ROLE OF INTESTINAL EPITHELIUM IN INFLAMMATORY BOWEL DISEASE: EFFECT OF CYTOKINES AND GLUCOCORTICOIDS ON CXCL8 AND CXCL10 GENE EXPRESSION AND NF-κB SIGNALLING IN INTESTINAL EPITHELIAL CELL LINES.

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ABBREVIATIONS

5-ASA	5-amino salicylic acid
AP-1	activator protein 1
APC	antigen presenting cells
ATF-2	activating transcription factor 2
ATP	adenosine tri phosphate
CARD	caspase recruitment domain
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
CD	Crohn's disease
COPD	chronic obstructive pulmonary disease
COX	cyclooxygenase
Dex	dexamethasone
DLR	dual luciferase reporter
EDTA	ethylene diamine tetraacetic acid
FCS	fetal calf serum
GC	glucocorticoid
GM-CSF	granulocyte macrophage colony stimulating factor
GR	glucocorticoid receptor
GRE	glucocorticoid receptor responsive element
hsp	heat shock protein
IBD	inflammatory bowel disease
IκB	inhibitor kappa B
ICAM-1	intercellular adhesion molecule-1
IEC	intestinal epithelial cell
IFNγ	interferon gamma
IKK	inhibitor kappa B kinase
IL-1α	interleukin 1 alpha
IL-1β	interleukin 1 beta
IL-2	interleukin 2
IL-6	interleukin 6
IL-10	interleukin 10

IL-8	interleukin 8
IL-1R	IL-1 receptor
iNOS	inhibitor of nitric oxide synthase
IP-10	interferon gamma regulated protein 10
IRAK	IL-1 receptor-associated kinase
IRF-1	interferon regulatory factor 1
LAR	luciferase assay reagent
LB	Luria- Bertani
LP	lamina propria
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MCP-1	monocyte-chemoattractant protein 1
MEK	mitogen activated protein kinase kinase
MHC	major histocompatability complex
NF-κB	nuclear factor kappa B
NO	nitric oxide
NOD-2	nucleotide-binding oligomerization domain containing 2
PBS	phosphate buffered saline
PDTC	pyrolidinedithiocarbamate
PMA	phorbol myristyl acetate
SEM	standard error of means
TGFβ	transforming growth factor beta
TNFα	tumor necrosis factor alpha
TNFR 2	tumor necrosis factor receptor 2
SARS	severe acute respiratory syndrome
STAT1	signal transducer and activator protein 1
UC	ulcerative colitis

1. SUMMARY

Background and Aims: Activation of intestinal epithelial cells (IEC) may occur either by luminal antigens stimulation or by inflammatory signals deriving from immune cells in the close vicinity. Production of chemokines by the intestinal epithelium contributes to mucosal infiltration of inflammatory cells in inflammatory bowel disease (IBD). Little is known about the intestinal epithelial expression and secretion of CXCL10 (IP-10) involved in recruiting T-cells and monocytes. Especially, the role of IL-1β, one of the most abundant cytokines in IBD mucosa, has not been established yet. We therefore aimed to evaluate the expression and regulation of CXCL8/Interleukin-8 (IL-8) and CXCL10/Interferon gamma regulated protein 10 kda (IP-10), two of the most abundant chemokines in inflammatory bowel disease (IBD), in epithelial cell lines stimulated with proinflammatory cytokines (IL-1β, IFN γ and TNF-α). NF-κB is known to play a major role in the pathophysiology of IBD. This makes NF-κB a potential phamocological target to treat IBD. So we aimed at studying the effects of two inhibitors of NF-κB on IL-1β induced CXCL8 and CXCL10 gene expression in IECs. Glucocorticoids are known to be effective in regulating the cytokine mediated chemokine gene expression in immune cells. These kinds of study were lacking in relation to IECs. So we also aimed at evaluating the role of glucocorticoids in cytokine mediated CXCL8 and CXCL10 gene expression in IECs.

Methods: Dose dependency and time kinetic analysis of CXCL8 and CXCL10 mRNA expression and protein secretion following cytokine stimulations were performed in Caco-2, HT-29 and DLD1 human colonic epithelial cell lines by real-time quantitative PCR, northern blotting and ELISA, respectively. To characterize the role of the transcription factor NF-κB in CXCL10 gene regulation, transient transfections with CXCL10 gene promoter constructs, gel shift and supershift assays were performed. The effects of two different NF-κB inhibitors (PDTC and BAY11-7082) on IL-1β induced CXCL8 gene expression in Caco-2 and HT29 were studied by transient transfections, real-time PCR and ELISA. Glucocorticoid mediated effects on cytokine induced CXCL8 and CXCL10 gene expression in Caco-2 and HT29 cell lines were studied by real-time PCR and ELISA.

