Mucosal Toll-like receptor 3-dependent synthesis of complement factor B and systemic complement activation in inflammatory bowel disease

Short title: complement activation in IBD

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Abstract:

Background: Recent studies link Toll-like receptor 3 (TLR3) to the pathogenesis of inflammatory bowel disease (IBD). Screening the poly(I:C) response in an intestinal epithelial cell line, we found complement factor B mRNA (*CFB*) potently upregulated and went on to further study local and systemic activation of *CFB* in ulcerative colitis (UC) and Crohn's disease (CD).

Methods: In a transcriptome analysis of poly(I:C) stimulated HT-29 cells, we found *CFB* highly upregulated downstream of TLR3. We sought to confirm *CFB* upregulation in a microarray gene expression analysis on colonic biopsies from an IBD population (n=133). Immunohistochemical staining and *in situ* hybridization was done to identify cellular sources of complement factor B protein (fB) and *CFB*. Systemic complement activation was assessed in plasma (n=18) using neoepitope-based ELISA.

Results: Complement factor B mRNA and protein were abundantly expressed in the colonic epithelial cell line, and synthesis enhanced by the poly(I:C) TLR3 ligand. In inflamed vs. normal colonic mucosa of ulcerative colitis (UC) and Crohn`s disease (CD) *CFB* mRNA was the most significantly overexpressed gene and the mRNA abundance ratio was among the 50 highest.. Epithelial cells were the dominating site of fB expression. Systemic complement activation was significantly higher in active than in non-active IBD.

Conclusion: This study is the first to link TLR3 to activation of the alternative complement pathway, and complement factor B is potently upregulated locally in IBD in addition to having a possible central role in systemic complement activation. This suggests a prominent role for complement in IBD pathogenesis.

Introduction:

Inflammatory bowel disease (IBD), with the specific disease entities ulcerative colitis (UC) and Crohn's disease (CD), is characterized by chronic or recurrent inflammation in the gastrointestinal tract. The precise cause of the breakdown of intestinal homeostasis seen in IBD is unknown. However, the homeostasis is critically dependent on complex interactions between intestinal microbiota, epithelial cells and the immune system and requires diverse regulatory mechanisms.[1] These mechanisms are profoundly disturbed in IBD.

The complement system is an important part of the innate immune system and the first-line host defense. The main effector functions of complement activation are opsonization, chemotaxis, phagocytosis and induction of inflammatory mediators.[2] The initiation of complement activation can start through three different pathways; the classical, the lectin and the alternative pathway. The activation induces a cascade reaction, and these three pathways converge to activate C3, resulting in the generation of the C3 convertases. The C3 convertases are composed of C4b2a from the classic and lectin pathway, and C3bBb from the alternative pathway. The common pathway culminates in the terminal C5b-9 complement complex (TCC), which causes inflammatory responses in sub-lytic dose and in higher concentrations lysis of cells, in particular red cells and Neisseria bacteria. Activation products from this cascade reaction, like the anaphylatoxins C3a and C5a, attract phagocytic cells, facilitate phagocytosis and contribute to the inflammatory process by activating mast cells and endothelial cells, causing increased permeability of blood vessels. The complement activation products C3a, C5a and TCC are also known to be involved in the pathogenesis of autoimmune and chronic inflammatory diseases like rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis and Guillain-Barré syndrome.[3-5]

In previous studies we have focused on TLR3 signaling in colonic epithelial cells in the setting of inflammatory bowel disease.[6, 7] TLR3 signaling is interesting as it can be initiated from both viral RNA; either genomic dsRNA or intermediate RNA made during viral replication. Also, there is evidence of TLR3 signaling induced by endogenous RNA.[8, 9] Screening the effect of the TLR3 ligand poly(I:C) in HT-29 cells, we found a potent upregulation of complement factor B mRNA. This is highly interesting as complement factor B (fB) serves as the predominant protein of the alternative pathway amplification loop, and by this fB fuels activation of complement irrespective of which pathway that initiated the reaction.[10-12]

Although scarce, there is some previous evidence that complement is involved in IBD. Local activation of complement in intestinal mucosa and increased complement factors and inhibitors in circulation have been reported. [13-18] However, local source(s) and regulation of complement factors have not been addressed. Also, there are no previous reports on whether complement is activated in the systemic circulation in IBD. To address this issue we examined the regulation, expression and location of complement factor B in colonic mucosa *in vitro* and in UC and CD and sought evidence for systemic complement activation in patients with active IBD.

