Useful GI Function Tests and Molecular Tools for Veterinary Clinicians

Karin Allenspach, Dr.med.vet., FVH, DECVIM-CA, PhD, FHEA
Royal Veterinary College
London, United Kingdom
Email: kallenspach@rvc.ac.uk

Abstract

The mucosal immune system is at the forefront of defense against invading pathogens but, at the same time, must maintain tolerance toward commensals and food antigens in the intestinal lumen. The interplay between the innate immune response and commensal microorganisms is essential in this process. Great progress has been made to identify some of the genetic predispositions underlying inflammatory bowel disease (IBD) in certain breeds, such as the German Shepherd Dog. Some immunological markers, such as cytokine measurement, immunohistochemistry for p-glycoprotein expression, perinuclear anti-neutrophil cytoplasmic antibodies and PCR for antigen receptor rearrangement, are discussed for their clinical usefulness in the diagnosis and management of IBD.

Glossary of Abbreviations
ARD: Antibiotic Responsive Diarrhea
CCECAI: Canine Chronic Enteropathy Clinical Activity Index
DCs: Dendritic Cells
IBD: Inflammatory Bowel Disease
NOD2: Nucleotide Oligomerization Domain 2
PARR: Polymerase Chain Reaction for Antigen Receptor Rearrangement
PRR: Pathogen-Recognition Receptors
TLRs: Toll-Like Receptors

Introduction

Among the causes of chronic enteropathies in dogs, adverse reactions to food, idiopathic inflammatory bowel diseases and antibiotic responsive diarrhea (ARD) are common. These disorders are retrospectively diagnosed by their response to treatment.[1] The clinician faced with a case usually performs an extensive workup to exclude extragastrointestinal causes as well as treatable disorders, such as pancreatic diseases, chronic parasitic or bacterial infections, and tumors. After taking intestinal biopsies and reaching a tentative diagnosis, the gold standard approach to treatment is a trial therapy with elimination diet, antibiotic treatment for several weeks and finally immunosuppressive treatment with corticosteroids.

The last decade brought advances in knowledge about the pathogenesis of IBD in people; specifically, the interplay of innate immunity receptors with commensals of the intestinal microbiome is now implicated in the disease. Molecular studies have identified specific imbalances in the microbiome of people with IBD. In addition, genetic polymorphisms associated with an increased risk of development of IBD have been identified. These data promise to help in the development of new treatment options for IBD, including probiotics and targeted molecular treatment strategies. This article reviews the newest findings in canine IBD and discusses how they could lead to the development of new therapeutic targets.

Histology and Assessment of T-Cell Infiltration

Sampling of intestinal biopsies is considered an essential step to exclude neoplastic causes and confirm the presence of intestinal inflammation. However, the interpretation of intestinal biopsies is difficult and subject to controversy. In several recent studies looking at conventional histological interpretation of intestinal biopsy samples, no correlation of clinical activity with histological grading either before or after therapy was found.[1,2] In addition, total lymphocyte counts as well as the number of infiltrating CD_3 cells in the lamina propria were not good markers for clinical activity of disease, as there was no difference in cell counts before and after treatment.[2]

These findings suggest that the type and degree of histological infiltrates in canine IBD may not be as helpful as in human medicine, where the clinical scores correlate well with the histological grading. Therefore, a new grading scheme for the histological interpretation of endoscopically obtained biopsies from dogs and cats with IBD has recently been published by the WSAVA working group. The findings from this group suggest that microarchitectural changes seem to be significantly more important than cellular infiltrates when assessing histological severity of disease. However, so far, there is limited information on how well this new grading system correlates with clinical disease. Further prospective studies will assess this grading system in conjunction with clinical findings and outcomes in dogs and cats with IBD.

Evidence of Innate Immunity Hyper-Responsiveness in Canine IBD

Toll-like receptors (TLRs) are upregulated in the intestine of humans with Crohn's disease and ulcerative colitis. These receptors are responsible for recognizng specific microbe-assisted patterns of bacteria, viruses and fungi and are expressed on immune cells as well as intestinal epithelial cells. They form an important part of the barrier of the intestine toward the antigens in the intestinal lumen as they are intricately involved in the decision-making process of the gut immune system as to whether an antigen is self or non-self. The change in TLR expression may be either a consequence of the ongoing stimulation of TLRs by an altered

From the Purina Companion Animal Nutrition Summit: *The Gastrointestinal Tract in Health and Disease*; Lisbon, Portugal; March 22-24, 2012.

microbiota or may be a causal factor contributing to the pathogenesis of disease. Most human studies show that the mRNA and protein expression of TLR2 as well as TLR4 are increased in the intestines of people with active IBD.

In a recent clinical study at the Royal Veterinary College (RVC), London, we were able to show that dogs of any breed with clinically severe, active IBD express higher levels of TLR2 receptors in the duodenum compared to healthy dogs when measured by real-time PCR (polymerase chain reaction) in endoscopic biopsies. In addition, TLR2 expression was correlated with the clinical severity of IBD using the Canine Chronic Enteropathy Clinical Activity Index (CCECAI).[6,7] However, TLR4 expression levels were similar to those in healthy canine intestines.

