Enhanced Expression of CXCL10 in Inflammatory Bowel Disease -Potential Role of Mucosal Toll-like Receptor 3 Stimulation.

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Supported by grants from the Liaison Committee between the Central Norway Regional Health Authority (AEØ), The Norwegian Cancer Society (NJN and MB) and NTNU (AvBG). This work has also received funding from a research grant from The Liaison Committee between St. Olav`s University Hospital and the Faculty of Medicine, NTNU. The microarray analysis was supported by the Functional Genomics Programme (FUGE) of the Norwegian Research Council.

Background:

We explored the gene expression in colonic biopsies of active and inactive inflammatory bowel disease (IBD), in an extensive material of ulcerative colitis (UC) and Crohn`s disease (CD). The chemokine CXCL10 and its receptor CXCR3 were among the upregulated genes. This study examines the expression of CXCL10 and the mechanisms for its release in patients with UC or CD, and in intestinal epithelial cell (IEC) lines.

Methods: A microarray gene expression analysis was done on colonic biopsies (n= 133) from patients with IBD. Biopsies were studied with immunohistochemistry for CXCL10 and CXCR3 expression. Mechanisms for CXCL10 release in peripheral mononuclear cells (PBMCs) and in the colonic epithelial cell lines HT-29 and SW620 were studied upon pattern recognition receptor (PRR) stimulation.

Results: CXCL10 and CXCR3 mRNA abundances were increased in biopsies from active UC and CD compared to inactive disease and controls. CXCL10 was mainly localized to mucosal epithelial cells, with increased immunostaining in active IBD. CXCR3 positive cells were scattered in the lamina propria. CXCL10 was secreted from the colonic epithelial cell lines, in response to the toll-like receptor 3 (TLR3) ligand polyinosinic: polycytidylic acid (poly(I:C)). This ligand also induced a marked release of CXCL10 in PBMCs from IBD patients and controls.

Conclusions: We identify CXCL10 and CXCR3 as upregulated genes in colonic mucosa in active IBD. The TLR3-ligand poly(I:C) markedly increased release of CXCL10 in colonic epithelial cell lines, suggesting a TLR3 (dsRNA) mediated CXCL10 release from mucosal epithelial cells in IBD patients.

Key words: ulcerative colitis, Crohn‘s disease, poly(I:C), CXCL10, TLR3

Introduction

Chemokines direct the recruitment and migration of circulating leukocytes to inflammatory sites, and are important for the differentiation of secondary lymphoid tissue. They can be produced locally, by resident cells or by infiltrating leucocytes. The chemokines determine the composition of leukocytes in inflammation, and are thus central factors in the perpetuation of the inflammatory process (1).

In active ulcerative colitis and Crohn`s disease the immunological homeostasis is broken and the mucosa is characterized by influx of both innate immune cells (neutrophils, macrophages, dendritic cells and natural killer T-cells) and the adaptive immune cells (B-cells and T-cells). The balance/composition of subgroups of T-cells, especially Th1, Th2, Th17 and Treg, has been of particular interest (2-4). One highly relevant chemokine in this context is CXCL10 (formerly IP-10: interferon-γ induced protein 10).

CXCL10 is a typical CXC-chemokine where two of the four cysteine (C) residues that are located at the N-terminal part of the protein are separated by one amino acid (X). CXCL10 is a ligand for the receptor CXCR3, which is also the case for CXCL9 (former MIG) and CXCL11 (former I-TAC). Binding of ligand to CXCR3 is crucial for chemotaxis of these receptor positive cells to inflammatory sites (5). CXCL10 is regarded as a chemoattractant preferentially for activated T-cells (6) and particularly Th1 cells (7).

In a large-scale microarray study on endoscopic biopsies from diseased and non-diseased colon of UC and CD patients compared to healthy colonic mucosa from control individuals, we found a markedly enhanced and highly significant upregulation of CXCL10 mRNA and also a more modest overexpression of the CXCR3 receptor. Bearing in mind the central role of the innate immune system in these diseases we carried out a series of studies on CXCL10 expression in a patient material and in colon-derived intestinal cell lines with a panel of pattern recognition receptor-ligands covering the toll-like receptors (TLR) 1-9, nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and the cytokines IL10 and IL1β, to explore the sources and regulation of CXCL10.

Materials and methods

Clinical material

Patients admitted to the Gastrointestinal Endoscopy Unit, Department of Gastroenterology and Hepatology, St. Olav's University Hospital, were included in the study. The patients had ulcerative colitis or Crohn's disease, or underwent colonoscopy due to gastrointestinal symptoms. Healthy controls were included among those who had colonoscopy

with normal macro- and microscopic findings, and where clinically indicated supplementary examination did not reveal any significant disease. The UC group also included patients with associated primary sclerosing cholangitis (PSC). Patients using immunomodulants such as azathioprine or TNF alpha blockers were excluded. Colonoscopic biopsies were collected from macroscopically maximally inflamed colonic mucosa, normal biopsies always from the hepatic flexure. Four adjacent biopsies were taken from each location and either snap frozen and stored on liquid nitrogen, or fixed in 4% buffered formaldehyde. Hematoxylin-eosin stained sections were examined by an experienced pathologist to verify the presence or absence of inflammation. Serum was stored at -80^{o}C and used for analysis of chemokines and high-sensitivity CRP (hsCRP). PBMCs were obtained from heparinized peripheral venous blood by centrifugation (800g for 20 min at room temp.) on Lymphoprep mononuclear cell separation medium (Axis-Shield, Oslo, Norway) and stored in liquid nitrogen.

Ethical considerations

The study was approved by the Regional Medical Research Ethics Committee, and was registered in the Clinical Trials Protocol Registration System (identifier NCT00516776). All patients and control individuals gave informed consent.

Gene expression analysis

Microarray gene expression analysis was done on 133 colonic biopsies. Frozen biopsies were homogenized, and RNA extracted using the Ambion *mir*Vana mRNA isolation kit (Ambion, Austin, TX). Total RNA quantity and quality was assessed using NanoDrop Spectrophotometer (Thermo Scientific, Stockholm, Sweden) and Bioanalyzer capillary electrophoresis (Agilent Technologies, Santa Clara, CA). All RNA samples were of high quality (RIN > 7). Microarray analysis was performed using Illumina human HT-12 expression BeadChips (Illumina, San Diego, CA) on an Illumina BeadStation.

Histological and Immunhistochemical examination

Formaldehyde-fixed colonic biopsies were embedded in paraffin, and 4 µm sections cut and prepared for histological and immunohistochemical examination. Biopsies were stained with hematoxylin-eosin and evaluated by an expert pathologist assessing inflammation. The samples were classified as “normal”, “chronic inflammation” or “chronic active

inflammation", based on mucosal mononuclear and neutrophilic cellular infiltration, respectively.

