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Suppression of NK cells and regulatory T lymphocytes in cats naturally infected with feline infectious peritonitis virus

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ABSTRACT

A strong cell-mediated immunity (CMI) is thought to be indispensable for protection against infection with feline infectious peritonitis virus (FIPV) in cats. In this study, the role of natural killer (NK) cells and regulatory T cells (Tregs), central players in the innate and adaptive CMI respectively, was examined during natural FIPV infection. When quantified, both NK cells and Tregs were drastically depleted from the peripheral blood, mesenteric lymph node (LN) and spleen in FIP cats. In contrast, mesentery and kidney from FIP cats did not show any difference when compared to healthy non-infected control animals. In addition, other regulatory lymphocytes (CD4+CD25−Foxp3+ and CD3+CD8+Foxp3+) were found to be depleted from blood and LN as well. Phenotypic analysis of blood-derived NK cells in FIP cats revealed an upregulation of activation markers (CD16 and CD25) and migration markers (CD11b and CD62L) while LN-derived NK cells showed upregulation of only CD16 and CD62L. LN-derived NK cells from FIPV-infected cats were also significantly less cytotoxic when compared with healthy cats. This study reveals for the first time that FIPV infection is associated with severe suppression of NK cells and Tregs, which is reflected by cell depletion and lowered cell functionality (only NK cells). This will undoubtfully lead to a reduced capacity of the innate immune system (NK cells) to battle FIPV infection and a decreased capacity (Tregs) to suppress the immunopathology typical for FIP. However, these results will also open possibilities for new therapies targeting specifically NK cells and Tregs to enhance their numbers and/or functionality during FIPV infection.

1. Introduction

Feline infectious peritonitis virus (FIPV) is a coronavirus causing FIP that is characterized by polyserositis, vasculitis and severe lymphopenia (Addie et al., 2009). This disease has fascinated cat researchers for already half a century (Holzworth, 1963) but up until now, it cannot be treated/controlled in an efficient way. None of the conventional antivirals and therapeutics have been found effective against FIPV infection (Hartmann and Ritz, 2008). At present, it is the most important viral cause of death in cats. The current hypothesis states that FIPV arises through mutation from the avirulent coronavirus feline enteric coronavirus (FECV) (Chang et al., 2010). Additionally, FIPV is very immune evasive, especially toward the humoral branch of immunity (Pedersen and Boyle, 1980; Cornelissen et al., 2007; Dewerchin et al., 2008). About 30 years ago, Pedersen and Black (1983) already stated that a strong cell-mediated immune response (CMI) is key to surviving a FIPV infection. Cats with a strong CMI would survive, while

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cats with weaker CMI would develop disease (Pedersen and Black, 1983).

Natural killer cells (NK) are part of the innate branch of CMI and play major roles in anti-viral control (Brandstadter and Yang, 2011). They can lyse virus-infected cells directly, secrete pro-inflammatory cytokines or modulate ensuing innate and adaptive immune responses (Jonjic et al., 2008). Regulatory T cells (Tregs) on the other hand are central players in the adaptive branch of CMI. They are characterized by the expression of CD4, CD25 and Foxp3 and function to maintain an immunological balance that allows to resolve pathogen infection while minimizing immunopathology. Virus-induced enhancement of Treg function and frequencies may lead to persistent infection and possibly pathogen induced pathology while subversion of Tregs (virus or host induced) may cause damaging immunopathology (Belkaid and Rouse, 2005; Li et al., 2008). Foxp3 can also be expressed in other cells than classical Tregs, such as CD4+CD25− cells, CD8+ T cells and CD21+ B cells. These cells have been shown to be implicated in tumor immunology and autoimmunity, where they have an immunosuppressive role (Han et al., 2009; Leavy, 2010; Mauri and Bosma, 2012).

Since both NK cells and Tregs represent important players in CMI and given their crucial role in the development of adaptive immune responses (Kos and Engleman, 1996; Robbins et al., 2007), it is worth to dissect their roles during a FIPV infection. A strong innate NK cell-mediated immune response is often responsible for an acute viral control, exemplified during FIV and cytomegalovirus infections (Zingoni et al., 2005; Howard et al., 2010), while Treg enhancement suppresses anti-viral responses (Li et al., 2008). NK cell deficiencies regularly lead to severe recurrent viral diseases (Wood et al., 2011) and Treg deficiencies lead to damaging pathology (Luhn et al., 2007).

In order to investigate the role of both NK cells and Tregs during a FIPV infection, NK cells, classical Tregs and other Foxp3+ T cell subsets were quantified in immunological compartments (blood, mesenteric lymph node (LN), spleen) and infection sites (mesentery and spleen) of both healthy and FIPV-infected cats. Subsequently, NK cell phenotype and functionality was determined and compared between FIPV-infected cats and healthy cats.

2. Materials and methods

2.1. Antibodies

Monoclonal antibodies against feline CD3ε (NZM1) and against feline CD56 (SZK1) were kindly provided by Dr. Yorihiro Nishimura (Tokyo University, Japan) (Shimojima et al., 2003; Nishimura et al., 2004). Monoclonal antibodies recognizing feline CD25 were produced by the hybridoma 9F23.3 (North Carolina State University, Raleigh, North Carolina, USA). Monoclonal antibodies FE5.4D2, CA16.3E10, YFC120.5 and CA2.1D6 recognizing feline CD8β, canine CD11b, human CD16 and canine CD21 respectively, were acquired from AbD serotec (Dusseldorf, Germany). Monoclonal antibodies CAT30A and 3-4F4 against feline CD4 were purchased from Veterinary Medical Research and Development (VMRD, Pullman, USA) and SouthernBiotech (Birmingham, USA), respectively. A monoclonal antibody DREG56, cross-reactive with feline CD62L, was purchased from Acris antibodies (Hereford, Germany). A monoclonal antibody (10A12) against the N-protein was generated earlier (Dewerchin et al., 2006). During each staining, appropriate isotype-matched controls were used. Conjugated secondary antibodies [Molecular Probes (Invitrogen, Carlsbad, USA)] were goat anti-mouse IgG1 Alexa Fluor 647, goat anti-rat Alexa Fluor 488, goat anti-mouse IgG2a Alexa Fluor 488 and goat anti-mouse IgG3 fluorescein isothiocyanate (FITC). When primary antibodies from the same IgG1 isotype were used, the second primary antibody was labeled with Zenon Alexa Fluor® 488 Mouse IgG1 (Invitrogen, Carlsbad, USA).

2.2. Animals

Eight cats, feline leukemia virus- and feline immunodeficiency virus-negative and strongly suspected of naturally occurring FIP by clinicians (based on cat profile, clinical signs and blood and/or exudate examination) were

Table 1
Cats included in this study. The upper part shows the healthy animals (HC) while the lower part shows the FIP-diseased animals.

Cat	Age (years)	Gender	Breed	A. Effusion	Lesions
HC1	2	♂	European shorthair	N.A.	N.A.
HC2	7	♀	Persian shorthair	N.A.	N.A.
HC3	6	♂	European shorthair	N.A.	N.A.
HC4	2	♂	European shorthair	N.A.	N.A.
HC5	1	♀	European shorthair	N.A.	N.A.
HC6	3	♂	European shorthair	N.A.	N.A.
FIP1	1	♂	European shorthair	+	M K S
FIP2	2	♂	British shorthair	+	M K S
FIP3	5	♂	European shorthair	+	M Li I
FIP4	1	♀	European shorthair	−	K S Lu
FIP5	4	♀	European shorthair	+	K Li
FIP6	1	♂	British shorthair	+	M I
FIP7	2	♀	European shorthair	+	M K I
FIP8	7	♂	European shorthair	+	M K

N.A., Not applicable; A. Effusion, Abdominal effusion present; Lesions: M, Mesentery; K, Kidney; S, Spleen; Li, Liver; I, Intestines; Lu, Lungs.

used in this study. As control animals, six healthy, conventionally housed, feline leukemia virus-, feline immunodeficiency virus and feline coronavirus-negative cats, that had to be euthanized for health-unrelated problems, were selected (Table 1). All owners gave prior informed consent. FIPV infection was confirmed by performing immunohistochemistry using a monoclonal antibody against the N-protein (10A12) in combination with IgG1-specific FITC (Invitrogen) on sections of lesions on internal organs (e.g. kidney, intestine). An appropriate isotype-matched antibody served as control (13D12) (Nauwynck and Pensaert, 1995). The presence of lesions was determined macroscopically (Table 1).

