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Regulation of epithelial cell expressed C3 in the intestine – Relevance for the pathophysiology of inflammatory bowel disease?

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ABSTRACT

The complement system not only plays a critical role in efficient detection and clearance of bacteria, but also in intestinal immune homeostasis as mice deficient for key complement components display enhanced intestinal inflammation upon experimental colitis. Because underlying molecular mechanisms for this observation are unclear, we investigated the crosstalk between intestinal epithelial cells (IEC), bacteria and the complement system in the course of chronic colitis.

Surprisingly, mouse intestinal epithelial cell lines constitutively express high mRNA levels of complement component 3 (C3), Toll-like receptor 2 (Tlr2) and Tlr4. Stimulation of these cells with lipopolysaccharide (LPS), but not with flagellin, LD-muramyldipeptide or peptidoglycan, triggered increased C3 expression, secretion and activation. Stimulation of the C3aR on these cell lines with C3a resulted in an increase of LPS-triggered pro-inflammatory response. Tissue biopsies from C57BL/6J mice revealed higher expression of C3, Tlr1, Tlr2 and Tlr4 in colonic primary IECs (pIECs) compared to ileal pIECs, while in germ-free mice no differences in C3 protein expression was observed. In DSS-induced chronic colitis mouse models, C3 mRNA expression was up-regulated in colonic biopsies and ileal pIECs with elevated C3 protein in the lamina propria, IECs and the mucus. Notably, increased C3b opsonization of mucosa-attached bacteria and decreased fecal full-length C3 protein was observed in DSS-treated compared to untreated mice. Of significant interest, non-inflamed and inflamed colonic biopsy samples from CD but not UC patients displayed exacerbated C3 expression compared to controls.

These findings suggest that a novel TLR4-C3 axis could control the intestinal immune response during chronic colitis.

1. Introduction

Due to its main functions in detection, opsonization and elimination of bacteria as well as of apoptotic or malignant cells, the complement system is crucial for the efficient clearance of invading bacteria and self-derived danger – and thus, in essence for tissue homeostasis. However, uncontrolled and sustained complement activation evokes severe inflammatory processes and results in tissue damage as seen in inflammatory bowel diseases (IBD) (Ricklin et al., 2010). Although IBD has been linked to genetic variants of genes belonging to the innate immune system, the exact aetiology of IBD remains unresolved. Ulcerative colitis (UC) is restricted to the colon and presents severe

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mucosal inflammation that is accompanied by mucosal ulcerations. Crohn's disease (CD) in contrast is characterized by discontinuous, transmural inflammation that may affect all layers of the intestine throughout the gastrointestinal tract (Rosenstiel et al., 2009).

The complement system is an effective part of the innate immune system. It is capable of reacting quickly to danger derived from dangerous non-self such as bacteria and self (for example in form of apoptotic or infected cells) and then effectively removing such danger via mediating phagocytic uptake and/or direct lysis of opsonized target cells. In addition, complement also engages in a vital functional cross-talk with other biological sensor and effector systems such as the TLRs (Hajishengallis and Lambris, 2010), the coagulation system (Markiewski et al., 2007) and the adaptive immune system (Dunkelberger and Song, 2010). Both the complement and TLRs provide a rapid response to microbial structures, *e.g.* bacterial lipopolysaccharides (LPS). The two systems work together synergistically and antagonistically to optimally regulate the innate immune response positively and negatively (Hajishengallis and Lambris, 2010). If several systems of the innate immune system independently detect a pathogen, this is considered as verification of recognition, resulting in an amplification and regulation of the antimicrobial and inflammatory immune response (Ricklin et al., 2010). Detection of pathogen-associated molecular patterns (PAMPs) such as LPS, zymosan, cytosine phosphatidyl guanine (CpG) DNA leads to a synergistic increase of pro-inflammatory cytokines (TNF, interleukin-1β and −6) by complement and TLR activation. In particular, the signaling pathways of TLR2, TLR4 or TLR9 intersect with those driven by the anaphylatoxin receptors C3aR and/or C5aR at the level of activation of the Mitogen-activated protein kinase (MAPK) pathways, and here primarily the Extracellular signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK) levels (Ricklin et al., 2010; Zhang et al., 2007). Furthermore, activation of TLR4 has been demonstrated to induce up-regulation of complement component 3 (C3) and factor B (fB) expression (Pope et al., 2010).

