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Relationship of the mucosal microbiota to gastrointestinal inflammation and the presence of small cell intestinal lymphoma in cats

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Relationship of the mucosal microbiota to gastrointestinal inflammation and the presence of small cell intestinal lymphoma in cats

by

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Karin Allenspach-Jorn
Austin Viall

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

Bacto1080	Bacteroides
CRC	Colorectal cancer
Cy-3	Cyanine dye 3
Ebac1790	Enterobacteriaceae
Erec482	Clostridium
Eub338	Eubacterium (total bacteria)
Faecali698	Faecalibacterium
FeLV	Feline leukemia virus
FISH	Fluorescence in situ hybridization
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
Fuso0664	Fusobacterium
GI	Gastrointestinal
H&E	Hematoxylin-eosin
IBD	Inflammatory bowel disease
IHC	Immunohistochemistry
IQR	Interquartile range
Hel717	Helicobacter
LSA	Lymphosarcoma
max	Range maximum

min	Range minimum
NF-κB	Nuclear factor kappa B
PARR	PCR for antigen receptor rearrangement
rs	Spearman correlation coefficient
T_4	Thyroxine
TLR	Toll like receptor

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ABSTRACT

The gastrointestinal (GI) microbiota in healthy cats is altered in IBD. Little research has been performed to identify whether specific bacterial groups are associated with small cell GI lymphoma (LSA).

Fourteen cats with IBD and 14 cats with small cell GI LSA were included in this retrospective case control study. A search of the medical records was performed to identify cats diagnosed with IBD and with GI LSA. Bacterial groups identified by FISH in GI biopsies were compared between cohorts and correlated to $CD11b^+$ and NF-κB expression.

Fusobacterium spp. were higher in cats with small cell GI LSA in ileal and colonic adherent mucus, and combined colonic compartments compared to cats with IBD. *Bacteroides* spp. were higher in ileal adherent mucus and 3 combined ileal compartments of cats with small cell GI LSA. There were significant correlations between *Fusobacterium spp.* totals and $CD11b^+$ cell and NF-κB expression.

The bacterial alterations appreciated might be influential in development of small cell GI LSA, and should drive further studies to elucidate the effects of microbial-mediated inflammation on GI cancer progression.

CHAPTER 1. INTRODUCTION

Alterations in the Microbiome Relative to Gastrointestinal Disease Progression and Inflammatory Mediators

Diagnosis of small cell gastrointestinal (GI) lymphoma (LSA) in cats increased six-fold over a four-decade period spanning from 1964 through 2004, highlighting the growing clinical importance of this disease.[1] Frequent identification of the disease continues today, most notably in cats with chronic weight loss, vomiting, decreased appetite and diarrhea. Small cell GI LSA closely resembles feline inflammatory bowel disease (IBD) in its clinical presentation,[2] with both diseases having poorly defined etiologies. Various risk factors including genetic and molecular alterations,[3-5] diet,[3,6,7] and chronic inflammation[8-10] have been linked to the development of both disorders. Still other studies have suggested an association between chronic mucosal inflammation and the progression of IBD to pronounced dysplasia in the GI tract.[11-14]

Although the microbiota is intimately involved in GI homeostasis and immune balance, several studies in animal models and humans with cancer incriminate bacterial-induced chronic intestinal inflammation as promoting a local environment favorable for malignant transformation, including lymphomagenesis.[11-14] Increased numbers of mucosal-adherent bacteria were observed in cats with duodenal IBD,[10] while invasive bacteria were observed within blood vessels and serosa of cats with large cell lymphoma.[9] These collective observations suggest that altered microbial composition (e.g. dysbiosis) is associated with development of benign mixed mucosal inflammation (lymphoplasmacytic enteritis with IBD)

and GI lymphoid malignancy.[9,10,15-18] Microbial imbalances have also been implicated in the pathogenesis of approximately 20% of all human malignancies, including GI neoplasia such as colorectal adenocarcinomas and gastric carcinomas.[19-21] Both chronic, high-grade inflammation of disorders such as feline IBD, and lower grade smoldering inflammation attributable to pro-inflammatory bacteria (e.g. *Helicobacter* spp., Enterobacteriaceae, *Fusobacterium* spp.) might drive a tumor-permissive milieu characterized by mucosal infiltration with specialized effector cells, including $CD11b^+$ myeloid cell derived macrophages, as observed in humans, and rodent models.[9,19,21,22]

Differentiated $CD11b^+$ myeloid cells play an important role in tumor progression and angiogenesis in rodent models and humans with colorectal cancer (CRC).[19,23] Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a protein complex of transcription factors, is involved in immune regulation, DNA transcription, and pro-inflammatory cytokine and chemokine production.[24] This protein complex is also involved in activation of effector cells through toll-like receptors (TLRs) in response to GI micro-organisms. Dysregulation of the activation of NF-κB can lead to overproduction of pro-inflammatory cytokines and cell transformation, triggering the development of cancers in humans.[25-27]

We hypothesized that pro-inflammatory bacteria, including Enterobacteriaceae and *Fusobacterium* spp. are abundant in the intestinal biopsies of cats with small cell GI LSA relative to cats with IBD. An objective of this study was to investigate the potential

relationship between mucosal bacteria and the mucosal expression of NF-κB and infiltrating $CD11b^+$ immune cells in GI biopsies of cats with small cell GI LSA versus GI biopsies of cats with IBD.

CHAPTER 2. MATERIALS AND METHODS

Study Design

This study was a retrospective study involving 3 study centers, VDx Veterinary Diagnostics Laboratory, Davis CA; the University of Camerino Veterinary Teaching Hospital, Italy and Iowa State University, Ames IA. The medical records of each facility were reviewed to identify cats having GI endoscopic or laparoscopic derived diagnosis of histologic IBD or small cell GI LSA from January 2010 to January 2015. Fourteen cats with IBD and 14 cats with small cell GI LSA were included in the study. Inclusion criteria was comprised of complete medical histories, the performance of hematologic and serum biochemical analysis, and measurement of serum T4 in all cats. Serology for Feline Leukemia Virus (FeLV) and Feline Immunodeficiency Virus (FIV) infections, diagnostic imaging, and serum cobalamin levels were also assessed in most cats. Cats were also either tested for intestinal parasites via fecal examinations, dewormed or had deworming performed in the face of negative fecal examinations before biopsies were collected. All cats in both study cohorts tested negative for intestinal parasite ova on fecal examinations. Histopathologic examination of intestinal biopsy specimens after hematoxylin-eosin (HE) staining and immunophenotyping (e.g. IHC stains for CD21, CD79a [B-lymphocytes] and CD3 [T-lymphocytes]) was performed on each cat to determine a final diagnosis of IBD or small cell GI LSA. In some instances, there was overlap in immunohistopathologic features necessitating performance of polymerase chain reaction for antigen receptor rearrangement (PARR) to confirm a final diagnosis of IBD versus small cell GI LSA.

Fluorescence in Situ Hybridization

Formalin-fixed embedded tissue sections were mounted on glass slides and evaluated by fluorescence in situ hybridization (FISH) as previously described.[8] In brief, paraffin-embedded tissue specimens were deparaffinized using an automated system by passage through xylene (3 x 10 min), 100% alcohol (2 x 5 min), 95% ethanol (5 min) and lastly 70% ethanol (5 min). The slides were next immediately transported in deionized water to the DNA testing laboratory where they were air dried prior to hybridization. FISH probes of interest, 5'-labeled with either Cy-3 or FITC were reconstituted with DNAse-free water and diluted to a working concentration of 5ng/μL (Table 1).