2.3. Target cell line

Crandell feline kidney cells (CRFK) were cultured in MEM medium (GlutaMAX) supplemented with 5% fetal calf serum (FCS) (Greiner Bio-one, Kremsmuenster, Germany), 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, 0.1 mg ml^{-1} kanamycin and 2% lactalbumin (Invitrogen). To use CRFK as target cells, cells in suspension (1×10^6 ml^{-1}) were stained with carboxyfluorescein succinimidyl ester (CFSE) for 10 min at 37 °C.

2.4. Cell isolation from blood and tissue with subsequent processing

Cells from blood and tissue were isolated according to Vermeulen et al. (2012). Traditionally, mononuclear cells from blood are isolated over Ficoll-Paque, which typically produces a variable yield. Additionally, blood analysis by clinical labs takes some time to be performed, which can have a deleterious effect on cell viability during transport and subsequently on cell frequencies and functions (Bull et al., 2007). That is why in these experiments, the absolute amount of cells was calculated with a combination of several methods. First, 10 ml of blood was taken from *the vena jugularis* in heparin (15 U ml^{-1}) (Leo, Zaventem, Belgium). Then, a blood smear was prepared and subsequently stained with a diff-quick staining (Gomez-Ochoa et al., 2012). This staining provided the percentage of the lymphocyte population in the total white blood cell population. This percentage was then applied to the total white blood cell count from a diagnostic analysis. This gave accurate absolute lymphocyte counts in the whole blood. Finally, blood mononuclear cells were separated on Ficoll-Paque (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Staining of the mononuclear cells (vide infra) then allowed identification and absolute quantification of all lymphocyte subsets. One cm^2 of kidney cortex, containing lesion tissue if present, and most of the mesentery was dissected from all cats and weighed afterwards. Cells in tissue were isolated by passing subsequently through tissue grinders (250 μM mesh) (Sigma–Aldrich, St. Louis, Missouri, USA) and cell strainers (70 μM mesh) (Becton, Dickinson and Company, New Yersey, USA). After isolation, cells were counted, frozen (PTLPD81, Orthodyne, Alleur, Belgium) and stored at −196 °C in liquid nitrogen.

2.5. Quantification and phenotyping of natural killer cells

Phenotyping of NK cells was performed as previously described (Vermeulen et al., 2012). Briefly, a minimum of 1×10^6 isolated cells was stained at 4 °C for the surface molecules CD8, CD11b, CD16, CD25, CD62L in combination with CD56 and CD3. Analysis was done on a FACScanto flow cytometer using FACSDiva software (BD Biosciences, Mountain View, California, USA).

2.6. Quantification of Foxp3+ subsets

Frozen isolated cells (1×10^6) were thawed and immediately stained for phenotypic analysis in RPMI supplemented with 1 mM ethylenediaminetetraacetic acid (EDTA). Cells were incubated for 20 min at 4 °C while gently shaking the cells, both with the primary and dye-conjugated secondary antibodies. Cells were washed with cold RPMI with EDTA and centrifugated at 300 × g for 10 min at 4 °C. After staining of surface molecules (CD3, CD4, CD8, CD21 and CD25) cells were fixed with the fixation/permeabilization kit optimized for staining of intracellular Foxp3. Cells were then stained with anti-Foxp3 antibody, directly conjugated with AF647. Analysis was done on a FACScanto flow cytometer using FACSDiva software (BD Biosciences, Mountain View, California, USA).

2.7. Natural killer cell purification

As previously described, NK cells were identified through CD3 and CD56 staining followed by cell sorting on a FACS ARIAIII flow cytometer (BD Biosciences) (Vermeulen et al., 2012). Typical NK (CD3−CD56+) cell counts were between 5×10^3 and 2×10^4 cells ml^{-1} blood or μg tissue, while NKT (CD3+CD56+) cell counts varied between 5×10^2 and 2×10^3 cells ml^{-1} blood or μg tissue. Purity of the sorted cell populations was routinely >97%.

2.8. NK functionality assay

The functionality assay was performed as previously described, with minor modifications (Vermeulen et al., 2012). Briefly, 5×10^4 target cells (CFSE-stained CRFK) were seeded in V-bottomed 96-well plates (Nunc, Langenselbold, Germany). Subsequently, target cells were cocultured for 4 h with a varying amount of activated NK cells (activated with rHu IL-2 for 18 h (Invitrogen)). Evaluated effector/target cell ratios were: 0–1–5–10. The percentage of lysed cells was calculated as:

$$\%\text{lysis} = 100 \times \frac{(\text{number lysed target cells with effector cells} - \text{number lysed target cells in control})}{\text{Number of target cells}}$$

2.9. Statistical analysis

Statistical analyses were performed with SPSS 20.0 (SPSS Inc., Chicago, IL, USA). For all data, differences between medians were assessed by Mann–Whitney *U* tests. Differences were considered significant when $P \leq 0.05$.

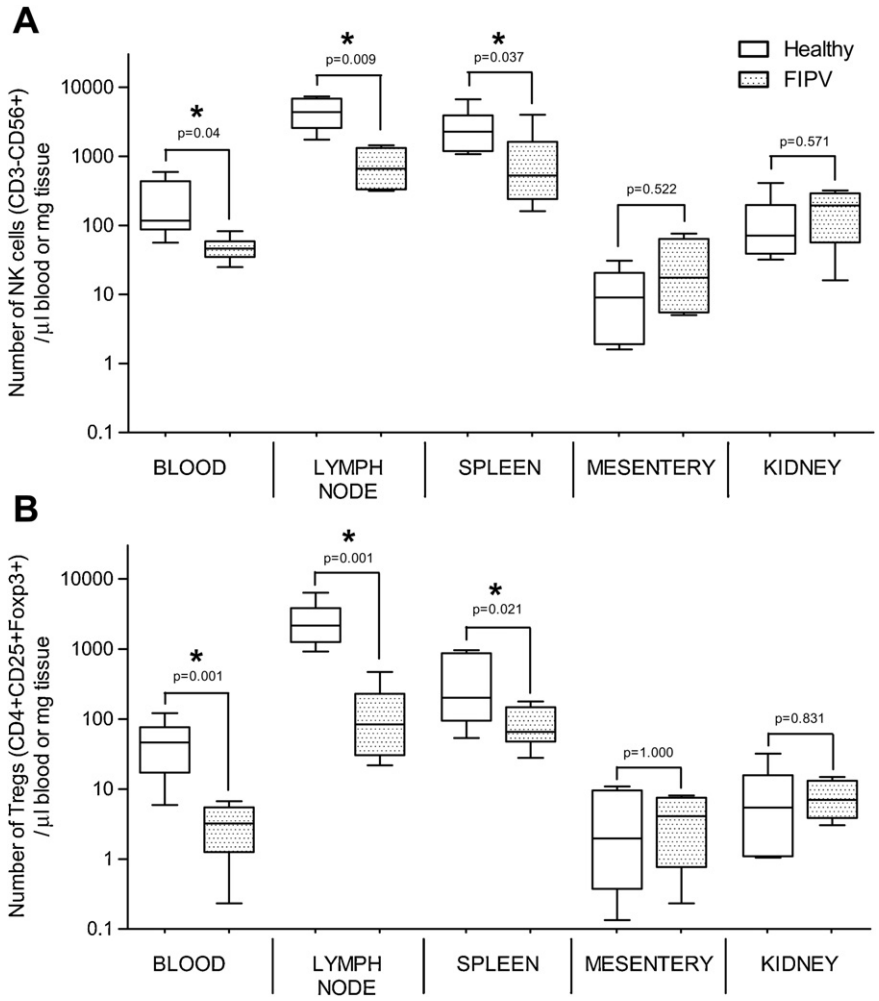

Fig. 1. Absolute number of NK cells (CD3−CD56+) and Tregs (CD4+CD25+Foxp3+) for healthy control cats and cats naturally infected with FIPV. Values are depicted as box-and-whisker plots where the whiskers refer to the minimum and maximum values ($n = 6$ for healthy cats; $n = 8$ for FIPV cats). Comparison of numbers was done for healthy and FIPV-infected cats in each compartment. Significant differences ($P \leq 0.05$) are indicated with an asterisk.