The same biopsies were further used for immunohistochemistry. For CXCL10 / CXCR3 immunohistochemistry, five sections from each of the sample groups active UC (UCa), inactive UC (UCi) active CD (CDa) inactive CD (CDi) and controls (C) were stained. These commercially available antibodies were used: CXCL10 goat polyclonal antibody (cat. nr. AF-266-NA, R&D Systems, Minneapolis, MN) dilution 1:5, CXCR3 mouse monoclonal antibody (cat. nr. ab64714. Abcam plc, Cambridge, UK) dilution 1:250). Detection was done using Dako EnVision peroxidase kit and Dako DAB+ chromogen (Dako, Glostrup, Denmark). Sections were counterstained with hematoxylin. For CXCL10, the degree of staining of the epithelium was assessed by two independent examiners as "no to little"=0 or "moderate to strong"=1. CXCR3 was quantified by counting positive cells in 4 different fields pr. slide (magnification x40). If total number positive cells for a sample was below the median for all samples, it was denoted 0, if above the median then denoted 1, then tested using Fischer Exact Test.

Culture and stimulation of intestinal epithelial cells

Two intestinal epithelial cell lines were used; HT-29 from a human colorectal adenocarcinoma, and SW620, from a lymph node metastasis of a human colorectal adenocarcinoma (Cat.nr. HTB-38 and CCL227, ATCC Manassas, VA). The cells were cultured at 37°C, 5% CO_2 in RPMI supplemented with fetal bovine serum 10%, glutamine 2mM and gentamicin 0.05%. They were detached from the culture flasks using trypsin/EDTA, resuspended in medium, and counted in a Countess Automated Cell Counter (Life Technologies, Grand Island, NY). Stimulation experiments were done in triplicate with 20000 cells per well in 96-well, flat-bottom plates incubated overnight at 37°C, 5% CO_2. Medium was then removed and fresh medium or TLR-ligand/cytokine was added. The ligands, target receptor in parenthesis, were the lipopeptide Pam3CysSK4(P3C) (TLR2/1), 300ng/mL, Lipomannan (LM) (TLR2/6), 30ng/mL, synthetic double-stranded RNA mimic, polyinosinic: polycytidylic acid (poly(I:C)) (TLR3), 50µg/mL , lipopolysaccharide (LPS) (TLR4), 100ng/mL , Flagellin (TLR5), 100ng/mL, the antiviral compound R848 (TLR7/8), 100ng/mL (all from InvivoGen, Toulouse, France), unmethylated CpG dinucleotides (TLR9), 10 µM (TibMolBiol, Berlin, Germany), the peptidoglycan component muramyl dipeptide (MDP) (NOD2) 1µg/mL (InvivoGen, Toulouse, France), and the recombinant cytokines IL-10,

100ng/mL , IL-1β, 100ng/mL (both from PeproTech, Rocky Hill, NJ). The cells were incubated at 37°C, 5% CO_2 for 20h before supernatant was harvested and stored at -20°C. CXCL10 in supernatans was quantified using an ELISA human Duo-Set (R&D, Abigdon, UK) according to manufactures instruction..

The viability of cells was determined by a colorimetric method; the MTT- (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Promega, Nacka, Sweden) (0.5mg/ml) in 10%FCS/RPMI was added to the adherent cells and incubated at 37°C, 5% CO for 3 hours. Supernatants were replaced by alkaline isopropanolol with 0.25% 1M NH_3OH. The plate was stirred for 30 min and optical density was measured at 570 nm using the luminometer function of Walla Victor3™ 1420 Multilabel Counter by Perkin Elmer™.

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TLR3 siRNA transfection of the intestinal epithelial cell line HT-29.

HT-29 cells were detached by trypsination and seeded at 20000 cells per well using culture medium without antibiotics. Transfection took place initially after plating cells using the transfection agent Lipofectamin, RNAiMAX (Ambion, Invitrogen Dynal, Oslo, Norway) and TLR3-siRNA (sense-GAACUGGAUAUCUUUGCATT, antisense-UGGCAAAGAUAUCCAGUUCTT) (5nM) or a non-silencing control (Sense-UUCUCCGAACGUGUCACGUdTdT, Antisense-ACGUGACACGUUCGGAGAAdTdT) (5nM) (both from Qiagen, Sollentuna, Sweden). The cells were incubated at 37°C and 5% CO_2 for 24h before they were stimulated with poly(I:C) (5ug/ml) for 20h. Supernatant was collected and kept at -20°C until CXCL10 content was determined by ELISA, while remaining cells were either assayed by MTT assay, or washed and lysed and stored at -80ºC until total cellular RNA was isolated for qRT-PCR. siRNA knockdown of TLR3 was confirmed by qRT-PCR in HT-29 cells after TLR3 siRNA transfection and poly-IC stimulation.

RNA isolation and qRT-PCR

Total cellular RNA was isolated from lysed cells using Ambion mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol. RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (Saveen Werner AB, Malmö, Sweden). Reverse transcription was performed using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) following the manufactures recommendations. cDNA was analysed for TLR3 transcripts by quantitative real time PCR using StepOnePlus™ Real-Time PCR System and StepOne™ software v2.1 (Applied Biosystems, Foster City, CA).

TaqMan gene expression assays for TLR3 (Hs01551078_m1) and GAPDH (Hs99999905_m1), and TaqMan Fast Real-Time PCR Universal PCR Master Mix were used (all purchased from Applied Biosystems, Foster City, CA). TLR3 expression was normalized to the housekeeping gene GAPDH and calculated relative to the negative control; HT-29 cells treated with non-silencing RNA.

CXCL10 and hsCRP in serum.

CXCL10 in serum was quantified using ELISA as described for the studies on intestinal epithelial cells. hsCRP analyses were done with a commercially available immunoturbidometric assay (Tina-quant, Roche, Indianapolis, IN).

Stimulation of PBMCs.

PBMCs from 39 randomly chosen subjects (control=9, UC=10, CD=10, UC+PSC=10) were thawed. No one used corticosteroids or other immunosuppressive medication at the time of sampling (Table 2). PBMCs (0.5 mill per well in flat-bottomed 96-well plates) were incubated in RPMI 1640 with glucose and L-glutamine (Life Technologies, Paisley, U.K.) supplemented with pooled 10% human A+ serum from blood donors (100µl/well). The PBMCs were stimulated with PRR-ligand/cytokine or medium as control. The same ligands were used here as for stimulation of cell lines, and the concentrations were: p3c:300ng/mL, Lipomannan: 30ng/mL poly(I:C): 50µg/mL, LPS: 10ng/mL, Flagellin: 100ng/mL, R848: 100ng/mL, CpG: 10 µM, MDP: 100ng/mL, IL-10: 100ng/mL, IL-1β: 100ng/mL. Supernatant was harvested after 6h and frozen at -20ºC. Supernatant was assayed for the following cytokines using the Bio-Plex multiplexing system (Bio-Rad, Hercules, CA) according to the manufacturer`s instructions; IL-1β, IL-1rα, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, FGF-β, G-CSF, GM-CSF, IFNγ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-bb, RANTES, TNFα and VEGF. In addition, CXCL10 release from stimulated PBMCs was analysed in supernatant by ELISA as described for IEC stimulation. PBMCs from 5 healthy donors were isolated as in the patient material, but not frozen, and CXCL10 was quantified in the supernatants.

