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Title: Differential expression of key regulators of Toll-like Receptors in Ulcerative Colitis and Crohn's disease: A role for Tollip and PPARγ?

Short Title: Reduced expression of Tollip and PPARγ in IBD

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Abbreviations: Toll-Like Receptor (TLR), Inflammatory Bowel Disease (IBD), Ulcerative Colitis (UC) and Crohn's Disease (CD).

Summary

The innate immune system is currently seen as the likely initiator of events which culminate in the development of Inflammatory Bowel Disease (IBD) with Toll-Like Receptors (TLRs) known to be involved in this disease process. Many regulators of TLRs have been described and dysregulation of these may also be important in the pathogenesis of IBD. The aim of this study was to perform a coordinated analysis of the expression levels of both key intestinal TLRs and their inhibitory proteins in the same IBD cohorts, both Ulcerative Colitis (UC) and Crohn's Disease (CD), in order to evaluate the potential roles of these proteins in the pathogenesis of IBD. Of the six TLRs (TLRs 1, 2, 4, 5, 6 and 9) examined, only TLR4 was significantly increased in IBD, specifically in active UC. In contrast, differential alterations in expression of TLR inhibitory proteins were observed. A20 and SOCS1 were increased only in active UC whilst IRAKm and Bcl-3 were increased in both active UC and CD. In contrast, expression of both PPARγ and TOLLIP was decreased in both active and inactive UC and CD and at both mRNA and protein levels. In addition, expression of both PPARγ and A20 expression was increased by stimulation of a colonic epithelial cell line Caco-2 with both TLR ligands and commensal bacterial strains. These data suggest that IBD may be associated with distinctive changes in TLR4 and TLR inhibitory proteins, implying that alterations in these may contribute to the pathogenesis of IBD.

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Introduction

The incidence of inflammatory bowel diseases (IBDs) such as Crohn's disease (CD) and Ulcerative Colitis (UC) has been steadily increasing over recent years. Although the aetiology of these diseases remains largely unknown, there is an accumulating body of evidence suggesting that, in genetically susceptible individuals, intestinal inflammation in IBD results from an alteration in the balance between resident microbes in the gut and the host immune response at the mucosal barrier [1].

The innate immune system is currently seen as the likely initiator of events which culminate in the development of IBD [1]. A family of innate immune sensors, the Toll-like receptors (TLRs), are essential in the host defence against pathogens. TLRs are found to be expressed on most intestinal immune cell types, including dendritic cells and intestinal epithelial cells (IECs), and as such play an essential role in the recognition of pathogenic infection in the intestine[2]. Ten TLR family members have been identified in humans, each of which responds to different pathogen-associated molecular patterns such as peptidoglycan (TLR2), viral dsRNA (TLR3), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5) viral ssRNA (TLR7/8) and unmethylated CpG DNA (TLR9). Whilst the specific ligand for TLR10 is unknown, it has recently been shown to mediate the inflammatory response to both bacteria and viruses [3, 4]. Activation of most TLRs results in the recruitment of the signalling adaptor molecule MyD88 and subsequent activation of signalling pathways which culminate in phosphorylation and degradation of IκB, and translocation of the transcription factor NF-κB to the nucleus. In contrast, TLR3 recruits the adaptor TIR-domain-containing adapter-inducing interferon-β (TRIF) to mediate signal transduction. TLR signalling through these pathways is important in the recruitment of inflammatory cells and in the production of inflammatory cytokines in the intestine [5].

TLR activation can, however, be a double-edged sword. Whilst activation of these receptors is essential for promoting the innate immune response to combat infection, sustained inflammatory signalling from TLRs can be detrimental and members of the TLR family have been shown to be involved in the pathogenesis of autoimmune, inflammatory and infectious disease. TLRs have been implicated in several gastrointestinal (GI) disorders including colon cancer, colitis and coeliac disease [5]. The expression pattern of several TLRs in the intestine has been investigated. TLR4, for example, is normally expressed throughout the intestine at

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low levels in both the epithelium and in lamina propria mononuclear cells but its expression is reported to be increased in IBD, indicating that increased TLR4 expression may contribute to the initiation and maintenance of intestinal inflammation [5, 6]. TLR2 and TLR8 have also been reported to be augmented in IBD, implying that these TLRs may also contribute to the excessive inflammation associated with IBD [1].

