ORIGINAL ARTICLE

Influence of NFκB inhibitors on IL-1β-induced chemokine CXCL8 and -10 expression levels in intestinal epithelial cell lines: glucocorticoid ineffectiveness and paradoxical effect of PDTC

Yeruva Sunil · Giuliano Ramadori · Dirk Raddatz

Abstract

Purpose Activation of intestinal epithelial cell (IEC) nuclear factor κB (NFκB) and the consequent chemokine upregulation are crucial events in inflammatory bowel disease (IBD) pathogenesis. Not much is known about the consequences of NFκB inhibition in terms of chemokine expression in intestinal cells. Therefore, we aimed to evaluate the efficacy of compounds known to disrupt the NFκB pathway on NFκB transcriptional activity and CXCL8 and CXCL10 gene expression in intestinal cell lines.

Methods The influence of NFκB inhibitors (dexamethasone, pyrrolidine dithiocarbamate (PDTC) and BAY 11-7082) on IL-1β-induced NFκB transcriptional activity was investigated by transient transfection of Caco-2 cells with an NFκB-secreted alkaline phosphatase reporter plasmid. Il-1β stimulated CXCL8 and CXCL10 mRNA and protein expression and was studied in Caco-2 and HT29 cells in the presence and absence of the NFκB inhibitors by quantitative real-time polymerase chain reaction and enzyme-linked immuno-sorbent serologic assay, respectively. To reveal alternative signalling cascades, experiments were also performed in the presence of the p38MAPK inhibitor SB 203580 and the ERK inhibitor PD 98059.

Results Dexamethasone did not downregulate chemokine expression sufficiently, probably due to a lack of glucocorticoid receptors in these cells. While BAY11-7082 inhibited chemokine expression, PDTC led to a paradoxical upregulation of CXCL8 in Caco-2 cells, which could be prevented by inhibition of p38MAPK.

Conclusion These data explain the frequent unresponsiveness of IBD to glucocorticoid treatment and suggest that alternative NFκB inhibition in IECs might be of use in IBD therapy. Drug development based on measuring anti-NFκB activity might be misleading and should therefore also include studies on relevant gene products.

Keywords NFκB · Intestinal epithelial cells · CXCL10 · CXCL8 · PDTC

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Introduction

In the past few years, there has been increasing interest in how cytokines, bacteria and bacterial polymers induce intestinal epithelial cell (IEC) gene expression. In IECs, gene expression must be tightly regulated to avoid overreaction to normal microbial flora while at the same time remaining responsive to harmful pathogens. Nuclear factor κB (NFκB) is a crucial player in maintaining intestinal barrier integrity since its tonic stimulation is essential for defensin production [1]. On the other hand, NFκB is involved in the transcriptional activation of several

genes involved in mucosal inflammation and in contrast to normal mucosa is activated, both in macrophages and in epithelial cells of inflamed intestinal mucosa [2], suggesting a prominent role in inflammatory bowel disease (IBD) pathogenesis.

The NFκB family is comprised of a group of transcription factors defined in part by their ability to bind a specific DNA sequence first identified in the enhancer of the immunoglobulin κ light chain gene [3, 4]. NFκB exists as a heterodimer comprising RelA (p65) and NFκB1 (p50) subunits in most cells; this heterodimer is the most potent gene transactivator of the NFκB family [5]. NFκB is activated by a variety of agents, such as cytokines, growth factors, T cell mitogens, oxidative stress, bacteria, viruses and their products. Upon activation by various stimuli, NFκB transcriptionally regulates many cellular genes involved in early inflammatory responses, including the chemokines CXCL8 [6] and CXCL10 [7].

CXCL8 (IL-8) is a proinflammatory CXC chemokine associated with the promotion of neutrophil chemotaxis and degranulation [8]. CXCL8 protein is secreted by a variety of cell types, including IECs [9, 10]. CXCL8 has also been shown to play an important but non-specific role in the pathogenesis of IBD, and its mRNA has been shown to be restricted to areas with histological signs of inflammatory activity and mucosal destruction [11].

CXCL10 is a CXC chemokine that binds to the CXCR3 chemokine receptor, expressed mainly by activated CD4$^+$ memory T cells, which produce a T helper cell 1 pattern of cytokine production [12]. CXCL10 is also known for its anti-angiogenic properties in cancers [13]. It has also been shown that IECs express CXCL10 mRNA, suggesting that the intestinal epithelium can play a role in modulating physiologic and pathologic T cell-mediated mucosal inflammation [14].

Increased NFκB activity was found in the IECs of IBD patients [2], suggesting that targeting NFκB activity in these cells might be an attractive goal for therapeutic intervention. Although glucocorticoids are known to inhibit NFκB activity [15], not much is known about their effect in IECs in terms of expression of inflammation-related genes. The same is true for other NFκB-inhibiting compounds like pyrrolidine dithiocarbamate (PDTC) and BAY11-7082, an irreversible inhibitor of IκBα phosphorylation. IL-1β is a potent inducer of NFκB. High concentrations of IL-1β are found both in Crohn's disease and ulcerative colitis intestine [16]. Previous studies have shown that CXCL8 [9] and CXCL10 [7] are upregulated by IL-1β in intestinal cells. We aimed, therefore, to test the efficacy of different inhibitors of NFκB on IL-1β-induced NFκB activity and CXCL8 and CXCL10 expression in an intestinal model system, utilising the established intestinal cell lines Caco-2 and HT29.

Materials and methods

Reagents

Recombinant IL-1β was purchased from Roche Applied Science (Mannheim, Germany). p38 MAPK inhibitor SB203580, MEK inhibitor PD98059 and BAY 11-7082, a specific inhibitor of NFκB, were purchased from Calbiochem (San Diego, CA, USA), and the NFκB inhibitor PDTC was purchased from Sigma-Aldrich (Germany).

Cell culture and stimulation protocols

The human colon adenocarcinoma cell lines Caco-2 and HT29 were obtained from DPZ (Braunschweig, Germany). Caco-2 cells were grown in Eagle's minimal essential medium (BioWhittaker) containing 20% foetal calf serum (FCS) supplemented with 100 U/ml each of penicillin and streptomycin and 1% non-essential amino acids at 37°C with 5% CO_2. HT29 cells were grown in Roswell Park Memorial Institute medium containing 10% FCS and 100 U/ml penicillin and streptomycin at 37°C with 5% CO_2.

