The protease inhibitor cystatin C down-regulates the release of IL-β and TNF-α in lipopolysaccharide activated monocytes

Susanne Thiesen Gren,*† Sabina Janciauskienė,* Salipalli Sandeep,‡ Danny Jonigk,§ Peter Hedly Kvist,* Jens Gammeltoft Gervien,* Katarina Häkansson,*‡ and Olof Grip†,†,‡

*Cellular Pharmacology and †Histology and Bioimaging, Novo Nordisk A/S, Malmö, Denmark; ‡Department of Clinical Sciences, Malmö, Lund University, Malmö, Sweden; and §Department of Respiratory Medicine and ‡Institute of Pathology, Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Member of the German Center for Lung Research, Hannover Medical School, Hannover, Germany

ABSTRACT

Human cystatin C, a member of the cysteine proteinase-inhibitory family, is produced by all nucleated cells and has important roles in regulating natural immunity. Nematode homologs to human cystatin C have been shown to have anti-inflammatory effects on monocytes and to reduce colitis in mice. In Crohn’s disease, pathogenic activated monocytes help drive inflammatory processes via the release of proinflammatory cytokines and chemokines. In particular, tumor necrosis factor-α-producing inflammatory monocytes have a central role in the intestinal inflammation in patients with Crohn’s disease. We investigated the potential of human cystatin C to regulate pathogenic activated monocytes and its potential as an Immunomodulator in Crohn’s disease. We found that cystatin C significantly decreased the lipopolysaccharide-stimulated release and expression of interleukin-1β and tumor necrosis factor-α in monocyte and peripheral blood mononuclear cell cultures from healthy donors, whereas interleukin-6 and interleukin-8 levels were unchanged. A similar reduction of interleukin-1β and tumor necrosis factor-α was also seen in peripheral blood mononuclear cell cultures from patients with Crohn’s disease, and in particular, tumor necrosis factor-α was reduced in supernatants from lamina propria cell cultures from patients with Crohn’s disease. Further investigation revealed that cystatin C was internalized by monocytes via an active endocytic process, decreased phosphorylation of the mitogen-activated protein kinase pathway extracellular signal-regulated kinase-1/2, and altered surface marker expression. The ability of cystatin C to modulate the cytokine expression of monocytes, together with its protease-inhibitory function, indicates that modulation of the local cystatin C expression could be an option in future Crohn’s disease therapy. J. Leukoc. Biol. 100: 000–000; 2016.

Introduction

Cytokines and chemokines are produced by a broad range of cells, including immune cells, such as monocytes and macrophages [1]. Cytokines and chemokines are important in health and disease, specifically, in immune and inflammatory responses, such as host responses to infection [2, 3]. Key innate proinflammatory cytokines and chemokines, including IL-1β, IL-6, TNF-α, and IL-8, are produced by monocytes and macrophages in response to pathogen innate stimulation through activation of TLR-2 and TLR-4 by LPS and Fcγ receptors in immune complexes [4–6]. These cytokines are involved in various biologic and inflammatory processes, such as cell proliferation, phagocytosis, regulation of cytokine and chemokine production, and recruitment of leukocytes, mainly neutrophils. A defective cytokine regulation disrupts homeostatic conditions and has an important role in the pathogenesis of many autoimmune and chronic inflammatory diseases, such as RA and CD [3, 7]. CD is a chronic inflammatory condition of the gastrointestinal tract that causes ECM degradation and mucosal destruction. Cytokines produced by intestinal macrophages, such as IL-1β, IL-6, and TNF-α [8, 9], are important in driving and maintaining intestinal inflammation [10]. The role of cytokines in the pathogenesis of CD is substantiated by the fact that antibodies that block TNF-α are now an established treatment. However, this therapy shows only partial efficacy in patients with CD. Novel agents targeting other cytokines are currently being tested in clinical trials, highlighting that cytokine blockade is an important field in the therapy of CD [10]. Given the redundancy

---

**Abbreviations:** AF647 = Alexa Fluor 647, AV/Cys = Acanthochelenteron vitaeae cystatin, CASP1/3 = caspase 1/3, CD = Crohn’s disease, cysC = cystatin C, ECM = extracellular matrix, HLA-DR = human leukocyte antigen D related, IHC = immunohistochemistry, LP = lamina propria, LPC = lamina propria cell, MFI = median fluorescence intensity, PD-L1/2 = programmed death ligand 1/2, RA = rheumatoid arthritis, ROI = region of interest

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

1. These authors contributed equally to this work
2. Correspondence: Department of Clinical Sciences Malmö, Lund University, Malmö, Sweden. E-mail: olof.grip@med.lu.se

The online version of this paper, found at www.jleukbio.org, includes supplemental information.
of the cytokine network and the complexity and heterogeneity of CD, targeting 1 specific cytokine may only have limited therapeutic efficacy. Therefore, other pathogenic mechanisms, such as ECM degradation, epithelial barriers, and mucosal destruction, which also have important roles in disease pathology, are being considered as drug targets.