It is clear, therefore, that the intensity and duration of the TLR response must be tightly controlled in order to maintain the balance between appropriate and inappropriate activation of the immune system in the intestine. Several of the expanding family of TLR Inhibitory Proteins have been found to be strongly expressed within and to be essential in the regulation of TLRs in the GI tract, namely Single Immunoglobulin receptor related (SIGIRR), A20, PPARγ, IRAK-m and Tollip [2]. Of these, SIGIRR$^{-/-}$ mice [7], PPARγ $^{+/-}$ mice [8] and mice with an IEC-specific deficiency in A20 [9] have all been shown to have increased susceptibility to intestinal inflammation. In addition, PPARγ is currently being explored as a potential therapeutic target for IBD, given the observation that PPARγ ligands have been shown to reduce the severity of experimental colitis [8]. However, despite the clear possibility that these TLR inhibitory proteins may be involved in pathogenesis of IBD, only a few reports have investigated expression of these in IBD cohorts, with fewer, if any, reports examining expression of multiple TLRs and their inhibitory proteins in the same cohort of IBD patients. The aim of this study therefore, was to perform a coordinated analysis of the expression levels of both key intestinal TLRs and their inhibitory proteins in both UC and CD in order to evaluate the potential role of these in the pathogenesis of IBD.

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MATERIALS AND METHODS

Reagents

LPS, Flagellin and Pam3Csk4 were purchased from Invivogen (San Diego, CA). Antibodies for SIGIRR (ab25875), Tollip (ab187198) and PPARγ (ab19481) were obtained from Abcam (Cambridge, MA, USA). The antibody for A20 (59A426) was obtained from Novus Biologicals (Abingdon, Oxford, UK). Rabbit anti-mouse immunoglobulin /HRP and goat anti-mouse/HRP were purchased from Dako Corp (Carpinteria, CA). Donkey anti-rabbit immunoglobulin /FITC was purchased from Dako Corp. The PPARγ agonist, Troglitazone, was purchased from Calbiochem (San Diego, CA.).

Study population

The study protocol, including all procedures, was approved by the University College Cork Clinical Research Ethics Committee of the Cork Teaching Hospitals. All patients were recruited from specialty gastroenterology clinics at Cork University Hospital, Cork, Ireland. Biopsies were obtained from patients with IBD with either active or inactive UC or CD, as diagnosed by conventional clinical, endoscopic and radiological criteria. The determination of active/inactive was made at time of endoscopy with no grading of the severity of the activity. Biopsies were obtained from healthy controls undergoing routine screening. The demographics of the study population are shown in table 1.

Biopsy Collection

Following prior administration of a single Fleet's enema (Lynchburg, Va., USA) 1hr prior to the procedure, subjects were placed in the left lateral position and a flexible sigmoidoscopy performed with a flexible video-colonoscope. Biopsies were taken from a pre-defined and consistent location immediately proximal to the junction of the sigmoid colon and rectum using a jumbo biopsy forceps (FB-5OU-1; Olympus Corporation, Tokyo, Japan). Biopsies were taken from same place in UC (rectosigmoid junction) and from active lesions in CD, and from rectosigmoid junction in controls. All biopsies were processed immediately.

Biopsy Processing

For RNA expression analysis, biopsies were immediately placed and stored overnight in RNALater (Ambion, Poole, Dorset, UK) at 4ºC. Following overnight saturation, the biopsies

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were placed in fresh RNALater and stored at -80ºC until further analysis. One biopsy per patient was processed for immunofluorescence (IF) and one for immunohistochemistry (IHC). For IF, biopsies were fixed in 4% paraformaldehyde overnight, washed in PBS, and then placed in 30% sucrose overnight and stored at -80ºC until further analysis. For IHC, tissues were fixed in 10% neutral buffered formalin and paraffin embedded.

Quantitative Real Time PCR (qRT-PCR)

Total RNA was isolated using the GenElute Mammalian Total RNA Mini kit (Sigma-Aldrich, St. Louis, MA) according to the manufacturer's instructions. cDNA synthesised using M-MLV Reverse Transcriptase (Sigma-Aldrich), random hexanucleotide primers, RNasin (40 U) and dNTPs (500 mM) (Promega, WI, USA). RT-PCR for all genes was performed using the LightCycler480 System (Roche Diagnostics, West Sussex, UK). Individual PCR primer pairs and probes were designed using the Roche Universal Probe Library Assay Design Centre (https://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp) (Table II). The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in the gene expression determined from the real-time quantitative PCR experiments. For human samples pooled control and IBD biopsy cDNA was used as the calibrator control for analysis of differential gene expression. For cell lines, transcript levels were normalised to the amount of GAPDH or β-actin mRNA and expression levels shown as fold induction relative to untreated.

