Veterinary Immunology and Immunopathology 146 (2012) 87–91

Contents lists available at SciVerse ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Short communication

# Gene expression of selected signature cytokines of T cell subsets in duodenal tissues of dogs with and without inflammatory bowel disease

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ARTICLE INFO

*Article history:*
Received 11 December 2011
Received in revised form 16 January 2012
Accepted 16 January 2012

*Keywords:*
Interleukin-17
Adaptive immunity
Canine chronic enteropathy
Quantitative reverse-transcriptase polymerase chain reaction

ABSTRACT

Inflammatory bowel disease (IBD) is a common cause of chronic diarrhoea in dogs. In people, specific cytokine patterns attributed to T cell subsets, especially T helper cell [Th]1, Th17 and regulatory T(reg) cells have emerged in IBD. In contrast, no specific involvement of a distinct T cell subset has been described so far in canine IBD. Thus, the aim of the present study was to assess gene expression of signature cytokines in duodenal tissues from 18 German shepherd dogs with IBD (group 1), 33 dogs of other breeds with IBD (group 2) and 15 control dogs (group 3). Relative quantification of IL-17A, IL-22, IL-10, IFNγ and TGFβ was performed. Expression of IL-17A was significantly lower in groups 1 and 2 compared to group 3 ($p = 0.014$), but no difference in the expression of IL-22 ($p = 0.839$), IFNγ ($p = 0.359$), IL-10 ($p = 0.085$) or TGFβ ($p = 0.551$) across groups was detected. Thus, no clear evidence for the involvement of Th-17 signature cytokines in canine IBD at the mRNA level could be shown. The contribution of specific T cell subsets to the pathogenesis of canine IBD warrants further investigation.



## 1. Introduction

In recent years, the role of different T cell subsets, and specifically T-helper (Th) cells in the pathogenesis of human inflammatory bowel disease (IBD) has been intensively investigated. IBD is a complex disease in which genetic predisposition, composition of the gut microbiota, environmental triggers and dysregulation of innate immunity play a role (Sartor, 2006). Recent research has highlighted the importance of Th17 cells in the pathogenesis of IBD, demonstrating increased numbers of these cells in the lamina propria of human IBD patients (Fujino et al., 2003) and mouse models of colitis (Ivanov et al., 2007). The inflammation occurring in human IBD, specifically Crohn's disease (CD), is thought to be driven by Th1 cells producing IFNγ and Th17 cells producing interleukin (IL)-17 and IL-22 (Brand et al., 2006). Impaired regulation of immune responses by regulatory T (Treg) cells is also part of the diseases' pathogenesis (Maul et al., 2005), and these cells have recently been characterised in the dog (Pinheiro et al., 2011). IBD is believed to be the most common cause of chronic gastrointestinal signs in dogs, and although the clinical and histological picture is different from that seen in humans, the molecular pathogenesis is thought to be similar in both diseases. However, so far, no clear cytokine expression profile has been associated with canine IBD (De Majo et al., 2008; German et al., 2000) and – to the

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doi:10.1016/j.vetimm.2012.01.013

authors' knowledge - the relevance of Th17 cells for the disease has not been assessed so far in dogs. Discrepancies in the results of studies investigating cytokines in canine IBD could be due to different techniques used, differences in breeds of dogs included, or sample sizes. To obtain more insight into the potential role of a specific cytokine pattern in the pathogenesis of canine IBD, the current study assessed the expression of signature cytokines of Th1 (IFNγ), Th17 (IL-17A, IL-22) and Treg cells (IL-10 and TGFβ) in endoscopic duodenal biopsies of dogs with IBD by quantitative reverse-transcriptase PCR.

## 2. Materials and methods

### 2.1. Study population

A total of 66 duodenal biopsies from dogs were evaluated. Dogs in group 1 consisted of 18 German shepherd dogs (GSDs) diagnosed with IBD (7 female, 11 male) with a median age of 4 years (range 1–11 years). Group 2 comprised 33 dogs of other breeds than GSDs diagnosed with IBD (see Table 1), with 21 females and 12 males and a median age of 7 years (range 1–17 years). Control samples were obtained from 5 healthy greyhounds and 10 healthy beagles. Of those, 7 were male entire, 4 female entire and 4 female spayed, with a median age of 3 years (range 1–8 years). Using Kruskall–Wallis test, median age was not different across groups ($p = 0.156$).