2.10. Animal welfare

This study was performed according to animal welfare guidelines. Under the application EC2012/043, this research was positively evaluated by the ethical committee of the Faculty of Veterinary Medicine, Ghent University

3. Results

3.1. Lymphocyte frequencies during FIPV infection

In order to evaluate NK frequencies, cells extracted from blood, mesenteric lymph node, spleen, mesentery and kidney were stained with mAbs against CD56 and CD3. Fig. 1A shows the absolute numbers in each compartment in healthy and FIPV-infected cats. For blood, an average of $212 \pm 198\ \mu l^{-1}$ and $48 \pm 19\ \mu l^{-1}$ NK cells was found in healthy and FIP cats respectively. Average NK cell counts in 1 mg lymph node (LN), spleen, mesentery and kidney of healthy (H) and FIP (F) cats were $4631 \pm 2233\ mg^{-1}$ (H) and $790 \pm 504\ mg^{-1}$ (F), $2748 \pm 2049\ mg^{-1}$ (H) and $1047 \pm 1456\ mg^{-1}$ (F), $12 \pm 11\ mg^{-1}$ (H) and $29 \pm 33\ mg^{-1}$ (F) and $126 \pm 138\ mg^{-1}$ (H) and $181 \pm 125\ mg^{-1}$ (F), respectively.

To quantify classical Tregs, cells were triple stained for CD4, CD25 and Foxp3. Treg staining (CD4+CD25+Foxp3+) (Fig. 1B) in the blood of healthy and FIP cats revealed an average of $50 \pm 38\ \mu l^{-1}$ and $3 \pm 2\ \mu l^{-1}$ Tregs, respectively. In LN, spleen, mesentery and kidney, the average Treg counts for healthy and FIP cats were $2780 \pm 1804\ mg^{-1}$ (H) and $140 \pm 154\ mg^{-1}$ (F), $403 \pm 384\ mg^{-1}$ (H) and $87 \pm 56\ mg^{-1}$ (F), $4 \pm 4\ mg^{-1}$ (H) and $4 \pm 3\ mg^{-1}$ (F) and $9 \pm 12\ mg^{-1}$ (H) and $8 \pm 5\ mg^{-1}$ (F), respectively.

Since general lymphopenia is a hallmark of FIPV infection, T and B cell counts were determined in blood and all tissues with CD3 and CD21 stainings (Fig. 2). Results revealed significantly different T cell counts between healthy and FIP cats in blood $2206 \pm 1160\ \mu l^{-1}$ (H) and $509 \pm 618\ \mu l^{-1}$ (F), LN $43{,}859 \pm 22{,}551\ mg^{-1}$ (H) and $13{,}371 \pm 15{,}881\ mg^{-1}$ (F) and spleen $17{,}198 \pm 12{,}956\ mg^{-1}$ (H) and $3924 \pm 1704\ mg^{-1}$ (F), while significantly different B cell counts were only seen in blood $912 \pm 489\ \mu l^{-1}$ (H) and $266 \pm 239\ \mu l^{-1}$ (F).

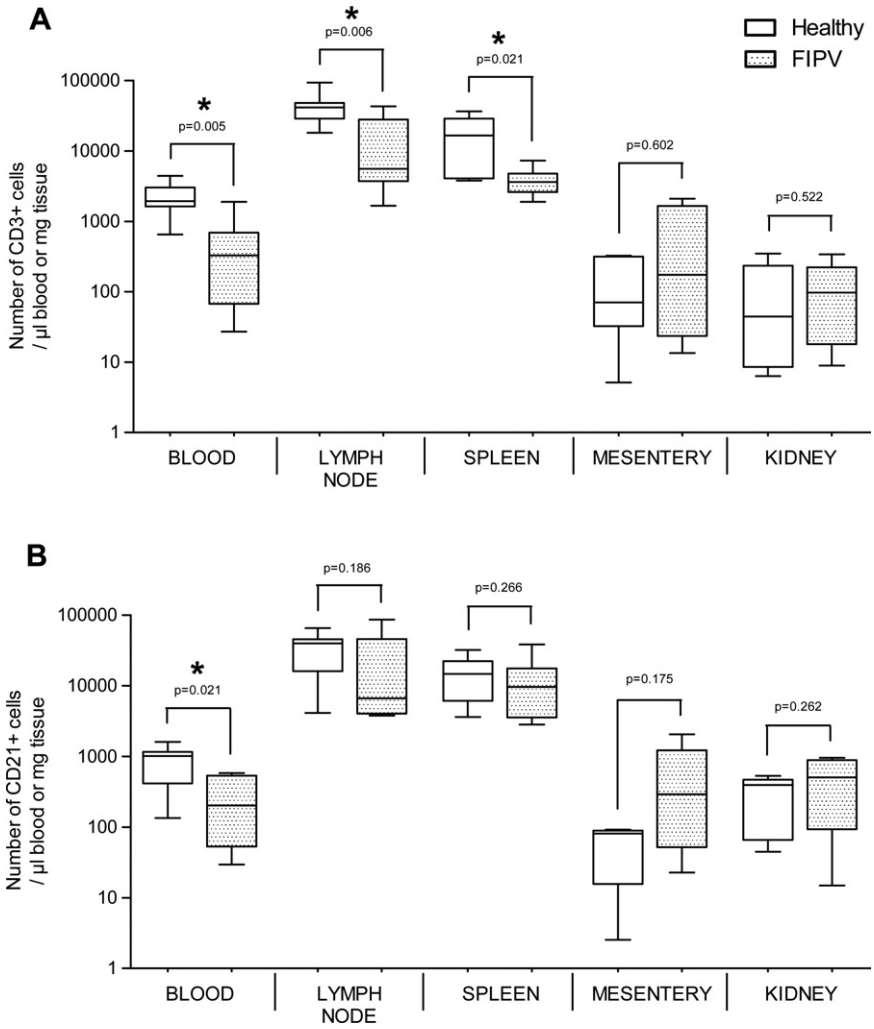

Fig. 2. Absolute number of T cells (CD3+) and B cells (CD21+) for healthy control cats and cats naturally infected with FIPV. Values are depicted as box-and-whisker plots where the whiskers refer to the minimum and maximum values ($n = 6$ for healthy cats; $n = 8$ for FIPV cats). Comparison of numbers was done between healthy and FIPV-infected cats in each compartment. Significant differences ($P \leq 0.05$) are indicated with an asterisk.

3.2. Assessment of NK and Treg lymphopenia

To compare the degree of lymphopenia in the NK cell compartment to other lymphocyte populations, cell ratios were determined relatively to the amount of T and B cells (Fig. 3). Results revealed significant differences only between ratios determined on cells isolated from the mesentery—NK/T 0.276 ± 0.071 (H) and 0.093 ± 0.009 (F) and NK/B 0.510 ± 0.240 (H) and 0.072 ± 0.019 (F).