Statistical Analysis

Microarray data analysis was done using Bioconductor (8) in the R software environment. Differential expression was determined using linear models with least squares regression and empirical Bayes moderated t-statistics (9). P-values were adjusted for multiple

comparisons using Benjamini Hochberg false discovery rate correction. The Kruskal-Wallis test was used for analysis of differences in levels of proteins in supernatant and serum. If significance, the Mann-Whitney U-test was used to test differences between pairs of subjects. For correlation between protein serum levels the Spearman Rank Correlation test was used. Fischer test was used to evaluate differences in immunohistochemical staining between groups. Calculations were performed by PASW Statistics version 18. A difference was considered significant if $p<0.05$ (2-sided).

Results

Clinical material

The 133 biopsies enrolled in the microarray analysis were sampled from 112 subjects (Table 1). Altogether 21 subjects with IBD had two biopsies taken; one from an area of active and one from inactive disease. Five of the subjects included in UC group also have associated PSC. There were no differences in age, duration of disease between the patient groups. The difference found regarding medication is as expected according to clinical practice, with more patients with UC receiving 5-ASA/S-ASA as compared to CD patients and more patients with CD receiving systemic corticosteroids compared to UC patients.

CXCL10 and CXCR3 are overexpressed in biopsies from active UC and active CD.

Microarray gene expression analysis showed a significant overexpression of CXCL10 and CXCR3 mRNAs in active UC and CD, both when compared to healthy controls and to nondiseased mucosa from UC or CD patients (Fig. 1A). Average CXCL10 mRNA change in diseased UC mucosa vs healthy controls was, $\log_2$, 2.58 (fold change 5.98), and diseased CD mucosa vs healthy controls, $\log_2$, 2.25 (fold change 4.76). CXCR3 changes were more modest but significant, $\log_2$, 0.39 (fold change 1.31) for UC vs healthy controls, and $\log_2$, 0.54 (fold change 1.45) for CD. In biopsies from nondiseased mucosa from UC and CD patients we found no significant change in CXCL10 or CXCR3 expression compared to healthy controls. The mRNAs of the cytokines IL1β, CXCL8, TNFα and IFNγ were also overexpressed in active UC and active CD vs controls and non-inflamed UC/CD, so also CXCL9 and CXCL11 (Fig. 1B). Strong correlations were found when comparing the mRNA levels of CXCL10 and CXCR3 to the proinflammatory cytokines IL1β, CXCL8, TNFα and IFNγ and also the chemokines CXCL 9 and CXCL11 (Table 2).

Colonic epithelial cells are an important source of CXCL10

Representative results from immunohistochemical examination of CXCL10 and CXCR3 in diseased mucosa of UC and CD patients and also controls are shown in Fig. 2. CXCL10 was localised to the epithelial lining with only scattered positive cells in the lamina propria. Staining was seen both as a diffuse staining of cytoplasm and as a granular staining related to epithelial cell nuclei. The cytoplasmatic staining was significantly increased in patients with active UC and active CD vs controls (for both $p<0.05$, 2-sided). No difference was found comparing inactive UC vs controls and inactive CD vs controls. When comparing the granular staining, no differences were observed between the patient groups and controls. The CXCR3 immunoreactive cells were scattered in the lamina propria. However, no significant difference in numbers of CXCR3 positive cells between patient groups and controls could be detected.

The TLR3 ligand poly(I:C) induces CXCL10 release from intestinal epithelial cells.

Release of CXCL10 from intestinal epithelial cells was examined in the HT-29 and SW620 cell lines, which were stimulated with 8 different PRR-ligands, covering TLR 1-9, and NOD2 in addition to cytokines IL10 and IL1b (Table 3). The TLR3-ligand poly(I:C) induced a massive CXCL10 release (Fig.3). No other ligands induced release of CXCL10 in these cells. MTT assay was done on both cell lines and viability was not influenced by any of the ligands used.

To ensure the CXCL10 release was mediated via TLR3, siRNA transfection of HT-29 cells was done and TLR3 levels analysed using quantitative RT-PCR. The CXCL10 release following transfection was reduced by 50.0% compared to non-transfected poly(I:C) stimulated HT-29 cells (Fig. 4). The TLR3 mRNA level, using qRT-PCR, was profoundly upregulated (218.4 %) after poly(I:C) stimulation and knockdown of TLR3 by siRNA transfection reduced its mRNA abundance by 87.7% (siRNA TLR3 vs siRNA control). MTT assay shows preserved viability of cells independent of transfection reagent / siRNA.

CXCL10 release in PBMC.

We explored the CXCL10 release from PBMCs upon PRR-ligand stimulation using the same ligands as in the intestinal epithelial cell lines. PBMCs from 39 randomly chosen subjects were assayed; controls (n=9), UC (n=10), UC +PSC (n=10) and CD (n=10) (Table 4). There was a significant difference in use of 5-ASA/sulphasalazine between UC and CD

patients which reflects clinical practice, however there was no significant difference in constitutive CXCL10 release from PBMCs whether these drugs were used or not. Following a 6-hour- stimulation of PBMCs, we analyzed the supernatant using the Bio-Plex assay as described. We found a constitutive CXCL10 release from PBMCs. Moreover, the nucleic acid ligands poly(I:C) (TLR3), R848 (TLR 7/8), and CpG (TLR9) considerably induced CXCL10 release in PBMCs after 6 hours stimulation in both controls (Fig. 5) and IBD patients. However, we could not find any differences in CXCL10 release between controls and UC or CD when assessing these four CXCL10 inducing ligands. Interestingly, the constitutive release of CXCL10 from PBMCs was inversely correlated with the serum levels of CXCL10 in IBD patients, p=0.013, rho=-0.396 The same response pattern, regarding induction of CXCL10 release, was found in freshly isolated PBMCs from five healthy controls, showing that cryopreservation of PBMC did not influence these cellular responses. CXCL10 in serum showed a considerable inter-individual variability. There was no significant difference in CXCL10 levels between patient groups, other than a trend towards a higher level in UC patients with PSC as compared to healthy controls and to UC patients without PSC (both p=0.080).

