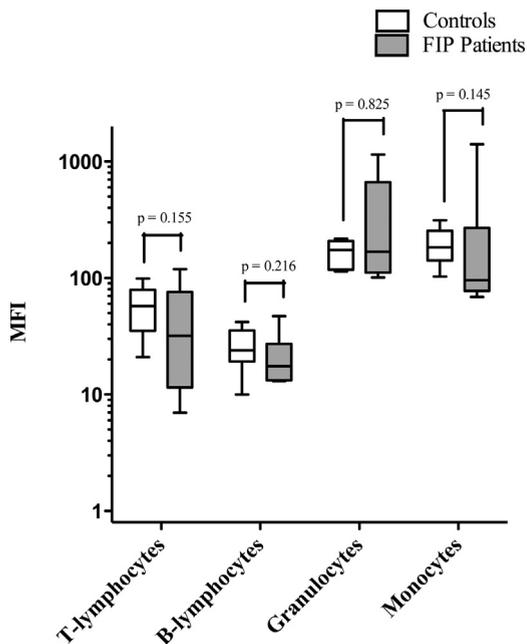


Fig. 6. Expression level of ICAM-1 on leukocyte subpopulations from controls and FIP patients. Expression of ICAM-1 (CD54) is unimodally distributed on all the leukocyte population. Expression level of CD54 was compared between controls and FIP patients using the logarithmic MFI parameter of the entire leukocyte subpopulation.

human leukocytes for this purpose (Hamblin et al., 1992). In addition, it is well known that excessive formaldehyde fixation can cause permeation of the cell membrane (Macey and McCathy, 1993). This would implicate that not only adhesion molecules expressed at the membrane surface but also intracellular storage pools of adhesion molecules become accessible for antibodies. Therefore it was first ascertained that the technique conserved the plasma membrane integrity, evidenced by a lack of cell staining with antibodies to the cytoplasmic antigen vimentin. Taken together, except for any possible changes due to the process of venipuncture itself, we believe that our findings represent the true state of the adhesion molecules on the surface of leukocytes from the circulating pools.

Patients included in our study were selected with great care in attempt to avoid potential confounding factors. Since glucocorticoids have been reported to affect the production of various cytokines, which in turn can affect the expression of adhesion molecules, it was verified that FIP cats had not received any glucocorticoid or other immunosuppressive therapy (Torsteinsdottir et al., 1999). In addition, as the exact influence of other pathogens on adhesion molecule expression is unknown, it was confirmed that FIP patients were not affected by intercurrent infections with FeLV or FIV.

Adhesion molecules coordinate the various phases of leukocyte adherence to endothelium in a stepwise fashion through a regulated mechanism of ligand binding. The present study aimed to assess the expression of principal adhesion molecules involved in the leukocyte–endothelium interaction. We have demonstrated that the majority

of adhesion molecules displayed an unimodal fluorescence distribution. Therefore changes in expression level (MFI), rather than distribution (% positive cells), appear to be an appropriate tool to determine whether there are alterations of expression associated with FIPV infection. The first step of extravasation, the characteristic leukocyte rolling phenomenon, is mediated by endothelial selectins that recognize sLex carbohydrate moieties on the terminal domains of glycoproteins on the leukocyte surface. Our results show considerable intersubject variation in the expression of sLex on all leukocyte populations. This wide biological range of sLex expression levels was not only seen in the patient group but also in the control group that was considered to be a relatively homogeneous group of young adult SPF cats. These findings are consistent with a study of Rebeck and Finn demonstrating that there is inherent variability in the expression of adhesion molecules (especially L-selectin) on granulocytes between and within healthy humans (Rebeck and Finn, 1994). The second step in the adherence cascade involves firm attachment of leukocytes on the endothelium and is largely mediated by the interaction between integrins and members of the Ig supergene family. LFA-1 and Mac-1 are β_2 -integrins that promote the tight adherence of leukocytes to the endothelial ligand ICAM-1. LFA-1 is found on all leukocytes, whereas Mac-1 is only present on myeloid cells. In this study we demonstrate that granulocytes and monocytes of FIP cats display significantly more Mac-1 and circulating lymphocytes express higher densities of LFA-1 than control subjects. Increase in integrin expression is regulated by at least two mechanisms and can occur rapidly within minutes or gradually over a time course of 12–72 h. The rapid alterations are achieved by their mobilization from intracellular storage pools to the cell surface. In circulating granulocytes and monocytes, but not in lymphocytes, significant amounts of Mac-1 (CD11b/CD18) are stored in intracellular vesicles, which upon cell activation are translocated within minutes to the membrane surface (Mazzone and Ricevuti, 1995). This undoubtedly allows the rapid and prompt responsiveness of these cells during the development of the inflammatory process and might explain their predominance in inflammatory infiltrates in FIP (Takano et al., 2009a). In addition to these rapid changes in cell-surface expression, quantitative alteration in integrin receptors can occur with longer stimulation (hours to days) and involves *de novo* integrin subunit synthesis. Lymphocyte adhesion relies greatly on LFA-1 (CD11a/CD18). Since little or no CD11a/CD18 is stored intracellularly, up regulation of these adhesion molecules depends on this second mechanism, which is a substantially slower process (Mazzone and Ricevuti, 1995). These factors may underline the relatively slow lymphocyte responsiveness to activation, which in turn may be reflected in their later accumulation during the development of FIP lesions (Kipar et al., 1998). Consistent with their original name (“very late antigens”), most leukocyte β_1 integrins increase their expression gradually after several days of activation. VLA-4 is unique among β_1 integrins in its ability to bind, in addition to matrix proteins, the endothelial ligand VCAM-1. The latter is a member of the Ig supergene family that resides on