Results: CXCL8 mRNA was quickly induced by IL-1β and TNFα within 40 minutes and reached to a maximum expression within 1 hour in Caco-2, HT29 and DLD1 cells. CXCL10 mRNA induction by IFNγ treatment followed a delayed characteristic with a maximum expression after 8 hours in Caco-2 cells, whereas in HT29 and DLD1 cells the expression reached its maximum after 24 hours. TNFα was capable of upregulating CXCL10 mRNA in the same timely manner with a lower effect than IFNγ in Caco-2 and HT29. Remarkably, IL-1β was the strongest and earliest inducer of CXCL10 mRNA in Caco-2. There was a strong synergistic effect of either TNFα or IL-1β with IFNγ both on CXCL10 mRNA and protein expression in all the cell lines. Experiments with a specific NFκB inhibitor and transfection experiments with a NFκB binding defective, CXCL10 gene promoter construct revealed that, CXCL10 gene induction by IL-1β and its synergism with IFNγ in Caco-2 cells is mediated through NF-κB. Unexpectedly PDTC, a non specific inhibitor of NF-κB, enhanced IL-1β induced CXCL8 gene expression in a cell specific manner. Glucocorticoids were ineffective in inhibiting cytokine mediated CXCL8 and CXCL10 gene expression in Caco-2 and HT29 cell lines.

Conclusions: Our data demonstrate that IL-1β is a strong and early inducer of CXCL8 gene expression and protein secretion in colonic epithelial cells. CXCL10 gene expression can be differentially regulated by proinflammatory cytokines and underline that the interplay between epithelial cells and immune cells is important in intestinal inflammation. Depending on the cellular context and utilizing the NF-κB pathway, IL-1β alone and/or in synergism with IFNγ, may play a major role in the induction of CXCL10 gene in IECs, suggesting IL-1β to be a promising target in treating IBD (e.g. in case of failing anti-TNFα strategies). Usage of pharmacological inhibitors to NF-κB should be given attention, as we show that the non specific inhibitor of NF-κB, PDTC, inhibits IL-1β induced NF-κB activity and enhances IL-1β induced CXCL8 gene expression in a cell specific manner, a phenomenon which might be explained by the activation of alternative proinflammatory pathways. Glucocorticoids, which are known to be anti-inflammatory in function, were ineffective in inhibiting cytokine mediated CXCL8 and CXCL10 gene expression in IECs, a phenomenon which might contribute to the known steroid unresponsiveness that occur in many patients with IBD.

2. Introduction

2.1. Etiology, prevalence and pathogenesis of Inflammatory Bowel Disease (IBD)

Among the few complex and enigmatic diseases, IBD offers many challenges to both clinicians and researchers. IBD exists in two major forms known as 1) Crohn's disease (CD) and 2) Ulcerative colitis (UC). Both diseases are clinically characterized mainly by diarrhea and abdominal pain in a varying degree, depending on the localization and intensity of intestinal inflammation. Histologically UC is characterized by continuous inflammation restricted to the mucosa, while CD is characterized by transmural inflammation. The highest incidence and prevalence rates of both forms of IBD have been reported from northern Europe, the United Kingdom and North America. In recent years the incidence of IBD in southern Europe, Asia and much of the developing world has been rising. As many as 1.4 million persons in the United States and 2.2 million persons in Europe suffer from this disease (Loftus, 2004). Different factors are responsible for the outcome of IBD, like environmental factors, familial and genetic factors, microbial agents, intestinal immune and non immune systems etc (Fiocchi, 1998; Katz et al., 1999) **(Fig 1)**. Most believe that intestinal bacterial flora is vital for persistence of the inflammatory process (Sartor, 2005). IBD is thought to result from improper and ongoing activation of the mucosal immune system. This could be due to the defects in both barrier function of intestinal epithelium and the mucosal immune system. In patients with IBD and in murine models significant progress has been made in characterizing immune-cell populations and inflammatory mediators responsible for this disease (Podolsky, 2002).