The diagnosis of IBD was made based on appropriate clinical signs (vomiting and/or diarrhoea, weight loss) of at least 3 weeks' duration, exclusion of other causes of chronic gastrointestinal signs and the presence of lymphoplasmacytic and/or eosinophilic inflammation on histopathological review of duodenal biopsies. Written owner consent was obtained for all dogs included into the study. Additionally, the study was approved by the Royal Veterinary College Ethics and Welfare Committee. One mucosal biopsy was placed immediately into RNAlater (Ambion, Huntingdon, UK) and stored at −80 °C until further use. Euthanasia of the control dogs was performed for reasons unrelated to the present study; duodenal mucosal biopsies were obtained with endoscopic forceps immediately *post mortem* and stored in the same manner as described above.

**Table 1**
Breeds of dogs suffering from IBD used in the study.

| Breed | Number of cases |
|---|---|
| Mixed breed | 4 |
| Cavalier King Charles Spaniel | 3 |
| Cocker Spaniel | 3 |
| Rottweiler | 3 |
| Boxer | 2 |
| Golden Retriever | 2 |
| Staffordshire Terrier | 2 |
| Yorkshire Terrier | 2 |
| Basset | 1 |
| Bearded Collie | 1 |
| Border Collie | 1 |
| Cairn Terrier | 1 |
| Dobermann Pinscher | 1 |
| English Pointer | 1 |
| Irish Setter | 1 |
| Labrador | 1 |
| Rhodesian Ridgeback | 1 |
| Tibetan Spaniel | 1 |
| Weimaraner | 1 |
| Breed not stated | 1 |

### 2.2. RNA extraction and cDNA synthesis

Total RNA was extracted using the Mammalian Total RNA extraction Kit (Sigma–Aldrich, Gillingham, UK). Twenty-five milligrams of tissue were homogenised into 0.5 ml lysis solution, using the Mixer Mill MM300 (Retch, Leeds, UK). Subsequently, an in-solution DNase digestion step (Turbo DNase, Ambion, Austin, USA) was performed. cDNA was synthesised using oligo$(dT)_{15}$ primers and the Improm II reverse transcriptase enzyme (Promega, Southampton, UK). Controls were performed without reverse transcription. None of the samples showed evidence of amplifiable genomic DNA with this assay.

### 2.3. Generation of positive controls

To obtain positive controls suitable for subsequent qPCR analysis, we cloned parts of the canine sequences for IL-17A, IL-22, IL-10, IFNγ, TGFβ and the two reference genes previously shown to be most stably expressed in canine endoscopic intestinal samples (Peters et al., 2007), glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and succinyl dehydrogenase (SDHA). To do so, primers were either designed based on published gene sequences using the Primer3 software (Rozen and Skaletsky, 2000) or, in the case of GAPDH and SDHA, published primers were used (McMahon et al., 2010) (see Table 2). Primers were optimised for annealing temperature and, if necessary, $MgCl_2$ concentration using standard PCR (Immolase polymerase, Bioline, London, UK) with the following steps: enzyme activation at 95 °C for 7 min, followed by 35 cycles of 94 °C for 30 s, annealing temperature (see Table 2) for 30 s, elongation at 72 °C for 1 min and a final extension step of 72 °C for 7 min. Reactions were performed in 25 μl total volume and contained 1 μl of canine cDNA, 800 pmol of cytokine specific primers and 2.5 mM $MgCl_2$ (with the exception of IFNy, where 4.5 mM $MgCl_2$ were used). Amplified fragments were visualised on a 1.5% agarose gel and purified using the Wizard SV Gel and PCR clean-up system (Promega). Purified fragments were cloned into the pGemT-Easy vector (Promega) following the manufacturer's instructions. Plasmid DNA was extracted from several colonies using the PureYield Plasmid Minprep system (Promega), sequenced (Source Bioscience, Cambridge, UK) and confirmed against published sequences (Table 2).