Immunofluorescence

Single biopsies were embedded in OCT embedding medium and stored at -20ºC. Cryostat sections (8μm thick) were mounted on poly-L-lysine coated glass slides. Slides were incubated with PBS with 0.1% Triton X100 (PBS-T) for 30 minutes. Non-specific binding sites were blocked by incubating with PBS-T +5% donkey serum (Sigma-Aldrich) for 1h at room temperature in a humidified chamber. Primary antibodies were diluted 1:1000 and incubated with the tissue sample overnight at 4ºC in a humidified chamber. Slides were washed x3 with PBS-T prior to incubation with secondary antibody for 1h. Both primary and secondary antibodies were diluted in PBS-T with 5% donkey serum. Controls were treated as above omitting the primary antibodies. Immunofluorescence was visualised on an Olympus BX51 microscope using an Olympus DP71 camera.

Immunohistochemistry

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Immunohistochemical staining was performed in 4-μm thick formalin-fixed, paraffin-embedded whole-section slides. Sections were deparaffinized in xylene and rehydrated prior to analysis. Antigen retrieval was performed by microwave irradiation in 0.01 M citrate buffer, pH 6.0. Slides were washed twice for 5 min in a wash buffer containing 50 mM Tris-Cl, pH 7.6; 50 mM NaCl; 0.001% saponin. Endogenous peroxidase was quenched with 3.0% hydrogen peroxide in methanol for 10 min. Nonspecific binding was blocked using 5% normal serum in wash buffer for 1 hr. Sections were incubated overnight at 4°C with primary antibody. The PPARγ antibody was diluted 1:1000 and the A20 antibody diluted 1:50. Primary antibody binding was localized using a biotinylated secondary antibody, and visualised using avidin-conjugated HRP and DAB substrate, contained within the Vectastain ABC detection kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with haematoxylin and mounted. Parallel negative controls were performed for each antibody, using rabbit IgG instead of the primary antibody.

Bacterial Culture

Lactobacillus salivarius subsp. salivarius was obtained from ATCC (Rockville, MD, USA) and was cultured at 37° under anaerobic conditions for 24 hr in de Man–Rogosa–Sharpe broth (Oxoid, Basingstoke, UK). *Escherichia coli HB101K-12* (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) was cultured in lysogeny broth at 37° under aerobic conditions for 24 hr with constant shaking. *Bacteroides fragilis* CIT01, kindly provided by Dr Jim O'Mahony, Cork Institute of Technology, was cultured at 37° under anaerobic conditions for 24 hr in brain heart infusion broth (Oxoid) supplemented with 0·05%l-cysteine hydrochloride (Sigma, Dorset, UK). Plate counts were performed for each strain with the respective agar plates to enumerate the bacterial number.

Prior to incubation with mammalian cells, bacteria were washed with PBS by two steps of centrifugation (4000 x g for 5 min) and diluted in PBS for stimulating at a ratio of bacteria:cells of 10:1.

Cell Culture

Caco2 colon epithelial cells were obtained from the ATCC (Rockville, MD, USA). Cells were maintained in DMEM containing 10% FCS and Penicillin/Streptomycin. Cells were

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seeded at $2x10^5$ cells/ml unless otherwise stated, cultured overnight, and then either stimulated with TLR ligands, pretreated with Troglitazone (10µM) for 1h prior to TLR ligand stimulation or co-incubated with bacteria for 4hr as per the figure legends. Following co-incubation, cells were washed twice in cold PBS and RNA isolated as above.

Statistical Analysis

All qRT-PCR expression data was subjected to a One-way Anova with Bonferroni post-Test. Values of $p < 0.001$ are indicated by three asterisks (***). Values of $p < 0.01$ are indicated by two asterisks (**). Values of $p < 0.05$ are indicated by one asterisk (*). All tests were done using Graph Pad statistical software

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Results

TLR4 expression is increased in Ulcerative colitis and Crohn's Disease

Given the link between IBD and the commensal bacterial flora, we initially investigated expression of the TLRs known to be involved in bacterial sensing in both UC and CD; namely TLRs 1, 2, 4, 5, 6 and 9 [5]. We performed quantitative RT-PCR for these TLRs on biopsy tissue from 21 Healthy Controls (HC), from 10 UC patients with signs of active disease, from 12 UC patients with inactive disease, from 10 patients with active CD patients and from 9 patients with inactive CD. We were able to detect expression of all six TLRs using qRT-PCR in the colonic biopsies from all subjects (Figure 1A). Some inter-patient variation in expression of TLRs was observed with some TLR transcripts not being detectable in all samples. Of these we only observed a statistically significant increase in TLR4, where expression was increased 13 fold in active UC compared to Healthy controls ($p < 0.001$) (Figure 1). Whilst a slight increase in expression of TLR4 was seen in the inactive UC population and in both CD populations in comparison to HC, it was not statistically significant. The remaining TLRs were unchanged in their expression compared to healthy controls. Given that, of the bacterial sensing TLRs, only TLR4 was observed to have altered expression, we subsequently investigated expression of one of the TLRs known to be involved in viral sensing, TLR8. Expression of TLR8 was also seen to not be significantly altered in either UC or CD (Figure 1B).