In 2001, seminal studies identified the gene encoding NOD2 (nucleotide-binding oligomerization domain containing 2), a gene susceptible for Crohn's disease, as being important in white but not Asian patients (Hugot et al., 2001; Ogura et al., 2001). NOD2 is also known as CARD15 (Caspase Recruitment Domain 15) and was mapped on to chromosome 16q. NOD2/CARD15 is expressed by many cells including intestinal epithelial cells and is known to function as an antibacterial factor in human intestinal epithelial cells (Cario, 2005; Hisamatsu et al., 2003). Of the 60 NOD2/CARD15 allelic variants identified, three variants R675W, G881R and

3020insC and approximately 17 other polymorphisms appear to be disease-associated in CD (Lesage et al., 2002). Mutations in the NOD2/CARD15 can contribute to the pathogenesis of CD in various ways. These mutations can lead to hyporesponsiveness (act as a dominant negative) or hyperresponsiveness (act as a dominant active) to bacteria, finally resulting in the chronic inflammation of intestine (Abreu, 2003).

Regardless of how exactly immune-cell populations and inflammatory mediators ultimately promote chronic gut inflammation, it is becoming increasingly evident that the immune system plays a crucial role in disease pathogenesis. Because the inflammation is localized primarily in the intestinal tract in IBD, researchers have focused on the intestinal lumen as the site for antigenic trigger. One of the important conceptual advances in IBD research has been the proposal that failure to regulate normally protective cell-mediated immune responses in the intestinal and colonic mucosa results in sustained activation of the mucosal immune system and the uncontrolled overproduction of proinflammatory cytokines and mediators (Laroux. et al., 2001).
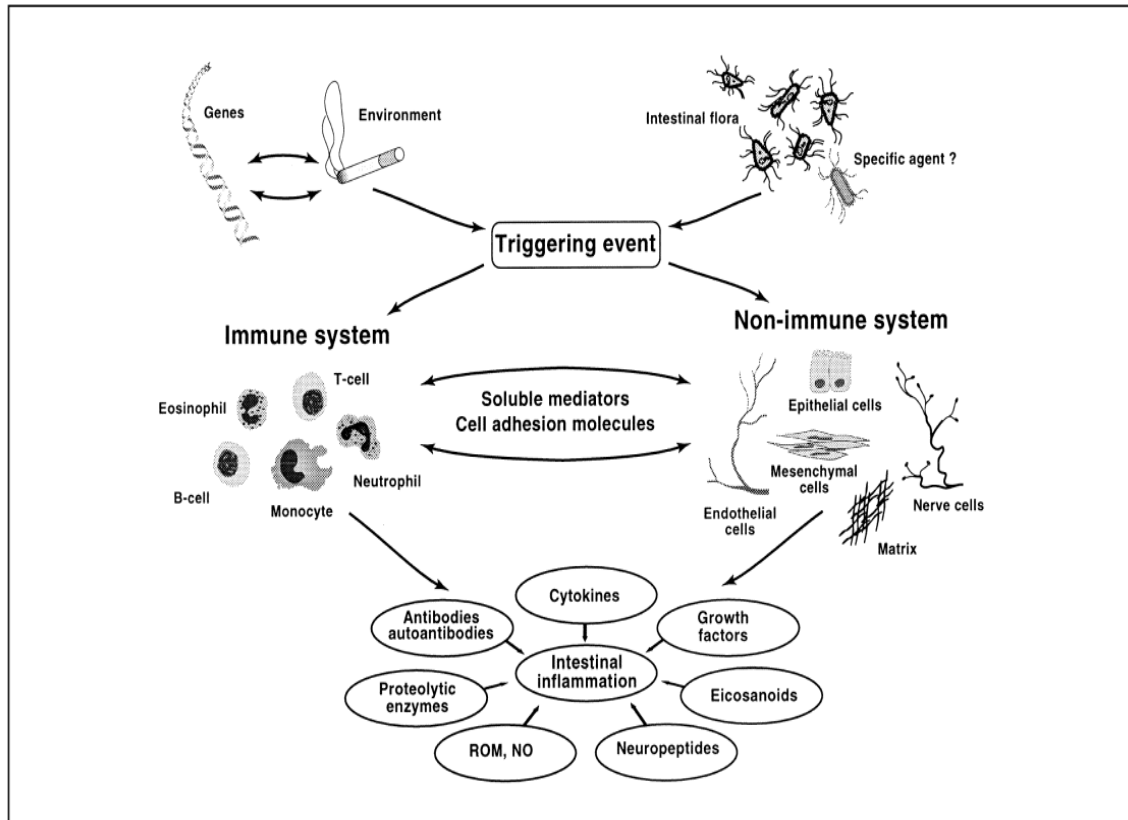
Figure 1: Possible causes for the pathogenesis of inflammatory bowel disease