### 2.4. Quantitative polymerase chain reaction

Primers used in standard PCR were tested for qPCR and, where not suitable, new qPCR primers were designed as described above (Table 2). Published primer sequences were used for IL-17A (Maccoux et al., 2007). Each qPCR reaction was performed in 20 μl, contained 200 nM of each primer and 1 μl of cDNA in addition to SsoFast Evagreen Supermix (Bio-Rad, Hemel Hampstead, UK). The reaction involved an enzyme activation step at 95 °C for 5 min,

**Table 2**
Characteristics of primers used in conventional and quantitative PCR.

| Gene | Forward primer (5′–3′) | Reverse primer (5′–3′) | Annealing temperature (°C) | NCBI accession number | Used in standard (S) or quantitative (Q) PCR |
|---|---|---|---|---|---|
| IL-17A | CTGAGCCTGGTGGCTATCAT | CGAACAATAGGGGTGACACA | 59.5 | NM_001165878.1 | S |
| IL-17A | CACTCCTTCCGGCTAGAGAA | CACATGGCGAACAATAGGG | 60 | NM_001165878.1 | Q |
| IL-22 | TCCAGCAGCCCTATATCACC | TTGGCTTAGCTTGTTGCTGA | 60.2 | XM_538274.2 | S, Q |
| IFNγ | TTCAGCTTTGCGTGATTTTG | CTGCAGATCGTTCACAGGAA | 54.3 | NM_001003174.1 | S, Q |
| IL-10 | TCTGTTGCTGCCTGGTCCT | TGATGTCTGGGTCGTGGTT | 54.3 | NM_001003077.1 | S, Q |
| TGFβ | GCATGTGGAGCTGTACCAGA | TAGTACACGATGGGCAGTGG | 55 | NM_001003309.1 | S, Q |
| GAPDH | GATTGTCAGCAATGCCTCCT | CGGTTGCTGTAGCCAAATTC | 55 | XM_003435649.1 | S |
| GAPDH | GGAGAAAGCTGCCAAATATG | ACCAGGAAATGAGCTTGACA | 55 | XM_003435649.1 | Q |
| SDHA | GGACAGAGCCTCAAGTTTGG | GGCATCCTTCCGTAATGA | 55 | XM_535807.3 | S, Q |

followed by 40 cycles of 94 °C for 15 s, annealing temperature (see Table 2) for 10 s, elongation at 72 °C for 10 s and an additional melting step of 80 °C for 5 s before each plate reading (to melt primer dimers). Each reaction was carried out in triplicate and reaction efficiency was determined for each gene using 10-fold dilutions ($10^7$ molecules $\mu l^{-1}$ to $10^1$ molecules $\mu l^{-1}$) of plasmids containing the cloned amplicon. Melting curves were generated to ensure a single amplicon had been produced. IL-17A, IL-22, IL-10 and TGFβ were assessed in all 3 groups; however IFNγ expression could only be measured in groups 2 and 3 owing to limited availability of samples from group 1.

Gene expression was quantified by averaging the triplicate measurements for each biological sample for all genes, following normalisation of the expression ratio of each target gene to the geometric mean of the two reference genes, as described recently (Vandesompele et al., 2002).

### 2.5. Statistical analysis

Statistical analysis was performed using Graph Pad Prism software package (Version 5, Graph Pad Software Inc., La Jolla, CA, USA). Data were tested for normal distribution using histograms and the D'Agostino and Pearson omnibus normality test. Comparison of non-normally distributed data between groups was performed using the Kruskall Wallis test (for comparison of all 3 groups) or the Mann–Whitney *U* test (when only 2 groups could be evaluated). If significant differences across all groups were observed, a Dunn's multiple comparison test was performed as a post hoc test.

## 3. Results and discussion

In the present study, we aimed to identify the potential role of T cell subsets specific cytokines in the pathogenesis of canine IBD. Interleukin-17A mRNA expression was significantly different across groups ($p = 0.014$). Expression was significantly lower in duodenal samples from respective groups 1 and 2 than the control group (see Fig. 1 and Table 3). Interleukin-22 ($p = 0.839$), IL-10 ($p = 0.085$) and TGFβ expression ($p = 0.551$) was not different across the groups (see Table 3). Furthermore, there was no difference in the expression of IFNγ between groups 2 and 3 ($p = 0.359$; see Table 3).