Similar to NK cells, Treg ratios were determined relatively to the total amount of T (CD3+, CD4+ and CD8+) and B cells (Fig. 4). No significant differences could be seen in any of the compartments with the exception of the LN. Here, FIPV Treg ratios to all lymphocyte subsets were significantly lower when compared to healthy cats: Treg/CD3 0.059 ± 0.039 (H) and 0.020 ± 0.026 (F), Treg/CD4 0.066 ± 0.036 (H) and 0.032 ± 0.048 (F), Treg/CD8 0.096 ± 0.055 (H) and 0.030 ± 0.033 (F) and Treg/CD21 0.062 ± 0.033 (H) and 0.019 ± 0.020 (F).

3.3. Other Foxp3+ regulatory subsets

Several recent studies found important roles for other Foxp3 expressing lymphocytes in different immunological contexts. To examine the role of these subsets, Foxp3 expression was analyzed in CD4+CD25−, CD3+CD8+ and CD21+ cell populations (Fig. 5). When the absolute numbers of Foxp3+ cells were compared between healthy and FIP cats, significantly lower CD4+CD25−Foxp3+ numbers could be seen in the blood $8.7 \pm 9.2\ \mu l^{-1}$ (H) and $2.6 \pm 3.1\ \mu l^{-1}$ (F) as well as in the LN compartment $1188.3 \pm 815.5\ mg^{-1}$ (H) and $71.6 \pm 49.1\ mg^{-1}$ (F) of FIP cats. The same compartments also had a significantly lower amount of CD3+CD8+Foxp3+ in FIP cats, in blood: $8.9 \pm 9.3\ \mu l^{-1}$ (H) and $2.5 \pm 3.1\ \mu l^{-1}$ (F) and in LN: $170.1 \pm 200.9\ mg^{-1}$ (H) and $9.6 \pm 7.2\ mg^{-1}$ (F). Kidney tissue of FIPV-infected cats on the other hand, had significantly higher amounts of CD3+CD8+Foxp3+ cells in FIP cats $1.1 \pm 0.5\ mg^{-1}$ (H) and $9.1 \pm 10.3\ mg^{-1}$ (F).

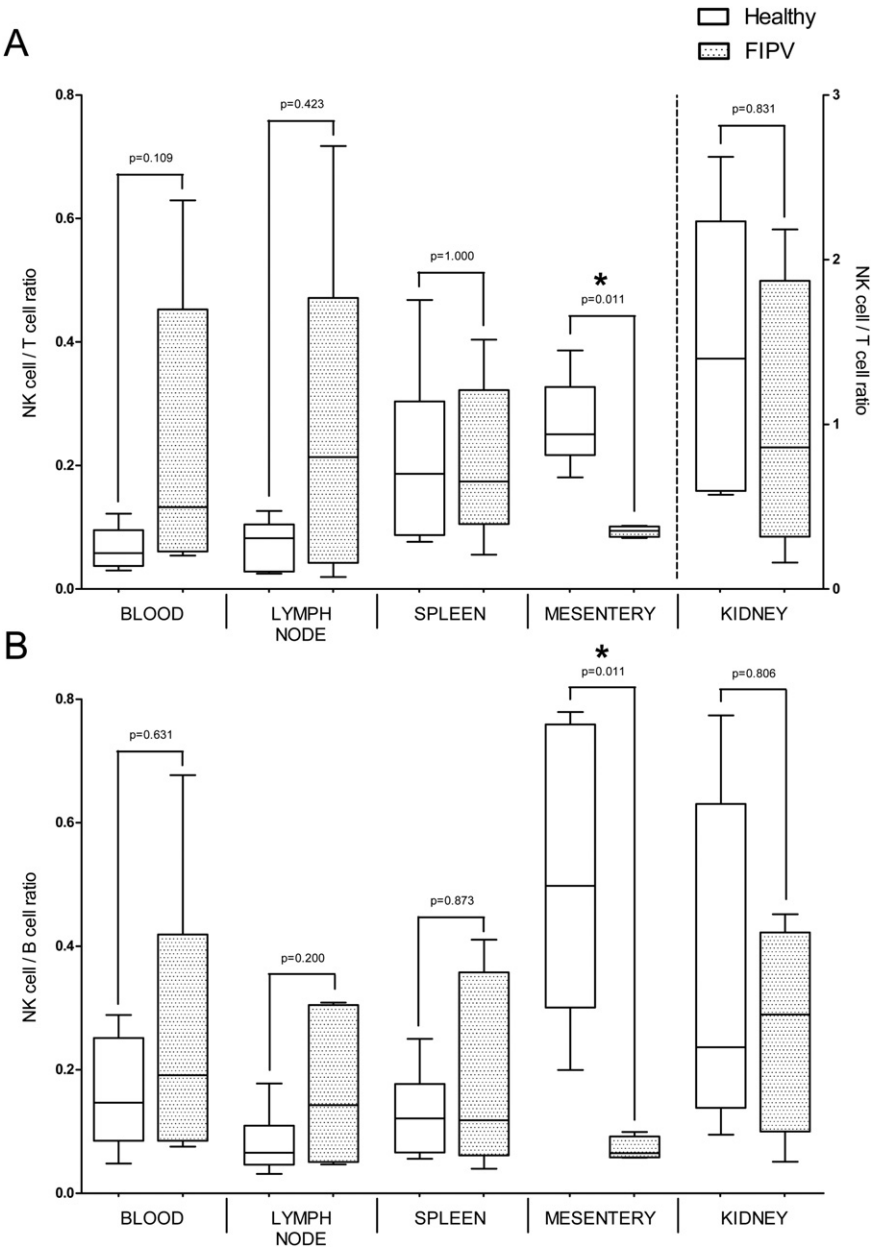

Fig. 3. Cell ratio of NK cells (CD3−CD56+) relative to T cells (A) and B cells (B) for healthy control cats and cats naturally infected with FIPV. Values are depicted as box-and-whisker plots where the whiskers refer to the minimum and maximum values ($n = 6$ for healthy animals and $n = 8$ for FIPV-infected animals). Comparison of numbers was done for healthy and FIPV-infected cats in each compartment. The value right of the dotted line is plotted on the right *Y*-axis. Significant differences ($P \leq 0.05$) are indicated with an asterisk.

3.4. Phenotypical analysis of NK cells

Determining the cell phenotype can provide much information on the functional, migratory and/or activation status of a cell. NK surface phenotype was performed on cells extracted from blood, lymph node and spleen of healthy and FIPV-infected cats. Cells from mesentery and kidney were too low in numbers to allow this analysis. Surface molecules that were co-evaluated on NK cells (CD56+CD3−) were CD8, CD11b, CD16, CD25 and CD62L (Fig. 6).

When CD3 expression was compared between healthy and FIP cats, only CD56+ cells extracted from LN showed a significant difference $33.5 \pm 10.6\%$ (H) and $19.7 \pm 9.4\%$ (F) (Fig. 6). No differences could be detected in CD8 cell surface expression. CD11b expression however was fourfold higher on blood NK cells from FIP cats $11.9 \pm 9.2\%$ (H) and $44.4 \pm 25.3\%$ (F). Staining for CD16 showed that a significantly higher number of NK cells isolated from blood and LN expressed this Fcγ receptor in FIP cats (blood $76 \pm 12\%$ (H) and $90.9 \pm 9.5\%$ (F) and LN $70.1 \pm 6.5\%$ (H) and $80.5 \pm 8.2\%$ (F)). The activation marker CD25 was also expressed on a