For total bacterial counts, Eub338-FITC was used.[28] For other analyses, specific probes targeting *Clostridium* spp. (Erec482),[29] Bacteroides-Prevotella group (Bacto1080),[30] *Fusobacterium* spp. (Fuso0664),[31] Enterobacteriaceae (Ebac1790),[32] *Helicobacter* spp. (Hel717),[33] and *Faecalibacterium* spp. (Faecali698),[34] were labeled with Cy-3 and were applied simultaneously with the universal bacterial probe Eub338-FITC. This probe array was selected to identify specific bacterial groups and individual bacterial species previously shown to be relevant in the pathogenesis of intestinal cancers in humans, rodents, and dogs.[9,14,19,22,23,27,35-54]

Tissue sections were bathed in 30μL of DNA-probe-hybridization buffer mix in a hybridization chamber maintained at 54°C overnight (12 hours). Buffer 1 (20mM Tris, 0.9M

NaCl, 0.1% SDS, 20% Formamide, 10% Dextran sulfate) was used for the hybridization of Eub338, Erec482, Bacto1080, Fuso0664, Ebac1790 and Faecali698. Buffer 2 (20mM Tris, 0.9M NaCl, 0.1% SDS, 40% Formamide) was used for the hybridization of Hel717. Washing was then performed using wash buffer (hybridization buffer without SDS), the slides were rinsed with sterile water, then allowed to air-dry, and mounted with SlowFade Gold mounting media and 22x22-1 glass cover slip. Probe specificity was confirmed in pilot studies by combining the irrelevant probe non-Eub338-FITC with Eub338-Cy-3, and through hybridization experiments with pure bacterial isolates to screen for non-selective hybridization.

In Situ Quantification of Mucosal Bacteria Associated with Biopsy Specimens

Bacteria were visualized and quantified using an Eclipse TE2000-E fluorescence microscope. Images were captured with a CoolSnap EZ camera and bacterial enumeration was performed using MetaMorph software. A minimum of 3 separate hybridized intestinal tissue sections were evaluated for their bacterial content. Bacterial quantification was performed in 10 representative mucosal fields of each hybridized tissue section of formalin-fixed, paraffin embedded biopsy specimens. A hybridized tissue section was first visualized at 200x magnification to locate the center of each tissue section. Then surrounding fields of bacterial populations were subsequently selected, and quantified using a 60x Plan Apo oil objective with an optional 1.5x multiplier lens. Bacterial counts were obtained randomly in 3 mucosal fields in 2 of the 3 hybridized tissue sections, and randomly in 4 views on the third

hybridized tissue section, accounting for 10 views for bacterial quantification in total. The ten fields counted included bacteria located within 4 well-defined mucosal compartments: (1) bacteria found within free mucus, (2) bacteria localized within adherent mucus, (3) bacteria attached to the surface epithelium, and (4) bacteria invasive within the mucosa (Figure 1).

Immunohistochemical Assessment of the Number of CD11b$^+$ Cells and Mucosal NF-κB Expression

The number of mucosal CD11b$^+$ myeloid cells, and nuclear factor – kappa B (NF-κB) expression were quantified in intestinal tissues of all 28 cats. This included 14 cats diagnosed with IBD and 14 cats diagnosed with small cell GI LSA. For IHC evaluation, paraffin embedded tissue sections were rehydrated and neutralized for endogenous peroxidases with 3% hydrogen peroxide for 5 minutes followed by rinsing for 5 minutes in distilled water. For antigen retrieval, the heat-induced epitope retrieval (HIER) method was selected, on the basis of previous experiences showing better results with antigens, such as CD11b$^+$, that might require more efficient breakage of methylene bridges formed during formalin fixation and allowing antibody to bind. In brief, slides were incubated in an universal antigen retrieval buffer, used in a water bath set to 140°F and allowing the slides to incubate in retrieval solution overnight. Non-specific binding was blocked by incubation of slides for 10 minutes with a protein-blocking agent before application of the primary antibody. Slides were then incubated overnight in a moist-chamber with the following primary antibodies: canine cross-reactive monoclonal antibody against NF-κB (p65)[55] diluted 1∶50, and a goat polyclonal

to $CD11b^+$ (anti-$CD11b^+$ antibody - ref. N° ab62817) diluted 1∶50. The immunoreaction with streptavidin–immunoperoxidase was visualized with 3,3′-diaminobenzidine substrate. Tissues were counterstained with Mayer's hematoxylin (Figure 2). For negative IHC controls, the primary antibodies were omitted. Sections of feline and canine spleen and tonsil served as positive control tissues for both NF-κB (p65) and $CD11b^+$ cell staining. Finally, isotype controls were used as negative controls to help differentiate eventual non-specific background signal from specific antibody signal, by the use of primary antibodies lacking specificity to the target, but matching the class and type of the primary antibody used in the IHC studies.

For assessment of intestinal NF-κB (p65) and $CD11b^+$ cells, the cells were quantified in select compartments of the GI tract biopsies (small intestine: villi, basal crypt area, villus-crypt junction; large intestine: apical crypt area, basal crypt area). All cellular types were evaluated using a light microscope (Carl Zeiss), a×40 objective, a×10 eyepiece, and a square eyepiece graticule (10×10 squares, having a total area of 62,500 μm^2). Ten appropriate fields were chosen for each compartment and arithmetic means were calculated for each intestinal region. Results were expressed as IHC positive cells per 62,500 μm^2. Cells on the margins of the tissue sections were not considered for evaluation to avoid inflation of positive cell numbers.

The numbers of mucosal NF-κB (p65) expressing cells and $CD11b^+$ cells were quantified using an image-analysis system consisting of the previously mentioned light microscope

(Carl Zeiss) attached to a Javelin JE3462 high-resolution camera and a personal computer equipped with a Coreco-Oculus OC-TCX frame grabber and high-resolution monitor. Computerized color-image analysis was performed by using Image-Pro Plus software. The area of each biopsy in all cross sections in every cat was recorded. For each cat, the total bioptic area was calculated as the sum of the areas of all fields in all bioptic cross sections on one slide. The NF-κB (p65) expressing cells and $CD11b^+$ cells were counted per section, and stained cell densities were expressed as the number of mononuclear cells per square millimeter of analyzed bioptic area.[56]

Statistical Analysis

Tabular data were organized by probe, organ, mucosal compartment and disease group. Mean, median, standard deviation, minimum, and maximum values were calculated from the absolute bacterial counts. Normality of data was assessed visually by histograms and Q-Q plots. If data were not found to be normally distributed, they were log-transformed and reassessed. Median values were compared among groups for significance using the Wilcoxon rank sum test. Analyses were considered significant at $P < 0.05$. All graphs were constructed using non-log-transformed, actual raw data.

Associations between bacterial numbers (identified by FISH) and the numbers of $CD11b^+$ cells and NF-κB expressing cells were assessed using Spearman's rank correlation and tested for significance. Significance was determined as $P < 0.05$. Sample size for enrollment was

calculated after consultation with a statistician and was based on the desired power and variability in the number of $CD11b^+$ cells and NF-κB expressing cells observed in IBD versus small cell GI LSA feline cohorts. Randomization of 12 cats per group would give a power of 80% to detect differences in expression of $CD11b^+$ cells and NF-κB expressing cells between cohorts at the .05 significance level. All analyses were made using SAS 9.4.

Table 1 Oligonucleotide probes used for fluorescence in situ hybridization (FISH) and bacterial quantification.