cytokine-activated endothelium (Carlos and Harlan, 1994). Our results show that feline neutrophils, like their human counterparts, express relative low levels of VLA-4 confirming the prevailing view that VLA-4 plays a relatively minor role in neutrophil adhesion. In contrast, monocytes and lymphocytes express moderate to high levels of VLA-4 and its interaction with VCAM-1 has been proposed as an alternative emigration pathway for these cells. In our study, an increased expression of the α_4 subunit (CD49d) of VLA-4 on B-lymphocytes from FIP patients was demonstrated. This is consistent with the immuno-histochemical findings of Kipar et al. and Berg et al., who both examined the cellular composition of FIP lesions. They showed that in lesions without extended necrosis, B-cells and plasmacells progressively infiltrate granulomas, hence replacing macrophages and forming a broad outer rim (Berg et al., 2005; Kipar et al., 1998). Since VLA-4 is critically involved in monocyte interaction with the endothelium, it was of interest that, despite this cell type predominates in FIP granuloma's, the expression levels of CD49d were significantly lower on monocytes from FIP patients compared with healthy controls. Meerschaert and Furie showed that monocyte adhesion on human EC monolayers was only inhibited completely when EC were preincubated with a combination of mAbs against the β_2 integrins and VLA-4 (Meerschaert and Furie, 1995). These *in vitro* observations suggest that CD11/CD18 and VLA-4 represent alternative pathways for the transendothelial migration of monocytes. Given that the availability of at least one of these pathways is sufficient to support normal levels of migration, together with our findings, makes us assume that monocyte extravasation in FIP largely depends on β_2 integrin-ICAM-1 interaction. ICAM-1 is a member of the Ig supergene family member that is not only expressed on endothelial cells but also on lymphocytes and monocytes/macrophages. It is well known that homotypic and heterotypic lymphocyte adhesion, mediated by interaction of ICAM-1 and CD11a,b/CD18, facilitates lymphocyte functions including antigen recognition, lymphocyte costimulation, and cytotoxicity. In addition, ICAM-1 has been shown to play an essential role in monocyte-lymphocyte communication (Hubbard and Rothlein, 2000). Since the central role of ICAM-1 in the immune response, we explored the expression of this molecule in FIP. No differences were noted in the expression of ICAM-1 on lymphocytes and monocytes between patients and healthy controls. To date, published data on ICAM-1 expression on granulocytes is limited. Most recently, it was reported that human neutrophils do express ICAM-1 and it is up regulated by *in vitro* stimulation with endotoxin and tumor necrosis factor (TNF)- α . It was further speculated that up regulation of ICAM-1 may allow neutrophils to assist each other in reaching the final destination through their increased 'stickiness' via CD11a,b/CD18-ICAM-1 interaction (Wang et al., 1997). Our flow cytometric analyses with the anti-CD54 monoclonal antibody demonstrated that feline granulocytes express moderate amounts of ICAM-1. The expression on granulocytes in the FIP group was characterized by a high variability between patients that might be responsible for the lack of significant difference with the control group. This variability might

correlate with the stage of disease. However, this hypothesis could not be thoroughly assessed since in spontaneously occurring FIP it is not possible to determine the moment of infection nor to have an exhaustive follow-up, because cats were euthanatized just after blood sampling.