Other studies have found that only a subgroup of dogs with IBD (the ones responding only to steroid administration) showed an increased expression of TLR2, TLR4 and TLR9, compared to healthy intestines when expression was measured by real-time PCR. In further studies looking at German Shepherd Dogs, we found that TLR4 expression was 60-fold higher in the duodenum, ileum and colon of dogs with IBD compared to samples from healthy dogs; however, TLR2 and TLR9 were similarly expressed.[8] These data show that it is important to look at similar phenotypes of dogs when choosing cases for such studies, as the results vary depending on the severity of disease, the treatment response and the specific breed of dog.

In addition, care must be taken to compare studies using real-time PCR as the standardization method depending on the reference genes, which need to be carefully chosen for each study. TLR2 has recently been shown to be overexpressed in the diseased intestine in mouse models of IBD.[9] TLR2 in this context is implicated in the homeostasis and repair of intestinal tissue after injury. It is therefore possible that the high expression of TLR2 found in dogs with IBD in the studies mentioned above could be a marker of intestinal inflammation, and its physiological action is to downregulate ongoing inflammation. TLR5 expression was consistently downregulated in the intestines of German Shepherd Dogs with IBD as compared to healthy dogs.[8]

In mice and humans, TLR5 is highly expressed in the healthy small intestine, with CD11c+ dendritic cells (DCs) in the lamina propria mucosa expressing most TLR5. It is believed that this tolerogenic phenotype of DCs induces T regulatory cells and stimulates the production of anti-inflammatory cytokines, such as IL-10 in response to flagellin. In contrast, in intestinal inflammation characterized by the upregulation of Th1– and Th17 cytokines, CD11c- DCs express low levels of TLR5 but high levels of TLR4. In this context, TLR4 is thought to be upregulated to compensate for the low TLR5 expression.

It could be speculated that the differentially low expression of TLR5 and high expression of TLR4 seen in the intestines of the German Shepherd Dogs in our study indicate a similar compensatory role of TLR4, as gram-negative flagellated bacteria can also be recognized through binding of LPS by TLR4. Figure 1 shows the current concept of how IBD develops in people and in dogs.[10]

PCR for Antigen Receptor Rearrangement to Diagnose Intestinal Lymphoma

Polymerase chain reaction for antigen receptor rearrangement (PARR) amplifies the highly variable T or B cell antigen receptor genes and is used to detect a clonally expanded population of lymphocytes. In a recent study at the RVC, we prospectively evaluated the accuracy of PARR in diagnosing lymphoma from biopsies obtained endoscopically compared to the gold standard of histopathology and clinical outcome (determined by follow-up information of at least two years) (Gajanajake, et. al. Abstract. ECVIM.2008).

Samples from 39 dogs were included in the study. Five dogs had a diagnosis of lymphoma, of which four were positive on PARR. One dog was diagnosed with an intestinal carcinoma, three with a gastric carcinoma (with concurrent inflammation in the intestine), and 30 with inflammatory bowel disease. Five dogs with IBD and two dogs with carcinoma were positive on PARR. Of the five dogs with IBD that were positive on PARR, four were clinically well on follow-up but one had been euthanized due to the development of jaundice. This indicated a sensitivity and specificity of 80% and 79%, respectively, for PARR to correctly identify cases of canine gastrointestinal lymphoma when

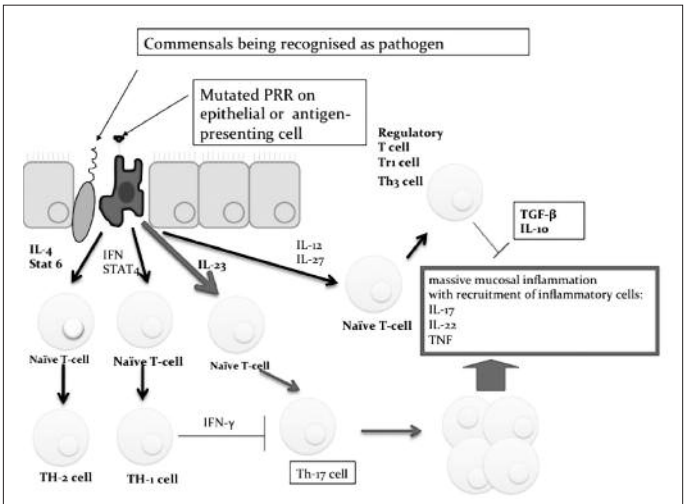

Figure 1. Proposed pathogenesis of inflammation in canine and feline IBD.[13] In the case of IBD, a primary defect in the recognition of commensals or pathogens by innate immunity receptors may play a role. Mutations in pathogen-recognition receptors (PRR) lead to misrepresentation of commensals as pathogens, which results in production of IL-23, driving naïve T cells to differentiate into Th17 cells. These Th17 cells now produce large amounts of proinflammatory cytokines, such as IL-17, and TNF. This leads to tissue destruction and epithelial cell injury, which lets even more antigens pass through to the lamina propria. This inflammatory response cannot be counter-regulated anymore by regulatory T cells, which leads to the characteristic inflammatory pattern seen in IBD.

compared to histopathology and clinical outcome as a gold standard. The data derived from this pilot study indicate a noteworthy false positive rate (7/36 cases) for PARR when used on endoscopic biopsies to diagnose cases of canine intestinal lymphoma. The conclusion that a positive PARR test on an endoscopic biopsy means a diagnosis of lymphoma must therefore be made cautiously in a clinical situation.