Probe	Target	Sequence (5' → 3')
Eub338	*Eubacteria*	GCT GCC TCC CGT AGG AGT
Erec482	*Clostridium coccoides-E. rectale cluster*	GCT TCT TAG TCA RGT ACC G
Ebac1790	*Enterobacteriaceae*	CGT GTT TGC ACA GTG CTG
Bacto1080	*Bacteroides* spp. *Prevotella* spp.	GCA CTT AAG CCG ACA CCT
Hel717	*Helicobacter* spp.	AGG TCG CCT TCG CAA TGA GTA
Faecali698	*Faecalibacterium* spp.	GTG CCC AGT AGG CCG CCT TC
Fuso0664	*Fusobacterium* spp.	CTT GTA GTT CCG CYT ACC TC

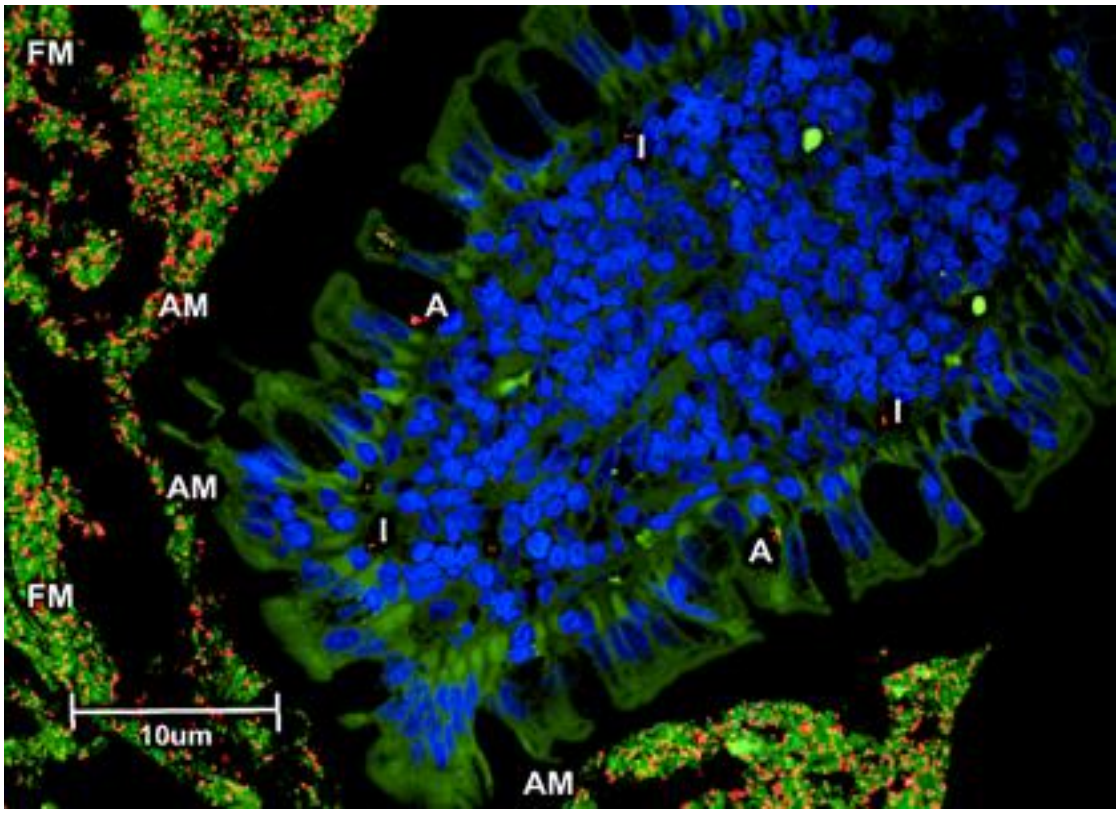

Figure 1 Mucosal compartments for bacterial quantification. FM, bacteria within free mucus within the GI lumen; AM, bacteria within adherent mucus adhered to the epithelia; A, bacteria directly attaching to surface epithelia; I, bacteria invasive within the mucosa. Three-color fluorescence in situ hybridization (FISH) of *Bacteroides* spp. identified in ileal biopsies of a cat with small cell GI LSA.
Legend: Cy-3-labeled *Bacteroides* spp. (labeled orange) localized within adherent mucus of an ileal biopsy specimen. The mucus is also occupied by other bacteria (total bacteria labeled green with FITC-Eub). The dark blue structures are epithelial cell nuclei stained with DAPI.

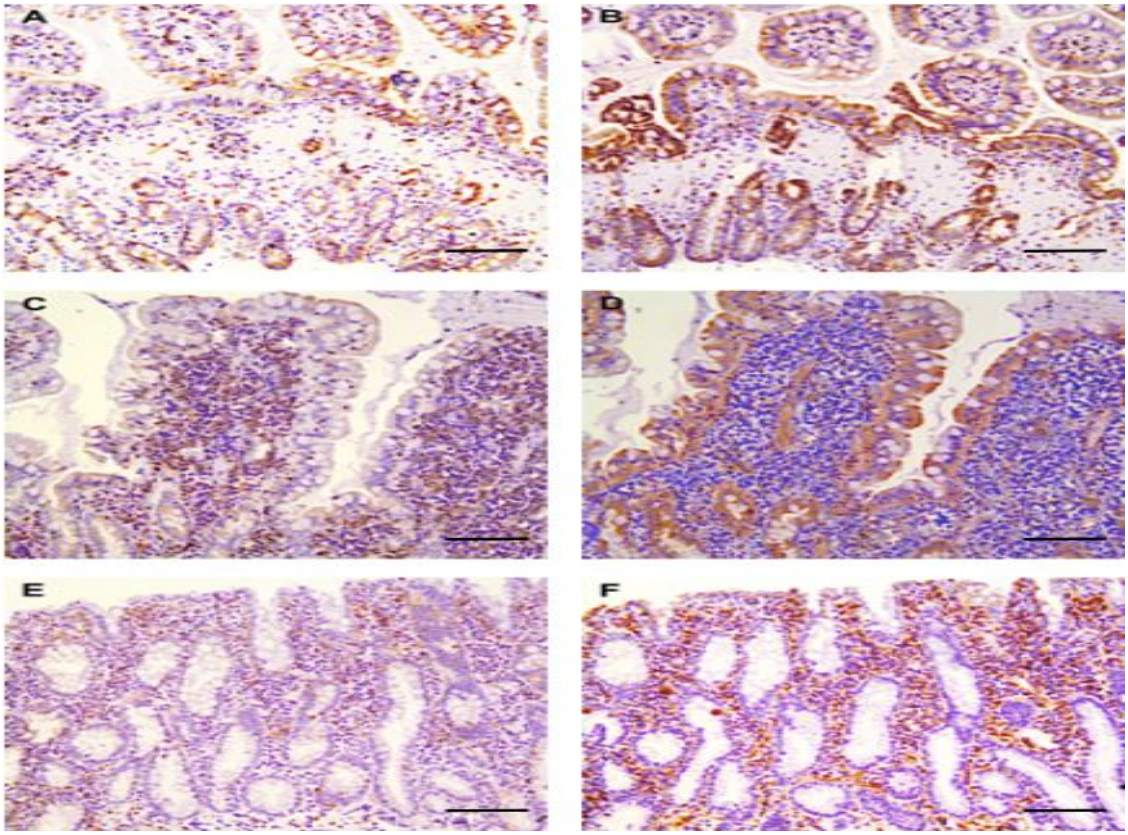

Figure 2 IHC staining for $CD11b^+$ and NF-κB primary antibodies in ileal and colonic biopsies of cats with IBD and small cell GI LSA. (A) $CD11b^+$myeloid cell staining in the ileum of a cat with IBD. Cross sectional view of ileal villi after IHC staining with a goat polyclonal antibody to $CD11b^+$ as represented by the brown chromophore staining amongst the epithelium and in the lamina propria. (B) NF-κB staining in the ileum of a cat with IBD. Cross sectional view of ileal villi after IHC staining with a canine cross-reactive monoclonal antibody against NF-κB as represented by the brown chromophore staining amongst the epithelium and in the lamina propria. (C) $CD11b^+$ staining in the ileum of a cat with small cell GI LSA. Longitudinal view of ileal villi after IHC staining with a goat polyclonal antibody to $CD11b^+$ as represented by the brown chromophore staining amongst the epithelium and in the lamina propria. (D) NF-κB staining in the ileum of a cat with small cell GI LSA. Longitudinal view of ileal villi after IHC staining with a canine cross-reactive monoclonal antibody against NF-κB as represented by the brown chromophore amongst the epithelium and in the lamina propria. (E) $CD11b^+$ staining in the colon of a cat with small cell GI LSA. Longitudinal and cross sectional views of colonic mucosa and lamina propria after IHC staining with a goat polyclonal antibody to $CD11b^+$ as represented by the brown chromophore staining amongst the epithelium and in the lamina propria. (F) NF-κB staining in the colon of a cat with small cell GI LSA. Longitudinal and cross sectional views of colonic mucosa and lamina propria after IHC staining with a canine cross-reactive monoclonal antibody against NF-κB as represented by the brown chromophore staining amongst the epithelium and in the lamina propria. All scale bars, 200 μm