The combination of environmental and genetic factors in coordination with normal enteric or pathogenic microbes leads to the activation of intestinal immune and non immune systems. Both systems reciprocate through the release of soluble mediators and expression of cell adhesion molecules. This results in the multiplication of intestinal inflammation and tissue damage. Adapted from (Katz et al., 1999)

2.1.1. Cytokines in IBD

Cytokines play a major role in the modulation of the immune system. They are rapidly synthesized and secreted by inflammatory cells upon stimulation and in turn induce the production of adhesion molecules and other inflammatory mediators (Rogler and Andus, 1998). The role of cytokines in the mucosal immune system has been studied intensively (Elson et al., 1995; Sartor, 1994) and were found to influence the nature of mucosal immune responses. Ample evidences were found for a disturbed balance between proinflammatory and antiinflammatory mediators in IBD. Expression levels if proinflammatory cytokines tend to be constitutively elevated in IBD. Reports of experimental colitis in IL-2 and IL-10 knockout mice suggest an active participation of proinflammatory cytokines in IBD (Davidson et al., 1996; McDonald et al., 1997) .

High concentrations of IL-1β found both in CD and UC intestine (Mahida et al., 1989), were largely ascribed to local mononuclear cells (Youngman et al., 1993). Increased production of IL-1β was also seen in other forms of gut inflammation (Gionchetti et al., 1994) and above all it is also present in normal mucosa (Raddatz et al., 2005). In contrast to IL-1β, TNFα serum protein and mRNA content have been variable. Even though there are controversial opinions about TNFα elevation in IBD patients (Akazawa et al., 2002; Stevens et al., 1992; Su et al., 2002), this cytokine has been extensively targeted for clinical investigations to block its activity as a novel form of therapy for CD. The mechanism mediating the beneficial effects of a chimeric anti-TNFα antibody is still unclear (Targan et al., 1997). Circulating levels of IL-6, a major acute phase reactant, are distinctively higher in patients with active CD but not in patients with UC (Mahida et al., 1991). Mucosal mRNA levels of IFNγ are significantly higher in patients with active UC than in controls and patients with inactive CD (Sawa et al., 2003). In active Crohn's disease, IFNα and especially IFNγ have been found to be elevated in the intestinal mucosa (Breese et al., 1993; Fais et al., 1991), however without increased concentrations in the circulation (Nielsen et al., 1988).

2.1.2. Chemokines in IBD

Chemokines, a set of low-molecular weight cytokines (7-10 kDa), directing migration and activation of leukocytes, play a major role in the perpetuation of inflammatory processes (MacDermott, 1999). They are not only involved in inflammatory process but also in wound repair, organ development and tumor metastasis etc **(Fig 2)**. The final composition of leukocytes present in the inflammed intestine is most likely due to both, secreted chemokines and the relative expression of chemokine cell surface receptors on different immune cell types. There are more than 50 chemokines today that are classified into four categories, CXC, CC, C and CX3C chemokines (Bacon et al., 2002). Cytokines are distinguished from chemokines based on the anility of latter to bind to G protein coupled serpentine receptors (Bacon et al., 2002). Chemokines are also classified into constitutively secreted and inducible ones (Laing and Secombes, 2004). Homeostatic chemokines regulating basal leukocyte trafficking and the organization of the lymphoid tissue are constitutive chemokines, whereas inflammatory molecules responsible for mediating the recruitment of leukocyte effector population to the sites of immune reaction and tissue injury are termed as inducible chemokines (Laing and Secombes, 2004).

Several chemokines have been investigated for their role in the patogenesis of both CD and UC and their expression is consistently increased during the active phase of the disease (MacDermott et al., 1998; Papadakis and Targan, 2000). Micro-array analysis with biopsies from CD and UC patients showed a clear upregulation of CXC chemokines and their receptors (Puleston et al., 2005). CXCL8 and CXCL10 genes, two of the main CXC chemokines, upregulation may occur in acute inflammation in response to pathogens, but they are also known to be over-expressed in IBD patients (Banks et al., 2003; Uguccioni et al., 1999).