Assay for activation of NFκB

Transient transfection with pNFκB-secreted alkaline phosphatase (SEAP; a reporter plasmid in which NFκB promoter elements are linked to the gene coding for secretory alkaline phosphatase) was used for assaying NFκB activity.

Caco-2 cells were plated in 24-well plates 24 h before transfection (Nunc, Roskilde, Denmark) at a density of 50,000/well in 1 ml medium. After 24 h, the cells were transfected using the non-liposomal formulation FuGENE (Roche Molecular Biochemicals, Mannheim, Germany). On the day of transfection, fresh medium was added. FuGENE was added to the plasmid DNA at a ratio of 3 μl/μg DNA. FuGENE was pre-diluted in 100 μl serum-free medium and added dropwise to the concentrated plasmid DNA. After 15 min at room temperature, the mixture was added to the cells. The cells received 200 ng pNFκB-SEAP (BD Biosciences, Clontech, Palo Alto, CA, USA)/well. Twenty-four hours after transfection, the cells were treated with PDTC (0.2, 2 and 20 μg/ml, which are equivalent to final concentrations of 1.2, 12 and 120 μM), BAY11-7082 (1, 10 and 100 μM), SB203580 and PD98059 for 1 h and then stimulated with IL-1β (1 ng/ml) for 6 h. Finally, cell supernatants were collected, and SEAP assays were performed as per the manufacturer's protocol.

RNA and protein expression studies

For stimulation, IECs were plated into 6-well plates at a density of 5×10^5 cells per well and grown until they

reached 70–80% confluence. One hour prior to the stimulation, cells were pre-treated with one of the following: dexamethasone (1 μM), PDTC (0.2, 2 or 20 μg/ml), BAY11-7082 (1, 10 or 100 μM), SB203580 (10 μM) or PD98059 (10 μM). Cells were then stimulated with IL-1β (1 ng/ml) for either 1 or 4 h for mRNA expression studies or for 24 h for enzyme-linked immunosorbent serologic assay (ELISA) assays.

RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The RNA concentration was determined photometrically using a Gene Quant RNA/DNA calculator (Pharmacia, Freiburg, Germany). RNA was subsequently used for real-time polymerase chain reaction (PCR). Reverse transcription of mRNA was performed using 1 μg of total cellular RNA, as described previously [17].

PCR was carried out using gene-specific primers (Invitrogen, Karlsruhe, Germany) for human CXCL8 (forward 5′-ATG ACT TCC AAG CTG GCC G-3′, reverse 5′-GCT GCA GAA ATC AGG AAG GC-3′), CXCL10 (forward 5′-CCA GAA TCG AAG GCC ATC AA-3′, reverse 5′-CAT TTC CTT GCT AAC TGC TTT CAG-3′) and β-actin (forward 5′-CTG GCA CCC AGC ACA ATG-3′, reverse 5′-CCG ATC CAC ACG GAG TAC TTG-3′) in an ABI Prism 7000 system.

PCR reactions were set up with Sybr® Green PCR Mastermix, containing 0.3 μmol/l of each primer and 1 μl of reverse transcription product in a 25-μl volume. A two-step amplification protocol was chosen, consisting of initial denaturation at 95°C for 10 min followed by 45 cycles of 15 s denaturation at 95°C and 30 s annealing/extension at 60°C. Finally, a dissociation protocol was performed to control for specificity of amplification products. Relative expression of each chemokine was then calculated using the comparative threshold-cycle method, as described earlier [18]. mRNA gene expression is presented as fold increase calculated in relation to unstimulated cells, after normalisation against β-actin. Glucocorticoid receptor (GR) mRNA content was quantified in Caco-2 and HT29 cells as described previously [18].

Supernatants of Caco-2 and HT29 cultures were collected 24 h after stimulation with IL-1β, and concentrations of CXCL8 and CXCL10 in the supernatants were measured by ELISA (Rnd Systems, Wiesbaden, Germany), following protocols provided by the manufacturer.

Statistical analysis

Statistical analyses were carried out using the Prism software packet (version 3.0, Graphpad Software, San Diego, CA, USA). Comparisons between two or more treatment groups were made with the unpaired t test or analysis of variance, where appropriate. In case of RNA

expression, a log transformation was performed beforehand. Statistical differences were regarded as significant at a p value below 0.05. Data are expressed as means±standard error of the mean.

Results

PDTC and BAY11-7082 inhibit IL-1β-mediated pNFκB-SEAP reporter gene activity in Caco-2 cells

In order to show whether PDTC and BAY11-7082 could function in inhibiting NFκB in Caco-2 cells, we performed reporter assays utilising an NFκB-SEAP reporter, which harbours NFκB binding elements. IL-1β treatment resulted in a 4.01±0.416-fold increase in reporter gene activity. This induction was inhibited in a dose-dependent manner by PDTC and BAY11-7082. Both stimulated and spontaneous NFκB activities were half-maximally inhibited by PDTC at a range between 0.2 and 2 μg/ml and by BAY11-7082 between 1 and 10 μM (Fig 1).

Effect of dexamethasone on cytokine-mediated CXCL8 and CXCL10 mRNA and protein expression

Chemokine mRNA and protein levels were induced by IL-1β. Dexamethasone served to reduce IL-1β-induced CXCL8 mRNA to 82.7±0.5% when compared to an untreated control. However, although there was a trend, there was no significant reduction of CXCL8 protein by dexamethasone treatment (Fig 2a). IL-1β-induced CXCL10 mRNA expression was reduced to 81.3±9.4% of control levels in the presence of dexamethasone. Also, CXCL10 protein levels were not significantly downregulated by dexamethasone (Fig. 2b). In HT29 cells, dexamethasone had no influence on CXCL8 or CXCL10 expression, either at the mRNA or protein level (data not shown).