CHAPTER 3. RESULTS

Final Histopathologic Diagnoses

Fourteen cats were diagnosed with IBD based on histopathologic assessment of the mucosal cellular infiltrate (lymphoplasmacytic) after H&E, immunophenotypic staining and PARR as an adjunct diagnostic in equivocal cases. GI biopsies in 9 of 14 of these cats exhibited a 30% to 60% heterogenous population of small to intermediate lymphocytes and plasma cells invading the lamina propria, with mild but varying numbers of neutrophilic and eosinophilic cellular infiltrates per any given 400x magnification field assessed after H&E staining via microscopy. The diagnosis of IBD in these cats was supported by IHC showing relatively evenly mixed CD3 and CD21 or CD79a cellular positivity without immunophenotypic evidence of clustering of either T-cell or B-cell populations. Sparse numbers of intraepithelial lymphocytes (i.e. approximately 0 to 20 per 100 villous enterocytes) were also appreciated in limited villous epithelium and less commonly in crypt epithelium. Additionally, PARR was performed on intestinal biopsies from the remaining 5 of these 14 cats to confirm diagnosis of a polyclonal lymphocyte mucosal population, as these biopsies were assessed as having areas of mild to moderate villous blunting and fusion with crypt effacement on histopathologic assessment, greater numbers of intraepithelial lymphocytes in both villous and crypt epithelium and rare, patchy areas of moderately heavy monomorphous lymphoid infiltrates of small to intermediate-sized CD3 staining lymphocytes.

Fourteen cats were diagnosed with small cell GI LSA based on H&E stains and immunophenotyping, with 1 cat requiring PARR to confirm a final diagnosis. Thirteen of these 14 cats showed histopathologic evidence of moderate to marked villous blunting and fusion with crypt effacement after H&E staining. Moderate to marked intraepithelial villous and crypt lymphocytes (i.e. more than 40 per 100 villous enterocytes) and diffuse heavy effacement of the lamina propria with a heavy monomorphous lymphoid infiltrate of small to intermediate-sized CD3 staining lymphocytes, occasionally with sparse CD21/CD79a positive cells, were also appreciated after IHC staining. The occasional sparse CD21/CD79a positive cells were suspected to represent a mild inflammatory background. The 1 cat that required PARR to confirm the diagnosis of small cell GI LSA was assessed as having similar villous and crypt histopathologic features but exhibited a mild number of eosinophils and neutrophils infiltrating the lamina propria, minimal intraepithelial lymphocytes and patchy areas of monomorphous lymphoid infiltrate with varying areas of mixed T and B-cell populations, suggesting possible polyclonality.

Of the 14 cats diagnosed with IBD, 7 were castrated male and 7 were spayed female. Nine of these cats were domestic shorthairs, and there was 1 cat each of domestic longhair, Lynx Point, Savannah, Siamese, and Ocicat. Of the 14 cats diagnosed with small cell GI LSA, 6 were castrated male, 7 were spayed female, and sex and neuter statuses were not reported in the medical record for 1 cat. Eleven of these cats were domestic shorthairs, and there was 1 cat each of domestic longhair, Himalayan, and Maine Coon. Histories were available for all 28 cats. Duration of clinical signs for each cat was reported as at least 3 weeks prior to

presentation. Clinical signs of vomiting (n = 24) and weight loss (n = 13) were noted most frequently with some cats also exhibiting variable hyporexia (n = 9), small bowel diarrhea, large bowel diarrhea, or both (n = 10) and hematochezia (n = 2). In general, cats with small cell GI LSA were older than cats with IBD (small cell GI LSA: mean 10.9, standard deviation 3.35; IBD: mean 7.4, standard deviation 3.68; $P = 0.009$). Serum T4 measurements were within reference range for all cats (n = 28). Cats in which FeLV and FIV status were assessed (10 cats with IBD, and 14 cats with small cell GI LSA) were all negative. Serum cobalamin measurements were available for 11 cats (5 with IBD and 6 with small cell GI LSA). One of the 5 cats with IBD with reported serum cobalamin concentrations had a cobalamin level less than 300ng/L and 4 of the 6 small cell GI LSA cats with reported serum cobalamin concentrations had cobalamin measurements less than 300ng/L. Abdominal ultrasound findings were available in 22 of the 28 cats (10 cats with IBD and 12 cats with small cell GI LSA). The remaining 6 cats either did not have abdominal imaging performed or the report was excluded from the medical records. Of the cats with abdominal ultrasounds performed, 10 had normal gastrointestinal wall thickness (7 cats with IBD and 3 cats with small cell GI LSA) and 12 had diffuse thickening of the GI muscularis (3 cats with IBD and 9 cats with small cell GI LSA). Five of the cats with IBD and 9 of the cats with small cell GI LSA had enlarged mesenteric lymph nodes. In the remaining cats with abdominal imaging performed, lymph nodes were noted as normal in size.

Quantification and Spatial Distribution of Mucosal Bacteria Determined by Fluorescence in Situ Hybridization

Only few bacteria were identified as invasive within the intestinal mucosal biopsies. Therefore, bacterial quantification was performed in the 3 other mucosal compartments (free mucus, adherent mucus, attaching to surface epithelia) (Table 2) and separately in adherent mucus (Table 3), in both the ileal and the colonic biopsies. Gastrointestinal biopsies of cats with small cell GI LSA had significantly increased numbers of total *Fusobacterium* spp. bacteria in the 3 combined mucosal compartments of the colon ($P = 0.017$) compared to GI biopsies of cats with IBD (Table 2). Total *Fusobacteria* spp. bacteria in the 3 combined mucosal compartments of ileal biopsies of cats with small cell GI LSA was not statisticaly significanct ($P = 0.059$) compared to ileal biopsies of cats with IBD (Table 2). Gastrointestinal biopsies of cats with small cell GI LSA had significantly increased numbers of total *Fusobacteria* spp. bacteria in the adherent mucus compartments of the ileum ($P = 0.046$) and colon ($P = 0.016$) versus GI biopsies of cats with IBD (Table 3). There was a significantly increased number of *Bacteroides* spp. bacteria in the 3 combined ileal mucosal compartments ($P = 0.034$) (Table 2) and in the ileal adherent mucus ($P = 0.036$) (Table 3) of GI biopsies of cats with small cell GI LSA compared to GI biopsies of cats with IBD. The numbers of total bacteria, *Clostridium* spp., Enterobacteriaceae, *Helicobacter* spp. and *Faecalibacterium* spp. were not significantly different when comparing the colonic and ileal biopsies of cats with IBD relative to the biopsies of cats with small cell GI LSA.

The amount of each species of bacteria evaluated were subjectively observed to be more prevalent in the adherent mucus layer versus the free mucus, attached to the surface epithelia, and invasive within the mucosa, including for *Fusobacterium* spp. and *Bacteroides* spp. bacteria (Figure 3 and 4).