Interleukin-8 (IL-8/CXCL 8)

CXCL8 commonly known as interleukin 8, is a CXC chemokine which consists of 72 amino acids in its mature form and is identified as a basic and heparin binding protein (Matsushima et al., 1988). It binds to CXCR1 and CXCR2 receptors which are present on different cells. CXCL8 protein is secreted by a variety of cells, including intestinal epithelial cells (Fusunyan et al., 1998; Kim et al., 2001). At the site of inflammation it is supposed to participate in the recruitment and transmigration of

neutrophils (Struyf et al., 2005). Besides chemoattracting the neutrophils, CXCL8 also induces neutrophils to release lysosomal enzymes, to change the cell shape and adhesion to endothelial cells (Hoch et al., 1996). Several studies confirmed the role of CXCL8 gene in association with various acute and chronic inflammatory conditions like Psoriasis, rheumatoid arthritis, gout, severe trauma, cardiopulmonary bypass and reperfusion lung injury etc (Finn et al., 1996; Hoch et al., 1996; Rodenburg et al., 1999). CXCL8 has also been shown to play an important but non specific role in the pathogenesis of IBD and its mRNA was shown to be restricted to areas with histological signs of inflammatory activity and mucosal destruction (Banks et al., 2003; Mazzucchelli et al., 1994; Uguccioni et al., 1999). Studies in human monocytes and lymphocytes revealed a differential regulation of CXCL8 gene expression by exogenous TNFα and IL-1β (Chaly et al., 2000). Studies in intestinal epithelial cells with bacterial infection indicated an activation of CXCL8 gene through NF-κB (Elewaut et al., 1999). The actual mechanisms of CXCL8 mRNA and protein production and the effect of different cytokines on the expression and secretion of CXCL8 in IECs are still unclear.

Interferon-gamma regulated protein 10 kDa (IP-10/CXCL10)

CXCL10 is another CXC chemokine which binds to the CXCR3 chemokine receptor, expressed mainly by activated CD4$^+$ memory T cells that produce a T helper cell 1 (Th 1) pattern of cytokines (Loetscher et al., 1996; Qin et al., 1998). It is also known for its anti-angiogenic properties in cancers (Proost et al., 2001). In animal models of tumor rejection, CXCL10 gene was found to elicit a potent thymus-dependent antitumor response *in vivo* (Luster and Leder, 1993). In endothelial cells CXCL10 is known to inhibit proliferation and induce apoptosis (Feldman et al., 2006). CXCL10 gene expression is known to be upregulated in diseases like SARS (Law et al., 2005) and may also contribute to the recruitment of specific subpopulations of T cells (Th1 type) from the blood stream into the synovial joints in rheumatoid arthritis patients (Hanaoka et al., 2003). In chronic obstructive pulmonary disease (COPD) patients, the expression levels of CXCL10 are increased and correlate with the degree of infiltration by CD8$^+$ T cells that produce IFNγ (Saetta et al., 2002). In the intestine of patients with inflammatory bowel disease, CXCL10 is known to be highly expressed. CXCL10 is produced by a variety of cells like neutrophils (Gasperini et al., 1999),

eosinophils when stimulated with IFNγ (Dajotoy et al., 2004), activated human bronchial epithelial cells (Sauty et al., 1999) and cultutred human keratinocytes (Boorsma et al., 1998) etc. Recently, it was also shown that intestinal epithelial cells either constitutively expressed CXCL10 mRNA or when stimulated with IFNγ (Dwinell et al., 2001). While CXCL10 gene regulation was studied in other cell models (Majumder et al., 1998), its regulation in IECs is still unclear (Dwinell et al., 2001; Yang et al., 1997).
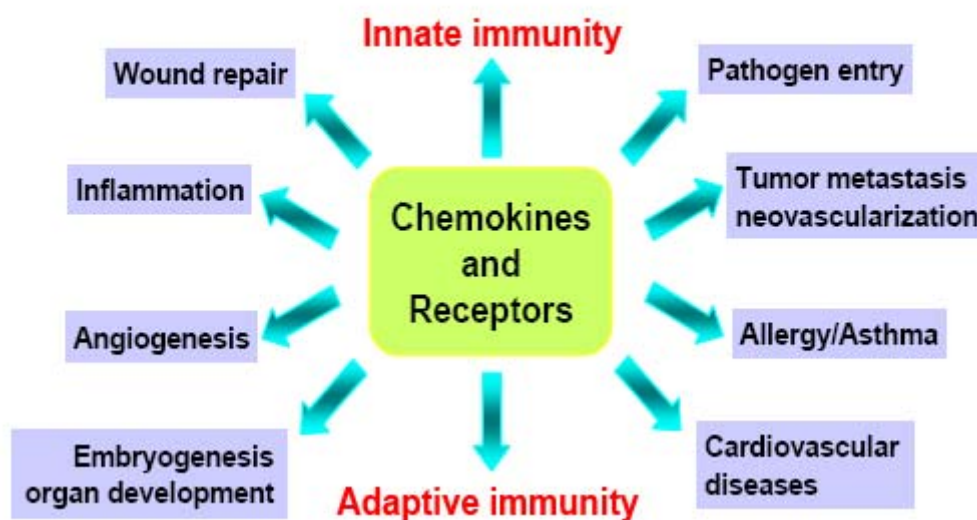
Figure 2: The role of chemokines and chemokine receptors in the pathophysiologic conditions