Discussion

The initial microarray gene expression analyses described in this study were done on endoscopic biopsies from a large, well controlled IBD biobank collected in a routine clinical setting. Assessing the results to generate hypotheses on the inflammatory processes in IBD, we observed a marked and highly significant increase in mRNA abundance of CXCL10 in endoscopic biopsies from diseased mucosa. Moreover, although to a lesser degree, its receptor CXCR3 was also upregulated. The mRNA levels of CXCL10 and CXCR3 were strongly correlated and they also correlate very well to other, more established markers of inflammation such as IL1β, CXCL8, and TNFα in inflamed mucosa, supporting that CXCL10 indeed has a role in the inflammatory process in IBD.

CXCL10 is known to be expressed in monocytes, fibroblasts, endothelium (10) and also in intestinal epithelial cells (11), thus the overexpression of CXCL10 in mucosal biopsies can derive from both epithelial, stromal and immune cells. Although somewhat controversial, the immune cells of the colonic mucosa have earlier been considered the main source of

CXCL10It has, however, also been shown that IFNγ and to a lesser degree IL1β and TNFα induce CXCL10 release from epithelial cells (11, 12). In our immunohistochemical studies (Fig. 2), the CXCL10 protein is localized mainly to the intestinal epithelium. CXCL10 positivity is clearly higher in inflamed UC and CD mucosa, as compared to healthy control individuals, and like mRNA abundance it is similarly increased in the two diseases. This points to the colonic epithelium as an important source of chemokine attracting CXCR3+ cells. In addition to the cytoplasmatic staining we see dense granula in relation to the nucleus. One previous study on keratinocytes reports granulas of CXCL10 located to the rough endoplasmatic reticulum (13). This finding is interpreted as a sign of de novo synthesis of CXCL10 in the colonic epithelial cells.

There are few studies on CXCL10 in the colonic mucosa of IBD patients, and these results seem somewhat inconsistent. One study (14) using immunohistochemistry on samples from UC patients with active inflammation vs controls showed an enhanced CXCL10 expression but solely in the lamina propria and not in the epithelial cells. Another study found a correlation between CXCL10 transcript level in colonic mucosa and endoscopic activity in UC (n=49) (15) . Manousou et al. found increased CXCL10 mRNA in colonic biopsies from CD patients (n=20) compared to UC(n=22) and controls(n=20). The CXCL10 mRNA level was found to be increased in 13 of 20 CD patients and 9 of 22 UC patients but also 6 of 20 controls.(16) In yet another report (17)) CXCL10 was found upregulated in inflamed mucosa from pediatric patients with active UC and CD (PCR, UC, n=6, CD, n=9). The gene expression results from these previous studies are consistent with our observations, however the localization of CXCL10 diverges between our observations and other studies. This discrepancy is difficult to explain, but the present material is large and well controlled. It includes both inflamed and noninflamed mucosa from patients and samples from healthy controls, and the immunohistochemical positivity correlates well with gene expression on mRNA level. Our results thus seem valid.

A paradigm in IBD pathogenesis has been that UC is characterized by an atypical Th2 inflammation and CD by an inflammation where Th1 cytokines dominate (2, 3). CXCL10 is considered as a chemokine that supports Th1-type inflammation and antagonizes Th2-type inflammation (18). Thus, the observation of a marked and similar increase of CXCL10 in both UC and CD is surprising. This supports the emerging view that separation of IBD into Th1/Th2 inflammation is to rigid, and that UC and CD show features characteristic of both types of inflammation. This suggestion is supported by the finding of overexpressed IFNγ (the classic Th1-cytokine) in our clinical material of IBD. IFNγ causes release of CXCL10

from several cell types (19). We found CXCL10 and IFNγ to be upregulated in both active UC and CD, IFNγ and CXCL10 correlating well. This analysis does not allow us to discern whether the primary event is induction of CXCL10 by IFNγ, or of the increase of IFNγ is seen as a result of IFNγ producing CXCR3+ cells influx as response to TLR3-mediated CXCL10 release.

CXCR3+ is mainly expressed on activated Th1-cells and also found on memory T-cells, NK cells, B-cells, mast cells and endothelial cells (18). There are inconsistent data about expression of CXCR3 in mucosa of active IBD (20, 21). Previous studies using immunohistochemical staining found an increased number of CXCR3+ cells in submucosa of CD but not in lamina propria, and not in UC patients (21). In this study we found CXCR3 mRNA to be, although highly significant, only modestly overexpressed in both UC and CD. We could, however, not find any difference in the number of CXCR3+ cells in mucosa of active IBD vs inactive IBD or controls. This may be due to internalization of receptor when arriving at the site of inflammation (22), or be caused by a "dilutional effect" since other immunological cells than CXCR3+ also infiltrate the mucosa. At least one previous study shows a similar picture. Papadakis et al found increased CXCR3 mRNA and at the same time lowered CXCR3 protein by "mean fluorescence intensity" in lymphocytes isolated from lamina propria in UC patients, but not from lamina propria in CD patients (20).

Having observed that CXCL10 is localized to intestinal epithelial cells, we chose to explore the CXCL10 regulation in two well-established epithelial cell lines derived from adenocarcinoma of the colon, using a panel of PRR ligands. Like colonic epithelial cells these cell lines do not express IFNγ, (23), the most established inductor of CXCL10, enabling us to examine the more direct effects of the PRRs on CXCL10 release. Interestingly, we found the TLR3 ligand poly(I:C) to be a potent inducer of CXCL10 release. We conclude from this that CXCL10 synthesis and release from colonic epithelium is a valid pathophysiological phenomenon and most likely regulated directly via a TLR3 mediated mechanism. This novel observation is highly interesting and may be relevant to the pathogenesis of inflammatory bowel disease.TLR3 has been suggested to be a sensor for endogenous RNA that is released during tissue necrosis. Cavassani et al (24) demonstrated that TLR3 regulates the inflammatory response during microbial septic peritonitis and ischemic bowel injury. Thus, it is possible that endogenous mRNA from damaged tissue induces TLR3 signaling and increases the level of CXCL10 in the epithelium, inducing homing of CXCR3+ cells to the mucosa thereby amplifying inflammation in the mucosa and further disturbing the mucosal homeostasis. Poly(I:C) is a synthetic analogue to double stranded RNA and mimics dsRNA

recognition (i.e. viral RNA) by the innate immune system. The observed effect of poly(I:C) thus implicates that exposure to viral material as well as endogenous mRNA might contribute to, or even induce, inflammation in IBD.