In CD and other autoimmune diseases, there is an unbalanced ratio between proteases and protease inhibitors [11–13]. For example, an up-regulated expression of matrix metalloproteases and cathepsin B, L, and S is found in CD, particularly in areas with mucosal destruction [12–15]. One of the protease inhibitors, the low-molecular-weight protein cysC (13.4 kDa) belongs to the type 2 cystatins within the cystatin protein superfamily [16]. CysC is present in a variety of human tissues, fluids, and secretions in varying concentrations, ranging from <0.5 mg/L in urine to 49 mg/L in seminal fluid [17, 18]. In patients with RA, systemic blood levels of cysC is decreased, which is hypothesized to contribute to the predisposition and acceleration of atherosclerosis and amyloidosis in patients with RA [19]. CysC is a potent inhibitor of cysteine proteases, such as cathepsin B, H, K, L, and S. These cathepsins have multiple roles in ECM degradation, bone resorption, antigen presentation, apoptosis, and inflammation [20, 21]. Hence, inhibitors such as cysC undoubtedly have an important role in regulating processes driven by cathepsins [20, 22, 23]. Apart from their role as protease inhibitors, cystatins have immunomodulatory functions [24–26]. Nematode homology of cystatins have been shown to have anti-inflammatory functions and have been investigated for their therapeutic potential [27–30]. In animal models of colitis, the nematode cystatin AVCys was found to express immunomodulatory functions by reducing inflammation and causing less mucosal erosion and infiltration of inflammatory cells [31]. In particular, inflammatory F4/80+Ly6Chigh monocytes and macrophages, which are hyperresponsive to TLR stimulation and account for the up-regulation of monocyte cytokines [32], are reduced in the colon of mice treated with AVCys, and levels of the chemokines MIP-1α/β, MCP-1/3, RANTES, and the cytokines IL-6 and IL-17A are suppressed [31]. These anti-inflammatory functions of the nematode cystatin AVCys are thought most likely to be independent of its cysteine protease-inhibitory effect, making the human cysC an interesting therapeutic target.

In this study, we investigated the role of human cysC as a modulator of the inflammatory cytokine responses induced by LPS-activated human monocytes isolated from healthy donors and patients with CD. Moreover, we analyzed possible modes of action by which cysC regulates the cytokine responses evoked by monocytes and investigated the expression in, and influence of, cysC on mucosal biopsies from patients with CD.

MATERIALS AND METHODS

Purification of PBMCs and monocytes

Buffy coats from healthy donors were purchased from the Clinical Immunology Blood Bank (Rigshospitalet, Copenhagen, Denmark), and PBMCs were obtained using Ficoll-Paque Plus (GE Healthcare BioSciences AB, Uppsala, Sweden) density-gradient centrifugation. Monocytes were isolated from PBMCs by positive selection using CD14 antibody-coated magnetic bead separation (CD14 human MicroBeads; Macs Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions.

Patient material

Blood samples were collected from patients with CD and active inflammation, which was confirmed by endoscopy at the Gastroenterology Outpatient Clinic of Skåne University Hospital (Malms, Sweden). Mucosal biopsies from patients with CD were obtained from macroscopically inflamed and non-inflamed mucosa (pale mucosa, distinct vascular pattern, and no friability) using large-capacity forceps (Radial Jaw 4; Boston Scientific, Natick, MA, USA) (Supplementary Table 1). The distance between the inflamed and non-inflamed area was >50 cm. Clinical routine histology confirmed inflammation and normal mucosa at the sites in question. Mucosal biopsies that showed no endoscopic or histologic signs of inflammation were likewise obtained from controls without inflammatory bowel disease who were referred for colonoscopy because of bowel discomfort, loose stool, or anemia. All samples were obtained with informed consent and in agreement with the approval of the Regional Ethics Committee in Lund, Sweden.

Purification of PBMCs from whole blood

Whole blood (5 ml) was collected in BD Vacutainer blood collection tubes containing 3.2% sodium-citrate (BD Biosciences, Franklin Lakes, NJ, USA). For PBMC purification, whole blood was layered on Ficoll-Paque Plus, and the PBMCs were obtained using density-gradient centrifugation.

Purification of LPCs

Biopsies were collected in medium (RPMI 1640 medium supplemented with 10% FCS and 1% penicillin + streptomycin [all from Thermo Fisher Scientific, Waltham, MA, USA]). Subsequently, epithelial cells were removed by incubation in HBSS (Thermo Fisher Scientific), supplemented with 1% penicillin + streptomycin and 1 mM EDTA (Thermo Fisher Scientific) for 45 min in a humidified incubator with 5% CO2 at 37°C. The biopsy specimens were washed with HBSS containing 10% FCS and then cut into very small pieces using a sterile scalpel. These finely divided biopsy specimens were incubated for 2 h in a humidified incubator in 5% CO2 at 37°C, stirred in medium containing 20 μl/ml DNase I (Sigma-Aldrich, St. Louis, MO, USA) and 0.13 Wüch tro units/ml Liberase (Roche Diagnostics GmbH, Mannheim, Germany), then passed through a 70 μm filter. The LPCs were washed and cultured as described below.