Adapted from (Le et al., 2004)

2.1.3. Role of intestinal epithelial cells in mucosal inflammation in IBD

In addition to its many homeostatic functions such as barrier protection, mucus production and reabsorption, the intestinal epithelium also plays an important role in regulating local inflammation and immune responses, through an interactive process with components of the immune system and also those which act directly to inhibit colonization of bacteria along the exposed surface (Pitman and Blumberg, 2000) **(Fig 3)**.

The intestinal mucosal immune system is an important location where decisions regarding host responses to enteric antigens are made. So, the mucosal immune compartment must be able to discriminate between harmless antigens derived from nutrients or bacterial flora on the one hand and harmful antigens on the other hand (tolerance vs clearance). Therefore, the induction and maintenance of mucosal tolerance is crucial to avoid inappropriate immune responses in the gut (Nagler-Anderson, 2001). This decision making process is a complex interplay between several cell types like T-cells, B cells, dendritic cells etc., present in the mucosa (Laroux et al., 2001) **(Fig 4)**.

Studies on bacterial-enterocyte interactions revealed that CXCL8 is one of the several host factors that are released by epithelial cells along with IL-1β, IL-6, TNFα, GM-CSF and several C-X-C and C-C chemokines (Eckmann et al., 1993; Yang et al., 1997). This mixture of cytokines and chemokines ensures that circulating immune cells are not only chemoattracted into epithelial/mucosal sites from the circulation but are activated and sustained on reaching the site of infection. IECs depending on their origin and maturity may have a different and distinct epithelial cell chemokine/cytokine responses (Yang et al., 1997). Proinflammatory cytokines IL-1β, TNFα and IL-6 are also part of the secretory response of epithelial cells following contact with pathogenic bacteria, but they are released at lower effective concentrations than chemokines. The importance of such a secretion is two fold. Primarily, these cytokines activate local macrophages, which participate in host protection against infection. Secondarily, these cytokines can themselves induce epithelial cells to produce chemokines, which will result in a multiplier effect of the initial stimulus. Epithelial cells do not only produce cytokines that affect other cells

but also respond to cytokines. One important additional way by which epithelial cells respond to their cytokine mileu involves the important cytokine IFNγ. Lymphocytes present in the close environment of epithelial cells produce IFNγ (Carol et al., 1998; Ebert, 1990; Lundqvist et al., 1996), which in turn acts on epithelial cells to increase expression of MHC class I and II antigens and also ICAM-1 and other adhesion molecules on the apical and basal surfaces of epithelial cells (Eckmann et al., 2000; Huang et al., 1996; Parkos et al., 1996). These mediators attract further inflammatory cells.
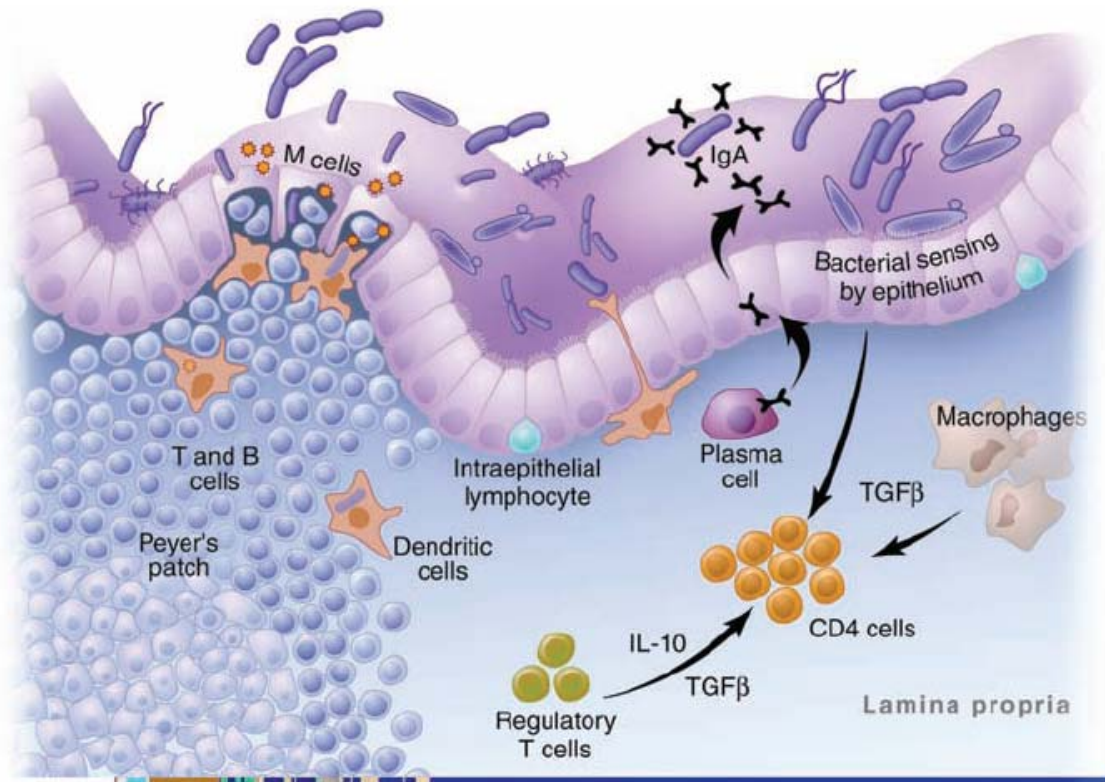
Figure 3: The Gut epithelial barrier