We also studied PBMCs, as a potential source of CXCL10 both in serum and intestinal mucosa which PBMCs may infiltrate in IBD. They are orchestrated by chemokines and are in an equilibrium with the other inflammatory cells present in the intestinal mucosa. In the PBMCs we see a constitutive release of CXCL10 which was inversely correlated to levels of CXCL10 in serum. PBMCs respond to a wider panel of PRR ligands than what we see in the intestinal epithelial cells. In PBMCs TLR3, 7, 8 and 9 –ligands all induce release of CXCL10, among these the TLR3 ligand is the most potent inductor. PBMCs thus behave differently from the HT-29 and SW620 cell lines, which do express TLR7,8 and 9 on mRNA and protein levels (25, 26) but only respond to TLR3 ligand stimulation. The contribution of PBMCs to local and systemic CXCL10 induction in IBD is still unclear .

Supporting the role of the CXCL10/ CXCR3 axis in IBD are observations suggesting that it may be involved in the pathogenesis of several other chronic inflammatory diseases such as psoriasis, rheumatoid arthritis, multiple sclerosis and atherosclerosis (27). In rheumatoid artritis a phase II study of anti-CXCL10 antibody (MDX-1100) has been completed showing clinical benefit (28). Moreover, in IBD anti CXCL10 antibody has been tested in several mouse models, where 3 out of 4 cases show benefit (29-32). Phase II studies of MDX-1100 in human UC and CD are ongoing and will show if neutralizing CXCL10 improves IBD in a clinical setting (33)

Our data suggest that CXCL10 has a central role in the inflammatory process of IBD. We find intestinal epithelial cells to be an important source of CXCL10 and poly I:C stimulation of IECs potently induces CXCL10 release via TLR3. This opens for the interesting possibility that CXCL10 can be induced by RNAs, i.e. from damaged tissue or viruses.

Acknowledgements:

We thank Kari Slørdahl, Bjørn Munkvold. Liv Ryan and Unni Nonstad at IKM, NTNU, who proved excellent technical assistance. .

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Tables:

TABLE 1. Characteristics of subjects enrolled in microarray analysis.

	Controls	UC	CD	p
Number of subjects	20	70	22	
Age, years (range)	46(26-71)	43(19-64)	42(26-63)	n.s
Female sex (%)	8 (40)	37(51)	7(53)	n.s
Duration of disease, years (range)	-	13(0-40)	7(1-28)	n.s
5-ASA/S-ASA (%)	0	45(64)	7(32)	0.000 [a]
Systemic corticosteroids (%)	0	8(17)	9(41)	0.001 [b]
hsCRP, mg/L (range)	1.2(0.3-12.9)	1.7(0.5-41.9)	3.7(0.3-17.5)	n.s

Age, duration of disease and hsCRP are given as median, and gender and medication as numbers.

[a] Significantly higher use of ASA/S-ASA in UC vs CD subjects.

[b] Significantly higher use if systemic corticosteroids in CD vs UC subjects.

TABLE 2. Correlations in microarray analysis of colonic biopsies

		CXCL10	CXCR3	IL1β	CXCL8	TNFα	IFNγ	CXCL9	CXCL11
CXCL10;	rho	1.000	0.619	0.810	0.850	0.804	0.789	0.899	0.576
CXCR3;	rho	0.619	1.000	0.531	0.536	0.615	0.566	0.697	0.411

All correlations are highly significant, $p<0.001$

TABLE 3.

Ligand	p3c	LM	poly(I:C)	LPS	Flagellin	R848	CpG	MDP	IL10
Receptor	TLR1/2	TLR2/6	TLR3	TLR4	TLR5	TLR7/8	TLR9	NOD2	IL10R

TABLE 4. Characteristics of subjects enrolled in stimulation of PBMCs.

	Controls	UC	CD	p
Number of subjects	9	20	10	
Age (range)	48(22-68)	40(20-65)	36(26-49)	n.s
Female sex (%)	5(55.6)	8(40)	3(30)	n.s.
Duration of disease(range)	0	12(1-38)	10(3-28)	n.s
5-ASA/S-ASA (%)	0	13(65)	3(30)	0.002 [a]

Systemic corticosteroids (%)	0	0	0	n.s.
hsCRP (range)	1.9(0.8-5.1)	1.6(0.6-31.5)	3.4(1.2-16.8)	n.s

Age, duration of disease and hsCRP are given as median, and gender and medication as numbers.
[a] Significantly higher use of ASA/S-ASA in UC vs CD subjects.

Figure legends

Fig. 1

Microarray-derived gene expression levels of A) CXCL9-10-11 and CXCR3 mRNA and B) IL1β, CXCL8, TNFα and IFNγ mRNA in colonic biopsies are shown as $\log_2$; mean and individual values plotted. UC –ulcerative colitis; CD –Crohn's disease; a – active, i – inactive. *p<0.001 vs control, #p<0.01 vs inactive disease.

Fig. 2

.Immunohistochemical staining for CXCL10 and CXCR3 in colonic biopsies from controls, active ulcerative colitis and active Crohn`s colitis. CXCL10 is located mainly in the epithelial cells, but scattered staining is also seen in the lamina propria. The staining of CXCL10 in epithelial cells is more intense in active ulcerative and active Crohn`s colitis than control. In addition to the cytoplasmatic staining of CXCL10 we see granular staining related to the nucleus. The CXCR3 positive cells are found scattered in the lamina propria and with no difference in diseased versus healthy control mucosa. Original magnification x20.

Fig. 3.

CXCL10 release from HT-29 (A) and SW620 (C) cells stimulated with PRR-ligands corresponding to TLR1-9 and NOD2 (see Table 3) or the cytokines IL10 and IL1b for 20 hours. Lowest standard for CXCL10 ELISA; 31.25 pg/mL, indicated by —. *p<0.05 vs medium. MTT-assays (B and D) show preserved viability of cells irrespective of ligand added. Data given as mean ± SEM.

Fig. 4.

CXCL10 release in response to poly(I:C) is impaired in HT-29 cells following TLR3 siRNA treatment.

HT-29 were left untreated or transfected with TLR3 siRNA, or non-silencing RNA (ns RNA) for 24hrs, before cells were stimulated with poly(I:C) or medium for 20hrs. CXCL10 content in supernatant was assessed by ELISA. $p<0.05$. The results shown as mean ± SD of triplicates.

Fig. 5

CXCL10 release from PBMCs in healthy controls stimulated with PRR-ligands corresponding to TLR1-9 and NOD2 or the cytokines IL10 and IL1β (see Table 3) for 6h. Cell supernatant was harvested and assayed for CXCL10 by muliplexing. Highest observed value in range at 42000 pg/mL indicated by —· 9 of 9 values for poly(I:C) were above range. 6 of 9 values for R848 were above range. Data given as mean ± SEM.