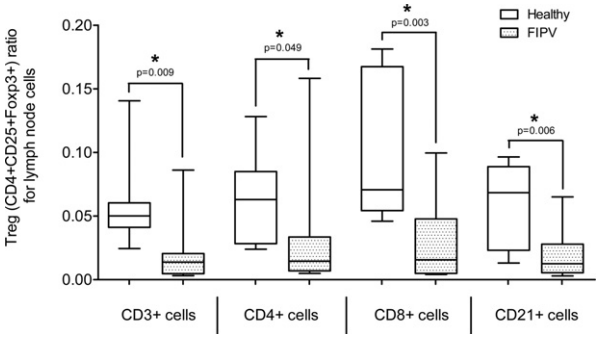

Fig. 4. Lymph node Treg cell ratio (CD4+CD25+Foxp3+) relative to CD3+ (T cells), CD4+, CD8+ and CD21+ cells (B cells) for healthy control cats and cats naturally infected with FIPV. Values are depicted as box-and-whisker plots where the whiskers refer to the minimum and maximum values ($n = 6$ for healthy animals and $n = 8$ for FIPV-infected animals). Comparison of numbers was done for healthy and FIPV-infected cats in the lymph node. Significant differences ($P \leq 0.05$) are indicated with an asterisk.

higher proportion of NK cells isolated from blood in FIP compared to healthy cats: 43.1 ± 18.8% (H) and 77.3 ± 14.6% (F). Finally, the cell adhesion molecule CD62L (L-selectin) was expressed three times more on blood- and LN-derived NK cells in FIP cats compared to healthy cats (blood 13.3 ± 12.4% (H) and 39.9 ± 17.5% (F) and LN 14.3 ± 7.1% (H) and 41.3 ± 21.5% (F)).

3.5. NK functionality

Analysis revealed that LN-derived NK cells from FIP cats were significantly less functional than NK cells from control cats (Fig. 7A). In the LN, for an E:T ratio of 1, 5 and 10, the percentage of lysed cells was 24.3 ± 5.6% (H) and 11.3 ± 5.2% (F), 41.6 ± 7.2% (H) and 25.1 ± 9.0% (F) and 61.3 ± 5.8% (H) and 35.4 ± 12.3% (F), respectively. In the spleen, no difference in NK cytotoxicity could be noted for E:T ratios 1 and 5 (Fig. 7B). Similar to NK cells, LN-derived NKT cells defined here as CD56+CD3+, isolated from healthy cats demonstrated a higher cytolytic activity (15.2 ± 6.0%) than NKT cells from FIP cats (1.8 ± 0.4%) at an E:T ratio of 1. Finally, NKT cells from spleen of both healthy and FIPV-infected cats were unable to lyse CRFK target cells

4. Discussion

With the recent development of several cross-reactive or cat-specific monoclonal antibodies, more extensive analysis of specialized lymphocyte subsets, such as NK cells and Tregs has become possible (Lankford et al., 2008; Vermeulen et al., 2012). Since these cells are involved in many virus-induced diseases and FIPV development is thought to be dependent on cell mediated immunity, this study aimed to investigate the role of these cells during a natural FIPV infection.

First, absolute numbers of NK cells and Tregs were quantified in FIPV-infected cats and compared to healthy cats. This showed a drastic decrease in NK cell numbers for FIP cats in all immunologic compartments evaluated, with a five-, six- and three-fold reduction in blood, LN and spleen, respectively. For Tregs, a 17-fold, 20-fold and 4-fold reduction was noted in blood, LN and spleen, respectively. Remarkably, mesentery and kidney did not show any difference in NK cell or Treg numbers while these tissues are often involved in FIPV pathology with the formation of severe vasculitis and granulomas (Addie et al., 2009).

NK cell lymphopenia is also seen during other viral infections, both human and feline. Explanations for this lymphopenia vary from virus-induced NK cell apoptosis to sequestration and altered trafficking of NK cells (Denney et al., 2010) (He et al., 2005; Mao et al., 2009; Howard et al., 2010). The Treg depletion that is seen during FIPV infection, somewhat contrasts other viral infections, which are nearly always associated with either an increase in Treg frequency or function (Keynan et al., 2008). One exception is HIV infection, which is characterized by decreased peripheral Treg numbers. However, the latter appears to result from Treg trafficking to lymph nodes and other places of viral replication (Andersson et al., 2005). More recent studies indicate that this Treg migration to sites of virus replication and lymphoid tissue is even vital for proper immune regulation (Belkaid and Tarbell, 2009; Campbell and Koch, 2011). In the current study, the opposite was noted, with extreme Treg depletion in all examined immunologic compartments. In addition, no evidence of elevated Treg numbers in inflamed tissue could be found. This depletion may likely contribute to the uncontrolled inflammatory responses that cause the typical immunopathology, which is lethal to FIPV-infected cats.

Besides regular Tregs (CD4+CD25+Foxp+), Foxp3+ regulatory cells also encompass CD4+CD25−, CD8+ and CD21+ regulatory cells (Morgan et al., 2005; Mizoguchi and Bhan, 2006; Noh et al., 2010). These cells have also been detected in cats (Lankford et al., 2008) and we verified the existence of these subsets in cats and detected a significant reduction of CD8+ Tregs and CD4+CD25− Tregs in both blood and LN of FIPV-infected cats. Curiously, a significantly elevated number of CD8+ Tregs was noted in the kidney of FIPV-infected cats. This could imply an immunoevasive strategy of FIPV, by upregulating CD8+ Treg numbers and thus suppressing immunity at sites of infection. These cells, currently a hot topic in human immunology, seem to be implicated in tumor immunology and autoimmunity (Han et al., 2009; Leavy, 2010; Mauri and Bosma, 2012).

Lymphopenia is commonly present in FIP cats in both the T and B cell population in all immunological compartments (blood, LN and spleen) (Haagmans et al., 1996; Takano et al., 2007). This was verified in our setup through staining of CD21 and CD3 molecules. Indeed, severe lymphopenia was noticed in the T cell compartment with a four-, three- and four-fold reduction in numbers in blood, LN and spleen respectively. B cells however, were only significantly lowered in the blood with a three-fold reduction. These results are in agreement with those from Paltrinieri et al. (2003) who found a similar reduction in both the T and B cell compartment in blood from FIP cats.

To evaluate if NK cells and Tregs were more sensitive to lymphopenia than T or B cells, their depletion was