Glucocorticoid receptor (GR) mRNA expression in Caco-2 and HT29 cells

GR was shown to be expressed in both cell lines. However, its expression level was extremely low (four orders of magnitude lower) in HT29 cells compared to Caco-2 cells (Fig. 3).

PDTC and BAY11-7082 effects on IL-1β-mediated CXCL8 mRNA expression and protein secretion in Caco-2 cells

PDTC, a known inhibitor of NFκB, was expected to inhibit IL-1β-induced CXCL8 mRNA expression, as CXCL8 expression is regulated by NFκB. To prove this, CXCL8
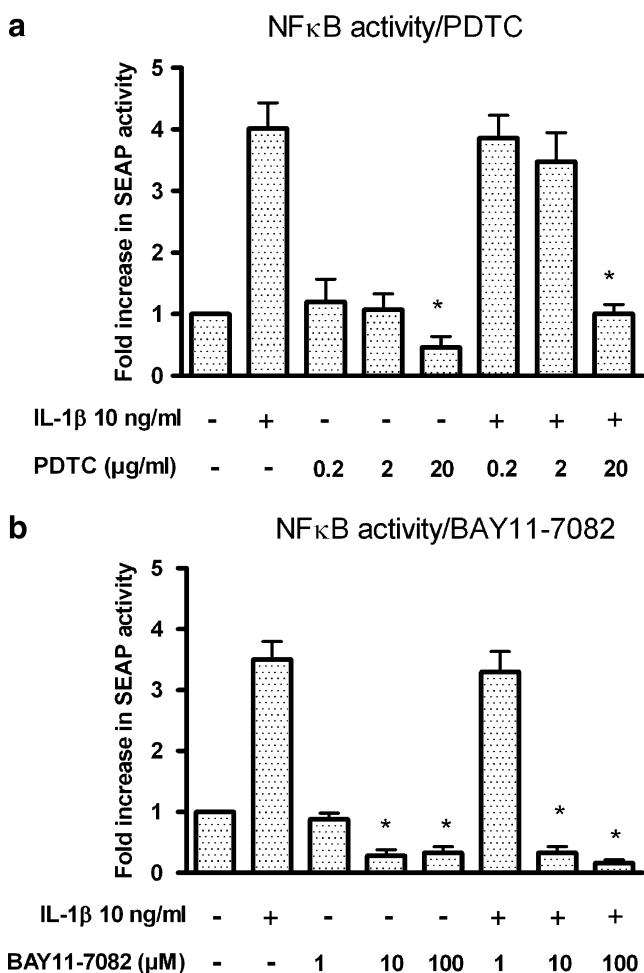
Fig. 1 Dose-dependent effects of pyrrolidine dithiocarbamate (PDTC) (**a**) and BAY11-7082 (**b**) on IL-1β-mediated pNFκB-secreted alkaline phosphatase (SEAP) reporter gene activity in Caco-2 cells. Caco-2 cells were transiently transfected with pNFκB-SEAP plasmid. Twenty-four hours after transfection, cells were pre-treated for 1 h with increasing concentrations of PDTC or BAY11-7082, as indicated. After 1 h, cells were stimulated with IL-1β or phosphate buffer solution as a control. Six hours after stimulation, cell supernatants were collected, and SEAP activity was measured. Data are shown as means±standard error of the mean of four individual experiments performed in duplicate for each sample. *Asterisk* corresponds to $p < 0.05$ compared to IL-1β stimulation alone

mRNA and protein expression levels were measured in IL-1β-stimulated Caco-2 cells pre-treated with PDTC.

Surprisingly, IL-1β induced CXCL8 mRNA expression was enhanced by PDTC in a dose-dependent manner. IL-1β led to a 117±9.1-fold increase in CXCL8 mRNA, which was enhanced to 150±21.6- and 262±62.35-fold increases in the presence of PDTC at 2 and 20 μg/ml, respectively. This observation was also confirmed at the protein level by ELISA of culture supernatants. PDTC alone did not stimulate CXCL8 expression (Fig. 4a).

In contrast, BAY11-7082, a specific inhibitor of IκB phosphorylation, inhibited IL-1β-mediated CXCL8 mRNA

expression and protein secretion in a dose-dependent manner. IL-1β-induced CXCL8 mRNA expression increased by 120.85±33.7-fold after 1 h. In the presence of BAY11-7082 at concentrations of 1, 10 and 100 μM, IL-1β-induced CXCL8 mRNA levels were inhibited to 119.02±31.35-, 8.19±3.73- and 0.97±0.15-fold increases, respectively. CXCL8 secretion induced by IL-1β reached 522.49± 46.68 pg/ml after 24 h. In the presence of BAY11-7082 (1, 10 and 100 μM), IL-1β-induced CXCL8 protein secretion was inhibited to 429.17±17.09, 328.98±21.81 and 244.84± 5.67 pg/ml, respectively (Fig. 4b).

Comparison of the effects of the NFκB inhibitor PDTC and MAP kinase inhibitors SB203580 and PD98059 on IL-1β-mediated CXCL8 mRNA expression and protein secretion in Caco-2 and HT29 cells

Since IL-1β-mediated CXCL8 gene expression is also under the control of MAP kinases, we examined the effect of the MAPK inhibitors SB203580 (p38 MAPK inhibitor) and PD98059 (MEK inhibitor) on IL-1β-induced CXCL8 gene expression in Caco-2 and HT29 cells and compared these effects with that of PDTC. Pre-treatment of both Caco-2 and HT29 cells with SB203580 (10 μM) led to a significant reduction in IL-1β-induced CXCL8 mRNA expression and protein secretion. In Caco-2 cells, IL-1β (1 ng/ml) increased CXCL8 mRNA levels by 19.75±2.52-fold, which was reduced to 7.52±0.77-fold in the presence of SB203580. IL-1β-induced CXCL8 secretion in Caco-2 cells was reduced from 286.79±32.99 to 55.50±34.09 pg/ml in the presence of SB203580 (10 μM). PD98059 also inhibited IL1-β-induced mRNA and protein expression by approximately 50%. A combination of both PDTC (20 μg/ml) and SB203580 (10 μM) did not inhibit IL-1β-induced CXCL8 mRNA expression or protein secretion in Caco-2 cells. However, PDTC-mediated enhancement of IL-1β-induced CXCL8 mRNA expression and protein secretion was inhibited by SB203580, suggesting a role for p38 MAPK (Fig. 5a).