Cell culture

Monocytes were prepared in complete medium (RPMI 1640 medium supplemented with 10% FCS, 1% penicillin + streptomycin) and 10 ng/ml M-CSF (endothoxin-tested, produced in-house) and seeded at a concentration of 2 × 10^5 cells/well in 96 round-bottomed wells (BD Falcon, BD Biosciences). PBMCs were seeded at a concentration of 10^5 cells/well in 1 ml RPMI 1640 medium supplemented with 10% FCS and 1% penicillin + streptomycin in 24 flat-bottomed wells (BD Falcon, BD Biosciences). LPCs were prepared in RPMI 1640 medium supplemented with 10% FCS and 1% penicillin + streptomycin seeded at a concentration of 2 × 10^5 cells/well in 96 flat-bottomed wells (BD Falcon, BD Biosciences). The cell cultures were incubated in medium alone, in the presence of 10 ng/ml LPS (Escherichia coli, Santa Cruz Biotechnology, Santa Cruz, CA, USA), 10 ng/ml LPS (E. coli), and 20 μg/ml recombinant human cysC (endotoxin ≤ 1 EU/μg; Biochrom AG, Berlin, Germany), alone or in combination, for 20 h at 37°C in 5% CO2. Alternatively, cells were cultured for 2, 6, and 20 h in the presence of 10 ng/ml LPS (E. coli) at 37°C in 5% CO2 to obtain a time estimate for the measured cytokine levels. Supernatants were harvested and kept at −20°C until analyzed.

Cytokine assays

Cell culture supernatants were quantitatively assayed for IL-1β, IL-6, IL-8, IL-10, TNF-α, and GM-CSF using the Bio-Plex Pro human singleplex assays (all from Bio-Rad Laboratories, Hercules, CA, USA). All standards and samples were obtained with informed consent and in agreement with the approval of the Regional Ethics Committee in Lund, Sweden.
were analyzed in duplicate according to the manufacturer’s instructions, using a Luminex Bio-Plex 200 system (Bio-Rad Laboratories).

Flow cytometry
Monocytes or PBMCs were washed once in PBS (Thermo Fisher Scientific). PBMCs were detached from the flat-bottomed culture wells using a cell scraper. The cells were subsequently washed in PBS containing 2% FCS (all from Thermo Fisher Scientific) before staining for 30 min at 4°C in the dark. The following antibodies were used for surface staining: PE-Cy7-conjugated anti-CD3 (SK7); PE-Cy7–conjugated anti-CD11c (B-ly6); Pacific Blue–conjugated anti-CD14 (M5E2); FITC–conjugated anti-CD36 (C838); APC-conjugated anti-CD40 (5C3); PE-conjugated anti-CD56 (B159); APC–conjugated anti-CD62L (DREG-56); FITC–conjugated anti-CD64 (10.1); PE-Cy5-conjugated anti-CD86, PE-conjugated anti-CD163 (GH/61); PE-conjugated anti-HLADR (TU36); all from BD Pharmingen from BD Biosciences; FITC-conjugated anti-TLR-2 (TL2.1) and APC conjugated anti-TLR-4 (HTA125) (all from eBioscience, San Diego, CA, USA); and PerCP-conjugated anti-CD16 (5G8), Live/Dead Fixable Near-IR (Molecular Probes; Thermo Fisher Scientific). PBMCs were stained with AF647-conjugated cysC (Alexa Fluor 647 microscale protein labeling kit; Thermo Fisher Scientific), which were labeled according to the manufacturer’s instructions. Cells were analyzed by flow cytometry on a BD LSRII (BD Biosciences). The data were analyzed using FlowJo 7.6 software (TreeStar, Ashland, OR, USA).

CASP1 activity assay
CysC was tested for its CASP1-inhibition activity in vivo with the Caspasel-inhibitor drug detection kit (Abcam, Milton, Cambridge, United Kingdom) according to manufacturer’s instructions. Fluorescence readings were taken at 400-nm excitation and 505-nm emission wavelengths on Tecan Infinite M200 plate reader (Männedorf, Switzerland) for 120 min, every 10 min at 37°C.

Quantification of ERK1/2 and p38 phosphorylation by flow cytometry
Monocytes were purified as previously described, and 2.5 × 10⁶ cells per stimulation were rested for 1 h in 250 μl IMDM containing 25 nM HEPES and GlutaMAX (Thermo Fisher Scientific) at 37°C before stimulation. The cells were either kept untreated or stimulated for 5 min, 15 min, or 25 min with 100 ng/ml LPS (E. coli), 100 ng/ml LPS (E. coli) + 20 μg/ml recombinant human cysC or 20 μg/ml recombinant human cysC alone. After stimulation, the reaction was stopped by adding 250 μl preheated (37°C) fixation buffer (Cytosix; BD Biosciences) for 10 min. The monocytes were subsequently washed twice in PBS containing 2% FCS and stained with Pacific Blue–conjugated anti-CD14 (M5E2) for 15 min in the dark at room temperature. The cells were washed once and incubated in the dark for 30 min on ice with Perm Buffer III (Phosflow, BD Biosciences). After permeabilization, the cells were washed twice in PBS containing 2% FCS and stained with PE-conjugated anti-ERK1/2 (pT202/pY204) or PE-conjugated anti-p38 MAPK (pT180/pY182) (Phosflow, BD Biosciences). The monocytes were analyzed for ERK1/2 or p38 phosphorylation by flow cytometry on a BD LSRII. The data were analyzed using FlowJo 7.6 software.

