Expression of Toll-Like Receptor 3 is enhanced in Inflammatory Bowel Disease and mediates the excessive release of Lipocalin 2.

Short title: TLR3 mediates excessive release of Lipocalin 2 in IBD

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SUMMARY:

Antimicrobial peptides may influence the pathogenesis and course of inflammatory bowel disease (IBD). We sought to clarify the role of the antimicrobial glycoprotein lipocalin 2 (LCN2) in the colon by determining its localization and regulation in IBD.

Following a microarray gene expression study of colonic biopsies from a large IBD population (n=133), LCN2 was localized using immunohistochemistry and in situ hybridization. Moreover, we examined the regulation of LCN2 in HT-29 cells with a panel of Pattern Recognition Receptors (PRRs) and sought evidence by immunohistochemistry that the most relevant PRR, the Toll-like receptor TLR3, was indeed expressed in colonic epithelium in IBD.

LCN2 was among the ten most upregulated genes in both active ulcerative colitis (UCa) and active Crohn`s disease (CDa) vs healthy controls. LCN2 protein was found in both epithelial cells and infiltrating neutrophils, while mRNA synthesis was located solely to epithelial cells indicating that de novo synthesis and thus regulation of LCN2 as measured in the gene expression analysis takes place in the mucosal epithelial cells. LCN2 is a putative biomarker in feces for intestinal inflammation, different from calprotectin due to its epithelial site of synthesis. LCN2 release from the colonic epithelial cell line HT-29 was enhanced by both IL-1β and the TLR3 ligand poly(I:C), and TLR3 was shown to be constitutively expressed in colonic epithelial cells and markedly increased during inflammation.

Key words: lipocalin 2, TLR3, ulcerative colitis, Crohn`s disease, inflammatory bowel disease.

INTRODUCTION:

The pathogenesis of inflammatory bowel disease (IBD) involves both genetic susceptibility and environmental factors. Genetic studies suggest an important role for the innate immune system in IBD pathogenesis [1].

The normal intestine shows a balance between factors activating host immunity like gut bacteria, dietary antigens, endogenous inflammatory stimuli, and the host defense. The host responds to maintain mucosal integrity and down-regulate the inflammatory response to avoid excessive inflammation [2]. Perturbed homeostasis between commensal bacteria and mucosal immunity is a critical determinant in the development of inflammation in IBD [3]. Although the precise dysfunction remains unclear, emerging evidence has revealed that host-derived antimicrobial peptides have a key role in determining the composition of gut commensal bacteria, and there is accumulating evidence of a dysregulated expression of antimicrobial peptides (e.g. defensins) in intestinal epithelial cells in IBD [4, 5].

Lipocalin 2 (LCN2), formerly known as neutrophil gelatinase associated lipocalin (NGAL), is an antimicrobial glycoprotein. It has high affinity for secreted bacterial siderophores, which scavenge for iron. By binding siderophores, LCN2 reduces available iron for bacterial growth and plays an important role in bacterial colonization [6, 7]. LCN2 was originally found in neutrophil granules, but respiratory and intestinal epithelial cells, endothelial cells and renal tubular cells also release LCN2 during inflammation and injury [8-11]. LCN2 has properties of an acute phase protein and is rapidly released into the systemic circulation in several inflammatory and infectious diseases [7, 9, 12]. There are studies suggesting a role for LCN2 as marker of disease activity in IBD [10, 13, 14].

From our large study on colonic mucosal gene expression in IBD, we show that *LCN2* is among the most over-expressed genes in active UC and CD. This led us to examine the localization and regulation of LCN2 in colonic biopsies. Since toll-like receptors (TLRs) are key mediators of intestinal innate host defense, we examined the effect of various TLR- and NOD-ligands on LCN2-release from colonic epithelial cell lines.

MATERIALS AND METHODS

Patient material

Patients undergoing colonoscopy for known or suspected IBD at the Gastrointestinal Endoscopy Unit, St. Olav's University Hospital, Trondheim, Norway were included in the study. Healthy controls were recruited among persons undergoing colonoscopy due to gastrointestinal symptoms and had no signs of gastrointestinal disease.