We then wondered whether this enhancement effect of PDTC was cell-line dependent, so we used HT29 cells to check the effect of PDTC on IL-1β-mediated CXCL8 mRNA expression and protein secretion. In the case of HT29 cells, PDTC did not inhibit IL-1β-induced CXCL8 gene expression. It also did not enhance CXCL8 expression, as was the case for Caco-2 cells. In HT29 cells, CXCL8 was induced 11.49±2.39-fold by IL-1β, which was reduced to 2.03±0.59-fold in the presence of SB203580 and to 2.26±0.59-fold in the presence of PD98059. IL-1β-induced CXCL8 protein levels in HT29 cells were reduced from 5,163.30±777.04 to 1,157.72±179.59 and 1,718.86± 166.67 pg/ml in the presence of SB203580 and PD98059, respectively (Fig. 5b).

Fig. 2 Influence of dexamethasone on IL-1β-induced CXCL8 (a) and CXCL10 (b) mRNA (*left panel*) and protein (*right panel*) expression. Caco-2 cells (5×10^5 per well) were plated into 6-well plates and grown for 24 h. Cells were then pre-treated with dexamethasone (1 μM) for 1 h before stimulation with IL-1β (1 ng). Cells were harvested at 1 (CXCL8) and 4 h (CXCL10), total RNA was isolated and real-time reverse transcription-polymerase chain reaction was performed. For measuring protein expression, stimulation was performed for 24 h. Data are shown as the fold increase compared to the baseline controls and represent the means of three individual experiments±standard error of the mean. *Asterisk* corresponds to *p<0.05* compared to controls
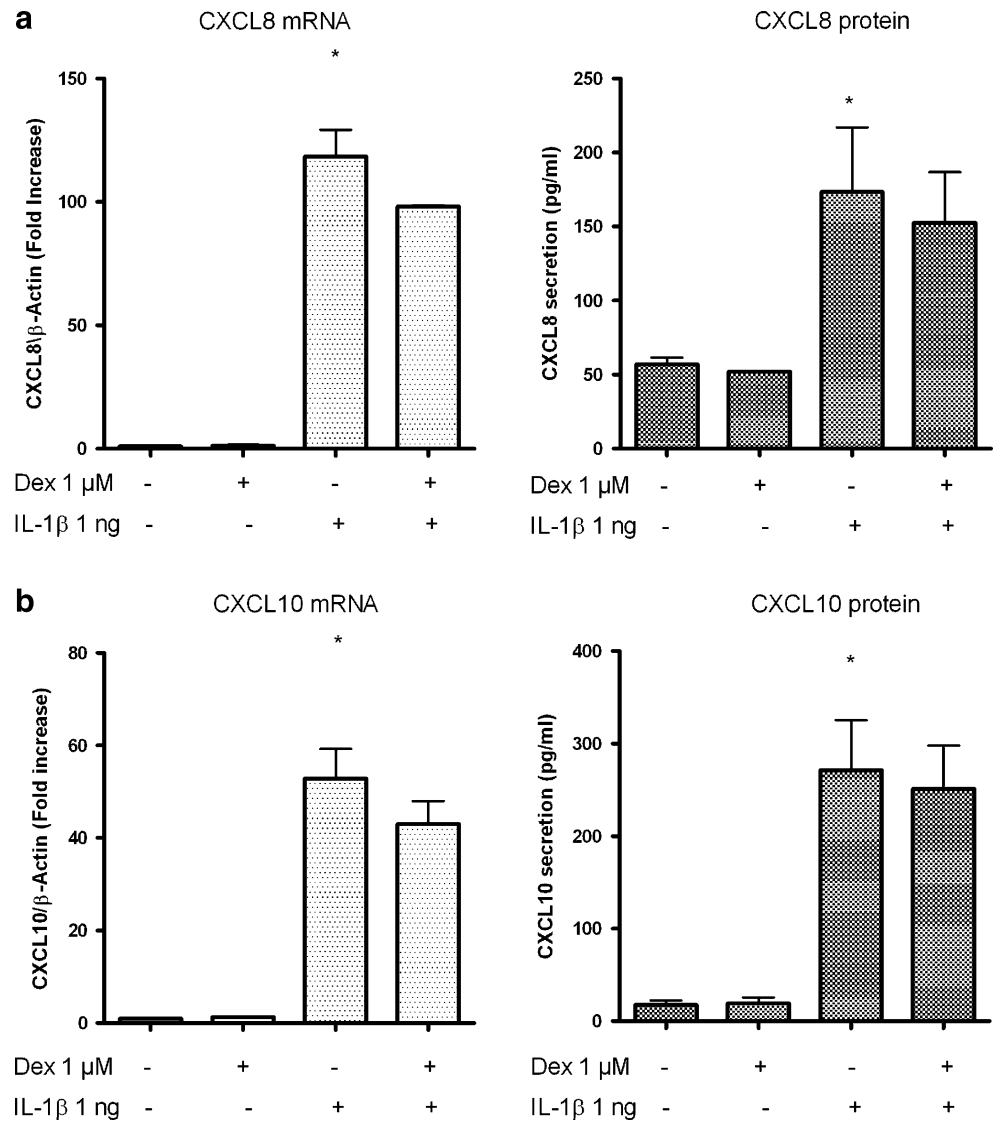
Comparison of the effects of the NFκB inhibitor PDTC and MAP kinase inhibitors SB203580 and PD98059 on IL-1β-mediated CXCL10 mRNA expression and protein secretion in Caco-2 and HT29 cells

In contrast to CXCL8, IL-1β-induced CXCL10 expression was downregulated by PDTC in Caco-2 cells. IL-1β (1 ng/ml) induced a 55.89±4.84-fold increase in CXCL10 mRNA, which was reduced to 45.42±4.66-, 9.51±0.72-, 17.03±3.21- and 9.94±1.30-fold increases in the presence of PDTC (20 μg/ml), SB203580 (10 μM), PD98059 (10 μM) and SB203580 (10 μM) together with PDTC (20 μg/ml), respectively (Fig. 6a). IL-1β-induced CXCL10 secretion in Caco-2 cells was reduced from 220.66±13.86 to 134.33± 28.42, 84.46±16.82, 150.66±6.06 and 93.21±23.21 pg/ml in the presence of PDTC, SB203580, PD98059 and SB203580 and PDTC together, respectively.