Complement factors are mainly produced in the liver (Alper et al., 1969; Torisu et al., 1972) with fluid phase and membrane-bound complement-regulatory proteins being also produced extra-hepatically and act ubiquitously. Further, non-hepatic production of complement factors, resulting in the presence of complement factors in the intestine, has previously been shown. Secretion of complement factors into the duodenum via the pancreatic compartment has been observed (Andoh et al., 1996), while intestinal epithelial cells (IECs) were identified to synthesize complement factors in the gut (Jain et al., 2014). Of note, changes in local complement production is associated with distinct inflammatory diseases. Laufer et al. found C4 in resections of adenocarcinomas of the colon by *in situ* hybridization in intestinal crypts. Further, in samples from CD patients, they detected *C4* mRNA transcripts in normal and inflamed tissues, while they observed *C3* mRNA production specifically in inflamed mucosal areas (Laufer et al., 2000). In addition, Sugihara et al. demonstrated a correlation between increased mucosal *C3* mRNA expression and NF-κB mediated IL-17 production in inflamed biopsies of IBD patients (Sugihara et al., 2010).

Complement factors were also directly detected in the intestinal lumen. In luminal aspirates from patients with bacterial infection in the small intestine, all complement factors up to and including C5 were detected, while complement components C6 to C9 were not (Riordan et al., 1997). Further, increased secretion of C3, C4, but not factor B (fB) was found in jejunal fluid of CD patients as compared with healthy control subjects (Ahrenstedt et al., 1990). Interestingly, IgG-triggered classical complement activation has been detected on the surface of intestinal epithelial cells from UC patients, while no C1q or C4c but strong C3b deposition was detected in CD patients (Halstensen et al., 1992). Additionally, higher C3 levels were observed in serum samples of CD patients as compared with those from UC patients or healthy controls (Hodgson et al., 1977a). Furthermore, in mouse models of chronic DSS-induced colitis, $C1q^{-/-}/MBL^{-/-}$ mice died early after disease onset, while $C5aR^{-/-}$ or $C3^{-/-}$ animals survived but displayed more severe intestinal inflammation and decreased survival rates in comparison to WT mice (Elvington et al., 2015; Johswich et al., 2009).

Together, these findings suggest that the complement system plays an important role in intestinal immune response during chronic inflammation. The interplay between IECs and intestinal bacteria, which is crucial for the maintenance of intestinal barrier function is disturbed during colitis. Thus, we assessed whether mucosal bacteria and/or IECs impact on intestinal complement production and activation under steady state and chronic inflammatory conditions.

2. Materials & methods

2.1. Study population

The study population for real-time quantitative PCR analyses included 21 individuals: 7 patients with UC (3 non-inflamed and in remission, 4 inflamed), 7 patients with CD (4 non-inflamed and remission, 3 inflamed) and 7 healthy controls (4 non-inflamed, 3 control colitis). Colonic biopsies were obtained during colonoscopy at the University Hospital Schleswig-Holstein, Campus Lübeck and active disease was defined by respective clinical, endoscopic and histological criteria. This classification was carried out in consideration of the drugs used in the treatment of IBD such as mesalamine, budenoside, prednisolone, azathioprine, 6-mecaptopurine and methotrexate. The endoscopies were part of regular patient management. All patients agreed to participation by giving informed consent at least 24 h before the procedure and the study was granted prior approval by the local ethics committee.

2.2. Intestinal epithelial cell lines

Murine small intestinal epithelial cell lines IEC-1 (Schwerk et al., 2013) and ModeK (Vidal et al., 1993) were kept in DMEM medium supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

IEC-1 or ModeK cells were stimulated with LPS (LPS-EB Ultrapure; InvivoGen, Toulouse, France), flagellin (from Salmonella typhimurium; Sigma-Aldrich GmbH, Munich, Germany), muramyldipeptide L-D isomer (LD-MDP; InvivoGen) or peptidoglycan (PDG, from Staphylococcus aureus; Sigma-Aldrich GmbH) at indicated concentrations or were left untreated for 24 h. After 24 h, cells were harvested and washed with ice-cold 1 x PBS. Afterwards, whole RNA extracts or whole protein lysates were prepared for further analyses.

2.3. Animal models and determination of clinical scores

Mice were maintained on a regular 12-h light-dark cycle under standard conditions and were provided with food and water ad libitum. All experiments were performed in accordance with the animal care guidelines of the University of Lübeck and after ethical evaluation by the state's animal welfare committee (license no.: V 242-7224.122-4 (14-1/15)). Procedures involving animals and their care were conducted in accordance with national and international laws and regulations.

Germ-free mice were maintained in plastic film isolators at the Central Animal Facility of the Hannover Medical School with food and water provided ad libitum and 12 h light-dark cycle.

Experimental chronic colitis was induced in C57BL/6J congenic wild type mice aged 12–14 weeks by administration of 2% (w/v) dextran sodium sulfate (DSS, molecular mass 40 kDa, TdB Consultancy, Uppsala, Sweden, Batch DB001-27) dissolved in drinking water for 5 days, followed by 5 days of normal drinking water, repeated for a total of three cycles. Control mice received water without DSS. Mice were sacrificed on day 30 of the experiment and clinical parameters were assessed. The modified disease activity index (DAI) was assessed as a combined score of weight loss, stool consistency (0 = solid,