Four adjacent endoscopic pinch biopsies were taken from non-inflamed mucosa of IBD patients or healthy controls at the hepatic flexure, and at site of maximally inflamed mucosa, if found. Three biopsies were snap frozen and kept on liquid nitrogen for molecular analyses and one formalin-fixed. Inflammation was confirmed on hematoxylin-eosin stained slides by an expert pathologist before including the sample in analysis.

Blood was drawn and serum prepared by 30–minute coagulation at ambient temperature before centrifugation at 2000 g, 4^{o}C for10 minutes, and stored at -80^{o}C.

Ethical considerations

All subjects gave informed written consent. The study was approved by the Regional Medical Research Ethics Committee (Ref. no. 5.2007.910), and registered in the Clinical Trials Protocol Registration System (identifier NCT00516776).

Gene expression analysis

Gene expression analysis is previously described [15]. Full data set from microarray analysis is available at ArrayExpress E-MTAB-184.

Histological examination, immunostaining and in situ hybridization

Formalin fixed, paraffin embedded biopsies were cut in 4 µm thick sections for routine histology, immunohistochemical examination and in situ hybridization. LCN2 and TLR3 immunohistochemistry was done on 25 randomly selected biopsies including healthy controls, inactive UC (UCi), active UC (UCa), inactive CD (CDi) and active CD (CDa) (5 from each group). Primary antibody, rabbit polyclonal anti human LCN2 (Ab 41105) was diluted 1:500. Primary antibody, mouse monoclonal anti human TLR3 (Ab 13915) was diluted1:50. Primary antibodies were from Abcam, Cambridge, UK. Secondary antibody was from Dako Real Envision (rabbit/mouse) and detection was done using Dako DAB+ chromogen (Dako, Glostrup, Denmark). Two independent examiners assessed the staining of the epithelium for both LCN2 and TLR3, as no to little staining, or moderate to strong staining. Fischer test was used to detect group differences. Three different types of negative control were made for the TLR3 staining. First, merely excluding primary antibody. Second, replacing primary antibody with non-human-immunized antibody of same isotype as the primary antibody for TLR3 used; mouse, monoclonal IgG1 (Dako, X0931, Glostrup, Denmark). Third, blocking primary antibody with recombinant human TLR3 peptide (Abnova P0506, Heidelberg, Germany).

In situ hybridization for *LCN2* mRNA was done on the same colonic biopsies as the immunohistochemical staining, using a custom RNAscope (Advanced Cell Diagnostics, Hayward, California, USA) kit according to the manufacturer's protocol.

Culture, stimulation and small interfering RNA transfection of intestinal epithelial cells

The human intestinal cell lines HT-29, HCT 116 (colorectal adenocarcinoma) and SW620 (lymph node metastasis of a colorectal adenocarcinoma), were used (Cat.no. HTB-38, CCL247 and CCL227, respectively, ATCC, Manassas, Virginia, USA). Medium for HT-29 and SW620 was RPMI with 10% fetal calf serum, glutamine 2 mM and gentamicin 0.05 %. Medium for HCT 116 was McCoy's medium (ATCC, Manassas, Virginia, USA) with 10% fetal calf serum, 1% L-glutamine and 1% penicillin-streptomycin. Cells were cultured at 37°C, 5% CO2. Trypsin/EDTA was used to detach the cells from the culture flasks. Cells were counted using Countess Automated Cell Counter (Life Technologies, Grand Island, New York, USA). Stimulation was done in triplicates with 20000 or 30000 cells per well on 96 well plates overnight. Medium was then replaced and ligand added. The ligands were the lipopeptide Pam3CysSK4(P3C) (TLR2/1) 300 ng/mL, Lipomannan (LM) (TLR2/6) 30 ng/mL, synthetic double-stranded RNA mimic polyinosinic:polycytidylic acid (poly(I:C)) (TLR3) 0.5, 5 or 50 µg/mL, lipopolysaccharide (LPS) (TLR4) 100 ng/mL , Flagellin (TLR5) 100 ng/mL, the antiviral compound R848 (TLR7/8) 100ng/mL (all from InvivoGen, Toulouse, France, except poly(I:C) from Amersham Bioscience, Piscataway, New Jersey, USA), unmethylated CpG dinucleotides (TLR9) 10 µM (TibMolBiol, Berlin, Germany), the peptidoglycan component muramyl dipeptide (MDP) (NOD2) 1 µg/mL (InvivoGen, Toulouse, France), and the recombinant human cytokines IL-10, 100 ng/mL and IL-1β, 100 ng/mL (both from PeproTech, Rocky Hill, New Jersey, USA), CXCL8 50 ng/mL (Invitrogen, Paisley, UK), TNFα 100 ng/mL) and IFNγ 1, 10 and 100 ng/mL (both from PeproTech, Rocky Hill, New Jersey, USA). The cells were stimulated for 20h before supernatant was harvested and stored at -20°C. LCN2 was analyzed in supernatant using a commercially available ELISA kit, DY 1757 (R&D, Abigdon, UK). The viability of cells was assessed by MTT-assay as previously described