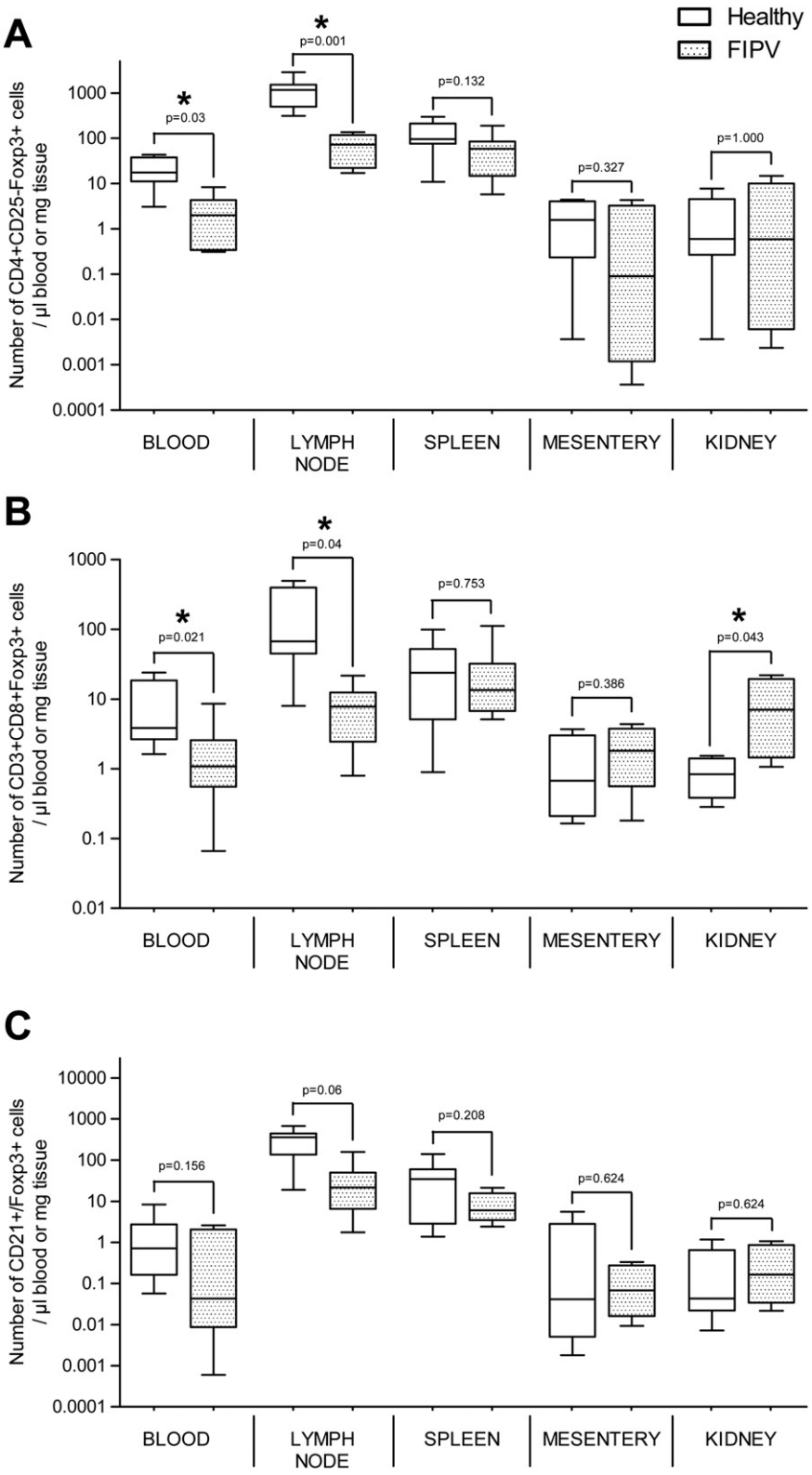

Fig. 5. Absolute quantification of several regulatory Foxp3+ subsets CD4+CD25−Foxp3+ (A), CD3+CD8+Foxp3+ (B) and CD21+Foxp3+. Values are depicted as box-and-whisker plots where the whiskers refer to the minimum and maximum values ($n = 6$ for healthy cats; $n = 8$ for FIPV cats). Comparison of numbers was done for healthy and cats naturally infected with FIPV in each compartment. Significant differences ($P \leq 0.05$) are indicated with an asterisk.

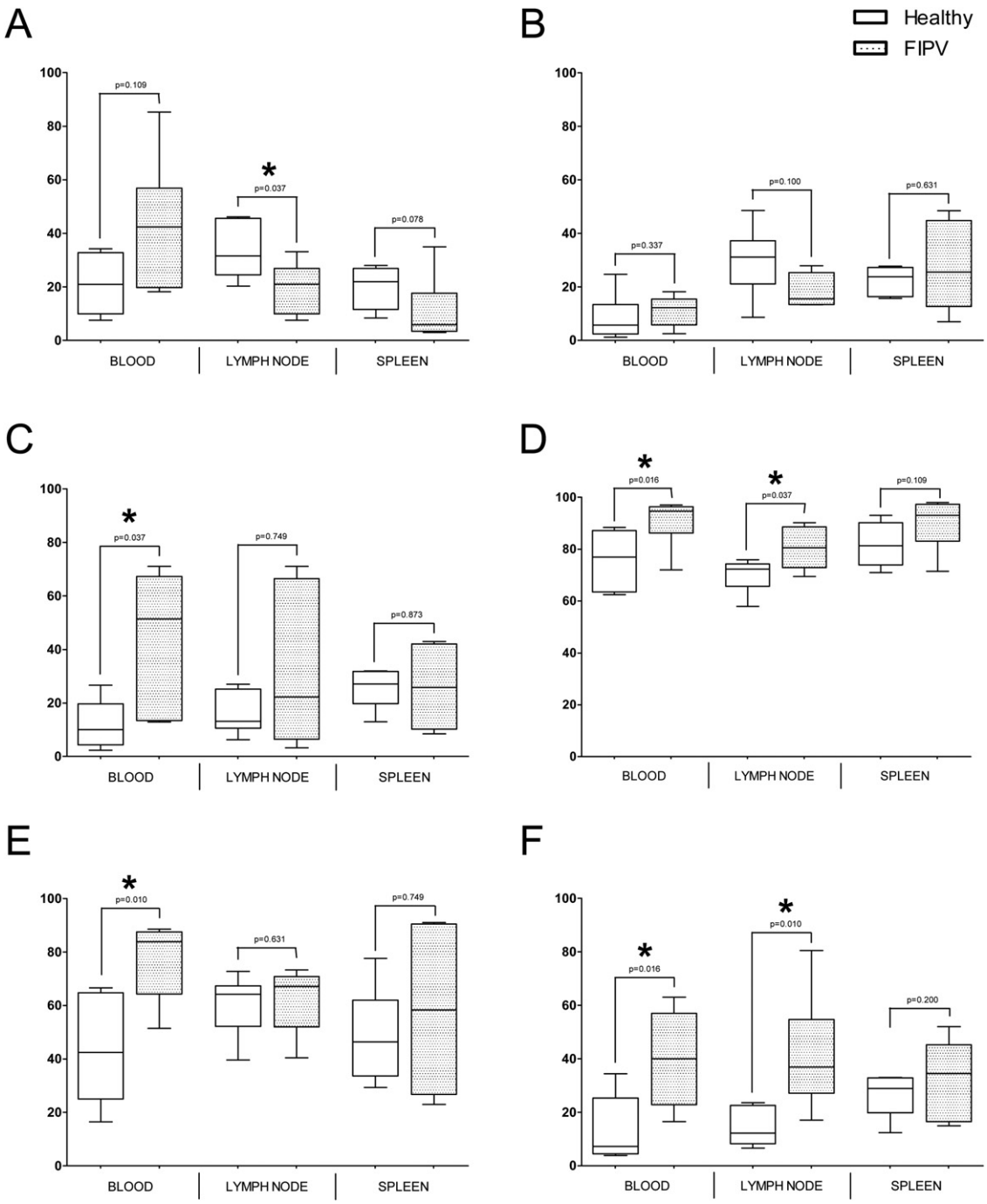

Fig. 6. Comparison of the expression level of CD3 (A) on CD56+ cells and of 5 other markers: CD8 (B), CD11b (C), CD16 (D), CD25 (E) and CD62L (F) on NK cells (CD3−CD56+) from blood, mesenteric lymph node and spleen between healthy cats and naturally infected FIPV cats. Values are depicted as box-and-whisker plots where the whiskers refer to the minimum and maximum values ($n = 6$ for healthy animals and $n = 8$ for FIPV-infected animals). Significant differences ($P \leq 0.05$) are indicated with an asterisk.

determined relatively to T or B cells. Except for NK cells originating from the mesentery and Tregs originating from the LN, no significant differences could be observed. In the LN, Treg depletion was even more pronounced than in other lymphocyte populations, which may further influence immune responses since Treg homing to lymphoid tissue has been shown to be crucial for proper immune functioning (Zhang et al., 2009). This indicates that the T and B cell depletion, typical for FIP cats, is also present to a comparable extent in the NK cell and Treg compartment. This depletion might be the result of apoptosis, like was found for T and B cells. Roles for several apoptosis-inducing factors (TNF-α, TRAIL and Fas ligand) have already been proposed (Takano et al., 2007), but need to be verified.