IHC
Sections (5 μm thick) were cut from paraffin blocks, floated onto salinized slides and dried for 30 min at 70°C and overnight at 60°C. Deparaffinization was performed using xylene, and rehydration was accomplished with graded ethanol. The sections were stained with 1:200 monoclonal anti-cysC antibody (2C8; Novus Biologicals, Littleton, CO, USA), followed by DAB staining at 1:200 for 15 min and hematoxylin staining for 3 min. All immunohistochemically stained slides were scanned using the Nanozoomer 2.0 (Hamamatsu Photonics K.K., Hamamatsu City, Japan) using a whole-slide scan at an original magnification of ×20. The following image analysis was performed using Visiopharm Integrator System software (Visiopharm, Hoersholm, Denmark).

RESULTS
CysC inhibits LPS-induced production of inflammatory cytokines
To determine the maximal levels of IL-1β, IL-6, IL-8, TNF-α, and GM-CSF released in the supernatants, monocytes were

www.jleukbio.org
activated with LPS for 2, 6, or 20 h. The maximal level of TNF-α was reached after 6 h and remained constant up to 20 h. The other cytokines—IL-1β, IL-6, IL-8, IL-10, and GM-CSF—reached their peak levels at 20 h (Supplementary Fig. 1).

To examine the modulatory effect of cysC on cytokine production, monocytes were cultured for 20 h with LPS, LPS plus cysC, or cysC alone. As expected, LPS significantly induced the release of all analyzed cytokines relative to control cells or cells treated with cysC (data not shown). As illustrated in Fig. 1A, levels of TNF-α and IL-1β were significantly reduced in the supernatants from monocytes stimulated with LPS plus cysC compared with monocytes stimulated with LPS alone. The expression of IL-1β and TNFα, but not IL-8, was also significantly decreased in the LPS plus cysC–treated cells compared with the LPS alone (Fig 1B). Under the same experimental conditions, cysC had no effect on the protein levels of IL-6 and IL-8 in LPS-activated monocyte cultures and only a minor, although not statistically significant, decreasing effect on the levels of IL-10 and GM-CSF. It is noteworthy that cysC alone significantly increased the levels of IL-6 and IL-8, whereas a minor increase in the levels of IL-1β and TNF-α was seen relative to controls (Fig. 1B and Supplementary Fig. 2).

To exclude cell death as a possible cause of the cytokine decrease seen in LPS- and cysC-stimulated monocytes, cell viability was assessed by flow cytometry. No difference in cell viability was found between monocytes stimulated with LPS and monocytes stimulated with LPS plus cysC (Supplementary Fig. 3A). Likewise, to exclude apoptosis as a possible cause of reduced cytokine levels, monocytes were examined for CASP3 activity. Very few apoptotic CD14+ monocytes were found in either LPS- or LPS plus cysC–stimulated cultures, and no difference in CASP3 activity was observed (Supplementary Fig. 3B). We thereby excluded cell death and/or apoptosis as a possible cause of the decreased levels of IL-1β and TNF-α in LPS- and cysC-stimulated monocyte cultures.

Figure 1. CysC reduces IL-1β and TNF-α in LPS-induced monocyte cultures. Modulation of cytokines by human recombinant cysC. (A) Monocytes were treated for 20 h with LPS or LPS plus cysC, and the concentrations of the indicated cytokines were measured in the supernatants. Data are shown as means ± sm of 4–6 donors. **P < 0.01. (B) mRNA levels of IL-1β, TNF-α, and IL-8 were determined in monocytes treated for 20 h with LPS, LPS plus cysC, or cysC alone (n = 3, 18 repeats).
**CysC alters the phenotype of LPS-induced monocytes**

A nematode cystatin, homologous with the type 2 cystatin family, is able to modulate the expression of human monocyte surface molecules [33]. To further characterize the effects of cysC, we assessed the expression of selected surface receptors using flow cytometry. MFI levels of monocyte-receptor expression were compared on control monocytes with cysC-stimulated monocytes and on LPS-stimulated monocytes with LPS plus cysC-stimulated monocytes (Fig. 2). CysC alone significantly down-regulated the expression of CD11c, CD36, and CD163 and up-regulated the expression of HLA-DR relative to controls. Compared with LPS-stimulated monocytes, LPS plus cysC-stimulated monocytes significantly decreased the expression of CD14 and increased the expression of CD36 and CD86. In contrast, the expression of CD11c, HLA-DR, and CD163 remained unchanged. A tendency toward increasing MFI levels of TLR-4 in LPS plus cysC-stimulated cultures was observed, although this did not reach statistical significance (Fig. 2).

---

**Figure 2. CysC alters the phenotype of LPS-induced monocytes.** Flow cytometry analyses of selected surface-marker expression on monocytes. Live, singlet cells were gated according to forward light scatter A and side light scatter A. Representative histograms of surface-marker expression on monocytes cultured in media alone (gray lines), with LPS (red line), LPS plus cysC (black line) or with cysC (gray, dashed line). Bar charts displaying the MFI values are depicted next to the histograms for each analyzed surface marker. Data are shown as means ± s.e.m of 3–6 donors. *P < 0.05, **P < 0.01, ***P < 0.001.
Compared with unstimulated cells, LPS-stimulated cells showed significantly lower expression of CD11c, CD36, CD86, CD163, and TLR-4 and higher expression of HLA-DR. Expression of CD40, CD62L, CD64, and TLR-2 was likewise assessed but did not show differences between any of the stimulations (data not shown).