In HT29 cells, CXCL10 mRNA expression and protein secretion induced by IL-1β were inhibited significantly by

PDTC. IL-1β-induced CXCL10 mRNA by 11.49±2.39-fold. In the presence of the inhibitors PDTC and SB203580, IL-1β-induced CXCL10 mRNA expression was reduced to 5.12±1.50 and 10.76±1.15, respectively. Surprisingly, PD98059, either alone or in the presence of IL-1β, led to an upregulation (up to 46.12±10.97-fold) of CXCL10 mRNA, but this effect was not seen at the level of IL1β-induced CXCL10 protein secretion. In the presence of PDTC, SB203580 and PD98059, IL-1β-induced CXCL10 protein secretion was reduced from 86.88±26.83 to 34.93± 10.83, 8.70±4.38 and 14.54±14.54 pg/ml, respectively (Fig. 6b).

Discussion

Steroids are known to inhibit NFκB activity, both by direct protein–protein interaction with the GR [19] and induction
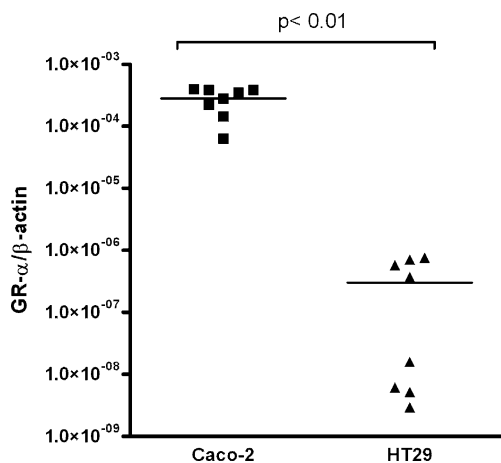
Fig. 3 Glucocorticoid receptor α mRNA expression in Caco-2 and HT29 cells. Cells (5×10^5 per well) were plated in 6-well plates, grown for 24 h and harvested. RNA was isolated and reverse transcribed to cDNA. Real-time polymerase chain reaction was performed with gene-specific primers for GRα. β-actin was used as an internal control. Data shown represent the GRα/β-actin mRNA ratios of four independent experiments performed in duplicate. The *horizontal bars* represent the means

of IκBα, an NFκB inhibitor, via GR activity [15]. The present data confirm earlier observations that colonic epithelial cells are largely unresponsive to glucocorticoids. Dexamethasone was not capable of significantly down-regulating CXCL8 or CXCL10 expression. This is in accordance with our previous findings showing only an incomplete transrepression of NFκB activity in transient reporter gene assays in Caco-2 cells stimulated with IL-1β, although the level of GR expression in those cells is comparable to peripheral blood mononuclear cells [20]. Nevertheless, the number of available GR molecules seems to be insufficient, since transrepression of NFκB can be restored by GR overexpression [20]. In HT29 cells, there is a clear lack of GRs, as suggested by the low abundance of GR mRNA (more than 1,000-fold less than in Caco-2 cells).

In contrast to steroid-sensitive IBD patients, where initial NFκB activity normalises, NFκB was shown to remain activated in IECs of IBD patients who are resistant to steroid therapy [21], suggesting that GR signalling might be disturbed, possibly due to a lack of functional GRs. Therefore, inhibiting NFκB with alternative compounds is of importance in treating IBD. Although 5-aminosalicylic acid is known to inhibit NFκB [22], it is of limited use in severe ulcerative colitis or Crohn's disease, and patients do not profit from its additional oral use when systemic steroids are given. Although PDTC and BAY11-7082 are not intended for pharmacological use in humans, they are paradigms for NFκB inhibitors and are widely used in experimental settings.

PDTC, a thiol-containing agent, is known to be a stable anti-oxidant and has been widely used to inhibit the activation of NFκB in a variety of cell lines. In the present study, both PDTC and BAY11-7082 inhibited IL-1β-induced NFκB reporter gene activity in a dose-dependent manner in Caco-2 cells, revealing their inhibitory effect on NFκB activation. However, treatment of Caco-2 cells with PDTC resulted in an dose-dependent enhancement of IL-1β-induced CXCL8 mRNA expression, rather than its inhibition, which seems to contradict previous studies that showed IL-1β was able to induce the CXCL8 gene via NFκB in Caco-2 cells [23]. ELISA experiments also showed that this effect was persistent at the level of protein synthesis and secretion. In contrast, IL-1β-induced CXCL8 expression and secretion were inhibited in a dose-dependent fashion by BAY11-7082.

Previous studies with PDTC revealed that it is not only involved in inhibiting NFκB but could also activate other signalling pathways, depending on the cellular context. In rat mesangial cells, PDTC induced gene expression of stromelysin through tyrosine kinase-mediated activation of the transcription factor AP-1 [24]. Additionally, ICAM-1 expression was upregulated by PDTC in human endothelial cells via activation of the AP-1 pathway [25]. In vascular smooth muscle cells, PDTC induced G1 phase cell-cycle arrest, partially by activating p38 MAPK [26]. Since IL-1β-induced CXCL8 gene expression was also shown to be regulated by p38 MAPK via the CXCL8 promoter in Caco-2 cells [27], we aimed to elucidate the role of p38 MAPK in PDTC- and IL-1β-induced CXCL8 gene expression.

As expected, inhibition of p38 MAPK with the inhibitor SB205380 led to an inhibition of spontaneous and IL-1β-induced CXCL8 expression and secretion in Caco-2 cells. SB203580 inhibited PDTC-mediated enhancement of spontaneous and IL-1β-induced CXCL8 expression, suggesting that PDTC activates the p38 MAPK pathway and, consequently, CXCL8 gene expression.