Number of Mucosal CD11b$^+$ Myeloid Cells and NF-κB Expression

Cats with small cell GI LSA had significantly increased numbers of CD11b$^+$ cells within the lamina propria of both ileal (median = 503; min 30, max 1985; $P = 0.012$) and colonic biopsies (median = 77; min 13, max 2858; $P < 0.0001$) versus cats with IBD (Figure 5). Cats with small cell GI LSA also had significantly increased expression of NF-κB in the lamina propria of both ileal (median = 750; min 69, max 2227; $P = 0.0055$) and colonic biopsies (median = 154; min 40, max 3581; $P < 0.0001$) when compared to cats with IBD (Figure 6). Additionally, data analysis showed a positive correlation between the total numbers of *Fusobacterium* spp. bacteria and the total numbers of CD11b$^+$ cells ($P = 0.009$; rs 0.476) as well as cells expressing NF-κB ($P = 0.004$; rs 0.523) as assessed by spearman correlation coefficients.

Table 2. Total bacteria in 3 combined compartments (free mucus, adherent mucus, attaching to surface epithelia). Data has been logged transformed to control for variability, and are reported as mean and standard deviation of the transformed data.

Probe	Group	Ileum	Colon
Eub338	IBD	5.52 ± 1.92	6.56 ± 0.65
	LSA	6.75 ± 0.37	6.55 ± 0.39
Erec482	IBD	4.95 ± 2.13	5.59 ± 1.23
	LSA	6.19 ± 0.77	5.86 ± 0.78
Ebac1790	IBD	4.36 ± 2.47	4.33 ± 1.87
	LSA	3.98 ± 1.75	4.80 ± 1.14
Bacto1080	IBD	5.64 ± 1.01	5.91 ± 1.59
	LSA	6.60 ± 0.38[a]	6.00 ± 1.17
Hel717	IBD	1.54 ± 1.60	1.97 ± 1.82
	LSA	2.07 ± 1.07	2.27 ± 1.71
Faecali698	IBD	4.62 ± 2.13	5.12 ± 1.71
	LSA	5.95 ± 0.75	5.35 ± 1.27
Fuso0664	IBD	4.60 ± 2.14	5.14 ± 1.27
	LSA	6.32 ± 0.52	6.14 ± 0.66[b]

IBD, inflammatory bowel disease cats; LSA, small cell gastrointestinal lymphoma cats. (total = 28 cats; IBD = 14 cats; small cell GI LSA = 14 cats).
[a]Mean *Bacteroides* spp. bacterial count in ileal biopsies of small cell GI LSA cats significantly higher than in ileal biopsies of cats with IBD ($P = 0.034$).
[b]Mean *Fusobacterium* spp. bacterial count in colonic biopsies of small cell GI LSA cats significantly higher than in colonic biopsies of cats with IBD ($P = 0.017$).

Table 3. Total bacteria in adherent mucus. Data has been logged transformed to control for variability, and are reported as mean and standard deviation of the transformed data.

Probe	Group	Ileum	Colon
Eub338	IBD	5.33 ± 2.04	6.51 ± 0.66
	LSA	6.72 ± 0.38	6.50 ± 0.41
Erec482	IBD	4.90 ± 2.11	5.51 ± 1.33
	LSA	6.09 ± 0.97	5.80 ± 0.81
Ebac1790	IBD	4.34 ± 2.46	4.25 ± 1.91
	LSA	3.67 ± 2.13	4.72 ± 1.18
Bacto1080	IBD	5.43 ± 1.23	5.76 ± 1.86
	LSA	6.57 ± 0.38[a]	5.95 ± 1.25
Hel717	IBD	1.45 ± 1.53	1.86 ± 1.70
	LSA	1.99 ± 1.10	2.04 ± 1.82
Faecali698	IBD	4.57 ± 2.13	5.03 ± 1.72
	LSA	5.94 ± 0.74	5.20 ± 1.59
Fuso0664	IBD	4.40 ± 2.18	5.06 ± 1.29
	LSA	6.28 ± 0.56[b]	6.10 ± 0.70[c]

IBD, inflammatory bowel disease cats; LSA, small cell gastrointestinal lymphoma cats. (total = 28 cats; IBD = 14 cats; small cell GI LSA = 14 cats).

[a]Mean *Bacteroides* spp. bacterial count in ileal biopsies of small cell GI LSA cats significantly higher than in ileal biopsies of cats with IBD ($P = 0.036$).

[b]Mean *Fusobacterium* spp. bacterial count in ileal biopsies of small cell GI LSA cats significantly higher than in ileal biopsies of cats with IBD ($P = 0.046$).

[c]Mean *Fusobacterium* spp. count in colonic biopsies of small cell GI LSA cats significantly higher than in colonic biopsies of cats with IBD ($P = 0.016$)