Materials and methods

Patient material

The IBD biopsy material (cohort I) was collected from patients coming for colonoscopy at the Gastrointestinal Endoscopy Unit, St. Olav´s University Hospital, Trondheim, Norway. Healthy controls were recruited among persons undergoing colonoscopy due to gastrointestinal symptoms, but where no evidence of gastrointestinal disease was found.

Endoscopic pinch biopsies from colonic mucosa were taken from non- inflamed mucosa of both IBD patients and healthy controls at the right flexure and, if inflammation was present, at the maximally diseased site. Four adjacent biopsies were taken at each site. One biopsy was fixed in formalin and later embedded in paraffin. Three biopsies were snap-frozen and stored in liquid nitrogen for molecular analyses. The formalin-fixed sample was haematoxylin-eosin stained and examined by an expert pathologist before including the material in the analysis.

Blood samples for complement analyses were drawn from patients with IBD at the Department of Gastroenterology and Hepatology, St Olav`s University Hospital (Cohort II). Control blood samples were drawn from healthy, age- and sex matched volunteers. Blood for plasma was collected on EDTA tubes and placed on ice until centrifugation for 15 min at 2500 x g, 4°C. Serum was obtained from blood drawn on gel-tubes and left at room temperature for 30 min for coagulation and subsequent centrifugation at 1880 x g for 10 min., 4°C. Samples were stored at -80°C.

Gene expression analyses

The gene expression analyses of human intestinal cells were done on poly(I:C) (polyinosinic:polycytidylic acid, TLR3 ligand) stimulated and unstimulated HT-29 cell cultures. Cell lysis and mRNA isolation were done according to instructions for the Ambion *mir*Vana mRNA isolation kit (Ambion, Austin, TX). Quantity and quality of mRNA was assessed using NanoDrop spectrophotometer (Thermo Scientific, Stockholm, Sweden) and Bioanalyzer capillary electrophoresis (Agilent Technologies, Santa Clara, CA). Microarray analysis was performed on an Illumina HiScan, using Illumina human HT-12 expression Bead-Chips (San Diego, CA).

Gene expression analysis of colonic biopsies has been described previously.[6, 19] The full data set is available at Array Express E-MTAB-184.

Quantitative real-time (qRT) PCR was used to confirm the regulation of complement factor B (*CFB*) mRNA found in the microarray analysis of colonic biopsies, of *CFB* in intestinal cell line experiments and also to confirm knockdown of *TLR3* upon transfection. Primer sequence for *CFB* was GCCAGACTATCAGGCCCATT forward, ACTTTGTCATAGCCTGGGGC reverse, GAPDH GCCGCATCTTCTTTTGCGTC forward, GATCTCGCTCCTGGAAGATGG reverse (Qiagen, Solentuna, Sweden). For *TLR3*, TaqMan Hs01551078_m1 and for *GAPDH*, Hs99999905_m1 (Applied Biosystems) were used. SuperScript VILO from Invitrogen was used for cDNA synthesis, and for the qrt-PCR Fast SYBR Green Master Mix or TaqMan Fast Real-Time PCR Fast Advanced Master Mix (all from Applied Biosystems, Foster City, CA, USA). To confirm the microarray results from colonic biopsies, five randomly chosen colonic biopsies from healthy controls and inflamed mucosa of UC and CD were used for PCR. StepOnePlus Real-Time PCR System and StepOne software v.2.1 (Applied Biosystems) was used for all PCRs. The ΔΔCt method was used to analyse the PCR results.[20]

Cell culture studies

The intestinal cell line HT-29 from colonic adenocarcinoma (American Type Culture Collection, Manassas, VA), was used to study responses to dsRNA. The cell line was grown in RPMI-1640 with 10% fetal calf serum, 2mM glutamine and 0.05% gentamicin, 37 °C, 5% CO2. Cells were detached using trypsin/ethylenediamine tetraacetic acid (EDTA) and counted using Countess Automated Cell Counter (Life Technologies, Grand Island, NY). Stimulation for microarray analysis was done in duplicates in 5 separate experiments. The ligand used was synthetic double stranded RNA mimic, poly(I:C) 5µg/mL for 6 hours. The supernatant was

harvested and cells washed in cold PBS and mRNA isolated before microarray analysis. To prove whether the poly(I:C) response was mediated by TLR3, transfection of HT-29 was done using lipofectamin, RNAiMAX (Ambion, Invitrogen Dynal, Norway) and *TLR3* small interfering RNA (siRNA), siTLR3.6 and siTLR3.8 or a control RNA (all 5 nM, from Qiagen, Solentuna, Sweden). In transfection studies the poly(I:C) stimulation time was 12 hours. Supernatant was harvested and stored at -20°C. Cells were lysed and mRNA isolated for PCR determining *CFB* mRNA and *TLR3* mRNA.