In contrast to recent findings in human IBD, duodenal samples of dogs with IBD included in this study did not show up-regulation of the Th17 related cytokines IL-17A and IL-22 compared to healthy canine tissues. Indeed, IL-17A expression was significantly lower in both diseased groups compared to control samples and IL-22 and IFNγ expression showed no differences across groups. Our data raise the possibility that the pathogenesis of canine IBD is different from that of human CD, in that neither Th1 nor Th17 cells seem to play a major role in driving intestinal inflammation; however, alternative explanations also have to be considered. Recent studies in human IBD have found that IL-17A can play an anti-inflammatory role in some instances (O'Connor et al., 2009; Shen and Durum, 2010) and may be important in maintaining epithelial barrier integrity (Kinugasa et al., 2000). Therefore, our data could also indicate that a lack of IL-17A leads to an increased permeability of the intestinal epithelial barrier in canine IBD, allowing bacteria to cross into the lamina propria and exacerbate underlying inflammation. Similarly, we speculate that failure to up-regulate IL-22 in IBD could enhance the inflammatory response. IL-22 has been described to be an anti-inflammatory cytokine in humans and mice, strengthening the epithelial barrier, promoting the production of mucin (Sugimoto et al., 2008) and inducing the production of antimicrobial peptides (Zheng et al., 2008).

We did not find any differences in the expression of IL-10 mRNA. This finding contrasts with one human study (Melgar et al., 2003), but is consistent with another, in which low IL-10 expression was detected in inflamed areas and intestinal granulomas of CD patients (Schreiber et al., 1995). Thus, IL-10 could act as an indicator of the loss of counter-regulatory anti-inflammatory mechanisms and/or the loss of oral tolerance in canine IBD. The fact that no difference in TGFβ expression was observed in the present study is interesting. Even though TGFβ is involved in both the development of Tregs and Th17 cells, the fact that it was not differentially expressed across groups in the current study reconciles with the finding that neither IL-10 nor Th-17-type cytokines are increased in canine IBD.

There are several limitations of the current study. Firstly, investigating cytokine profiles to determine a specific T cell subset involvement may not be ideal, even though some cytokines are considered "signature cytokines". Current research has highlighted the plasticity of Th17 cells, which can harbour characteristics of "classical" Th17 cells (producing IL-17, IL-22 and IL-23) or can switch to develop a "Th1-like" cytokine pattern, characterised by IFNγ synthesis (Lee et al., 2009). In addition, cytokines thought to be specifically produced by Th17 cells are also secreted by a large number of innate immune cells (Cua and Tato, 2010).