TLR regulatory proteins are differentially expressed in IBD

As minimal alterations in the expression levels of the TLRs examined were observed, we subsequently examined the expression of a range of TLR inhibitory proteins in the same tissue. As many of these have been described [10], we focussed predominantly on those that have been shown to play a regulatory role in the intestine examining expression of SIGIRR, A20, PPARγ, IRAKm, Tollip, SOCS1and Bcl-3 [2, 11]. We also examined expression of RP105 as this is reported to suppress TLR activation in B cells and B cells are a well-established intestinal immune cell type [12]. We observed significant alterations in expression of six of these. A20, SOCS1, IRAKm and Bcl3 were seen to have increased expression in IBD (Figure 2A). Of these A20 and SOCS1 expression was significantly increased in the active UC population ($p<0.01$). Expression of IRAKm was elevated in both active UC and active CD ($p<0.01$) and Bcl3 expression was strongly elevated in inflamed UC ($p<0.001$) and also

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increased in both active and inactive CD (p<0.01) (Figure 2A). In contrast to the findings in figure 2A, expression of both Tollip and PPARγ was found to be significantly deceased in IBDs relative to HC (Figure 2B). Indeed expression of Tollip was significantly decreased in both active and inactive UC (p<0.001) and also decreased in both active and inactive CD (p<0.01). PPARγ also showed strongly suppressed expression in active UC, inactive CD and active CD (p<0.001) with suppression also observed, albeit to a slightly lesser level, in inactive UC (p<0.01) (Figure 2B). Neither SIGIRR nor RP105 exhibited any alteration in expression in either UC or CD (Figure 2C).

Observations in alterations in expression of Tollip, SIGIRR and PPARγ were confirmed at the protein level using immunofluorescence for Tollip and SIGIRR and IHC for PPARγ (Figure 3). Expression of both Tollip and PPARγ was observed to be predominantly in the epithelial cells, whilst expression of SIGIRR was observed in both the epithelial cells and the immune cell populations. Protein expression of both Tollip and PPARγ was observed to be strongly decreased in both active UC and active CD tissues in comparison to healthy control (Figure 3A and 3B), with no robust alteration in the protein expression of SIGIRR observed (Figure 3C). The level of protein expression of A20 was also investigated by immunohistochemistry. Whilst expression of A20 was detected in the sections from healthy volunteers, this expression was seen to be predominantly localised to the immune cell populations. In both UC and CD populations, the expression of A20 was also detected in the epithelial cells (Figure 3D). H&E staining was also performed on sections from all three population groups, demonstrating increased cellular infiltration, angiogenesis and inflammation in the IBD cohorts (Figure 3E).

Taken together these results indicate an impaired expression of the TLR regulatory proteins PPARγ and Tollip in the colons of both UC and CD patients, with increased expression of A20 in the epithelial cells, relative to HC, which may contribute to the deleterious inflammation associated with IBD.

Stimulation of the Intestinal Epithelial Cell line Caco-2 with TLR ligands increases expression of PPARγ and A20.

As an aberrant response to the intestinal flora has been associated with the pathogenesis of IBD, we subsequently wished to determine the ability of TLR-mediated signals to effect

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expression of TLR regulatory proteins. Caco-2 colonic epithelial cells were selected for these experiments as expression of Tollip, PPARγ and SIGIRR had been noted as being particularly strong in the epithelium. Cells were stimulated with ligands for TLR4 (LPS), TLR5 (Flagellin) and TLR 2/1 (Pam3Csk4). None of these ligands induced a significant change in mRNA expression of either Tollip (Figure 4A) or SIGIRR (Figure 4B). Both LPS and Flagellin stimulation resulted in increased expression of PPARγ ($p<0.01$) whilst Pam3Csk4 had no effect (Figure 4C). In contrast, both Flagellin and Pam3Csk4 stimulation resulted in increased expression of A20 ($p<0.01$) (Figure 4D). These results indicate that TLR ligands selectively regulate expression of PPARγ and A20 in this colonic cell line.

Commensal bacteria *E.Coli K12* and *B. fragilis* increase expression of PPARγ and A20 in the Intestinal Epithelial Cell line Caco2.

As altered microbiota populations have been associated with IBD patients [13] and, as such, the composition of the commensal flora may account for the altered expression of some of the TLR inhibitors seen in Figures 2 and 3, we finally wished to investigate the ability of commensal bacteria to regulate expression of these proteins in the intestine. Caco-2 cells were co-incubated with three strains of commensal flora; *Lactobacillus Salivarius (L.Salivarius), E.Coli K12* and *Bacteroides Fragilis (B.Fragilis)* and mRNA expression of TLR regulatory proteins assessed. Similar to stimulations with TLR ligands, expression of Tollip was not seen to be increased with any of the Bacterial strains investigated (Figure 5A). Interestingly, expression of SIGIRR was seen to be significantly reduced following stimulation with *B.Fragilis* ($p<0.01$) (Figure 5B). Similar to findings in figure 4, the expression levels of PPARγ ($p<0.01$) and A20 ($p<0.001$) were increased following co-incubation with the bacterial strains *E.Coli K12* and *B.fragilis* (Figure 5C and D). These results indicate that expression of these proteins can be directly influenced by the commensal flora.