To validate the poly(I:C) induced TLR3 response, HT-29 cells were transfected for 24 hours using lipofectamin, RNAiMAX (Ambion, Invitrogen Dynal, Oslo, Norway) and *TLR3* small interfering RNA (siRNA) (sense-GAACUGGAUAUCUUUGCATT, antisense-UGGCAAAGAUAUCCAGUUCTT) (5 nM) or a control siRNA RNA (sense-UUCUCCGAACGUGUCACGUdTdT, antisense-ACGUGACACGUUCGGAGAAdTdT (5 nM), (Qiagen, Solentuna, Sweden). Then the cells were stimulated with poly(I:C) 5 µg/ml for 20 hours as described. The supernatant was collected and stored at -20°C for analysis of LCN2 using ELISA. The remaining cells were either lysed and used for *TLR3* qRT-PCR, or analysed by MTT-assay.

RNA isolation and *TLR3* qRT-PCR

RNA isolation and TLR3 qRT-PCR was done as previously described [15] using TaqMan gene expression assays for *TLR3* (Hs01551078_m1) and for the housekeeping gene *GAPDH* (Hs99999905_m1).

Serum analyses

LCN2 was measured in serum using a LCN2 ELISA kit based on the polyclonal rabbit antibody ab 954 developed by T. Flo, [16]. Samples were diluted 1:100.

A commercially available assay was used for high sensitivity measurement of C-reactive protein (CRP) in serum (Tina-quant, Roche, Indianapolis, Indiana).

In the LCN2 analysis in serum and plasma from healthy volunteers, serum was prepared by 30–minute coagulation at ambient temperature before centrifugation at 2000 g, 4ºC for10 minutes. Plasma was obtained by immediate centrifugation of citrate blood at 2200 g, 4ºC for 10 min. LCN2 was analyzed using a commercially available ELISA kit, DY 1757 (R&D, Abigdon, UK).

Statistical analysis

Microarray data analysis was done as previously described [17]. Other data were assessed for normality by Shapiro-Wilk test. The data from serum and supernatant were not normally distributed and thus tested by Kruskal-Wallis test, if significances were detected the Mann-Whitney U-test was used to detect group differences. Correlation between serum protein levels was done with the non-parametric Spearman Rank correlation. Calculations were performed by PASW Statistics version 20 and Graph Pad Prism 5.0. Differences of $p<0.05$ (two-sided) were considered significant.

RESULTS:

Clinical Material

The 133 colonic biopsies were taken from 112 subjects and are previously described [15]. Serum samples are from 223 subjects partly overlapping the biopsy material. There were no differences in patient characteristics between the groups (Table 1). However, aminosalisylic acid/sulphasalazine (5-ASA/S-ASA) was more often used in the UC group and CD patients used more systemic steroids, as expected from clinical practice.

Lipocalin 2 mRNA and protein expression in colonic biopsies.