Imbalance of the Intestinal Microbiota in Canine IBD

Molecular studies on the intestinal microbiome in dogs of different breeds with IBD have found that members of the families *Enterobacteriaceae* and *Clostridiaceae* were enriched in the diseased intestine. These bacteria are thought to contribute to the pathogenesis of disease in dogs as well as humans with IBD. There seems to be differences in the microbiome of different dog breeds that are predisposed to the development of IBD. It appears that German Shepherd Dogs with CE have a distinctly different microbiome from healthy dogs, as well as from other breeds of dogs presenting with IBD, with overrepresentation of certain traditionally labelled "beneficial" bacteria in the duodenum, specifically sequences of the order of *Lactobacilalles*. This may indicate why many German Shepherd Dogs respond to dietary and/or antibiotic treatment alone, whereas in other breeds with CE, immunosuppressive treatment is often necessary to control clinical signs.

Genetic Predisposition in German Shepherd Dogs with IBD

Over the last decade, numerous genes have been found to be associated with an increased risk of development of IBD in humans, many of them implicated in the innate immune response in the intestine.[11] Mutations in pathogen recognition receptors, such as nucleotide oligomerization domain 2 (NOD2), toll-like receptor 4 (TLR4), IL-23 receptor and others, have all been associated with IBD.[11] In dogs, it has long been obvious to clinicians that IBD seems to have a genetic component. This is particularly evident in breeds like the Boxer, which is predisposed to histiocytic ulcerative colitis. Another breed with a predisposition to the development of IBD is the German Shepherd Dog, which seems to be predisposed to antibiotic-responsive diarrhea. We recently found that several polymorphisms in TLR4 and TLR5 are significantly associated with IBD in German Shepherd Dogs.[12] One polymorphism in TLR5 also seems to be more widely implicated in the pathogenesis of IBD in dogs in general. The next step will be to evaluate correlations between such polymorphisms and the particular phenotype expressed in different breeds in order to make the genetic assays useful to the practitioner.

References

1. Allenspach K, et. al. Chronic enteropathies in dogs: evaluation of risk factors for a negative outcome. *J Vet Intern Med*. 2007;21(4): 700-708.

2. Schreiner NM, et. al. Clinical signs, histology, and CD3-positive cells before and after treatment of dogs with chronic enteropathies. *J Vet Intern Med*. 2008.22(5):1,079-1,083.

3. Day MJ, et. al. Histopathological standards for the diagnosis of gastrointestinal inflammation in endoscopic biopsy samples from the dog and cat: a report from the World Small Animal Veterinary Association Gastrointestinal Standardization Group. *J Comp Pathol*. 2008;138(Suppl 1):S1-S43.

4. Frolova L, et. al. Expression of Toll-like receptor 2 (TLR2), TLR4, and CD14 in biopsy samples of patients with inflammatory bowel diseases: upregulated expression of TLR2 in terminal ileum of patients with ulcerative colitis. *J Histochem Cytochem*. 2008;56(3): 267-274.

5. Szebeni B, et. al. Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. *Clin Exp Immunol*. 2008; 151(1):34-41.

6. McMahon LA, et. al. Expression of Toll-like receptor 2 in duodenal biopsies from dogs with inflammatory bowel disease is associated with severity of disease. *Vet Immunol Immunopathol*. 2010;135(1-2):158-163.

7. Burgener IA, et. al. Upregulation of Toll-like receptors in chronic enteropathies in dogs. *J Vet Intern Med*. 2008;22(3):553-560.

8. Allenspach K, et. al. Evaluation of mucosal bacteria and histopathology, clinical disease activity and expression of Toll-like receptors in German shepherd dogs with chronic enteropathies. *Vet Microbiol*. 2010;146(3-4):326-335.

9. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun*. 2000;68(12):7,010-7,017.

10. Cario E, Podolsky DK. Toll-like receptor signaling and its relevance to intestinal inflammation. *Ann NY Acad Sci*. 2006;1072: 332-338.

11. Shih DQ, Targan SR, McGovern D. Recent advances in IBD pathogenesis: genetics and immunobiology. *Curr Gastroenterol Rep*. 2008;10(6):568-575.

12. Kathrani A, House A, Catchpole B, Murphy A, German A, Werling D, Allenspach K. Polymorphisms in the tlr4 and tlr5 gene are significantly associated with inflammatory bowel disease in German shepherd dogs. *PLoS One*. 2010;5(12):e15740.

13. Allenspach K. Clinical immunology and immunopathology of the canine and feline intestine. *Vet Clin North Am Small Anim Pract*. 2011; 41(2):345-360.