Treatment of Caco-2 cells with a PPARγ agonist inhibits both LPS and Flagellin induced IL-6 production.

As expression of PPARγ had been observed to be strongly reduced in the IBD cohort (Figure 2B and 3B), with expression of PPARγ increased by both TLR and bacterial stimulation (Figure 4C and 5C), we wished to further examine the functional effect of PPARγ on TLR-induced responses in the Caco2 cell line. In order to study this, we utilised a well

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characterised PPARγ agonist Troglitazone. Pretreatment of Caco2 cells with troglitazone, prior to stimulation of the cells with either LPS or flagellin, was seen to inhibit the ability of these stimuli to cause an induction of the pro-inflammatory cytokine IL-6 (Figure 6), thereby confirming that PPARγ is a functional inhibitor of TLR-induced inflammation in intestinal epithelial cells. Taken together, these data indicate that the reduced expression of PPARγ seen in IBD may play a role in allowing excessive signalling from TLRs and therefore contribute to the intestinal inflammation associated with these diseases.

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Discussion

In the present study we have comprehensively investigated the expression levels of bacterial sensing TLRs and several of their inhibitory proteins in the same cohort of IBD patients. We have shown that of these TLRs, only TLR4 expression is increased in IBD, specifically in active UC. In contrast, we observed distinct alterations in the expression patterns of several TLR inhibitory proteins with a noticeable suppression in expression of two of these; PPARγ and TOLLIP in both UC and CD and in both active and inactive disease states. Moreover, we also investigated the regulation of expression of these in a colonic cell line and have shown that only PPARγ and A20 expression is altered by both TLR ligands and commensal bacterial strains.

Whilst many previous studies have investigated the expression pattern in TLRs in the intestine, these have been performed predominantly on intestinal cell lines or in murine tissues [1]. Those studies examining expression of TLRs in human IBD samples have usually investigated the expression of just one or two individual TLRs in either UC or CD. Here we have performed a comprehensive analysis of the TLRs shown to be involved in bacterial sensing (TLRs 1,2, 4, 5, 6 and 9), in both active and inactive CD and UC, and have found only TLR4 mRNA to be augmented, with this increase in expression restricted to the active UC cohort. This finding is in keeping with other reports also showing augmented expression of TLR4 in IBD [14-18] and indeed with the large body of literature supporting a role for TLR4 in the pathogenesis of IBD [19]. Interestingly, we observed no increase in the expression of TLR2, or of the TLR2 co-receptors TLRs 1 and 6, in any of our IBD populations. The role of TLR2 in IBD remains controversial with certain murine studies demonstrating a detrimental role for TLR2 in IBD whilst another study utilising multiple murine IBD models indicated that TLR2 plays no role in the pathogenesis of IBD [20, 21]. Similarly, TLR2 has been reported in some studies to be increased in both active UC and active CD [15, 18, 22] and similar to our findings, other studies have shown no change in TLR2 expression at either mRNA or protein level in IBD [14]. The lack of alteration in TLR2 expression seen here is suggestive of a lack of a role for TLR2 in the pathogenesis of IBD. We also observed no alterations in TLR5 expression in our cohort of IBD patients. This is in contrast to reports showing either decreased [23] or increased [17] expression of TLR5 in UC. Similarly, in contrast to one previous report [24], we also observed no alteration in the expression of TLR8. These differences in reported expression patterns could be due to varying demographics of the patient cohorts,

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their treatment regimens at time of biopsy or to handling of the biopsies post-surgery. It remains clear, however, that the consistent finding across all these reports is the augmented expression of TLR4, potentially highlighting the critical role of this TLR, as opposed to the others, in the pathogenesis of UC.