The supernatant was diluted 1:10 and analysed using ELISA CXCL10 for the ligands poly(I:C) and R848 showing 5 times higher CXCL10 release in poly(I:C) stimulated HT-29 comparing R848 stimulated HT-29 (data not shown).

Fig. 1A.

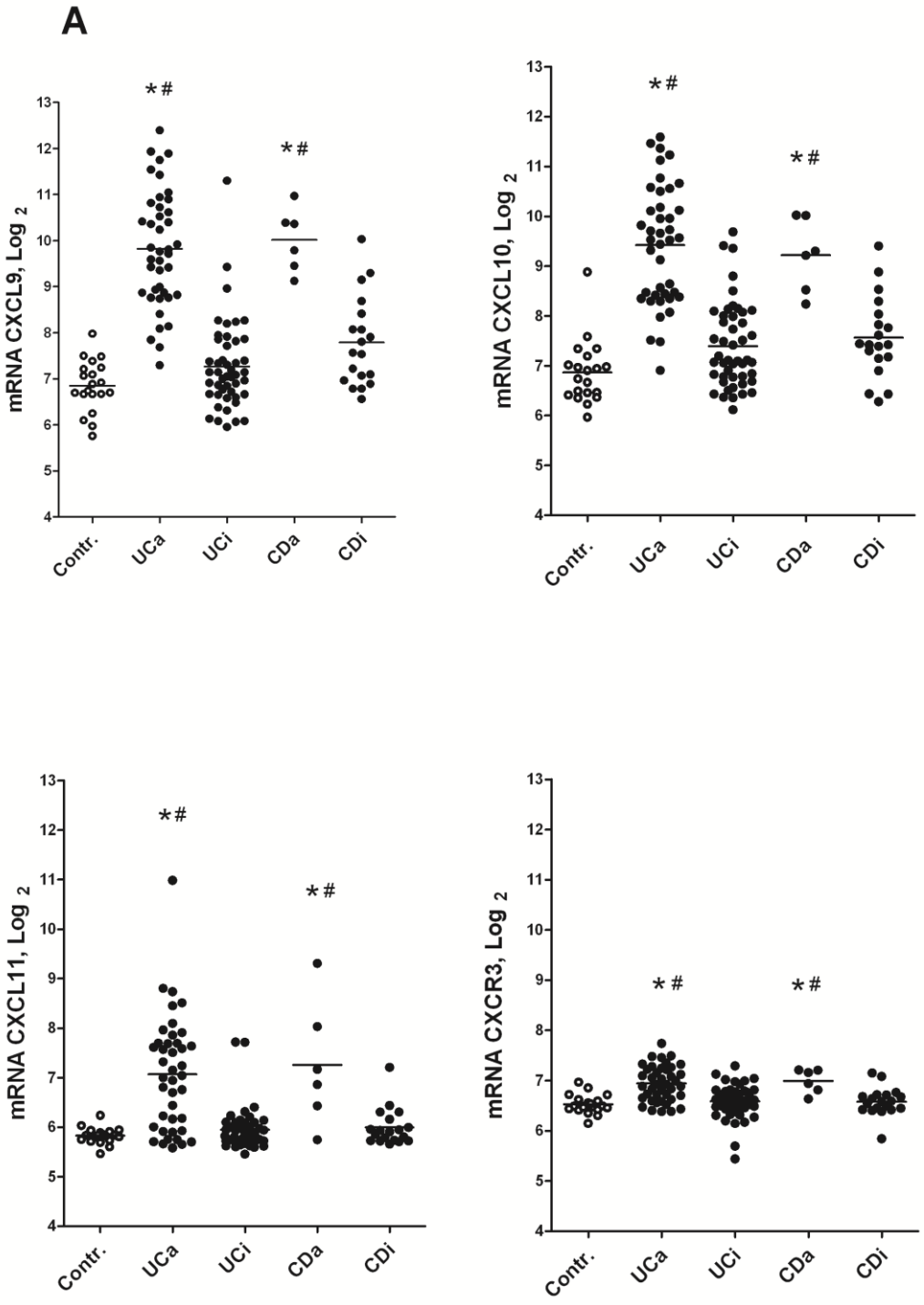

A
mRNA CXCL9, Log 2
mRNA CXCL10, Log 2
mRNA CXCL11, Log 2
mRNA CXCR3, Log 2
*#
Contr.
UCa
UCi
CDa
CDi

Fig. 1B.