The microarray analysis showed a remarkable overexpression of *LCN2* in biopsies from active UC (UCa) and active CD (CDa) compared to controls, or to biopsies from inactive UC (UCi) or inactive CD (CDi) (Fig.1). *LCN2* was among the ten most overexpressed genes comparing UCa or CDa to healthy controls [17]. Log_2 difference between UCa and controls was 4.10 (fold change 17.15), between UCa and UCi 3.56 (fold change 11.79), between CDa and controls was 3.85 (fold change 14.42), and between CDa and CDi 3.17 (fold change

9.00). These were highly significant. There was no significant difference in *LCN2* expression UCi or CDi vs controls, but a tendency to higher *LCN2* abundances could be seen.

The mRNA expression levels of IL-1β, CXCL8, TNFα and INFγ were also overexpressed in both UCa and CDa correlating strongly with LCN2. For IL-1β, rho=0.748, CXCL8, rho=0.729, TNFα, rho=0.542 and INFγ, rho=0.618 (all correlations $p<0.001$).

LCN2 immunohistochemistry of colonic biopsies showed strong staining of the epithelium in UCa and CDa (Fig. 2A), both enterocytes and goblet cells. Interestingly, goblet cell mucus stained positive in active disease. Moreover, LCN2 positive polymorphonuclear leukocytes were seen. Colonic epithelial cells in UCi or CDi were negative or weakly positive for LCN2. LCN2 epithelial staining was assessed as significantly increased in both UCa and CDa vs controls, and also in UCa vs UCi and CDa vs CDi (all $p<0.05$, two-sided). LCN2 immunostaining in epithelium was not significantly different in UCi vs control or CDi vs control.

In situ hybridization revealed *LCN2* induction in colonic epithelial cells in the same biopsies positive for LCN2 by immunohistochemistry (Fig. 2B). No infiltrating immune cells or other cells of the lamina propria or submucosa were positive for *LCN2*.

Stimulation of colonic epithelial cell lines and Lipocalin 2 release.

The dynamics of LCN2 regulation was further studied in the cell lines HT-29, HCT 116 and SW620. The LCN2 response to Pathogen Associated Molecular Patterns (PAMPs) is particularly interesting, thus we used a ligand panel covering TLR1-9 and included NOD2 due to its role in IBD. The HT-29 and SW620 cell lines were tested for the full panel, HCT116 with TLR3 ligand. We found that the HT-29 cells constitutively release LCN2, and the release was markedly enhanced by the TLR3 ligand poly(I:C) (Fig. 3). SW620 did not

release LCN2 at all. The HCT 116 cell line also dose-dependently released LCN2 after poly(I:C) stimulation (data not shown).

The correlation between *LCN2* and mRNA of the proinflammatory cytokines IL-1β, CXCL-8, TNFα and IFNγ in the microarray data was further studied by examining whether these cytokines release LCN2. IL-1β potently induced LCN2 release from HT-29 cells, and this effect was additive to poly(I:C) (Fig. 4). Neither CXCL8, TNFα nor IFNγ induced LCN2 release. HT-29 cells did not produce IL-1β, neither constitutively, nor upon stimulation with same ligands as used when assessing LCN2 release.

Silencing of TLR3 in poly(I:C) stimulated HT-29 cells.

Besides TLR3, dsRNA can also signal via at least three other sensors, melanoma differentiation-associated gene 5 (MDA-5), retinoic acid inducible gene-I (RIG1) and protein kinase R (PKR). The TLR3 mediated response to poly(I:C) in HT-29 cells was thus further explored using small interfering RNA (siRNA) for *TLR3*. Poly(I:C) increased LCN2 release 2.00 fold compared to the constitutive release from HT-29 cells, and TLR3 siRNA nearly abolished this response (Fig. 5).

In the same experiment poly(I:C) increased *TLR3* 9,53 fold. Further, siRNA transfection of poly(I:C) stimulated cells attenuated *TLR3* to 33.1% of cells transfected with non-silencing siRNA. HT-29 cell viability was unaltered by poly(I:C) or the transfecting reagents as assessed by MTT assay (data not shown).