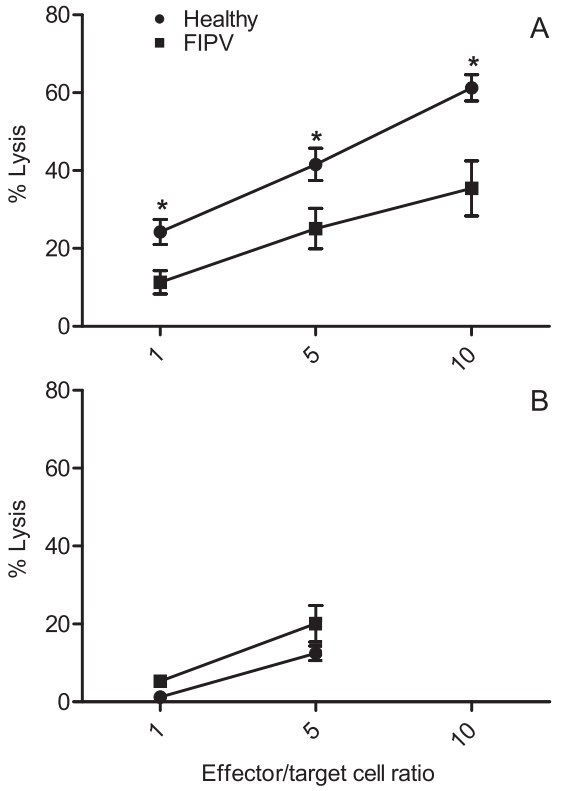

Fig. 7. Percentage of lysed target cells (CRFK) when cocultured with different amounts of NK cells isolated from (A) LN and (B) spleen. Values are shown as mean ± SEM and were compared between healthy and FIPV cats ($n = 3$). Significant differences ($P \leq 0.05$) are indicated with an asterisk.

In addition to cell frequency, cell phenotype also plays an important role in the functionality of a particular cell type (Morishima et al., 2006). Hence, the phenotype of NK cells originating from blood, LN and spleen were evaluated similar to Vermeulen et al. (2012) in healthy control and FIPV-infected cats. Due to low cell yield from the mesentery and kidney tissues, this analysis was not possible in these particular tissues.

A four-time increase in the percentage of blood-derived NK cells expressing CD11b might indicate that peripheral NK cells show a more mature phenotype in FIP cats, since CD11b expression is associated with maturation in both murine and human NK cells (Fu et al., 2011). In addition, FIPV NK cells could be more prone to migration to inflammatory sites or interaction with the complement system, since CD11b dimerizes with CD18 to form the adhesion molecule/complement receptor macrophage-1 antigen (Mac-1 or CR3) (Crozat et al., 2011). However, the latter seems unlikely since no significant higher amount of NK cells could be detected in FIPV-infected kidney or mesentery.

Similar to CD11b, FIPV infection elevated the percentage CD16+ blood-derived NK cells. So at least in terms of CD16-expression, peripheral NK cells from FIP cats seem to be better equipped for ADCC. Although the relative increase was significant, the five-fold reduction in absolute NK cell numbers seen earlier, in addition to the absence of viral proteins in 50% of FIPV-infected cells and the rapid internalization of cell-surface bound antibodies in infected cells, somewhat lowers the impact of this finding (Cornelissen et al., 2007; Dewerchin et al., 2008).

CD25 was also expressed on a higher percentage of blood-derived NK cells in FIP cats versus healthy cats. So even when NK cell numbers are significantly lower in FIP cats, their potential to proliferate appears to be much higher in comparison with normal healthy cats. This could indicate an attempt of the cat's immune system to counteract the lymphopenia typical for FIPV disease.

In addition to CD11b, CD16 and CD25, CD62L or L-selectin was also present on a higher percentage of blood-derived NK cells from FIP cats compared to healthy controls. The relative increase that was seen in our experiments could indicate that peripheral NK cells in FIP cats are more equipped to either migrate to the lymphoid tissues or the inflamed tissues that are ubiquitously present in an average FIPV diseased cat. However, NK cell enumeration did not reveal an increase in NK numbers in inflamed mesentery and kidney and even a major decrease in the LN. Another possibility might be that certain subsets (e.g. CD62L−) of NK cells are more sensitive to the FIPV induced lymphopenia, resulting in a relative increase in CD62L+ NK cell numbers. This is not necessarily a disadvantage for the FIPV diseased cats, since recent developments in human immunology indicate that CD62L could be used to define an intermediate state of NK cell maturation that performs both the cytotoxic as well as the cytokine producing functions, two functions that are normally divided between $CD56^{dim}$ and $CD56^{bright}$ NK subsets in blood (Juelke et al., 2010). This hypothesis however needs further examination with respect to feline NK cell maturation stages.

In the LN of FIP cats, significantly more NK cells expressed CD16 and CD62L. This increase was comparable with that in the blood. Similar results were seen in activated, inflamed human LN where significant increases in CD16+ NK cell frequencies were noted in comparison to non-inflamed LN (Romagnani et al., 2007). The authors envisaged a system where, very early during an immune response, immature NK cells are recruited to LN from where they recirculate after maturation (e.g. acquisition of CD16). Our findings (relative CD16+ NK cell increase in both blood and LN and a relative increase of CD62L+ LN NK cells) agree with this hypothesis, giving the LN a central role in FIPV immunity. Lastly, FIPV LN also showed a significant decrease in relative NKT cell numbers (CD3+CD56+). This enigmatic subset often plays a crucial role in promoting and enhancing early innate and adaptive responses as has been shown for herpes simplex virus (Grubor-Bauk et al., 2008). Virus-induced loss of these cells could compromise the host's capacity to control early virus infection and spread, resulting in higher viral loads as is the case for lymphocytic choriomeningitis virus (LCMV) and HIV (Tessmer et al., 2009).

Unlike blood- and LN-derived NK cells, splenic NK cells did not show any differences in phenotype. Although the spleen has major roles in developing both innate and cellular immunity, it also differs quite a lot from other lymphoid tissues, especially with regard to cell migration. After all, in contrast to lymph nodes and Peyers patches,

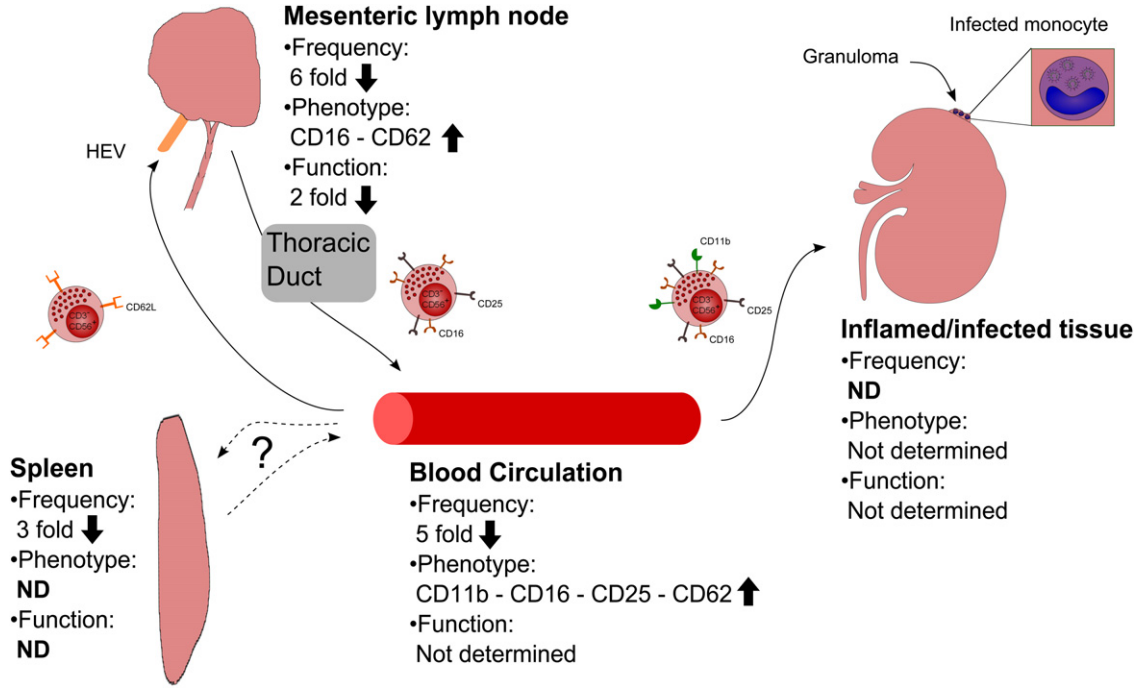

Fig. 8. Schematic overview of NK cell numbers, phenotype and function that are altered during an infection with FIPV. ND = not significantly different. HEV = high endothelial venule.

that mainly receive cells from the lymph fluid, the spleen monitors cells directly from the blood (Mebius and Kraal, 2005). This is also true for cat spleens, since they lack deep lymphatics (Blue and Weiss, 1981).