Given the relatively low alterations in TLR expression observed in our IBD cohorts, we subsequently hypothesised that dysregulation of expression of TLR inhibitory proteins may play a dominant role in facilitating the inflammation associated with IBD. To date, several of the expanding family of TLR inhibitory proteins have been found to be expressed in the GI tract and have been shown, predominantly using murine models, to functionally suppress both TLR responses and intestinal inflammation [2]. Similar to TLRs few, if any, studies have examined expression of more than one of these proteins at any time, therefore not providing a full overview of the relative importance of these proteins in an IBD population. Our studies have identified differential regulation of a number of these proteins in the same cohort of IBD patients. Expression of several of these was seen to increase in the active UC population (A20, SOCS1, IRAKm and Bcl-3), with both IRAKm and Bcl-3 also increased in the CD populations. These proteins are known to be induced by transcription factors, e.g.NFκB, which are activated by inflammatory signal transduction pathways. Thus, this upregulation of inhibitory proteins is likely a positive feedback mechanism to limit the inflammation seen in IBD. Clearly however, given the level of inflammation evident in both UC and CD, simultaneous upregulation of these proteins is not sufficient to contain the inflammatory response associated with IBD.

SIGIRR, described as an inhibitor of IL-1, IL-33 and TLR responses [25], has been shown in murine models of colitis to play an essential role in regulating intestinal inflammation [26]. In contrast to data shown by Kadota *et al*, wherein they reported reduced expression of SIGIRR in UC [27], we observed no alterations in the expression of the inhibitor protein SIGIRR in any of our cohorts by either qRT-PCR or by IF. Whilst it is possible that the difference in these results could be due to differing patient groups or different treatment regimes, it could also be due to the smaller patient cohort characterised in the study of Kodata *et al* [27]. Indeed, it is interesting that we also found no alteration of SIGIRR mRNA following TLR ligand stimulation, with a slight decrease of SIGIRR expression following stimulation with *B.fragilis*. Further work is necessary, especially in human samples, to clearly elucidate the importance of SIGIRR in regulating intestinal inflammation.

Conversely, PPARγ expression was down regulated in both UC and CD and also seen to be downregulated in both active and inactive populations. Whilst our findings for PPARγ in UC are in keeping with other reports [28, 29], to the best of our knowledge this expression pattern has not been previously observed for PPARγ in CD. This pattern of expression was shown at both mRNA and protein levels. Both the TLR4 ligand (LPS) and commensal bacteria were also seen to increase expression of PPARγ in an intestinal epithelial cell line. In addition, similar to data shown previously in the HT29 intestinal epithelial cell line [30], here we have confirmed a functional role for PPARγ as an inhibitor of intestinal inflammation, as treatment of cells with a PPARγ ligand reduced TLR-induced inflammation. It seems, therefore that under normal homeostatic conditions, signalling through TLRs by either pathogenic or commensal flora upregulates expression of PPARγ. In IBD, however, despite increased TLR4 expression, the level of the regulator PPARγ is suppressed. Whilst the etiology underlying this reduced expression of PPARγ remains unknown, it is tempting to speculate that there may be an innate inability of IBD patients to upregulate PPARγ in response to TLR stimuli. This may be due to specific promoter mutations or epigenetic regulation of the PPARγ gene. Irrespective of the mechanism, findings presented here further confirm the importance of PPARγ as a potential contributory factor in the pathogenesis of UC [30] and may extend its role to potentially also being a key regulatory protein in CD.

A similar suppression in expression of Tollip was observed in both UC and CD and in both active and inactive disease. Tollip has been shown to inhibit TLR responses in IECs [31]. Whilst the Tollip knockout mouse does not develop spontaneous colitis, Tollip deficiency aggravated spontaneous disease onset in IL-10 mice and increased susceptibility to DSS colitis [32]. Our data indicates individuals susceptible to the development of IBD may have an inability to upregulate Tollip expression and that this may contribute to intestinal inflammation. As we did not observe any effect on Tollip expression upon stimulation of cells with either TLR ligands or commensal bacteria, the mechanism of regulation of Tollip expression in the intestine remains unclear. The transcription factor Elf-1 has been recently shown to negatively regulate the Tollip gene in Caco-2 cells and as such it is a possibility that IBD patients may have elevated levels of this transcription factor [33].

As all members of our cohorts displayed reduced levels of both Tollip and PPARγ, it is tempting to speculate that that the combined reduction in both of these TLR repressor

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proteins facilitates the uncontrolled TLR-mediated inflammation associated with IBD. As such, it is possibly that therapeutically targeting these in isolation may not be sufficient to redress the inflammation in IBD. Further studies are required, particularly on human specimens, to fully elucidate the role of these proteins in the pathogenesis of IBD.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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Figure legends

Figure 1. TLR4 expression is increased in active UC as compared to Healthy Controls.

qRT-PCR analysis of TLR genes was assessed in UC, CD and Healthy controls (HC). Data

was normalized to a β-Actin and expressed using the $2^{-\Delta\Delta CT}$ method. Statistical analysis was

performed using one-way Anova with Bonferroni post-test. ** p<0.01

Figure 2. Expression of TLR inhibitory proteins is differentially altered in IBD

populations. qRT-PCR analysis of known TLR inhibitor genes was assessed in UC, CD and

Healthy controls (HC). A20, SOCS1, IRAKm and Bcl-3 mRNA was upregulated in IBD (A).