Localization of TLR3 in the colonic mucosa.

Having observed the TLR3 induced LCN2 response, colonic biopsies were examined by immunohistochemistry for TLR3, and TLR3 positivity was seen in the epithelium (Fig.6). TLR3 positivity significantly increased in UCa and CDa vs controls ($p<0.05$, two-sided), but

there was no significant difference in TLR3 staining of the epithelium between UCi or CDi vs controls. The staining of TLR3 was located to the nuclei/perinucleic areas. To ensure the specificity of the staining we added three different types of negative control in a repeated experiment of IHC for TLR3 as described in Materials and Methods. The TLR3 staining pattern was reproduced, but could not be visualized in any of the three controls as expected (data not shown). This nuclear/perinuclear pattern of staining for TLR3 has previously been found by others in fibrosarcoma cells[18].

Serum levels of Lipocalin 2.

As mucosal LCN2 was markedly increased in active IBD, it is potentially a marker of disease activity. Serum levels of LCN2 were significantly increased in CD patients compared to controls (Fig 7A), but not in UC patients. As the results in both the UC and CD groups are from patients with both active and inactive disease, these were recalculated restricting the data to patients with endoscopic results from the time of blood sampling and a significant increase in serum LCN2 in UCa vs UCi (Fig.7B) was found. In a further analysis of serum LCN2 in all included subjects we found a correlation to CRP, rho= 0.315 and $p< 0.001$ (two-sided).

This shows that LCN2 in serum may reflect IBD disease activity, but the strength of correlation was rather weak. We hypothesized that the weak correlation between serum LCN2 and CRP was caused by LCN2 leaking ex vivo from neutrophils, which store large amounts of this peptide. Thus, we examined whether technical procedures during blood sampling and preparation of serum could influence LCN2 levels. In a study in healthy volunteers, n=3, we found that LCN2 levels in plasma were markedly lower, 50.9% of that in serum.

DISCUSSION:

The present work is based on a microarray study of one of a large and well controlled material of colonic biopsies from an IBD-population obtained in a routine clinical setting. The

remarkably potent and robust *LCN2* response in active IBD seen in our own material makes it likely that this antimicrobial peptide has an important role in these diseases. Overexpression of *LCN2* in IBD has been shown previously, in variable degree and in materials of variable sizes [19-21].

Our immunohistochemical and in situ hybridization studies located LCN2 protein to epithelial cells and neutrophils, while ongoing synthesis was found only in the epithelial cells. Neutrophils are "prepacked" with LCN2 and further synthesis does not take place as the mature cells are released into peripheral blood. These findings are in accordance with previous studies [10, 22]. From our results we conclude that the profound upregulation of *LCN2* in microarray analysis is due to *LCN2* induction in the epithelium. Having localized inducible, de novo synthesis of LCN2 in colonic IBD to epithelial cells only, we wanted to explore if LCN2 could be induced by PRRs and found that TLR3 stimulation by the dsRNA-mimicking substance poly(I:C) induced LCN2 release in vitro. Supplementary studies with siRNA inhibition of TLR3 confirmed that the poly(I:C) response was indeed mediated via TLR3, strongly indicating that TLR3 has a central role in the LCN2 response in IBD.

Moreover, since the gene expression levels of several of the central proinflammatory cytokines were correlated to that of *LCN2* in the microarray study, we tested these for effect on LCN2 release. IL-1β potently induced LCN2 release in both cell lines, as also found in keratinocytes and a pneumocyte derived cell line, by Cowland et al [9]. We found the effect of IL-1β on LCN2 to be additive to poly(I:C). While the gene expression of the proinflammatory cytokines CXCL8, TNFα and INFγ also correlated well to *LCN2* in our initial microarray study, no effect could be shown on LCN2 release in this experimental system. These results suggest a dual mechanism of LCN2 release in vivo. The relative importance of TLR3 signaling vs IL-1β in induction of LCN2 is unknown, however since the

cell system tested does not produce IL-1β itself, TLR3 signaling represents a more direct effect on LCN2 induction in epithelial cells.