In addition to its many homeostatic functions the gut maintains an extensive and highly active immune system. The epithelium overlying contains specialized M cells that constantly transport gut bacteria and antigens from the gut lumen into the lymphoid tissue. Dendritic cells in the lamina propria (LP) reach through epithelial cells and also sample gut bacteria, there by activating $CD8^+$ T cells and $CD4^+$ T cells which results in intestinal inflammation. Then potentially tissue-damaging T cell responses can be inhibited by the antiinflammatory mediators released by T regulatory cells.

Adapted from (Macdonald and Monteleone, 2005)
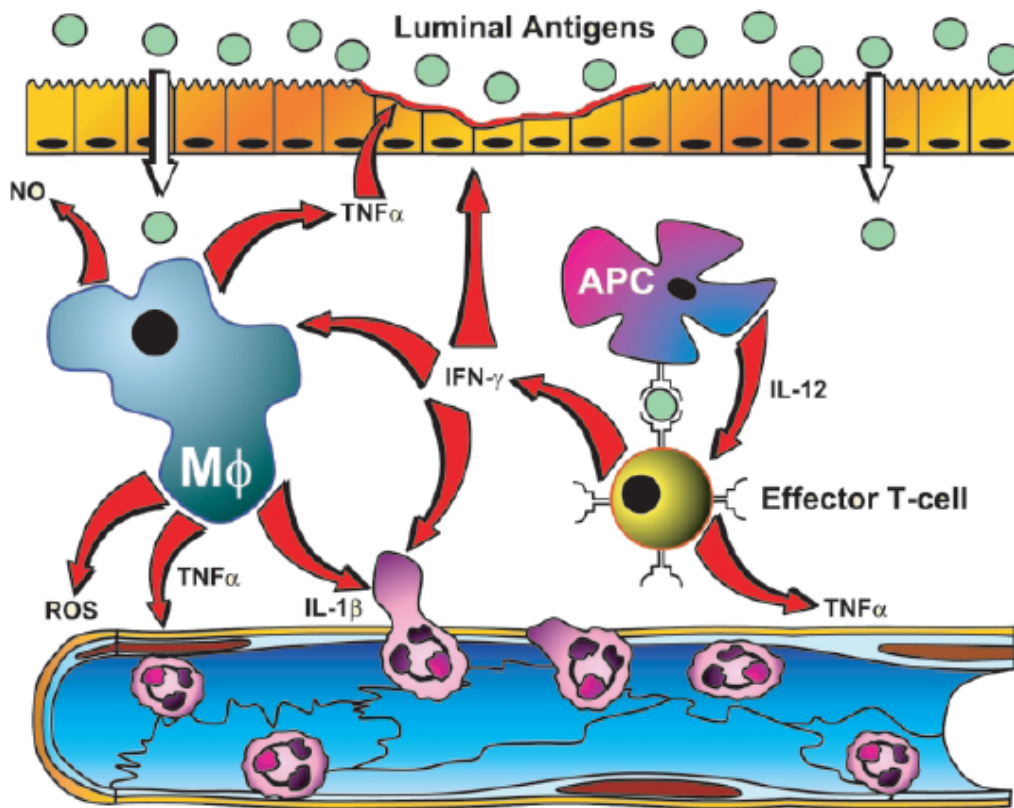
Figure 4: Intestinal immune response to enteric antigens