Altogether, our data indicate that cysC not only modulates the cytokine profile but also modifies the surface expression of phenotypic markers on monocytes.

**Uptake of cysC by monocytes**

To explore how cysC exerts its immunomodulatory effect on the cytokine release in LPS-induced human monocytes, we first determined whether monocytes could internalize cysC. Monocytes kept at 37°C had increased MFI expression of AF647-labeled cysC compared with monocytes kept at 4°C (Fig. 3A). Furthermore, cytochalasin D, a cytoskeletal inhibitor, decreased the MFI of AF647-labeled cysC, confirming that cysC was internalized by the monocytes (Fig. 3B). To examine the uptake mechanism of cysC by monocytes, increasing concentrations of unlabeled cysC were added to a fixed concentration of AF647-labeled cysC. Our results showed that unlabeled cysC was able to compete with labeled cysC in a concentration-dependent manner, suggesting a receptor-mediated uptake process (Fig. 3C).

To investigate whether monocytes are the main target cells for cysC, PBMCs from healthy donors were cultured with AF647-labeled cysC for 20 h, and the internalization of cysC was assessed by flow cytometry. The highest MFI expression of AF647-labeled cysC was associated with monocytes relative to granulocytes, T cells, B cells, NK cells, and NKT cells (Fig. 3D). We, therefore, concluded that monocytes are the cells within the PBMC fraction that take up cysC and that the uptake is an active endocytic-mediated process.

**Figure 3. CysC is actively taken up by monocytes.** Whether cysC is bound to, or taken up by, monocytes was assessed by flow cytometry ($n \geq 3$). Cells were gated according to forward forward light scatter A and side light scatter A on live, singlet cells. (A) Monocytes were incubated for 30 min with 10 μg/ml AF647-labeled cysC at 4°C (blue line) or 37°C (red line). A representative histogram from 1 donor is shown. (B) Representative histograms showing monocytes incubated with 10 μg/ml AF647-labeled cysC at either 4°C (blue line) or 37°C (red line). In addition, 10 μg/ml cytochalasin (black line in the histograms) was added for 30 min before the addition of 10 μg/ml AF647-labeled cysC at either 4°C or 37°C. Fluorescence minus one (FMO) control shows the monocytes incubated without cytochalasin and with unlabeled cysC. (C) Monocytes were incubated at 37°C for 30 min in medium alone (gray line) with 10 μg/ml AF647-labeled cysC (red line) or with 10 μg/ml AF647-labeled cysC and increasing concentrations (1 μg/ml [black line], 10 μg/ml [black, dotted line], 20 μg/ml [black, dashed line], or 40 μg/ml [gray, dashed line]) of unlabeled cysC. (D) PBMC cultures were incubated for 20 h with AF647-labeled cysC. Live, singlet cells within the PBMC fraction were identified according to their respective surface-marker expression of CD3, CD14, CD56, and CD16. The CD16+ cells were further assessed for their expression of CD66b. The cells were analyzed for their cysC uptake. Representative plots and histogram show monocytes (red line), granulocytes (black line), T cells (black, dashed line), NK cells (black, dotted line), and B cells that were determined according forward-side scatter properties and the lack of expression of CD3 and CD56 (gray line), and NKT cells (gray dashed line).
CysC decreases phosphorylation of ERK1/2
Monocyte stimulation with LPS induces phosphorylation of MAPKs, which consist of at least 3 pathways: JNK, ERK1/2, and p38. The MAPK further activates NF-kB, thereby activating the transcription of different cytokines [34]. Moreover, homologs to human cystatins have been shown to activate the MAPKs ERK1/2 and p38 [27, 35–37]. To elucidate how cysC modulates the LPS-induced cytokine release and expression of IL-1β and TNF-α, we chose to examine phosphorylation of the MAPKs ERK1/2 and p38. First, we determined when the maximal phosphorylation of ERK1/2 and p38 occurred during LPS stimulation compared with untreated cells. Upon LPS stimulation, no phosphorylation of ERK1/2 was detected after 5 min, whereas after 15 min, the phosphorylation increased and then decreased again after 25 min (Fig. 4A). Phosphorylation of p38 was already detectable after 5 min of LPS stimulation, although it increased further after 15 min, and then decreased after 25 min of stimulation (Fig. 4A). We, therefore, chose to look at the effect of cysC on MAPK phosphorylation after 15 min. As shown (Fig. 4B), cysC significantly decreased phosphorylation of ERK1/2 after 15 min, whereas phosphorylation of p38 was unaltered. Preincubation of monocytes with cysC for the 30 min before the 15 min stimulation with LPS had no effect on the phosphorylation of p38 (data not shown).

CysC has no effect on CASP1 expression and activity in vitro
CASP1 is a protease required for the cleavage of proinflammatory cytokines, such as IL-1β, into their active forms. Treating monocytes with LPS plus cysC did not alter CASP1 mRNA expression compared with the cells treated with LPS alone (Fig. 5A). Moreover, as illustrated in Fig. 5B, cysC did not inhibit CASP1 activity in vitro.