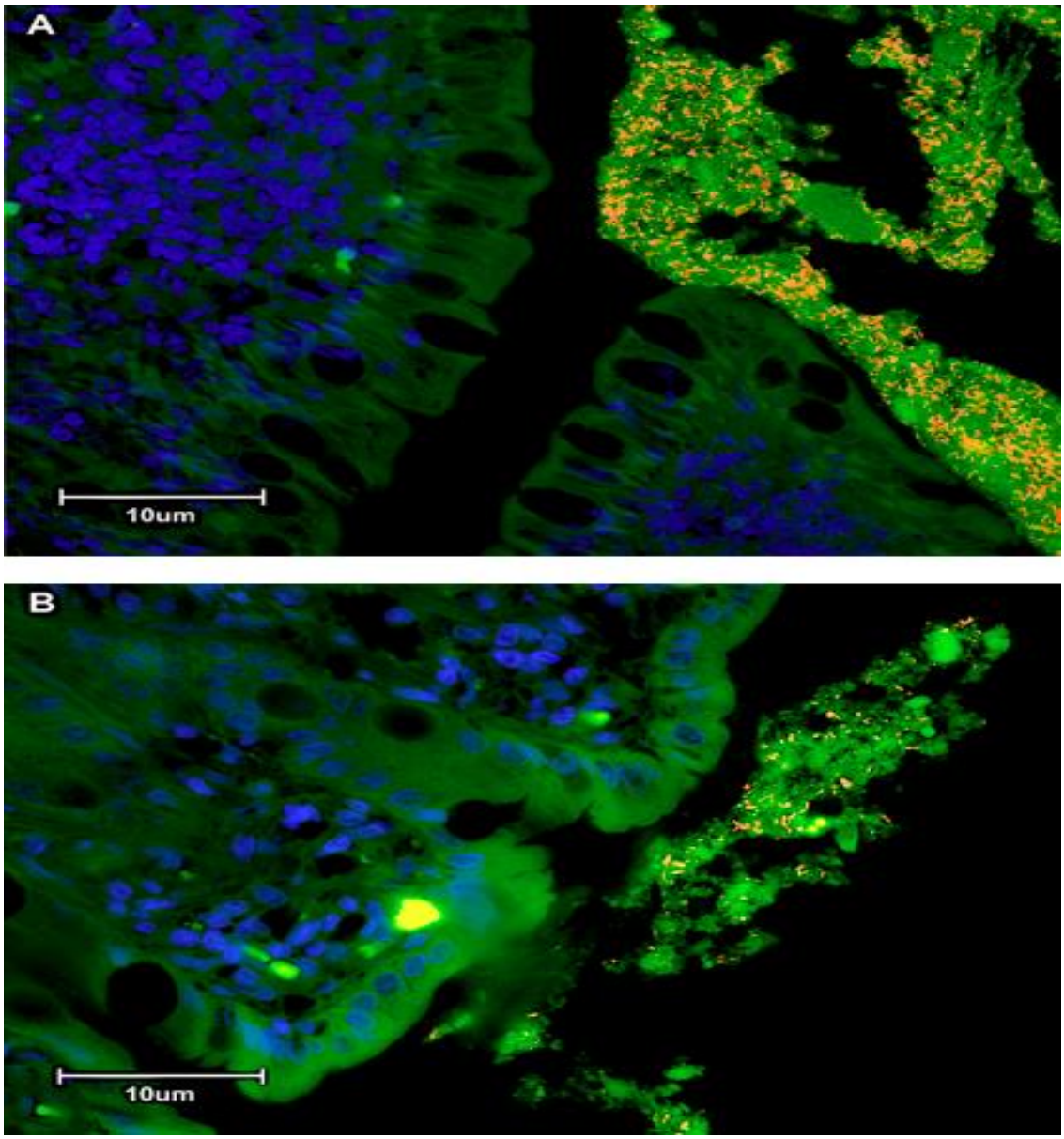

Figure 3.A Three-color fluorescence in situ hybridization (FISH) of *Fusobacterium* spp. identified in colonic biopsies of a cat with small cell GI LSA. Cy-3-labeled *Fusobacterium* spp. (labeled orange) localized within adherent mucus of a colonic biopsy specimen. The mucus is also occupied by other bacteria (total bacteria labeled green with FITC-Eub). The dark blue structures are epithelial cell nuclei stained with DAPI. Figure 3.B Three-color fluorescence in situ hybridization (FISH) of *Fusobacterium* spp. identified in colonic biopsies of a cat with IBD. Cy-3-labeled *Fusobacterium* spp. (labeled orange) localized within adherent mucus of a colonic biopsy specimen. The mucus is also occupied by other bacteria (total bacteria labeled green with FITC-Eub). The dark blue structures are epithelial cell nuclei stained with DAPI.

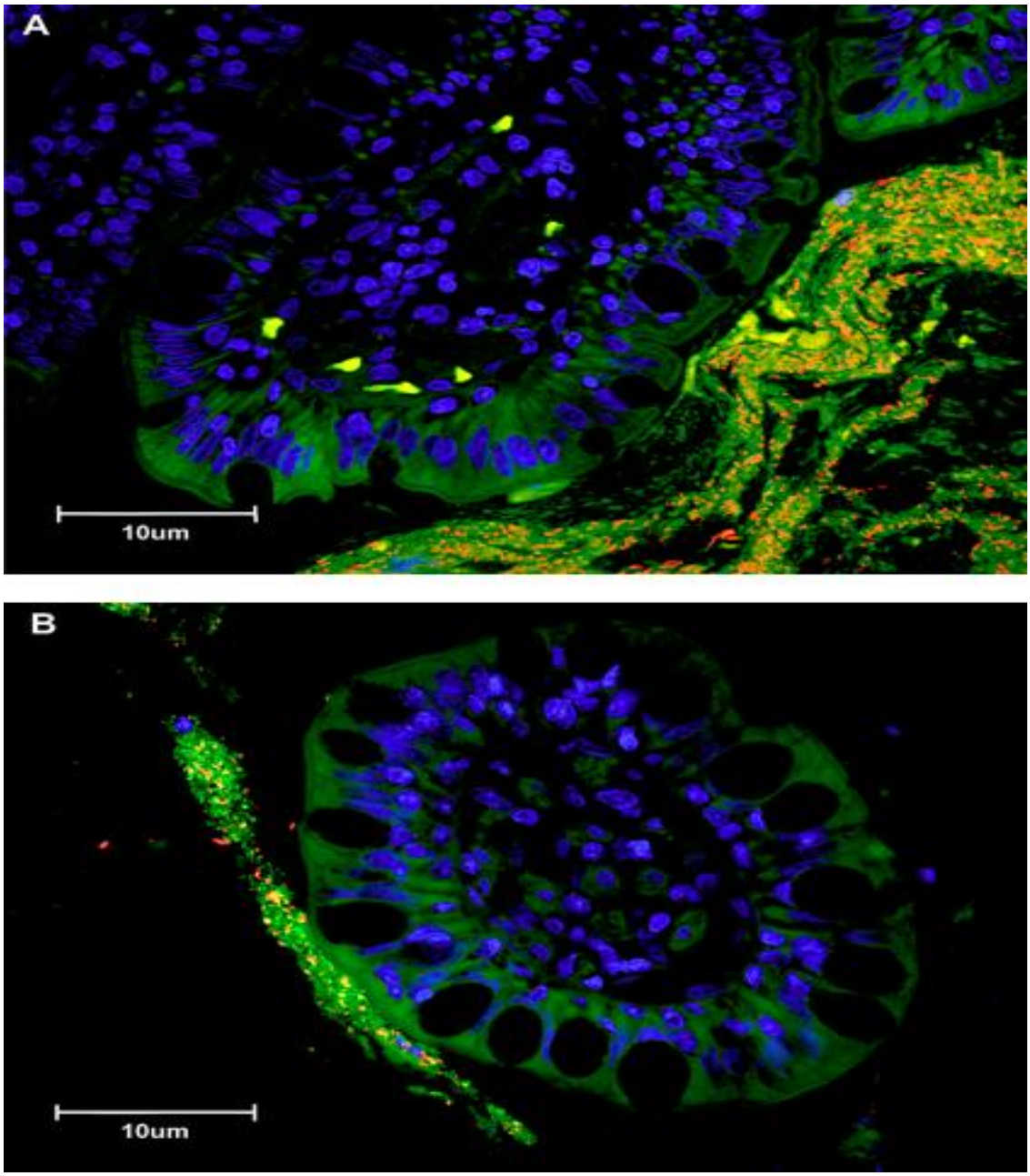

Figure 4.A Three-color fluorescence in situ hybridization (FISH) of *Bacteroides* spp. identified in ileal biopsies of a cat with small cell GI LSA. Cy-3-labeled *Bacteroides* spp. (labeled orange) localized within adherent mucus of an ileal biopsy specimen. The mucus is also occupied by other bacteria (total bacteria labeled green with FITC-Eub). The dark blue structures are epithelial cell nuclei stained with DAPI. Figure 4.B Three-color fluorescence in situ hybridization (FISH) of *Bacteroides* spp. identified in ileal biopsies of a cat with IBD. Legend: Cy-3-labeled *Bacteroides* spp. (labeled orange) localized within adherent mucus of an ileal biopsy specimen. The mucus is also occupied by other bacteria (total bacteria labeled green with FITC-Eub). The dark blue structures are epithelial cell nuclei stained with DAPI.