Tollip and PPARγ mRNA was downregulated in IBD (B) and RP105 and SIGIRR mRNA

displayed no alteration in expression (C). Data was normalized to a β-Actin and expressed

using the $2^{-\Delta\Delta CT}$ method. Statistical analysis was performed using one-way Anova with

Bonferroni post-test * p<0.05, ** p<0.01 and *** p<0.001

Figure 3. Protein expression of Tollip , PPARγ, SIGIRR and A20.

Tollip, PPARγ and SIGIRR protein expression were detected in colonic sections of both IBD

and HC. Tollip and SIGIRR expression was assessed by Immunofluorescence, magnification

20x exposure 3 s. (A and C) and PPARγ and A20 expression assessed by

immunohistochemistry, magnification 40x (B and D). H&E staining was performed (E). Data

shown are representative of 10 patients per group.

Figure 4. PPARγ and A20 expression is increased by TLR ligands

Caco-2 cells were stimulated with LPS (100ng/ml), Flagellin (100ng/ml) and Pam3Csk4

(1μg/ml) for 8 h. qRT-PCR analysis was performed on the stimulated cells and expression of

Tollip (A), SIGIRR (B), PPARγ (C) and A20 (D) assessed. Results shown are representative

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of three separate experiments. Values are shown as Mean ± SEM, (n=3). Statistical analysis was performed using one-way Anova with Bonferroni post-test. ** p<0.01.

Figure 5. SIGIRR expression is decreased, whilst PPARγ and A20 expression is increased, by stimulation with commensal bacterial strains.

Caco-2 cells were treated with three different bacterial strains *(L.Salivarius, E.Coli K12 and B.fragilis*) at a ratio of bacteria:cells of 10:1 for 8 hr. qRT-PCR analysis was performed on the stimulated cells and expression of Tollip (A), SIGIRR (B), PPARγ (C) and A20 (D) assessed. Results shown are representative of three separate experiments. Values are shown as Mean ± SEM, (n=3). Statistical analysis was performed using one-way Anova with Bonferroni post-test.** p<0.01 and *** p<0.001.

Figure 6. The PPARγ agonist, troglitazone, reduces LPS and Flagellin-induced IL-6 production.

Caco-2 cells were pretreated with Troglitazone (10μM) for 1 hr prior to stimulation with LPS (100ng/ml) or Flagellin (100ng/ml). qRT-PCR analysis was performed on the stimulated cells and expression of IL-6 assessed. Results shown are representative of three separate experiments. Values are shown as Mean ± SEM, (n=3). Statistical analysis was performed using one-way Anova with Bonferroni post-test. *** p<0.001.

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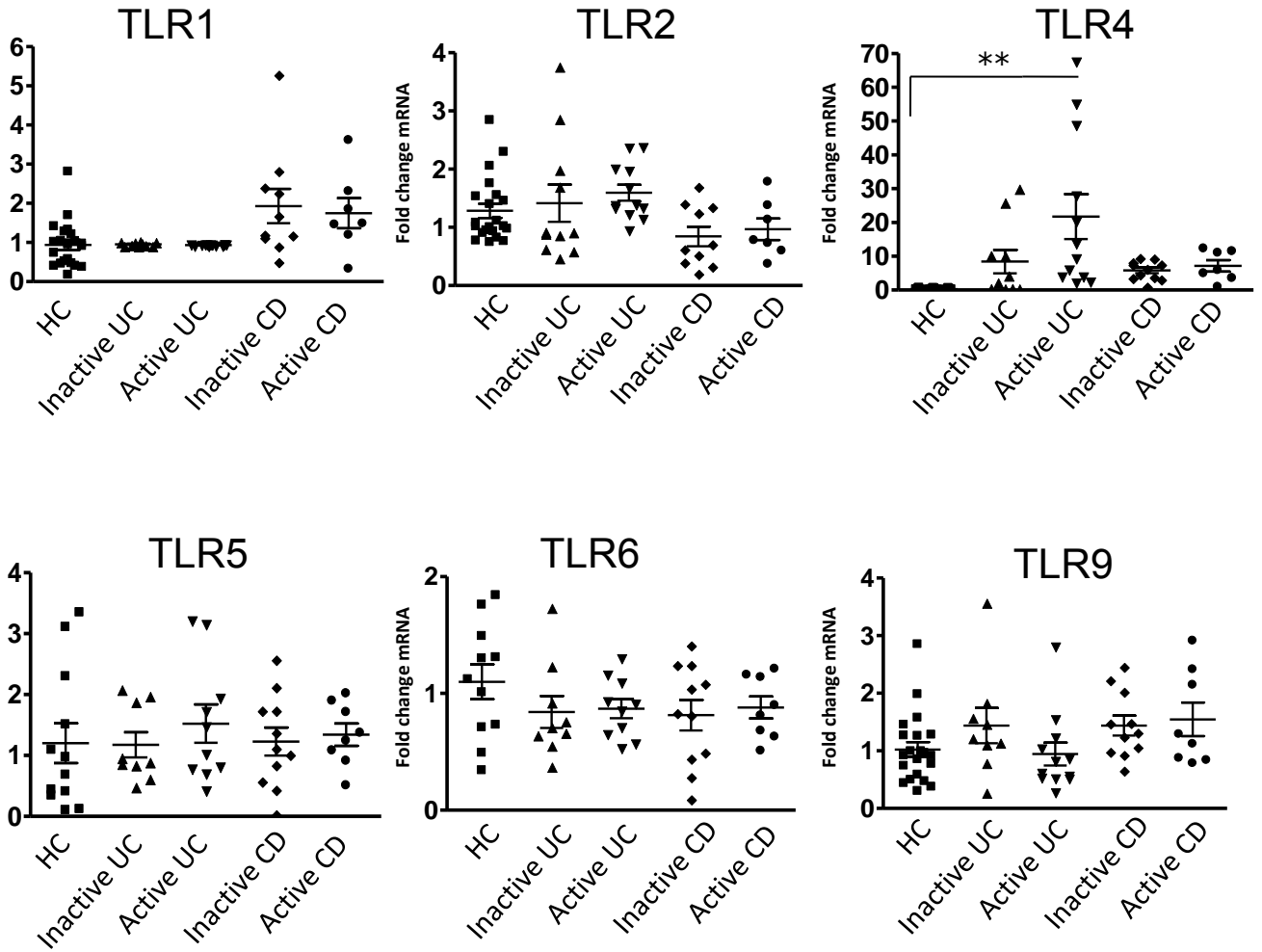Table I. Clinical characteristics of Study Populations