To further substantiate a role for TLR3 in IBD we examined its expression on protein level by immunohistochemistry of colonic biopsies. We found TLR3 to be constitutively expressed in the colonic epithelium. Changes in expression level are generally difficult to assess on histological sections and must be interpreted with caution. However, after examining sections from 25 biopsies, the results seem fairly solid. We found enhanced expression of TLR3 in mucosal biopsies with active inflammation compared to healthy controls and inactive IBD. This constitutive expression of TLR3 in colonic epithelium has previously been shown [23, 24]. In contrast to our findings, Cario et al [23] found unchanged expression of TLR3 in active UC vs inactive UC and reduced TLR3 in active CD vs inactive CD. They compared the fluorescence intensity in samples from a material of both inflamed and non-inflamed biopsies from ileum and colon in UC and CD patients. This heterogeneity might have biased their results. The material used in the present work is very well controlled and our assessment of TLR3 expression in colonic epithelium may be more robust. Thus, to the best of our knowledge, this is the first study that shows increased TLR3 expression in epithelial cells in active IBD.

Currently, there is scarce knowledge and some controversy around the role of TLR3 in gut inflammation in general. There is some proof of an upregulation of TLR3 in intestinal epithelial cells upon rotavirus infection, in vitro [25]. Previous studies indicate both protective and detrimental effects of TLR3 signaling on gut inflammation [26-28]. The presence of TLR3 and its action in the colonic epithelium is of potentially great interest in understanding the disease mechanisms of IBD. TLR3 senses dsRNA, which is found during replication of most viruses, in addition to the dsRNA viruses themselves. There is also evidence that TLR3 senses endogenous mRNA from damaged tissue and may maintain inflammation

independently of viral infection [27, 29]. Our studies show enhanced expression of TLR3 and a strong regulation of LCN2 in active IBD suggesting that TLR3, via binding of viral RNA or endogenous mRNA, has a role in the regulation of LCN2. The obvious role for LCN2 in this setting is as an antimicrobial peptide [7]. This opens for the interesting possibility of LCN2, with its antibacterial effect, to be a part of the mechanisms behind the altered microbiome that is associated with IBD.

A very interesting aspect of LCN2 in IBD is as a marker of inflammation. Previous studies have suggested serum LCN2 as a clinically useful marker of bowel inflammation [13]. Our results also showed that LCN2 levels in serum correlate with CRP. However, the clearly lower levels of LCN2 that we found in plasma strongly suggest that LCN2 release from neutrophil leukocytes contributes significantly to LCN2 levels in blood, and that the use of LCN2 in blood as a clinical marker of inflammation will depend critically on laboratory procedures.

Our results indicate that *LCN2* in the epithelium reflects the degree of local inflammation, as it correlates well to proinflammatory cytokines in the mucosa. Moreover, we saw strong LCN2 staining in both the cytoplasm of enterocytes and in the mucus of goblet cells. This suggests that secretion of LCN2 from goblet cells is an important mechanism for LCN2 release into gut lumen. Additionally, LCN2 from neutrophil leucocytes may leak into the gut lumen. This is interesting in a clinical setting, and LCN2 in feces has been found correlated to disease activity during gut inflammation in both humans and mice [14, 30]. Currently, fecal calprotectin is considered the best non-invasive marker of bowel inflammation. Calprotectin is found in neutrophils and to some extent in monocytes and thus reflects mucosal infiltration and shedding. As LCN2 is released from both neutrophils and activated epithelial cells it has a potential to be a more sensitive marker of disease activity than calprotectin, especially in chronic inflammation where neutrophils are scarce.

Our study has revealed that there is a markedly enhanced LCN2 expression in active IBD, most likely mediated via TLR3 signaling. TLR3 expression is enhanced in epithelium in active IBD, opening for the interesting view of viral or endogenous mRNA as factors contributing to altered immunologic homeostasis in IBD. LCN2 is a highly interesting protein in clinical practice, with potential as a marker of active IBD, measured in plasma or feces.

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