Immunostaining and in situ hybridization

Formalin-fixed colonic biopsies and pelleted HT-29 cells, embedded in paraffin and cut in 4 µm sections, were used for immunohistochemical (IHC) staining and in situ hybridization (ISH).

For IHC the sections were deparaffinised and rehydrated, treated with 3% hydrogen peroxide and boiled with citrate buffer pH 6. Mouse monoclonal antibody against human fB/ Bb was diluted 1:50 and sections incubated for 20 hours at 4˚C (A227, Quidel, San Diego, CA). Bound antigen was visualised by rabbit/ mouse secondary antibody; Envision-HRP kit and diaminobenzidine (DAB+) (both from Dako, Glostrup, Denmark). Isotype negative control was mouse IgG (X0931, Dako, Glostrup, Denmark) in the same concentration as the primary antibody. ISH for *CFB* mRNA was done on sections from the same colonic biopsies as the immunohistochemical staining. A commercially available kit, RNAscope 2.0, was used according to the manufacturer`s protocol. For positive and negative control the probes POLR2A and DapB were used (all reagents from Advanced Cell Diagnostics, Hayward, CA). All slides were counterstained with haematoxylin.

Measurement of complement components, cytokines and CRP.

The complement analyses were performed according to described methods.[21] Measurement of IL-6 in plasma was performed using multiplex cytokine immunoassay (Bio-Plex, Bio-Rad Laboratories, Hercules, CA), analyzed on a Multiplex Analyzer (Bio-Rad Laboratories). High-sensitivity CRP was analysed with an immunoturbidometric assay (Tina-quant, Roche, Indianapolis, IN) at Dept. of Laboratory Medicine at St. Olav`s University Hospital, Trondheim, Norway.

Statistical analysis

Statistical analysis of microarray data on colonic biopsies has been described previously [6]. Statistical analysis of microarray data on cell culture was done as the analysis of microarray data on colonic biopsies, but for this material the paired t-test was used. P-values were adjusted for multiple comparisons using Benjamini Hochberg false discovery rate correction.[22, 23] All quantitative RT-PCR results on biopsies and cell lysates were tested using unpaired and paired t-test, respectively, on results calculated by the ΔΔ Ct method. Complement activation products in plasma were assessed using the nonparametric Mann-Whitney U-test. Correlations between hsCRP in serum and complement activation products in plasma were done by the non-parametric Spearman`s rank correlation. Calculations were performed using PASW Statistics version 20 and GraphPad Prism version 5.0. Two-sided test, $p<0.05$ were considered significant.

Ethical considerations

The study was approved by the Regional Medical Research Committee (ref.no.5.2007.910). All subjects gave written informed consent. The study conforms with the principles outlined in the Declaration of Helsinki for use of human tissue or subjects and is registered in the Clinical Trials Protocol Registration System (identifier NCT00516776).

Results

Gene expression in poly(I:C) stimulated intestinal epithelial cells

CFB was among the most upregulated genes in the microarray analysis of poly(I:C)-stimulated cells together with chemokines like CXCL10 and CXCL11. The upregulation of *CFB* mRNA in HT-29 cells upon poly(I:C) stimulation was Log_2 5.953 (fold change 61.95), $p<0.001$. dsRNA is known to signal via TLR3 and also other pattern recognition receptors (PRR) like RIG-1and MDA-5. The relative involvement of these PRRs probably depends on the size of the poly(I:C) molecule[24] and other factors influencing the molecules' access to the intracellular compartments and receptor binding.

We thus performed knockdown of *TLR3* using siRNAs to test whether poly(I:C) induction of *CFB* was mediated by TLR3 (Fig.1). Post poly(I:C) *TLR3* mRNA abundance was 267% of control and siRNA transfection targeting *TLR3* reduced *TLR3* mRNA by 68%, 69% and 75% for siRNA TLR3.6, siRNA TLR3.8 and the combination of siRNA TLR3.6 and siRNA TLR3.8 respectively. Compared to controls, *CFB* mRNA increased to 2028% upon poly(I:C) stimulation. The reduction in *CFB* mRNA abundance post transfection was 61%, 53% and

63% for TLR3.6, TLR 3.8 and the combination, respectively. The transfection experiment was repeated altogether three times. We found a strong concordance between TLR3 and *CFB* mRNA levels in all experiments done. MTT assays show preserved viability of cells independent of reagents used.