	Ulcerative Colitis	Crohns Disease	Healthy Population
Male:Female	1:10 (total 22)	11:08 (total 19)	12:09 (total 21)
Median Age (range) (years)	42 (21-68)	34.89 (19-57)	50 (21-71)
Median duration of disease (range) (years)	3.3 (0.2-25)	4 (0.17-34)	
Medications			
5-ASA 0.3-1.3 g/day	12	8	
Topical treatment	8	0	
Anti-TNF therapies	0	2	
Oral steroid 10-35 mg/day	4	8	
Azathioprine 100-250 mg/day	3	2	
6-MP 50-100mg/day	2	1	
No medication	2	5	

Table II

qRT-PCR primers and corresponding Universal Probe Library (UPL) probe numbers

Gene	DNA Sequence (Sense 5'-3')	DNA Sequence (Antisense 5'-3')	UPL Probe Number
β actin	attggcaatgagcggttc	tgaaggtagtttcgtggatgc	11
TLR1	cctagcagttatcacaagctcaaa	tcttttccttgggccattc	79
TLR2	cgttctctcaggtgactgctc	tctcctttggatcctgcttg	14
TLR4	ctgcgtagaccagaaagc	ttcagctccatgcattgataa	33
TLR5	gacacaatctcggctgactg	tcaggaacatgaacatacaatctg	16
TLR6	tgaaacagtctcttttggtaaatgc	cagaatccatttgggaaagc	55
TLR8	tgtggttgtttctggattcaa	gctcgcatggcttacatga	79
TLR9	ccagaccctctggagaagc	gtaggaaggcaggcaaggt	81
A20	tgcacactgtgtttcatcgag	acgctgtgggactgactttc	74
RP105	tctgccggctgtaaagtcat	gtcagggatttcactgagacct	79
Bcl-3	gcctcagctccaatggtc	gaggagccatggggaatc	9
SOCS1	cccctggttgttgtagcag	gtaggaggtgcgagttcagg	36
SIGIRR	agctcttggatcagtctgctg	ggccctatcacagacacctg	36
Tollip	caacctcgtcatgtcctacg	gctggtacactgttggcatc	38
IRAK-m	agagctctgcgctgttctg	gctgcttgaaagtctctctctgc	26
PPARγ	tgacaggaaagacaacagacaaat	gggtgatgtgtttgaacttgatt	7
IL-6	caggagcccagctatgaact	gaaggcagcaggcaacac	7

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Figure 1

A.

B.

Figure 2   **Clinical Experimental Immunology**   **Page 26 of 54**

A.

B.

C.

Figure 3

Figure 4

A. Tollip

B. SIGIRR

C. PPARγ

D. A20

Figure 5

A. Tollip

B. SIGIRR

C. PPARγ

D. A20

Figure 6