Higher expression of adhesion molecules in patients is consistent with the general activation of peripheral leukocytes. However, this study has not determined the mechanisms underlying the altered expression. Since only a small proportion of circulating monocytes are infected with FIPV, together with the findings that granulocytes and lymphocytes, which are not susceptible for FIPV infection, show similar expression changes than monocytes indicates that direct effect of viral infection itself does not account for observed alterations. It seems more likely that indirect effects, such as complex changes in the cytokine milieu associated with advancing FIP disease are causing these altered cell surface molecule density. A variety of cytokines has been shown to influence adhesion molecules expression. Mainly tumor necrosis factor TNF- α , interleukine (IL)-1, and interferon (IFN)- γ have been implicated in the up regulation of integrins. Studies of cats with FIP have shown that cytokine expression is markedly altered as compared to healthy animals. Specifically it has been noted that the expression of the pro-inflammatory cytokines TNF- α , IL-1, and IL-6 are significantly increased in FIP, which very likely accounts for the activation of leukocytes in the circulation (Goitsuka et al., 1990; Kiss et al., 2004; Takano et al., 2009b). An additional or alternative explanation for our findings may be a difference in relative immaturity of leukocytes in FIP cats. The rate of migration of cells from the bone marrow through the blood to tissues is likely to be faster in FIP patients than in normal cats, resulting in the accumulation of less mature cells in the circulation. FIP hematology is often characterized by a neutrophilia accompanied with an increased count of left shifted or less mature ("band") neutrophils, providing evidence for an increased recruitment from the bone marrow. Differences in integrin expression are known to occur during leukocyte differentiation and altered surface levels may therefore reflect a different maturational level of cells in patients compared with controls (Elghetany, 2002; Kansas et al., 1990). Changes in the composition of FIP peripheral lymphocytes may also underlie the increased level of CD11a/CD18 expression we have seen on these cells. It has been shown that T-lymphocytes undergo phenotypic changes upon encountering specific antigens and this conversion from naïve to memory type involves increase of the level of expression of adhesion molecules (Sanders et al., 1988).

Whether the observed changes in cell adhesion molecule expression have functional consequences which contribute to the disease process can only be speculated. Whilst quantitative changes in expression have been associated with increased adhesion *in vitro*, it is recognized that adhesion *in vivo* can also occur without an increase in the level of surface expression. In fact, a body of data confirms that the regulation of the integrin-mediated adherence seems to be more complex than a pure quantitative change in expression of these molecules on the cell surface. It appears that under steady-state

conditions integrin molecules on the cell surface are in a relatively low functional active state and upon activation of the leukocytes these molecules convert to an active state resulting in a higher affinity for their respective ligands. These qualitative changes are believed to be at least equally important than quantitative changes for *in vivo* activity of many adhesion molecules (Arnaout, 1990). We examined only the level of surface expression of adhesion molecules and the affinity state of these molecules was not determined. However, since their key role in leukocyte adherence, increase of the number of adhesion molecules expressed on the cell surface may predispose leukocytes to increased adhesion. Because adherence is generally accepted as being a rate-limiting step for transmigration to occur, relatively high adherence is followed by relatively high transmigration. Adherent and diapedesing leukocytes may release proteases, toxic oxygen metabolites, and vasoactive substances, together leading to endothelial injury with subsequent loss of vascular integrity, edema, and tissue injury contributing to the hallmark lesions of FIP.

The recruitment of the characteristic leukocyte infiltrate in FIP is regulated by the expression of leukocyte adhesion molecules in conjunction with the reciprocal expression of their counter receptors on the vascular endothelium. Given that the site and disease specificity is believed to be related to endothelial expression of a combination of vascular adhesion molecules it would be of great value to assess the expression and distribution of these cytokine-inducible endothelial ligands at sites of FIP lesions in the future.