Complement factor B (fB) protein expression in intestinal epithelial cells

Poly(I:C) stimulated and unstimulated control HT-29 cells were studied for expression of fB protein by immunohistochemistry. fB protein was present in these cells and was clearly upregulated in parallel with fB mRNA by stimulation with this TLR3 ligand (Fig.2).

Complement factor B mRNA (*CFB)* expression in colonic biopsies

In the microarray gene expression study on colonic biopsies from IBD patients and healthy controls done previously[6, 19], we now show a significant upregulation of *CFB* in both active UC and CD vs. non-diseased mucosa and also vs. healthy controls (Fig.3). In active UC samples *CFB* was on average upregulated Log_2 2.49 (fold change 5.62) vs. controls. In active CD samples *CFB* was upregulated Log_2 2.35 (fold change 5.10) vs. controls. In non-inflamed mucosa there was no significant difference in *CFB* level vs. controls. The upregulation of *CFB* in colonic biopsies was confirmed by PCR in a random selection of biopsies; 5 from each group of active UC, active CD and controls. The relative abundance of *CFB* in active UC and active CD vs. controls was 8.42.

Immunohistochemistry and in situ hybridization of fB in colonic biopsies

Having shown that complement factor B mRNA and protein were upregulated in poly(I:C)/TLR3 stimulated HT-29 cells, and also that *CFB* was among the most significantly upregulated genes in colonic biopsies from active UC and CD, we further localized both mRNA and protein in colonic mucosa (Fig.4). In situ hybridization and immunohistochemical staining of colonic biopsies localized *CFB* mRNA and fB protein to the luminal epithelial cells with little or no staining of the epithelium of Lieberkuhn`s crypts. By immunohistochemistry we also observed a polarization of the staining, with fB located in the basal parts of the cells. Some positive cells were seen scattered in the lamina propria. Morphologically, most of the fB positive cells in the lamina propria were granulocytes, and some mononuclear cells. Some of the granulocytes were located intra-epithelially, suggesting that they were neutrophils[25] (Fig.2D). In situ hybridization and immunostaining were done on

serial sections, and staining overlapped nearly completely confirming the specificity of the immunohistochemical staining pattern.

Complement activation in systemic circulation

Subjects enrolled in the serum and plasma analyses are described in table 1. Two of the nine IBD patients had CD, seven UC. Four patients used 5-aminosalicylic acid p.o. and one 5-aminosalicylic acid enema. Otherwise, the patients were treatment naïve.

Complement activation products involved in all three initial and in the common pathway of complement activation were analyzed; C4bc, C3bBbP, C3bc and TCC. We found significantly increased levels of all four activation products, C4bc, C3bBbP, C3Bc and TCC, in IBD patients vs. controls (Figure 5A).

We analyzed CRP in serum with a high sensitivity method and could confirm increased levels of CRP in serum in IBD vs. controls. Serum-hsCRP correlated well with the complement activation products (Fig.6, A). The correlation coefficient ranged from 0.655 to 0.839 and all correlations were highly significant.

In the same cohort multiplex analysis of plasma was done. We found significantly increased IL-6 in diseased vs. controls (Fig.5B). Also, IL-6 correlated well with complement activation products in circulation with a correlation coefficient from 0.495 to 0.602 for all significant correlations (Fig.6, B).

Discussion

We here report, for the first time, that complement factor B (fB) is produced downstream of TLR3 in colonic epithelial cells. Moreover, in a gene expression study of a large material of colonic endoscopic biopsies we show overexpression of *CFB* and also in the same material find evidence of synthesis of fB in enterocytes. We also report, for the first time, an increase in complement activation products in systemic circulation. These observations demonstrate an intimate, and possibly important, connection between a receptor of the innate immune system in the colon and the complement system allowing the complement system to respond to potentially harmful agents.