To evaluate if the more activated NK phenotype in FIP cats leads to higher cytolytic capacities, a functional assay was performed on CRFK cells, similar to Vermeulen et al. (2012). This test was only performed on NK (CD3−CD56+) cells from LN and spleen, since NK cell counts in blood from FIP cats were too low. NKT cells (CD3+CD56+) were also included in the functional assay, since these cells have recently been shown to possess NK-like cytotoxic abilities (Kuylenstierna et al., 2011). Results revealed NK and NKT cells isolated from LN in FIP cats to be significantly less cytotoxic while splenic NK and NKT cells showed no differences in cytotoxicity. This indicates that, even though NK cells from FIP cats show a more activated phenotype and relatively more CD16+ cells, they display a reduced capacity to lyse target cells in vitro, in comparison with NK cells from healthy cats. Normally, viruses induce a NK response that is characterized by a higher cytolytic capacity (e.g. Herpes simplex virus 1 (HSV-1)) (Long et al., 2010). Some viruses however, have evolved to induce lower functionality in NK cells (e.g. HIV and Hepatitis C virus (HCV)) (Meier et al., 2005). One explanation for the lower cytotoxicity could be a proportional dysregulation of functional subsets, similar to the dysregulation seen in HIV-patients where less CD16+ NK cells (the cytotoxic subset) are detected. However, the opposite was found during FIPV infection, i.e. higher amounts of CD16+ cells. Another possibility could be a lowered granulation, a prerequisite for inducing cytolysis. To test this hypothesis, several antibodies against CD107a were tested. Unfortunately, none of these showed cross reactivity with the cat system. Finally, differences in NK cell homeostasis between several subsets could be present, through for example different survival or generation rates, which could lead to a proportional dysregulation similar as proposed for HIV infection (Meier et al., 2005). Splenic NK cells did not reveal any differences in functionality between healthy and FIP cats. This could be the consequence of the different anatomy of the feline spleen, resulting in different trafficking patterns, which could take the edge of any virus-induced alternations in frequency, phenotype or functionality (Blue and Weiss, 1981; Mebius and Kraal, 2005). Analysis of Treg functionality was not possible due to too low cell numbers in immunological compartments.

The impact of the characteristics of individual FIP cats on the results is difficult to assess. Tissue from FIP-mesentery and -kidney without lesions showed comparable results to tissue with lesions, both with regard to cell frequency and functionality. Similarly, results from the cat without effusion also showed no difference with the effusive cats. This indicates that both cell frequency and function, are not associated with the disease pattern (effusive–non effusive) that follows infection nor in the disease severity (presence of lesions). Possibly, there are other cellular immunity associated factors, such as cytokine production and cell-trafficking behavior, that determine which disease type follows infection with FIPV. In addition, the difference between effusive and dry FIP in practice is rather challenging with cats commonly possessing a mixture of the clinical signs typical for the

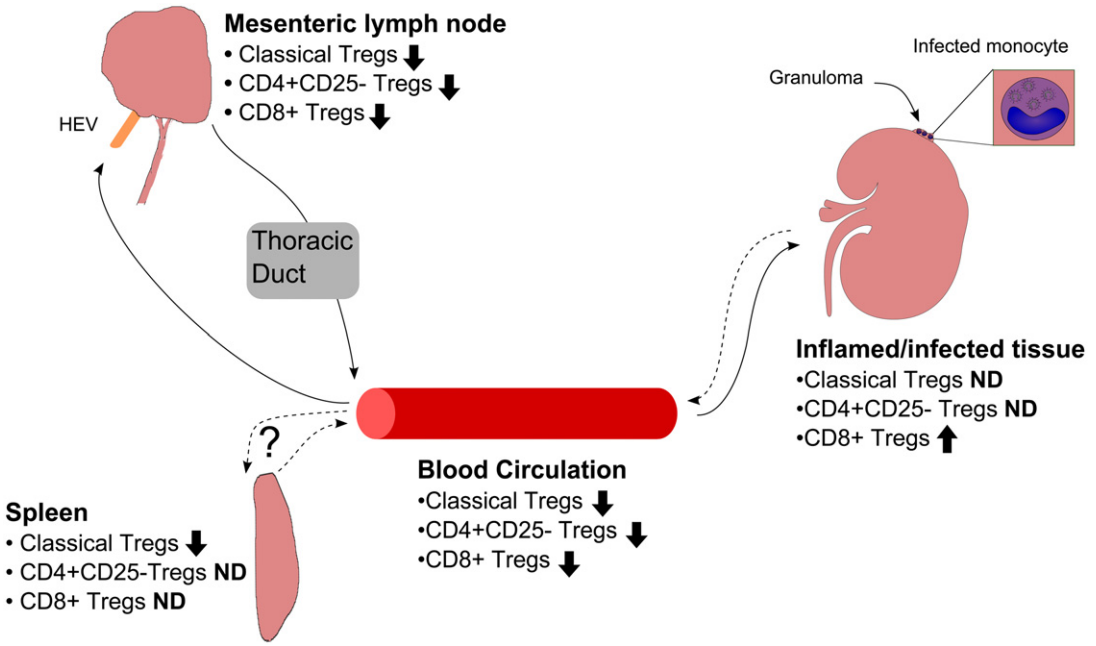

Fig. 9. Schematic overview of Foxp3+ regulatory cell numbers that are altered during an infection with FIPV. ND = not significantly different. HEV = high endothelial venule.

wet or the dry form (Berg et al., 2005; Hartmann, 2005). The severity of the clinical signs, with regard to the magnitude of granulomas and the affected organs is also very diverse. To draw conclusions from immunity studies, with regard to the type of FIP, would demand careful selection of animals, with clinical signs belonging exclusively to the dry or wet type.

In conclusion, FIPV infection is characterized by constitutive low amounts of NK cells in peripheral blood, mesenteric lymph nodes and spleen, while normal amounts of NK cells are found in infected tissue, despite the more migratory phenotype (i.e. higher expression of CD11b and CD62L). NK cells in FIP cats also showed a higher activation phenotype with higher expression of CD16 and CD25, but this did not translate in higher cytolytic activity, possibly due to virus-induced changes in NK cell biology or activation induced cell death (Fig. 8). Similarly, Treg frequencies are significantly reduced in all immunological compartments, while no reduction in numbers could be detected in inflamed tissue. In the mesenteric lymph node, the relative reduction in Treg numbers was even greater than for T and B cells (Fig. 9). Additionally, CD8+ and CD4+CD25− Tregs were also found to be depleted in blood and LN from FIPV cats while elevated CD8+ Treg numbers were observed in inflamed kidneys. The role of these findings however needs additional research.

5. Conclusion

This study determined for the first time that FIPV-induced lymphocyte depletion also encompasses the NK and Treg lymphocyte compartments. Additionally, despite their activated and migratory phenotype, NK cells were found to be less cytotoxic. These findings will translate in a reduced capacity of the innate immunity (NK cells) to battle acute virus infection and to aid ensuing immune responses and in a reduced capacity of the adaptive response (Tregs) to suppress the damaging inflammation that will lead to tissue damage. Further research, including more extensive cytokine and receptor profiling, will propose candidates for treatments that improve NK cell and Treg function and survival in order to strengthen the cats' immune response to FIPV infection

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