In summary, we have demonstrated that leukocytes from FIP cats show signs of activation in the peripheral circulation concerning adhesion. It is very likely that the priming of the blood leukocytes in FIP is one of the mechanisms behind the excessive recruitment and accumulation of immune cells resulting in granuloma formation and ultimately contributing indisputable to the pathogenesis of FIP disease. Our findings may provide reliable laboratory markers to support the ante mortem diagnosis of FIP which at present is still challenging. However, larger studies, including other pathological conditions, are warranted to evaluate the discriminating power of these parameters for FIP. In addition, detailed understanding of leukocyte interaction with vascular endothelial cells in FIP offers invaluable insights that could promote the emergence of novel therapeutic tools to interfere with leukocyte recruitment and subsequently limit tissue injury.

Competing interests

The authors declare that they have no competing interests.

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References

- Addie, D.D., Jarrett, O., 1992. A study of naturally occurring feline coronavirus infections in kittens. *Vet. Rec.* 130, 133–137.
- Arnaout, M.A., 1990. Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood* 75, 1037–1050.
- Barreiro, O., Sanchez-Madrid, F., 2009. Molecular basis of leukocyte-endothelium interactions during the inflammatory response. *Rev. Esp. Cardiol.* 62, 552–562.
- Berg, A.L., Ekman, K., Belak, S., Berg, M., 2005. Cellular composition and interferon-gamma expression of the local inflammatory response in feline infectious peritonitis (FIP). *Vet. Microbiol.* 111, 15–23.
- Carlos, T.M., Harlan, J.M., 1994. Leukocyte-endothelial adhesion molecules. *Blood* 84, 2068–2101.
- Ebnet, K., Vestweber, D., 1999. Molecular mechanisms that control leukocyte extravasation: the selectins and the chemokines. *Histochem. Cell Biol.* 112, 1–23.
- Elghetany, M.T., 2002. Surface antigen changes during normal neutrophilic development: a critical review. *Blood Cells. Mol. Dis.* 28, 260–274.
- Forsyth, K.D., Levinsky, R.J., 1990. Preparative procedures of cooling and re-warming increase leukocyte integrin expression and function on neutrophils. *J. Immunol. Methods* 128, 159–163.
- Goitsuka, R., Ohashi, T., Ono, K., Yasukawa, K., Koishibara, Y., Fukui, H., Ohsugi, Y., Hasegawa, A., 1990. IL-6 activity in feline infectious peritonitis. *J. Immunol.* 144, 2599–2603.
- Gorbalenya, A.E., Enjuanes, L., Ziebuhr, J., Snijder, E.J., 2006. Nidovirales: evolving the largest RNA virus genome. *Virus Res.* 117, 17–37.
- Hamblin, A., Taylor, M., Bernhagen, J., Shakoor, Z., Mayall, S., Noble, G., McCarthy, D., 1992. A method of preparing blood leucocytes for flow cytometry which prevents upregulation of leucocyte integrins. *J. Immunol. Methods* 146, 219–228.
- Herrewegh, A.A., Smeenk, I., Horzinek, M.C., Rottier, P.J., de Groot, R.J., 1998. Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. *J. Virol.* 72, 4508–4514.
- Holzworth, J., 1963. Some important disorders of cats. *Cornell Vet.* 53, 157–160.
- Hubbard, A.K., Rothlein, R., 2000. Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. *Free Radic. Biol. Med.* 28, 1379–1386.
- Jacobsegeels, H.E.L., Daha, M.R., Horzinek, M.C., 1982. Antibody, immune-complexes, and complement activity fluctuations in kittens with experimentally induced feline infectious peritonitis. *Am. J. Vet. Res.* 43, 666–670.
- Kaname, H., Mori, Y., Sumida, Y., Kojima, K., Kubo, C., Tashiro, N., 2002. Changes in the leukocyte distribution and surface expression of adhesion molecules induced by hypothalamic stimulation in the cat. *Brain Behav. Immun.* 16, 351–367.
- Kansas, G.S., Muirhead, M.J., Dailey, M.O., 1990. Expression of the Cd11/Cd18, leukocyte adhesion molecule-1, and Cd44 adhesion molecules during normal myeloid and erythroid-differentiation in humans. *Blood* 76, 2483–2492.
- Kipar, A., Bellmann, S., Kremendahl, J., Kohler, K., Reinacher, M., 1998. Cellular composition, coronavirus antigen expression and production of specific antibodies in lesions in feline infectious peritonitis. *Vet. Immunol. Immunopathol.* 65, 243–257.
- Kipar, A., May, H., Menger, S., Weber, M., Leukert, W., Reinacher, M., 2005. Morphologic features and development of granulomatous vasculitis in feline infectious peritonitis. *Vet. Pathol.* 42, 321–330.
- Kiss, I., Poland, A.M., Pedersen, N.C., 2004. Disease outcome and cytokine responses in cats immunized with an avirulent feline infectious peritonitis virus (FIPV)-UCD1 and challenge-exposed with virulent FIPV-UCD8. *J. Feline Med. Surg.* 6, 89–97.