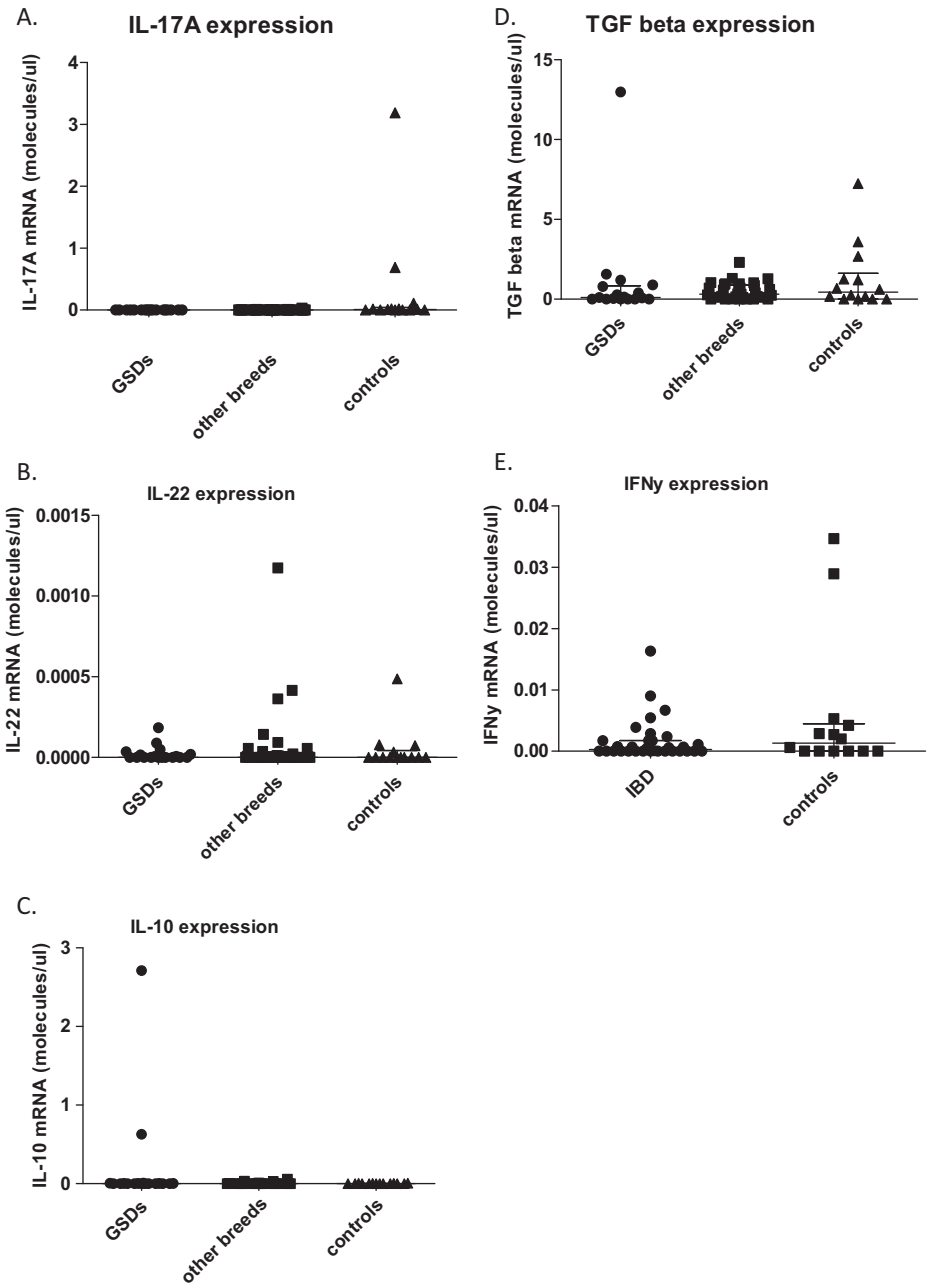


**Fig. 1.** Expression of selected cytokines in canine duodenal tissues measured by reverse transcriptase quantitative PCR. Data shown as relative expression to the geometric mean of two reference genes. GSDs = German shepherd dogs.

**Table 3**
Median (range) and *p*-values for the expression of selected cytokines in canine duodenal tissue.

| Gene | Group 1 (copies $\mu l^{-1}$) | Group 2 (copies $\mu l^{-1}$) | Group 3 (copies $\mu l^{-1}$) | *p*-value |
|---|---|---|---|---|
| IL-17A | $0.23^{-7}$ (0–0.00138) | 0 (0–0.29) | 0.009 (0–3.19) | 0.014* |
| IL-22 | 0 (0–0.00018) | 0 (0–0.0012) | 0 (0–0.00049) | 0.839 |
| IL-10 | 0.0017 (0–2.71) | 0.0015 (0–0.059) | 0 (0–0.006) | 0.085 |
| TGFβ | 0.1908 (0–12.98) | 0.3227 (0–2.303) | 0.4395 (0–7.25) | 0.551 |
| IFNγ | n.p. | 0.00028 (0–0.016) | 0.0013 (0–0.035) | 0.359 |

Group 1, German shepherd dogs with IBD; group 2, dogs of other breeds with IBD; group 3, control dogs; n.p., not performed.
* Indicates significant difference across groups.

We tried to avoid this problem by investigating cytokines produced by Th1, Th17 and Treg cells: the fact that none of the investigated cytokines were up-regulated could be interpreted to indicate that none of these Th cells is of particular importance in canine IBD. However, the expression of lineage-specific transcription factors, like RORγt or the aryl hydrocarbon receptor (Ivanov et al., 2006; Veldhoen et al., 2008), should be assessed in future studies.

Furthermore, using whole endoscopic biopsies is not ideal as the composition of the samples might vary. Laser microdissection of specific cell types could provide a solution to avoid this problem (Funke, 2011). Thirdly, assessing cytokines at the level of gene expression (mRNA) has the well-known draw-back of not necessarily correlating with cytokine protein production. Currently, this is complicated by the paucity of canine-specific monoclonal antibodies available, but future studies should also examine protein production when possible.

In conclusion, in contrast to the situation in human IBD, no clear Th1/Th17 cytokine pattern was found in canine IBD at the level of gene expression in the duodenal samples included in this study. Furthermore, there was no evidence of up-regulation of IL-10 and TGFβ mRNA in diseased duodenal tissue. The contribution of specific T cell subsets to the pathogenesis of canine IBD warrants further investigation in the future.

## Conflict of interest statement

The authors declare no conflict of interest.

## Acknowledgements

This study is funded through a Biotechnology and Biological Sciences Research Counsil (BBSRC) CASE Studentship with Protexin Veterinary (Somerset, UK). Consumables for this study were in part sponsored by the Morris Animal Foundation. The RVC manuscript approval number is VCS_00271.

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