CysC expression in the intestinal mucosa
In CD, there is an increased expression of proteases, such as matrix metalloproteinases and cathepsins [13, 38]. Proteases are important in the disease pathogenesis because they degrade ECM, thereby causing mucosal destruction and impaired barrier function. To balance that, ECM-degradation protease inhibitors are needed. To our knowledge, the expression level of cysC in the mucosa during pathogenic and in normal situations has never been examined. We, therefore, analyzed cysC expression in the mucosa of patients with CD and in controls using IHC. We found no increase in the density of cysC in patients with CD compared with controls (Fig. 6A). The cysC expression is illustrated by representative pictures from 1 control and 1 patient with CD (Fig. 6B).

Figure 4. CysC reduces phosphorylation of ERK1/2. Phosphorylation of the MAPK pathways ERK1/2 and p38 was assessed using flow cytometry. (A) To find the optimal phosphorylation time point, monocytes were kept untreated (gray line) or incubated with 100 ng/ml LPS for 5 min (black line), 15 min (black, dashed line), or 25 min (gray, dashed line) and stained for phosphorylation of ERK1/2 after 15 min. As shown (Fig. 4B), cysC significantly decreased phosphorylation of ERK1/2 after 15 min, whereas phosphorylation of p38 was unaltered. Preincubation of monocytes with cysC for the 30 min before the 15 min stimulation with LPS had no effect on the phosphorylation of p38 (data not shown).

Figure 5. CysC has no effect on CASP1 expression and activity in vitro. CASP1 is a protease required for the cleavage of proinflammatory cytokines, such as IL-1β, into their active forms. Treating monocytes with LPS plus cysC did not alter CASP1 mRNA expression compared with the cells treated with LPS alone (Fig. 5A). Moreover, as illustrated in Fig. 5B, cysC did not inhibit CASP1 activity in vitro.

Figure 6. CysC expression in the intestinal mucosa. In CD, there is an increased expression of proteases, such as matrix metalloproteinases and cathepsins [13, 38]. Proteases are important in the disease pathogenesis because they degrade ECM, thereby causing mucosal destruction and impaired barrier function. To balance that, ECM-degradation protease inhibitors are needed. To our knowledge, the expression level of cysC in the mucosa during pathogenic and in normal situations has never been examined. We, therefore, analyzed cysC expression in the mucosa of patients with CD and in controls using IHC. We found no increase in the density of cysC in patients with CD compared with controls (Fig. 6A). The cysC expression is illustrated by representative pictures from 1 control and 1 patient with CD (Fig. 6B).
CysC reduces IL-1β and TNF-α in cell cultures from patients with CD

Given the unaltered expression of cysC in CD compared with controls, we sought to investigate whether additional cysC could reduce the release of IL-1β and TNF-α by monocytes/macrophages contained in the LPC fraction isolated from the LP of patients with CD [8, 9]. As described above, in experiments using PBMCs from healthy donors, we found that cysC was predominantly taken up by monocytes (Fig. 3D). Comparable to monocyte cultures (Fig. 1A), cysC in particular reduced the cytokine levels of IL-1β and TNF-α in supernatants from LPS-activated PBMC cultures (Fig. 7A). Given the similar effect of cysC on the cytokine release of activated monocytes and in PBMC cultures, IL-1β and TNF-α were chosen as signature cytokines to monitor the cytokine release by PBMCs and LPCs from patients with CD. Similar to the findings in monocyte (Fig. 1A) and PBMC cultures (Fig. 7A) from healthy donors, cysC significantly reduced LPS-induced IL-1β and TNF-α release in PBMCs from patients with CD (Fig. 7B).

In addition, we analyzed the release of IL-1β and TNF-α in LPC cultures from patients with CD and active inflammation. Biopsies were taken from the inflamed and the noninflamed mucosa and LPCs were purified. In particular, a strong reduction in the levels of TNF-α was observed in LPC cultures stimulated with LPS plus cysC relative to LPS alone. Moreover, this effect of cysC on LPS-induced TNF-α release was observed in both LPC purified from inflamed and noninflamed mucosa (Fig. 7C). The levels of IL-1β were also reduced, although not to the same extent as seen in the monocyte (Fig. 1) and the PBMC cultures (Fig. 7A and 7B). We observed a marked difference in the basal cytokine levels in supernatants from inflamed and noninflamed LPC cultures, the highest levels were found in the inflamed cultures, as expected (data not shown). Because of the high bacterial load in the LP during intestinal inflammation, inflammatory macrophages, which are the macrophage subset that solely express TLRs [8, 39], might already be activated. Therefore, we also compared cysC-treated to nontreated control cultures. A similar reduction in IL-1β and TNF-α was observed as found in the LPS-stimulated cultures (Fig. 7D).

These results suggest that cysC may have a potential therapeutic effect in CD, given its ability to reduce TNF-α secreted in the LPC cultures. The effect of cysC is most likely mediated by the monocytes/macrophages in the LP because the cytokine profiles are similar in the monocyte, PBMC, and LPC cultures.

DISCUSSION

In this study, we demonstrated that recombinant human cysC reduces the proinflammatory cytokines IL-1β and TNF-α generated by LPS-activated monocytes from both healthy...
controls and patients with CD. Moreover, cysC reduces the levels of IL-1β and TNF-α in LPC cultures from patients with CD.