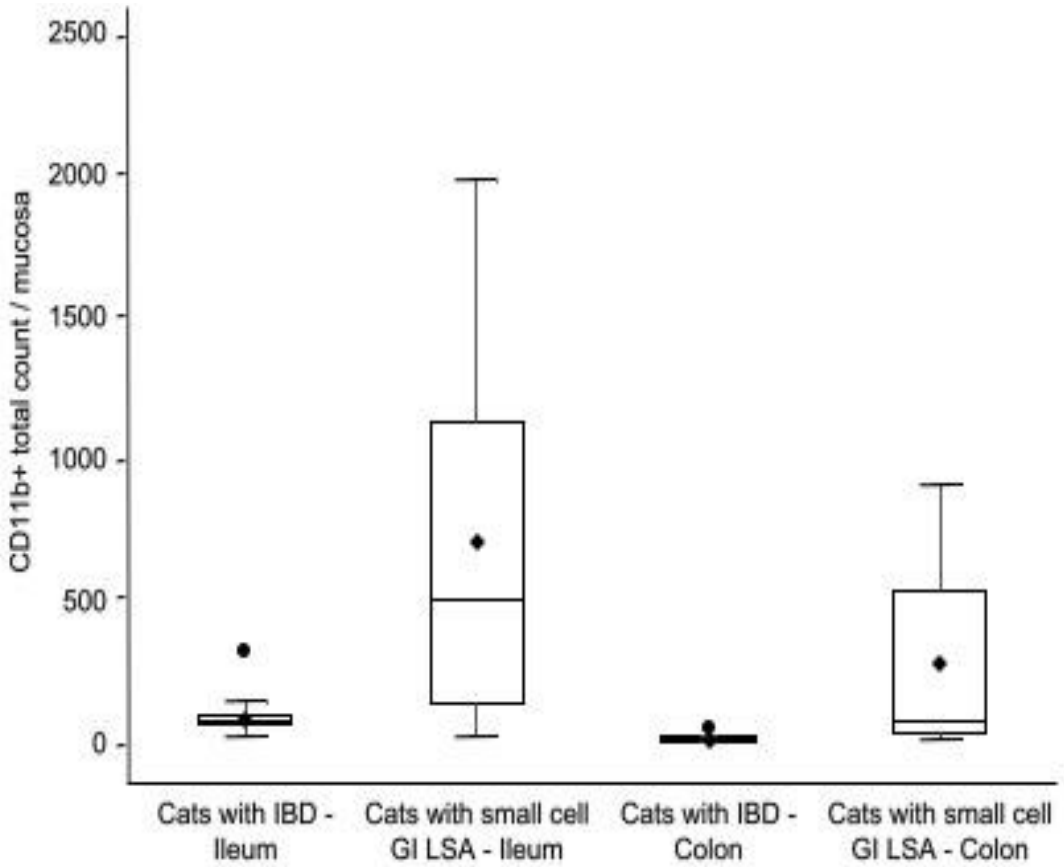

Figure 5 Mucosal $CD11b^+$ cellular expression in cats with IBD compared to cats with small cell GI LSA in the ileum and colon (total = 28 cats).
Boxes show lowest, median, and upper quartiles. Whiskers represent 1.5 of the interquartile range, with means denoted by the black rhombi and outliers denoted by the black circles. Median mucosal $CD11b^+$ myeloid cell counts are significantly higher in the ileum and colon of cats with small cell GI LSA compared to cats with IBD ($P < 0.05$).

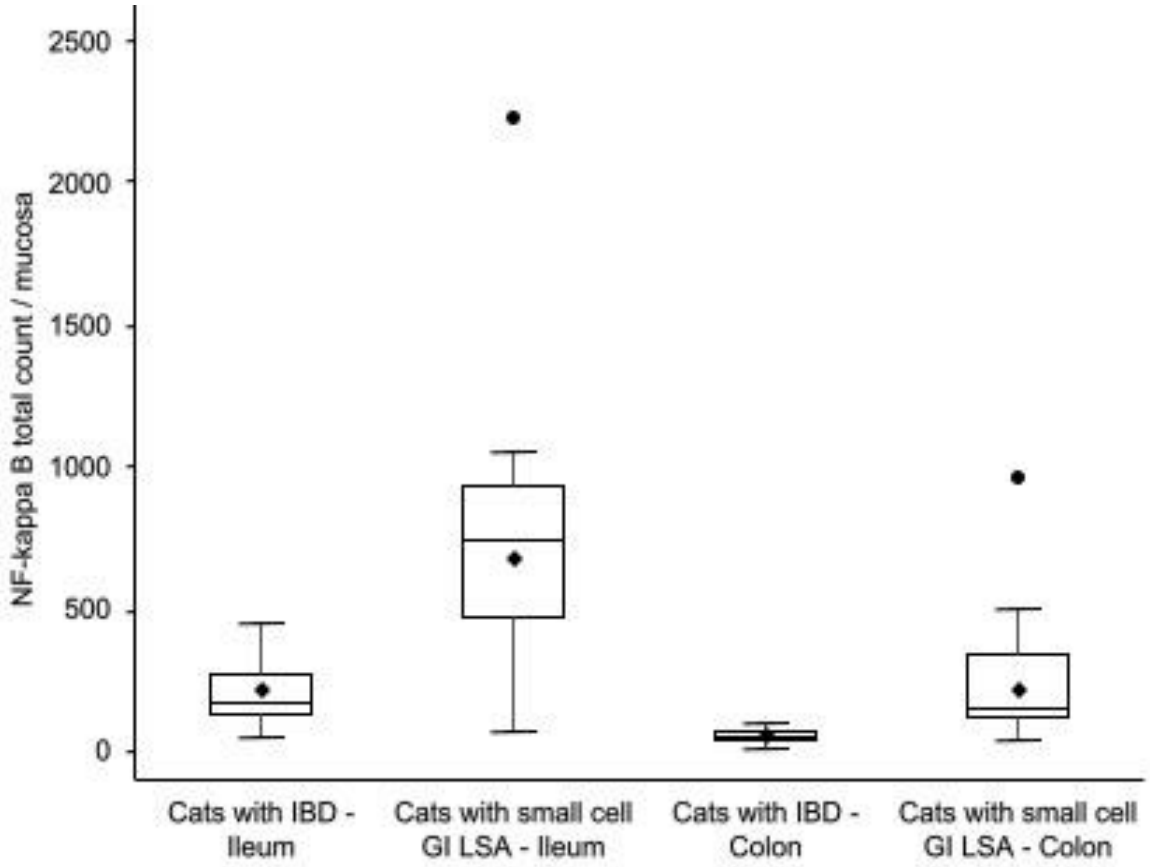

Figure 6 Mucosal NF-κB cellular expression in cats with IBD compared to cats with small cell GI LSA in the ileum and colon (total = 28 cats).
Boxes show lowest, median, and upper quartiles. Whiskers represent 1.5 of the interquartile range, with means denoted by the black rhombi and outliers denoted by the black circles. Median NF-κB mucosal expression is significantly higher in the ileum and colon of cats with small cell GI LSA when compared to cats with IBD ($P < 0.05$).

CHAPTER 4. DISCUSSION

Major bacterial phyla identified in feces of healthy cats include Enterobacteriaceae, *Bacteroides* spp., *Helicobacter* spp., *Fusobacterium* spp. and members of Firmicutes, including *Clostridium* spp. clusters IV and XIVa, and *Faecalibacterum* spp.[37,39,49,57] Compared to earlier studies performed in healthy cats, we chose to investigate the spatial distribution of the mucosal microbiota of ileal and colonic biopsies using FISH techniques in 2 important and clinically overlapping disease cohorts, cats with IBD and cats with small cell GI LSA. Our results indicate that the numbers of mucosa-associated *Fusobacterium* spp. and *Bacteroides* spp. were increased in the ileal biopsies of cats with small cell GI LSA compared to the ileal biopsies of cats with IBD, while the numbers of mucosa-associated *Fusobacterium* spp. bacteria were also increased in the colonic biopsies of cats with small cell GI LSA compared to cats with IBD. Furthermore, the numbers of *Fusobacteria* spp. in adherent mucus were strongly associated with increased numbers of $CD11b^+$ cells and up-regulated mucosal expression of NF-κB in GI biopsies of cats with small cell GI LSA compared to GI biopsies of cats with IBD.

In humans, some *Fusobacterium* spp., including *Fusobacterium nucleatum*, have been associated with an increased risk for colorectal and pancreatic cancers, as well as some oral squamous cell carcinomas.[38,42,44,52] *Fusobacterium nucleatum* numbers are significantly correlated with tumor size, and shortened survival times in a population of human Japanese patients with later stage CRC compared to earlier stages.[53] These findings, as well as

additional observations in rodent models and humans with CRC suggest that chronic inflammatory disease of the GI tract is a potential risk factor for the development of additional channels for amplified inflammatory processes, aneuploidy, high-grade dysplasia, metaplasia and loss of cellular proliferative checkpoints, all of which are processes that can be appreciated in malignancy.[13,19,54,58,59] The increased numbers of mucosal *Fusobacterium* spp. bacteria in GI biopsies of cats with GI LSA relative to GI biopsies of cats with IBD noted in the current study, although possibly just an effect of the GI malignancy, could also be associated with a potential progression from high grade chronic lymphoplasmacytic inflammation to inflammation-mediated lymphoid intestinal cancer.