These results might be explained by the presence of single AP-1 binding site in addition to the two NFκB binding sites in the *CXCL8* promoter [28]. Previously, it has been shown that IL-1β-mediated activation of p38 MAPK leads to AP-1 activation [29]. In addition to activation of transcription, post-transcriptional mechanisms contribute to the induction of CXCL8 gene expression [30]. Holtmann et al. have shown that the p38 MAP kinase pathway contributes to induction of CXCL8 synthesis by stabilising its mRNA [28]. Therefore, the activation of p38 MAPK by PDTC may lead to an enhancement of IL-1β-induced CXCL8 gene expression in Caco-2 cells by stabilising CXCL8 mRNA.

In HT29 cells, PDTC neither inhibited nor enhanced the IL-1β-induced CXCL8 expression and secretion, whereas SB203580 and PD98059 had inhibitory effects, suggesting

Fig. 4 Dose-dependent effects of pyrrolidine dithiocarbamate (PDTC) (**a**) and BAY11-7082 (**b**) on IL-1β-induced CXCL8 mRNA expression (*left panels*) and protein secretion (*right panels*) in Caco-2 cells. Caco-2 cells (5×10^5 per well) were plated in 6-well plates and grown for 24 h. Cells were then pre-treated either with 0.2, 2 or 20 μg of PDTC or 1, 10 and 100 μM BAY11-7082 per millilitre of culture medium for 1 h before stimulation with IL-1β (1 ng/ml). Cells were harvested after 1 h, and CXCL8 mRNA expression was measured by quantitative reverse transcription-polymerase chain reaction. For protein measurements, cell supernatants were collected after 24 h, and enzyme-linked immunosorbent serologic assays for CXCL8 protein were performed. Data shown represent the mean of four individual experiments± standard error of the mean performed in duplicate. *Single asterisk* corresponds to $p < 0.05$, and *double asterisks* correspond to $p < 0.005$ compared to IL-1β stimulation alone
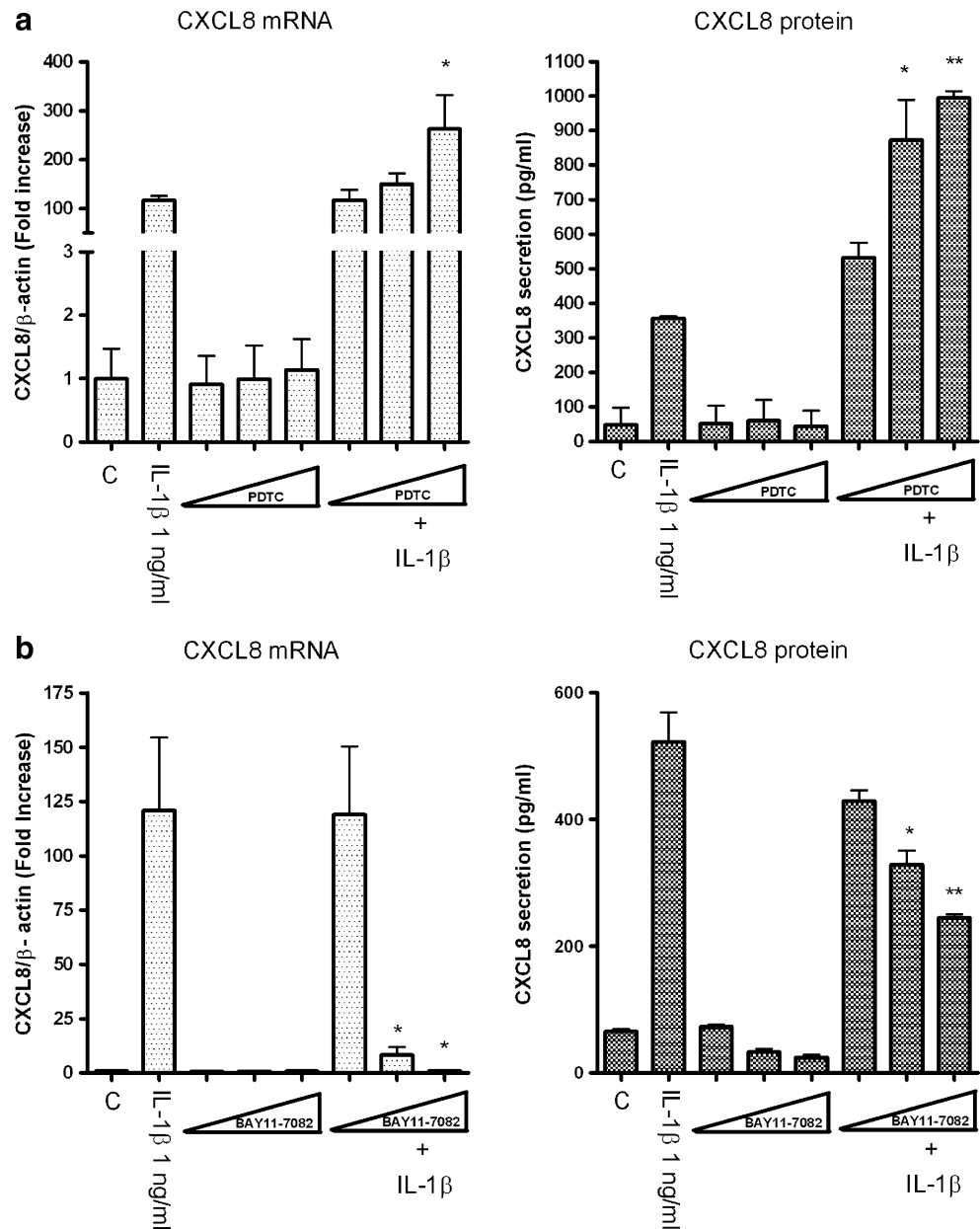
that the enhancing effect of PDTC on IL-1β-induced CXCL8 gene expression as cell-line dependent.