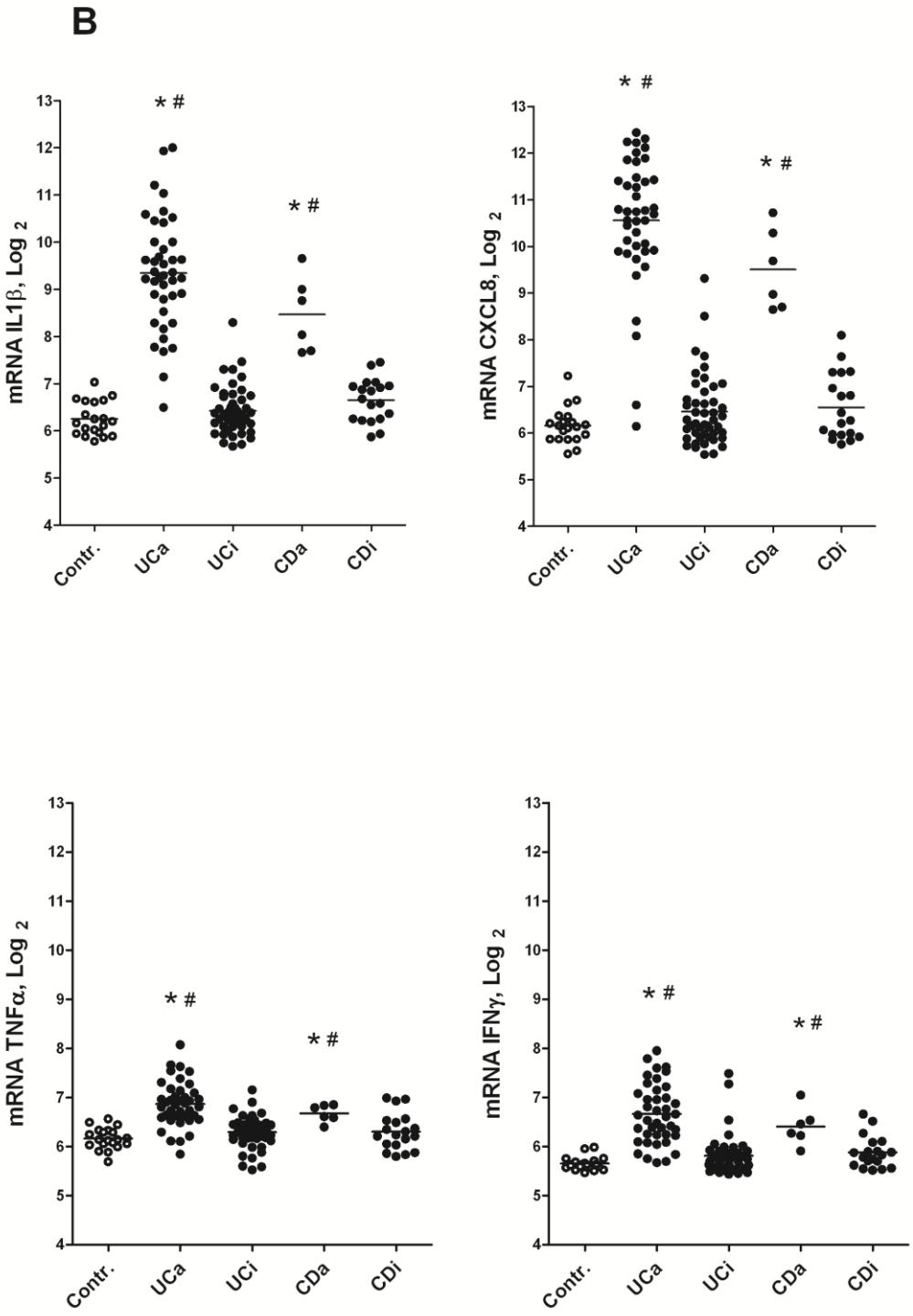

B
mRNA IL1β, Log 2
mRNA CXCL8, Log 2
mRNA TNFα, Log 2
mRNA IFNγ, Log 2
Contr.
UCa
UCi
CDa
CDi
* #

Fig. 2

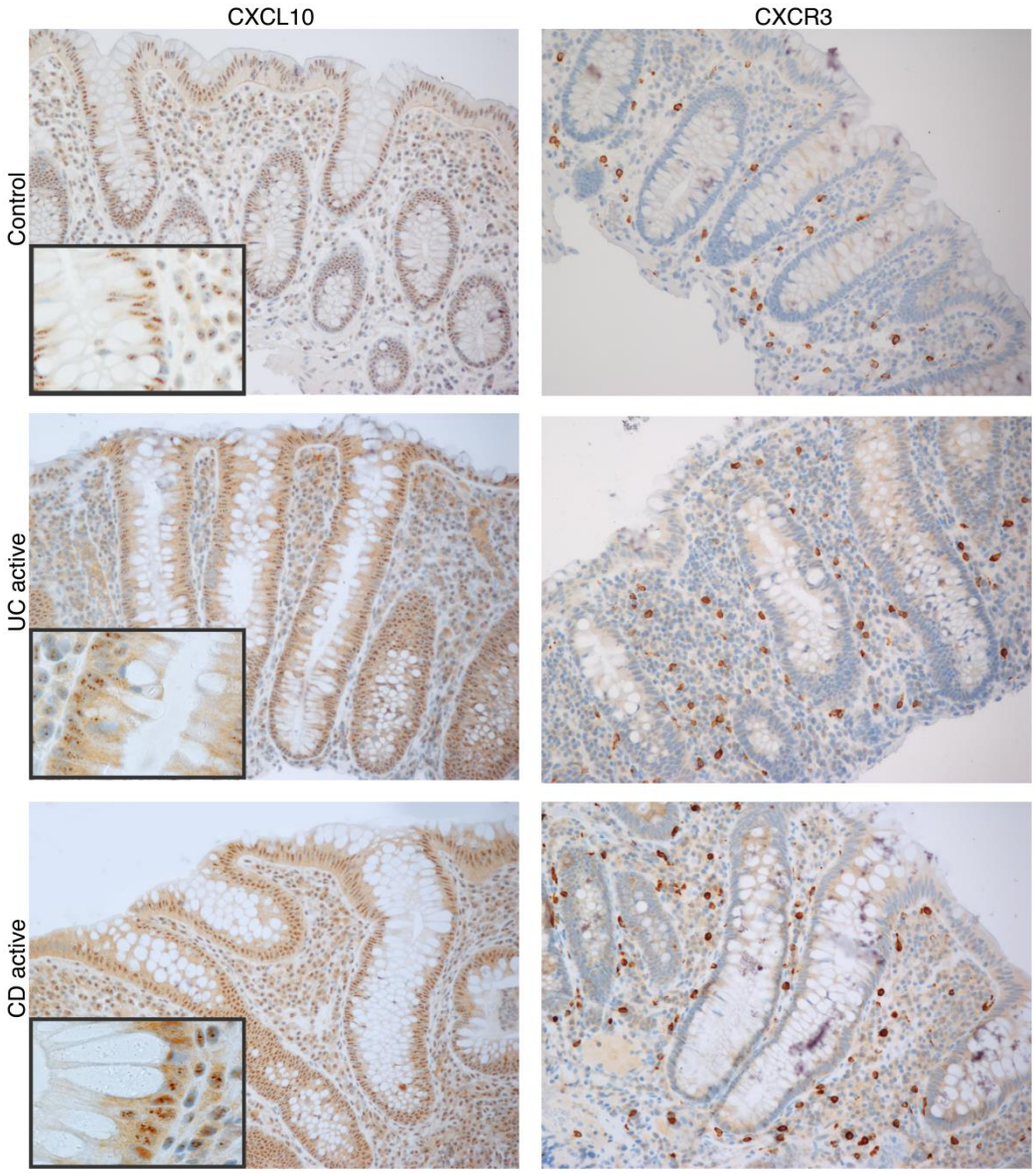


Fig. 3.