Although incompletely understood, GI inflammation-mediated progression to cancer development might involve bacterial toxin production, release of alarmins, gut biofilm modification, dysregulation of gut barrier function, suppression of anti-inflammatory mediators and transition through an element of high grade dysplasia to cell damage and aneuploidy.[13,19,35,40,60-62] *Fusobacterium* spp. is pro-inflammatory, exhibits adhesive and invasive properties via virulence factors (i.e., FadA, fusobacterium autotransporter protein 2 and fusobacterial outer membrane protein A), and promotes pro-oncogenic features which might contribute to accelerated tumor growth and tumor burden.[38,47,54] Increased *Fusobacterium nucleatum* is associated with an increase in adenoma formation and accelerated small intestinal adenocarcinoma development in rodent models of GI neoplasia.[41] Coupled with this was an expansion of $CD11b^+$ myeloid cells (i.e., dendritic cells, macrophages, and myeloid-derived suppressor cells) in the neoplastic tissues and an NF-κB-

driven pro-inflammatory response.[41] Similarly, our results also indicated increases in $CD11b^+$ and NF-κB expressing cells in GI biopsies of cats with small cell GI LSA, and there was a positive correlation between increased *Fusobacterium* spp. numbers and both $CD11b^+$ and NF-κB expressing cells. Furthermore, $CD11b^+$ myeloid cells might contribute to development of a tumor microenvironment,[19,63] and *Fusobacterium* spp. might induce toll like receptors (TLR), to stimulate NF-κB through the TLR4/MYD88 pathway.[54] Importantly, TLRs are found on both immune and non-immune cells, and can be expressed on tumors including human mantle cell lymphoma.[21,64]

There were also increased numbers of the Bacteroides-Prevotella bacteria in the ileal biopsies of the cats with small cell GI LSA compared to ileal biopsies of the cats with IBD. Prior studies have shown that increased *Bacteroides* spp. can be seen in healthy cats versus cats with IBD.[65,66] This suggests that progressive disease might be associated with a decreased *Bacteroides* spp. load. This difference in findings might be explained by the different sampling sites (mucosal versus fecal) that were used for *Bacteroides* spp. quantification,[67] or might be due to the different cohorts compared, considering that quantification of GI *Bacteroides* spp. in cats with GI small cell LSA, to the authors' knowledge, has not been previously evaluated. Also, as the *Bacteroides* spp. incorporates a number of different subspecies, there is also the possibility that cats with small cell GI LSA harbor increased numbers of enterotoxigenic *Bacteroides* spp. as compared to those with IBD, as occurs in humans.[68,69]

The additional 5 probes evaluated using FISH included *Clostridium* spp. (Erec482), Enterobacteriaceae (Ebac1790), *Helicobacter* spp. (Hel717), *Faecalibacterium* spp. (Faecali896) and total bacteria (Eub338). *Clostridium* spp. population and diversity have been shown to be decreased while Enterobacteriaceae bacteria is increased in humans, dogs and cats with chronic intestinal inflammation.[16,17,65,70-72] Utilizing 16S rRNA sequencing in cats with chronic enteropathies, *Faecalibacterium* spp. bacteria were decreased while Enterobacteriaceae numbers were increased in cats with diarrhea.[66] This variation of bacterial species also occurs in dogs with IBD.[72,73] Therefore, different inflammatory states are characterized by differences in the microbiota. The current study did not show any significant differences in *Enterobacteriaceae*, *Faecalibacterium* spp. and total bacteria (Eub338) in GI biopsies of cats with small cell GI LSA compared to GI biopsies of cats with IBD.

Helicobacter spp. induces host responses influencing risk of oncogenesis, and promotes chronic gut inflammation in humans and mice.[46] In a recent study, FISH showed that the presence of enterohepatic helicobacter was significantly associated with poorly differentiated large intestinal adenocarcinoma in cats.[48] Our results showed no significant difference in *Helicobacter* spp. bacterial counts in GI biopsies of cats with small cell GI LSA compared to GI biopsies of cats with IBD.

It should be noted that in this study the majority of bacteria evaluated were located in the adherent mucus of the GI biobsies, a finding consistent with a prior study.[10] Less bacteria was appreciated in the free mucus and attaching to the surface epithelium, although still subjectively substantial. In a number of the biopsies there was very minimal to no bacteria appreciated invasively in the mucosa.

Although there is clinical relevance in determining the short and long-term outcomes and response to treatment of the underlying enteropathy in cats with alterations in the GI microbiota, we were unable to fully assess the significance of the relationship of *Fusobacterium* spp. quantification and outcome due to the variation of treatments and the limitation on follow up for a number of these cats. This is worth assessing in future studies, as in human studies and rodent models, overabundance of *Fusobacterium* spp. in patients or rodents with CRC does appear to have a correlation with poorer prognostic outcome.[36,51,54]

There are a number of potential limitations to this study. These would include the retrospective study design using medical records completed by different clinicians and the small population of cats evaluated. As diagnosis of IBD and small cell GI LSA rely as much on prior history as diagnostic findings the authors included, at a minimum, the most pertinent historical findings that would be needed to make these diagnoses in light of findings associated with diagnostic testing performed. The administration of antibiotics that could have affected the microbiota would be another potential limitation. The authors attempted to include cats with no history of antibiotic administration 2 weeks prior to the acquisition of GI

biopsies but this information was not always reported in the medical records. Processing, including formalin-fixation and paraffin embedding of the biopsies, might have also played a role in the finding of bacteria contained in the adherent mucus compartment, but this is consistent with a prior study[10] and should not have caused a discrepancy in any 1 cat in the study relative to the study population as a whole. Another potential limitation was the inability to perform PARR on the formalin-fixed, paraffin embedded tissues of all cats. The authors acknowledge that both PARR and IHC, when utilized together could have reduced the underestimation of small cell GI LSA in this population and might have allowed for a more sensitive means of screening for small cell GI LSA amongst the cohorts.[2,74] Unfortunately, tissue specimens were sometimes too limited per each feline endoscopic biopsy sample to allow for this additional diagnostic, due to the extensive tissue requirement for both hybridization for FISH and $CD11b^{+}$ and NF-κB immunohistochemical assessment. Also, the expense of performing PARR on all samples in this study would have been beyond the scope of funds available for the study. The authors did attempt to circumvent this limitation by performing PARR on any sample that exhibited questionable lymphoid monomorphism. Furthermore, it has been suggested that although PARR is valuable as a differentiating diagnostic test, it should be considered as an adjunct to immunohistochemical and histologic diagnostics, as it also does have its limitations relative to false negatives.[2,74] It has been recommended that PARR be considered as part of a stepwise diagnostic strategy, indicating the importance of more advanced testing only as required in a true clinical setting.[2,74]

In conclusion, we showed that *Fusobacterium* spp. bacteria are increased in the ileum and colon of GI biopsies of cats with small cell GI LSA relative to GI biopsies of cats with IBD. We also showed that increased *Fusobacterium* spp. bacteria are positively correlated to increased $CD11b^{+}$ and NF-κB expression in neoplastic ileal and colonic biopsies versus ileal and colonic biopsies of cats with lymphoplasmacytic ileitis and colitis (idiopathic IBD). Future studies would be needed to attempt to further clarify whether increased *Fusobacterium* spp is a consequence of small cell GI LSA in cats or whether it plays a role in the development of the disease.

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