IL-1β is known to induce CXCL10 gene expression in Caco-2 cells through NFκB [7]. To find out if the enhancing effect of PDTC on IL-1β-induced CXCL8 gene expression was restricted to the CXCL8 gene, we investigated the role of PDTC on IL-1β-induced CXCL10 gene expression in Caco-2 and HT29 cells as well. Both real-time PCR and ELISA experiments revealed that PDTC inhibited IL-1β-induced CXCL10 gene expression in both Caco-2 and HT29 cells. The enhancing effect of PD98059 on IL-1β-induced CXCL10 gene expression in HT29 cells may be a result of the activation of other regulatory

pathways that stabilise CXCL10 mRNA. However, this effect was not seen at the level of protein synthesis. Inhibiting the p38 MAPK and MEK pathways resulted in the inhibition of IL-1β-induced CXCL10 gene expression in Caco-2 cells.

In conclusion, these data suggest that colonic epithelial cells are unresponsive to glucocorticoids. Among the tested compounds, only BAY11-7082 was able to downregulate CXCL8 and -10 in both cell lines. Although PDTC proved to inhibit NFκB-driven transcriptional activity, it led to an increase in CXCL8 expression in Caco-2 cells. This unexpected phenomenon is most likely due to activation of p38 MAPK, since it was sensitive to the MAPK inhibitor
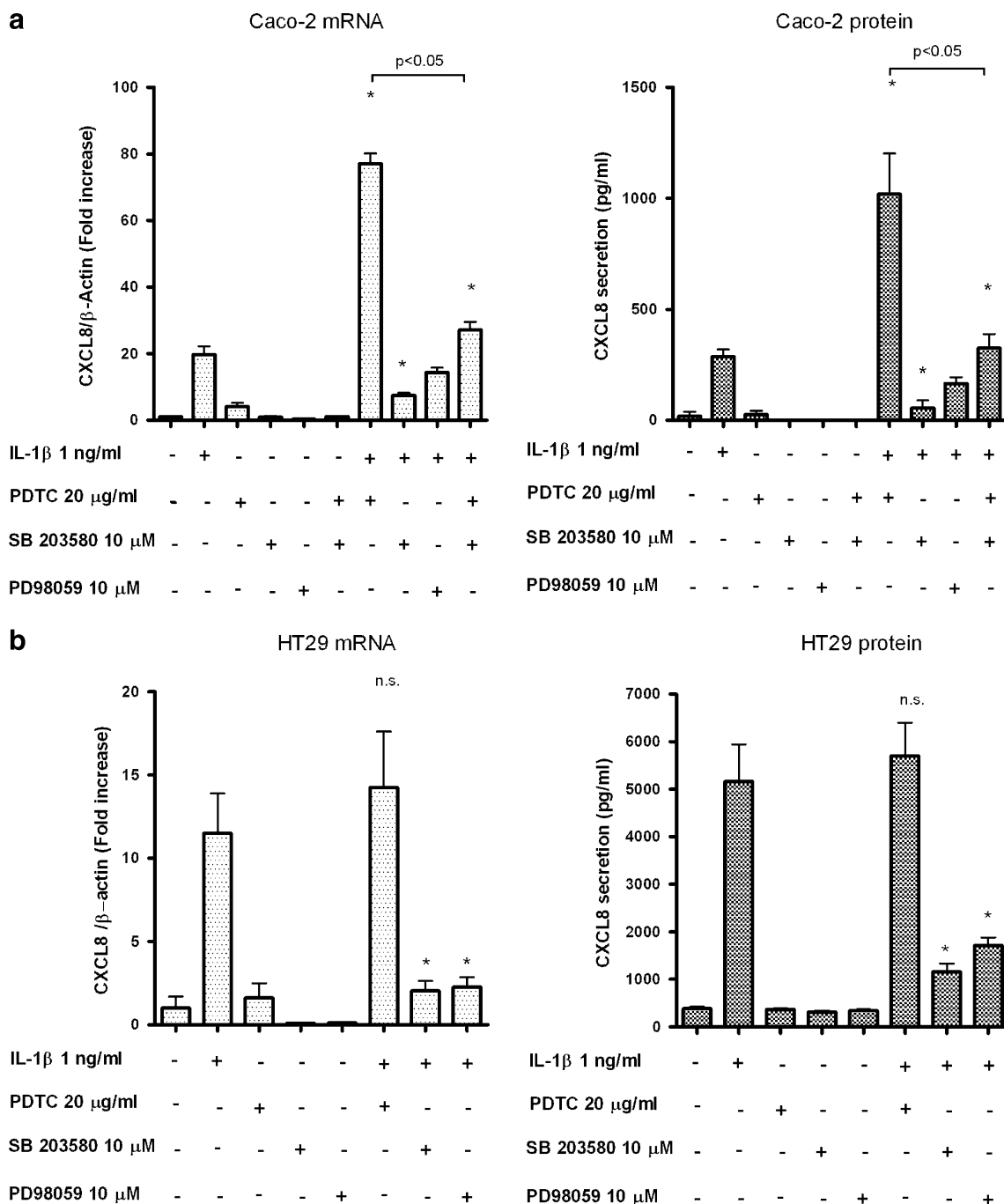
Fig. 5 Comparison of the effects of the NFκB inhibitor pyrrolidine dithiocarbamate (PDTC) and MAP kinase inhibitors SB203580 and PD98059 on IL-1β-mediated CXCL8 mRNA expression (*left panel*) and protein secretion (*right panel*) in Caco-2 (**a**) and HT29 (**b**) cells. Cells (5×10^5 per well) were plated in 6-well plates and grown for 24 h. Cells were then pre-treated with 20 μg/ml of PDTC, 10 μM of SB203580 or 10 μM of PD98059 for 1 h before stimulation with IL-1β for 4 h (for mRNA expression) or 24 h (for protein levels). CXCL8 mRNA expression was measured by quantitative reverse transcription-polymerase chain reaction. CXCL8 protein levels were measured from cell supernatants by enzyme-linked immunosorbent serologic assay. Data shown represent the mean of four individual experiments± standard error of the mean performed in duplicate. *Asterisk* corresponds to $p < 0.05$ compared to IL-1β stimulation, *ns* not significant