Previous studies have demonstrated that nematode-derived cystatins induce anti-inflammatory conditions by targeting mouse and human macrophages, promoting the release of IL-10 [28, 33, 37] and lowering the levels of IL-17A and IL-6 in a mouse model of colitis [31]. In this study, we did not find an increase in the IL-10 production or reduced secretion of IL-6 when cysC was added to the monocyte cell cultures. In contrast, a tendency toward lower levels of IL-10 and GM-CSF was observed. However, we found a significant decrease in the proinflammatory cytokines IL-1β and TNF-α. Moreover, cysC did not influence the levels of IL-6 and IL-8 in LPS-induced monocytes, indicating a specific effect of human recombinant cysC on IL-1β and TNF-α.

Cystatins can exert their functions both extracellularly and intracellularly [17, 20]. CysC has been shown to be internalized by various cancer cell lines [40, 41] and cells in ocular tissues [43]. Also, nematode cystatins have been shown to be actively internalized by macrophages [37]. CysC can also inhibit virus replication [43], indicating that cysC, apart from its extracellular functions, also has intracellular functions. Using labeled cysC, we show for the first time by flow cytometry that cysC is internalized mainly by the human monocytes within PBMCs. The internalization is shown to be an active process, most likely receptor mediated, given the ability of excess, unlabeled cysC to compete with labeled cysC. These results suggest that the effect of cysC on the cytokine release is mediated via an intracellular mechanism, which is in accordance with previous data [37, 40]. Preliminary data showed that blocking the CD36 (clone JC63.1) or the CD163 (RM3/1) scavenger receptors could not inhibit the effect of cysC on cytokine release and are, therefore, most likely not involved in the uptake of cysC (data not shown). Others have shown that cysC antagonizes TGF-β receptors [44], but whether it is involved in the internalization of cysC has not been elucidated, and putative receptors remain to be discovered.

The immune modulatory role of nematode cystatins, as well as chicken cystatin, has been shown to affect the MAPKs ERK and p38 and the NF-κB pathways [27, 35]. Given that stimulation of the TLR-4 induces activation and phosphorylation of the MAPKs ERK1/2 and p38 [34], we chose to focus our investigation on the effect of cysC on the MAPK ERK1/2 and p38 pathways. CysC does not have any effect on the phosphorylation of p38 but has a significant effect on ERK1/2 phosphorylation. Thus, we showed that cysC decreases phosphorylation of ERK1/2, whereas other authors suggest that cystatins activate the ERK1/2 and p38 pathways [27, 35–37]. However, these experiments were carried out under different experimental conditions, using either chicken cystatin [35, 36] or nematode cystatin [27, 37], explaining the discrepancies between these studies and our results. Also, the effect of the nematode cystatin is different from

Figure 7. CysC lowers IL-1β and TNF-α levels in LPCs from patients with CD. The influence of cysC on PBMCs from healthy controls and on PBMCs and LPCs from patients with CD was examined. (A) PBMCs were treated for 20 h with LPS or LPS plus cysC, and the concentrations of the indicated cytokines were measured in the supernatants (n = 6). Data are depicted as the fold change, which was calculated as the levels of cytokines measured in the supernatants of LPS plus cysC cultures against levels of cytokines measured in LPS-stimulated cultures. (B) PBMCs from patients with CD were treated for 20 h with LPS or LPS plus cysC, and the concentrations of IL-1β and TNF-α were measured in the supernatants (n = 4). (C and D) LPCs from patients with CD were treated for 20 h with LPS, LPS plus cysC, or cysC, and the concentrations of IL-1β and TNF-α were measured in the supernatants (n = 3). Data are shown as fold change. (C) Levels of IL-1β and TNF-α from LPS plus cyste–stimulated cultures were calculated against levels of cytokines measured in LPS-stimulated cultures. (D) In addition, levels of IL-1β and TNF-α from cysC-stimulated cultures were calculated against levels of cytokines measured in non-LPS-stimulated cultures.

www.jleukbio.org
the effect that we detected for human cysC, in which we see a reduction in IL-1β and TNF-α and no increase in IL-10. Our data suggest that human cysC inhibits the LPS/TLR-4 signaling cascade, leading to diminished transcription of specific cytokines via down-regulated phosphorylation of the ERK1/2 pathway. Selective inhibition of cytokines in LPS-induced monocytes via specific MAPK pathways has also been demonstrated in other studies using different compounds, for example, Nc-5a, a heteroglycan from the cyanobacterium Nostoc commune, estrogen, or MAPK antibodies [45–47], confirming that down-regulation of a particular MAPK pathway can contribute to specific cytokine modulations. Although we showed that cysC was internalized by monocytes and decreased phosphorylation of ERK, we cannot rule out that cysC could also have other modes of action, for example, by modulating the posttranslational processing of IL-1β and TNF-α. It is known that CASP1 activation and the release of mature IL-1β in response to TLR–NF-κB stimuli are tightly linked. Recent studies have suggested that inflammasome priming and activation by LPS requires the activation of ERK and that this is essential for CASP1 activation [48]. Our data indicate that cysC does not inhibit active CASP1 in vitro and has no effect on monocyte CASP1 mRNA. Still, the possibility cannot be excluded that cysC inhibits ERK1-mediated inflammasome activation, which is not dependent on active CASP1. However, that was not been investigated further in this study.