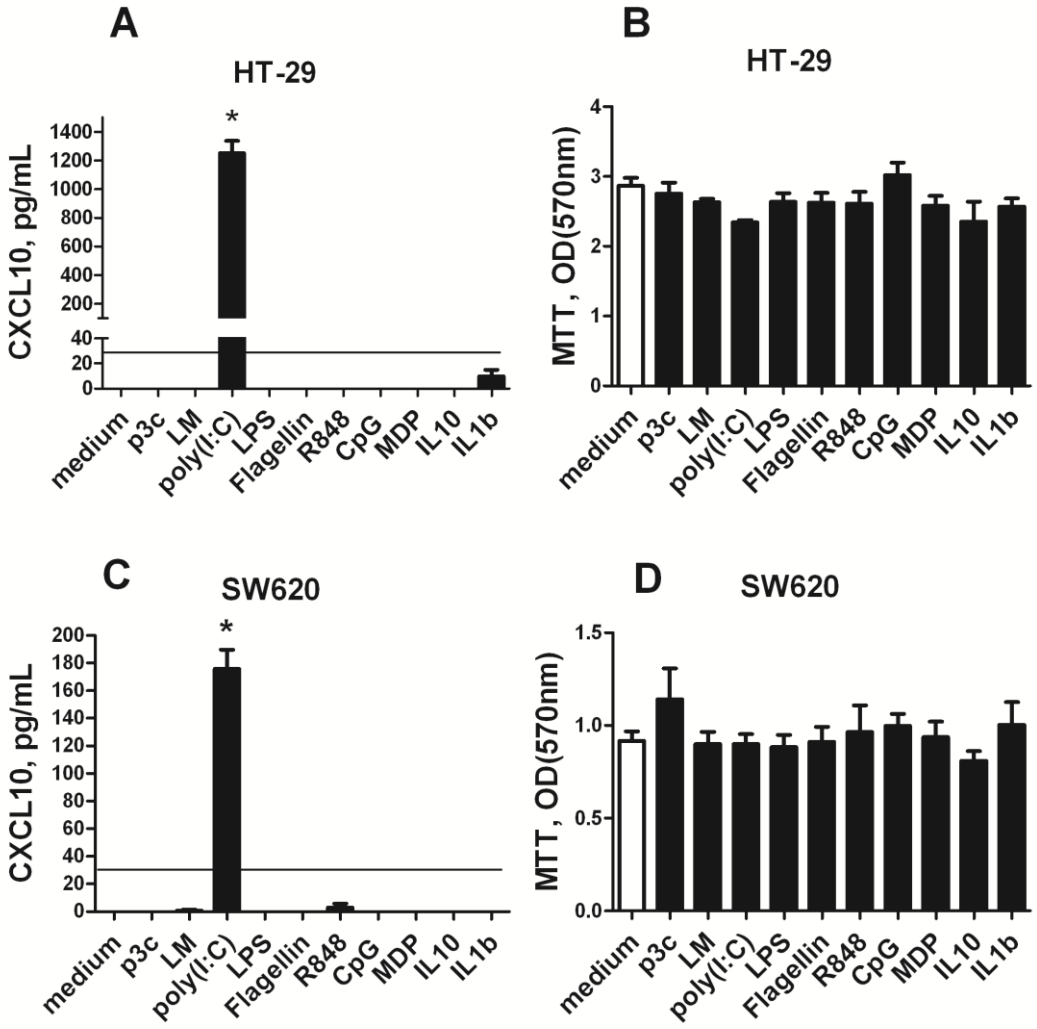

A
HT-29
CXCL10, pg/mL
1400
1200
1000
800
600
400
200
40
20
0
*
medium
p3c
LM
poly(I:C)
LPS
Flagellin
R848
CpG
MDP
IL10
IL1b
B
HT-29
MTT, OD(570nm)
4
3
2
1
0
medium
p3c
LM
poly(I:C)
LPS
Flagellin
R848
CpG
MDP
IL10
IL1b
C
SW620
CXCL10, pg/mL
200
180
160
140
120
100
80
60
40
20
0
*
medium
p3c
LM
poly(I:C)
LPS
Flagellin
R848
CpG
MDP
IL10
IL1b
D
SW620
MTT, OD(570nm)
1.5
1.0
0.5
0.0
medium
p3c
LM
poly(I:C)
LPS
Flagellin
R848
CpG
MDP
IL10
IL1b

Fig. 4

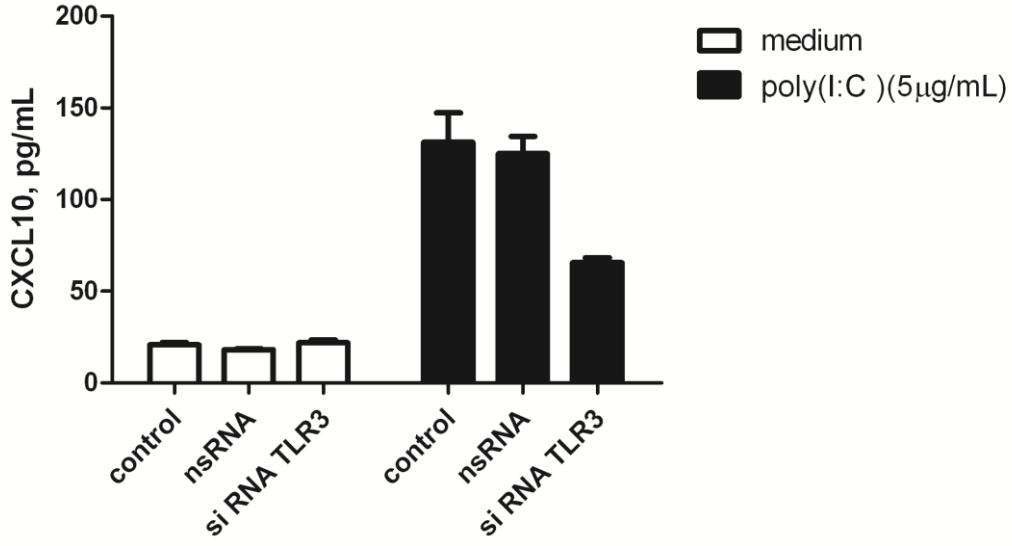
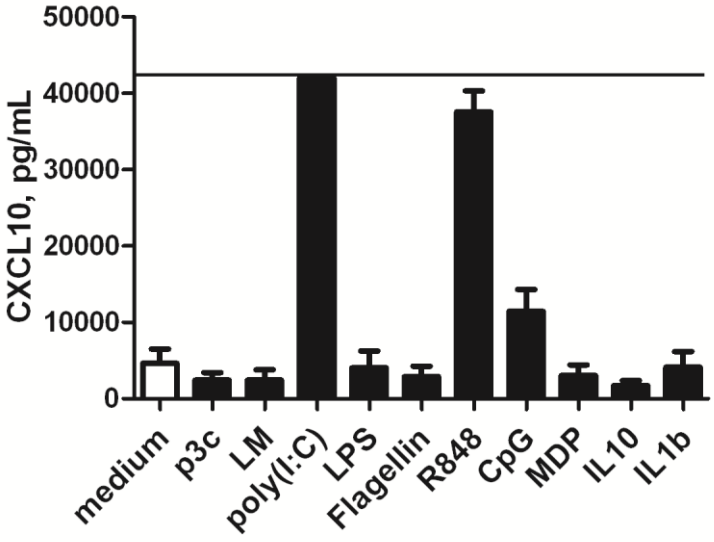

CXCL10, pg/mL
200
150
100
50
0
control
nsRNA
si RNA TLR3
control
nsRNA
si RNA TLR3
medium
poly(I:C)(5μg/mL)

Fig. 5


CXCL10, pg/mL
50000
40000
30000
20000
10000
0
medium
p3c
LM
poly(I:C)
LPS
Flagellin
R848
CpG
MDP
IL10
IL1b