SB203580. Application of NFκB inhibitors may be a useful adjunct to steroid therapy in cases of suspected epithelial chemokine expression. However, NFκB-inhibiting compounds might activate alternative inflammatory pathways, as has been shown in the case of PDTC. Therefore, drug-screening strategies based purely on measuring NFκB activity should be regarded cautiously and should include testing for relevant inflammatory gene products like CXCL8.
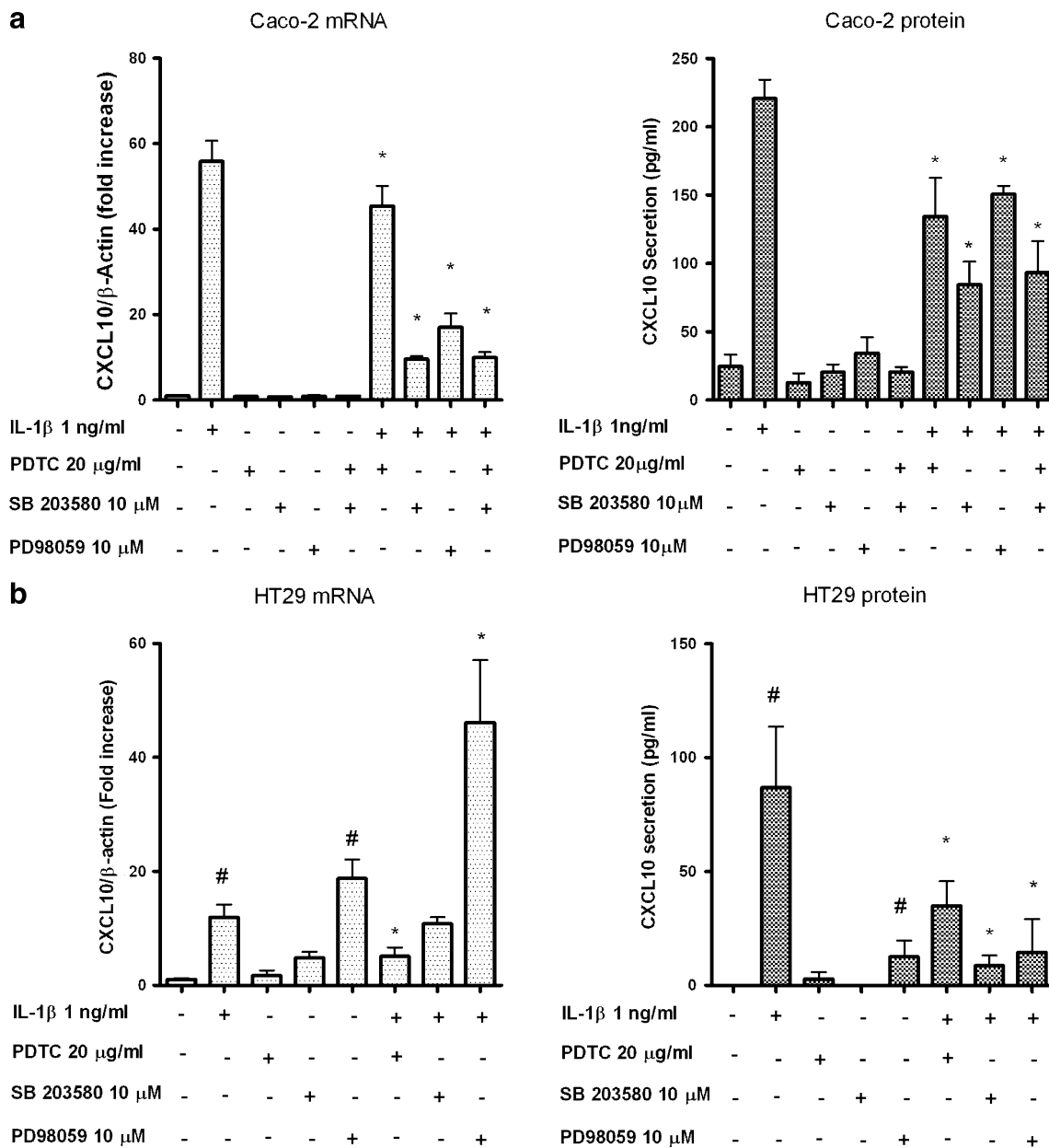
a

Caco-2 mRNA

Caco-2 protein

b

HT29 mRNA

HT29 protein

Fig. 6 Comparison of the effects of the NFκB inhibitor pyrrolidine dithiocarbamate and MAP kinase inhibitors SB203580 and PD98059 on IL-1β-mediated CXCL10 mRNA expression (*left panels*) and protein secretion (*right panels*) in Caco-2 (**a**) and HT29 (**b**) cells. Cell culture and stimulation conditions were as in Fig. 5. CXCL10 mRNA was measured by quantitative reverse transcription-polymerase chain reaction and CXCL10 protein by enzyme-linked immunosorbent serologic assay. Data shown represent the mean of four individual experiments±standard error of the mean performed in duplicate. *Asterisk* corresponds to *p<0.05* compared to IL1β alone, *number sign* corresponds to *p<0.05* compared to unstimulated controls

Recent studies in mouse conditional knockout models, in which NFκB signalling was ablated specifically in IECs, could show that a primary NFκB signalling defect in IECs disrupts immune homeostasis in the gastrointestinal tract, causing an inflammatory-bowel-disease-like phenotype [31], suggesting that a certain level of NFκB activation is necessary to keep epithelial barrier function. Remarkably partial disruption of NFκB signalling as achieved by

knocking out IKKβ, one important upstream activator of NFκB in macrophages resulted in a significant decrease in tumour size in a mouse model of colitis-associated cancer, probably by inhibition of paracrine growth factors. In the same model, IKKβ knockout in enterocytes, although not abolishing inflammation, prevented development of colonic cancer, via an increase in apoptosis [32]. Inhibition of NFκB may therefore be effective in prevention of colitis-

associated cancer. Nevertheless, keeping in mind the important role of NFκB in promoting intestinal barrier function and innate immune response care has to be taken employing NFκB inhibiting compounds in IBD therapy restricting its use to perpetuating inflammation with a state of NFκB overactivation.

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