Besides the effect on cytokine release, cysC also influences the phenotype of monocytes. It is known that LPS increases CD14 and HLA-DR expression [49, 50] and reduces expression of CD36, CD86, CD163, and TLR4 [50–53]. CysC significantly modulates the down-regulation caused by LPS of CD36 and CD86, with decreased CD14 expression, and shows a tendency to counteract TLR-4 down-regulation. This phenotypic expression pattern is similar to that of nonpathogenic, activated monocytes. However, cysC also has an effect on non-LPS-stimulated cells. CysC decreases CD11c, CD36, and CD163 in a manner similar to that in LPS-stimulated cells. This is in contrast to the observed anti-inflammatory–induced phenotype observed in LPS plus cysC–stimulated monocytes. CD163, a surface marker used to define anti-inflammatory type 2 macrophages, is shed from the cell surface upon LPS stimulation. Moreover, various cytokines regulate the expression of CD163. IL-6 and TNF-α suppress surface expression, whereas IL-10 increases the expression of CD163 on the cell surface [52]. In addition, the expression of CD36 can also be regulated by various cytokines and is down-regulated by LPS [53–55]. CysC induces the release of the proinflammatory cytokines IL-6 and IL-8 in nonpathogenically activated monocytes. This may explain the similar down-regulation of surface markers seen on monocytes stimulated with cysC alone and suggests that cysC alone might skew monocytes toward M1 antigen–presenting cells, thereby having a role in the early first-line of defense in acute inflammation. This is in line with a recent study showing that AVCystatin induces early and transient expression of both proinflammatory and anti-inflammatory markers on macrophages and that surface expression of MHC-II, CD40, CD80, CD86, FcγRII/III, PD-L1, PD-L2, and ICAM is up-regulated in response to AVCystatin [56]. CysC was initially thought to control peptide loading to the MHC II by regulating cathepsin S. Cathepsin S has a role in the removal of the MHC chaperone light chain and the formation of the MHC II-peptide complex [57]. However, cysC was later shown to influence neither MHC expression nor antigen peptide loading in mouse DC [58]. This is in accordance with our results that show that cysC does not influence the expression of HLA-DR in LPS-activated monocytes.

Currently, the most effective drugs used to treat CD are the anti–TNF-α therapies. Unfortunately, ~20% of patients with CD do not respond to these therapies, and a loss of response is seen in ≤45% of initial responders within the first year of treatment [59]. Considering the great unmet medical need, developing new therapies focused on dampening the inflammatory processes and hindering mucosal destruction and ECM degradation would be of great benefit.

In our study, we show that cysC, which hinders ECM degradation in light of its role as a protease inhibitor [20, 60], is not up-regulated in the mucosa of patients with CD. Other studies have shown that cathepsins are increased in CD [12, 13]. A disrupted homeostasis is, therefore, likely to occur between cathepsins and cysC, which may accelerate the degrading effect of the proteases on the ECM, causing mucosal destruction and impaired barrier function in CD. Moreover, besides its role as a protease inhibitor, we show that cysC displays immunomodulatory functions by inhibiting the release of IL-1β and TNF-α in pathogenically activated monocytes. Given the increase of newly recruited monocytes seen in the inflamed mucosa of patients with CD [5, 32], the increase in proinflammatory cytokines, and the ongoing ECM destruction [13], cysC or its regulation might represent an interesting candidate for pharmacologic intervention in CD therapy. Thus, further investigation on the effect of cysC is necessary to elucidate its biologic and immunologic function.

AUTHORSHIP
S.T.G. designed and performed the experiments, analyzed the data, and drafted the manuscript. S.J., J.G.G., K.H., and O.G. supervised the study and contributed to the experimental design. D.J. performed the immunohistochemistry staining, and P.H.K. analyzed the data.

ACKNOWLEDGMENTS
The authors wish to thank Thomas B. Rasmussen for valuable technical assistance and Magnus Abrahamson for introductory scientific discussions.

DISCLOSURES
S.T.G., P.H.K., J.G.G., and K.H. are, or have been, employed by Novo Nordisk A/S and own stock in Novo Nordisk A/S. This did not affect the authors’ interpretation of the data. This work was supported by Novo Nordisk A/S and grants from the Danish Ministry of Higher Education and Science (http://ufm.dk/en/research-and-innovation/funding-programmes-for-research-and-innovation/find-danish-funding-programmes/programmes-managed-by-innovation-fund-denmark/industrial-phd). The funders had no role in study design, data collection, or interpretation of data.
REFERENCES


inflammatory cytokine secretion by THP-1 monocytes through phosphorylation of ERK1/2 and Akt. Phytomedicine 21, 1451–1457.


KEY WORDS:
monocytes · cytokines · immunomodulator · inflammation · Crohn’s disease
The protease inhibitor cystatin C down-regulates the release of IL-β and TNF-α in lipopolysaccharide activated monocytes

Susanne Thiesen Gren, Sabina Janciauskiene, Salipalli Sandeep, et al.

*J Leukoc Biol* published online May 17, 2016
Access the most recent version at doi:10.1189/jlb.5A0415-174R