- Macey, M.G., McCarthy, D.A., 1993. Quantitation of adhesion molecules and other function-associated antigens on human peripheral blood leukocytes. *Cytometry* 14, 898–908.
- Macey, M.G., McCarthy, D.A., Vordermeier, S., Newland, A.C., Brown, K.A., 1995. Effects of cell purification methods on Cd11b and α -selectin expression as well as the adherence and activation of leukocytes. *J. Immunol. Methods* 181, 211–219.
- Mazzone, A., Ricevuti, G., 1995. Leukocyte CD11/CD18 integrins: biological and clinical relevance. *Haematologica* 80, 161–175.
- Meerschaert, J., Furie, M.B., 1995. The adhesion molecules used by monocytes for migration across endothelium include Cd11a/Cd18, Cd11b/Cd18, and α -4 on monocytes and Icam-1, Vcam-1, and other ligands on endothelium. *J. Immunol.* 154, 4099–4112.
- Nishimura, Y., Shimojima, M., Sato, E., Izumiya, Y., Tohya, Y., Mikami, T., Miyazawa, T., 2004. Downmodulation of CD3epsilon expression in CD8alpha+beta-T cells of feline immunodeficiency virus-infected cats. *J. Gen. Virol.* 85, 2585–2589.
- Pedersen, N.C., 1987. Virologic and immunologic aspects of feline infectious peritonitis virus infection. *Adv. Exp. Med. Biol.* 218, 529–550.
- Pedersen, N.C., Boyle, J.F., 1980. Immunological phenomena in the effusive form of feline infectious peritonitis. *Am. J. Vet. Res.* 41, 868–876.
- Rebuck, N., Finn, A., 1994. Polymorphonuclear granulocyte expression of CD11a/CD18, CD11b/CD18 and α -selectin in normal individuals. *FEMS Immunol. Med. Microbiol.* 8, 189–195.
- Sanders, M.E., Makgoba, M.W., Sharrow, S.O., Stephany, D., Springer, T.A., Young, H.A., Shaw, S., 1988. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN-gamma production. *J. Immunol.* 140, 1401–1407.
- Takano, T., Azuma, N., Hashida, Y., Satoh, R., Hohdatsu, T., 2009a. B-cell activation in cats with feline infectious peritonitis (FIP) by FIP-virus-induced B-cell differentiation/survival factors. *Arch. Virol.* 154, 27–35.
- Takano, T., Azuma, N., Satoh, M., Toda, A., Hashida, Y., Satoh, R., Hohdatsu, T., 2009b. Neutrophil survival factors (TNF-alpha, GM-CSF, and G-CSF) produced by macrophages in cats infected with feline infectious peritonitis virus contribute to the pathogenesis of granulomatous lesions. *Arch. Virol.* 154, 775–781.
- Torsteinsdottir, I., Arvidson, N.G., Hallgren, R., Hakansson, L., 1999. Monocyte activation in rheumatoid arthritis (RA): increased integrin, Fc gamma and complement receptor expression and the effect of glucocorticoids. *Clin. Exp. Immunol.* 115, 554–560.
- Wang, J.H., Sexton, D.M., Redmond, H.P., Watson, R.W.G., Croke, D.T., Bouchier-Hayes, D., 1997. Intercellular adhesion molecule-1 (ICAM-1) is expressed on human neutrophils and is essential for neutrophil adherence and aggregation. *Shock* 8, 357–361.
- Weiss, R.C., Scott, F.W., 1981a. Pathogenesis of feline infectious peritonitis – pathologic-changes and immunofluorescence. *Am. J. Vet. Res.* 42, 2036–2048.
- Weiss, R.C., Scott, F.W., 1981b. Pathogenesis of feline infectious peritonitis: nature and development of viremia. *Am. J. Vet. Res.* 42, 382–390.