The microarray gene expression ratios between inflamed and normal tissue were robust but not among the most impressive expression changes seen in the material. However, the *CFB* change is the statistically most significant one, making this a very consistent and most likely

important response to inflammation. Having confirmed the microarray results with quantitative RT-PCR, we further localized *CFB* mRNA to the epithelial cells in the colonic biopsies using the highly specific and sensitive RNAScope ISH method.[26] The fB protein was studied using IHC in serial sections and we confirmed that protein and mRNA resided in the same epithelial cells, preferentially in the luminally oriented ones with less positivity in the epithelium of the crypts of Lieberkuhn. Moreover, we observed fB positivity in granulocytes of the lamina propria and also in intraepithelial granulocytes which most likely are neutrophils due to their location. To our knowledge fB has previously not been described in granulocytes. We could, however, not find evidence for *de novo* synthesis of *CFB* in granulocytes using ISH. Possibly, the granulocytes are preloaded with fB upon release from bone marrow. Thus, it is likely that the increased *CFB* abundance in the gene expression studies is mainly due to upregulation of this mRNA in the epithelial cells during the inflammatory process. The liver is traditionally seen as the main site of synthesis of many complement factors. However, macrophages, monocytes and fibroblasts have also been found to synthesize complement factors and contribute as a local source for complement activation.[27, 28] Of possible relevance to the present results, in a recent study on sepsis in mice, fB was found to be induced in cultivated bone marrow macrophages after TLR2-3-4 stimulation.[29] In the present work, however, we could not find fB positivity in tissue macrophages or fibroblasts.

The present work thus finds that complement factor B is synthetized in cultured colonic epithelial cells stimulated by TLR3, and is strongly regulated in the colonic epithelium in active IBD. Previous reports from our laboratory show that TLR3 is indeed present in colonic epithelial cells.[7] Taken together with the HT-29 results, this makes it most likely that the colonic epithelium responds through TLR3 to inflammatory challenges by synthetizing fB. To our knowledge, previous studies have neither addressed the localization of fB in the inflamed colon, nor its induction upon TLR signaling. Ahrenstedt et al. found fB to be secreted from the jejunal mucosa in higher quantity in active IBD vs. controls[30], but the cellular source of fB was not addressed. Andoh et al. reported release of fB from intestinal epithelial cells *in vitro* upon stimulation with the proinflammatory ligands IL-1β, IL-6, and TNFα[31]. The same group found complement factor B in basal parts of the crypts of normal colonic mucosa[32]. This localization does not correspond to the findings in the present work. However, we suggest that our observation of complement factor B positivity in the same area of the mucosa, based on the combined use of immunohistochemistry and a very specific in situ hybridization method, is very robust.

The terminal complement complex (TCC) is the end product of the complement signaling cascade and is the complex responsible for cell lysis. However, it also has immunomodulating effects depending on the amount of TCC present. Previous studies found TCC deposited in both intestinal epithelium and endothelium in UC and CD.[13-15] The upregulation of fB in the colonic epithelium together with these previous observations strongly suggest a role for complement in the local inflammatory process in IBD. Considering also the results from our cell studies, it is very likely that it is to a major extent TLR3-fB driven.

Having given evidence for local expression and upregulation of the complement factor in the colonic mucosa during IBD, we further studied complement activation on a systemic level. We were able to demonstrate that such a systemic activation indeed takes place, which to our knowledge has not been seen in IBD patients previously. The significant increase in plasma levels of C4bc, C3bPBb, C3bc and TCC indicates activation of all pathways of the complement system. Moreover, the level of CRP correlated well with all complement activation products. This is interesting as CRP is an acute phase reactant, which acts as an opsonizing agent and also activates complement itself by binding the classical pathway factor, C1q. IL-6 correlated well with the complement activation products. IL-6 is derived from the site of inflammation, released to the circulation and is known as the main inductor for CRP in the liver. As CRP is able to induce complement activation, IL-6 may be the link between intestinal inflammation, CRP and complement activation.

Though there are no previous reports on systemic complement activation in IBD patients, there are studies showing increased levels of complement factors, inhibitors and "complement activity capacity" in plasma, which indirectly may indicate involvement of complement.[16-18] This systemic activation of complement may be interpreted as reflecting a low-grade whole body inflammatory reaction occurring in the IBD patients with the highest disease activity. The mechanisms for such general complement activation are unclear. It is possible that complement activation products from the bowel could leak into the circulation. However, this is in our opinion less likely since the half-life of the activation products is very short. An alternative explanation is that the damaged mucosal barrier in highly active IBD leads to the release of endogenous danger signals to the blood stream, activating complement systemically. This may, in turn, explain at least to some degree the inflammatory reaction seen in e.g. skin and synovial membranes in patients with very active IBD.

Both PRRs and complement are important upstream activators in inflammation, and modulate the adaptive immune system. Complement activation in IBD, both local and systemic, has largely been neglected as a possible target for treatment. However, the present observations make this a possible, novel option. In a mouse model investigating the role of complement in intestinal inflammation it is reported that fB knockouts and C3 knockouts were protected against the initial effects of dextran sodium sulfate (DSS) colitis.[33] C1q and mannose binding lectin (MBL) knockouts (blocked in classic and alternative pathways, respectively) exhibited more severe colitis than wild type mice, and all animals died 2 days post DSS. This shows both pathogenic and protective roles for complement in DSS-colitis, and more specifically gives evidence for a role of the alternative amplification pathway with subsequent terminal pathway activation and release of C5a in the colonic inflammation and injury. Moreover, there is emerging evidence of the effect of blocking complement components in rodent models of IBD. Blocking of C5a or its receptor was shown to attenuate colitis in rodent colitis models.[34-36] The emerging evidence of TLR and complement working together in inflammation suggests simultaneous inhibition of TLR/PRR and complement as a therapeutic modality[37] in severe cases of IBD.

Conclusion:

Complement fB was produced downstream of TLR3 in a colonic epithelial cells, and synthesis of *CFB* was enhanced at the site of inflammation in IBD. Moreover, we observed an enhanced activation of complement in the systemic circulation of patients with active IBD. The complement system together with relevant PRRs may represent novel therapeutic targets in the treatment of IBD.

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TABLES:

TABLE 1. Subjects enrolled in plasma complement analysis.

	Controls	IBD	p
Number of subjects	9	9	n.s.
Age (range)[1]	34 (23-66)	37 (25-65)	n.s
Female sex (%)	2 (22.2)	2 (22.2)	n.s.
Duration of disease (range)[1]	0	0 (0-12)	N/A
5-ASA (%)[1]	0	4 (44.4)	N/A
Systemic corticosteroids (%)	0	0	n.s.
hsCRP (range)	1.1 (0.2-4.9)	27.7 (0.2-222.6)	0.01 [a]

[1]Age, duration of disease and high sensitive C-reactive protein (hsCRP, mg/l) are given as median, and gender and medication as numbers. [a] Significantly higher serum hsCRP in IBD vs control subjects.

Figures legends

Fig.1. Relative changes in *TLR3* and complement factor B (*CFB*) mRNAs in poly(I:C) stimulated and siRNA transfected HT-29 cells, and MTT viability assay. Two different

siRNAs targeting TLR3 were used alone or in combination. Mean ± SD are shown. * $p<0.05$ vs contr. # $p<0.05$ vs poly(I:C) stimulated non-signaling (ns)RNA transfected cells.

Fig.2. Immunohistochemical staining of (upper panel) cell pellets of poly(I:C)-stimulated (A) and unstimulated (B) HT-29 cells. Isotype control of poly(I:C)-stimulated HT-29 cells (C). Immunhistochemical staining of (lower panel) colonic biopsy showing complement factor B (fB) positive staining of polymorphonuclear cells located in lamina propria (D) and staining of epithelial cells (inset). E shows in situ hybridization of complement factor B (*CFB*) serial section adjacent to D. Original magnifications x20 and x40 (insets).

Fig.3. Microarray-derived gene expression of complement factor B (*CFB*) mRNA in colonic biopsies shown as Log_2. UC: ulcerative colitis, CD: Crohn`s disease. a: active, i: inactive. Contr.: healthy control subjects. Means and individual values are shown. *$p<0.001$ versus controls, # $p<0.001$ versus inactive disease.

Fig.4. Immunohistochemical staining (IHC) and in situ hybridization (ISH) showing complement factor B protein (fB) and mRNA (*CFB*) expression in colonic biopsies in active UC and CD, and healthy controls. Serial sections from same biopsy were used to localize protein and mRNA. Original magnifications x20 and x40 (insets).

Fig.5. Complement activation products (A) and IL-6 (B) in plasma, CRP in serum (C). Data given as median and interquartile range. * $p<0.05$ vs control.

Fig.6. Levels of CRP (A) and IL-6 (B) correlated to the complement activation products C4bc, C3bPBb, C3bc and TCC in circulation. Significant correlations were found in all analyses except IL-6 vs TCC. Correlation coefficient and p-value are given.

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