In response to luminal antigens effector that are processed and presented by antigen presenting cells, effector $CD4^+$ T cells produce T helper-1 (Th1)-type cytokines. These cytokines may act on the gut epithelium directly and/or activate a resident macrophage (Mϕ) to release large amounts of proinflammatory mediators, cytokines as well as reactive oxygen species (ROS) and nitric oxide (NO), that finally result in the recruitment of additional leukocytes, multiplication of inflammation and subsequent tissue injury. Adapted from (Laroux et al., 2001)

2.1.4. Role of NF-κB in IBD

Nuclear factor κB (NF-κB) designates 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 (Baldwin, 1996; Grilli et al., 1993). NF-κB is comprised of subunits that can include cRel, RelA, RelB, p50 and p52 (Baeuerle and Henkel, 1994; Barnes and Karin, 1997). NF-κB exists as a heterodimer comprising RelA (p65) and NF-κB1 (p50) subunits in most of the cells, which is the most potent gene transactivator among the NF-κB family (Ruben et al., 1992) and is the major NF-κB protein found in the nucleus of cytokine stimulated IECs (Jobin et al., 1997). These p65 containing complexes which bind with high affinity to the consensus DNA sequences 5'-GGGPuNNPyPyCC-3' (p65/p50) or 5'- GGGPuNPyPyCC-3'(p65/c-Rel) leads to the activation of transcription (Parry and Mackman, 1994; Schmitz et al., 1995).

NF-κB is activated by a variety of agents like cytokines, growth factors, T cell mitogens, oxidative stress, bacteria, viruses and their products (Baldwin, 1996; Grilli et al., 1993). On activation by different stimuli, NF-κB transcriptionally regulates many cellular genes involved in early immune, acute phase and inflammatory responses, including IL-1β, TNF-α, IL-6, CXCL8, iNOS, COX-2 etc (Baldwin, 1996; Grilli et al., 1993) **(Fig 5)**. Of note IL-1β and TNFα are two main inducers of NF-κB. IL-1β or TNFα binds to its respective receptor and elicits a cascade of transductional signals that converge on NIK. Further activation of downstream signaling molecules takes place, which ultimately results in the activation of NF-κB **(Fig 6)**. This illustrates that the inducers and gene products of NF-κB activation are highly relevant to intestinal inflammation (Fiocchi, 1998; Sartor, 1997). In the past few years, there has been increasing interest in how cytokines, bacteria and bacterial polymers induce IEC gene expression. In IECs gene expression must be tightly regulated to avoid over reaction to normal microbial flora while at the same time remain responsive to harmful pathogens **(Fig 7)**. Intestinal mucosa is poised of active cell populations in continuous change from a proliferative and undifferentiated stage in the basal parts of crypts to mature surface villus epithelial cells (Que and Gores, 1996; Wong et al., 1999). Several studies showed that maturation of the cells from crypt bases to villus

surface involves substantial changes of cellular morphology, growth, proliferation and expression of biochemical markers (Louvard et al., 1992), however little is known about the alteration of immunological functions as IECs mature.

Differentiated HT-29 cells (cells with surface like character), when treated with IL-1β showed a lower IKK and NF-κB activity when compared to undifferentiated HT29 (crypt like cells) (Bocker et al., 2000). In differentiated IECs, bacterial invasion was reduced when compared to undifferentiated IECs (Coconnier et al., 1994). Studies in IECs stimulated with *Bacteroides fragilis* enterotoxin indicated that NF-κB can be a central regulator of chemokine gene expression and may be an important regulator of neutrophil migration (Kim et al., 2002). These studies indicate that a gradient of NF-κB activation is established along the crypt surface axis in response to stimulation by pathogens and proinflammatory cytokines.

NF-κB is involved in the transcriptional activation of different genes involved in mucosal inflammation. In addition, activation of NF-κB in IEC has been demonstrated *in vivo* (Rogler et al., 1998). In patients with ulcerative colitis and Crohn's disease, increased NF-κB activity was found in IECs along with macrophages (Andresen et al., 2005; Jobin et al., 1997; Rogler et al., 1998). Activation of NF-κB in patients with active inflammatory bowel disease suggests that regulation of NF-κB activity is a very attractive target for therapeutic intervention and is known to be targeted by different agents **(Fig 8)**.
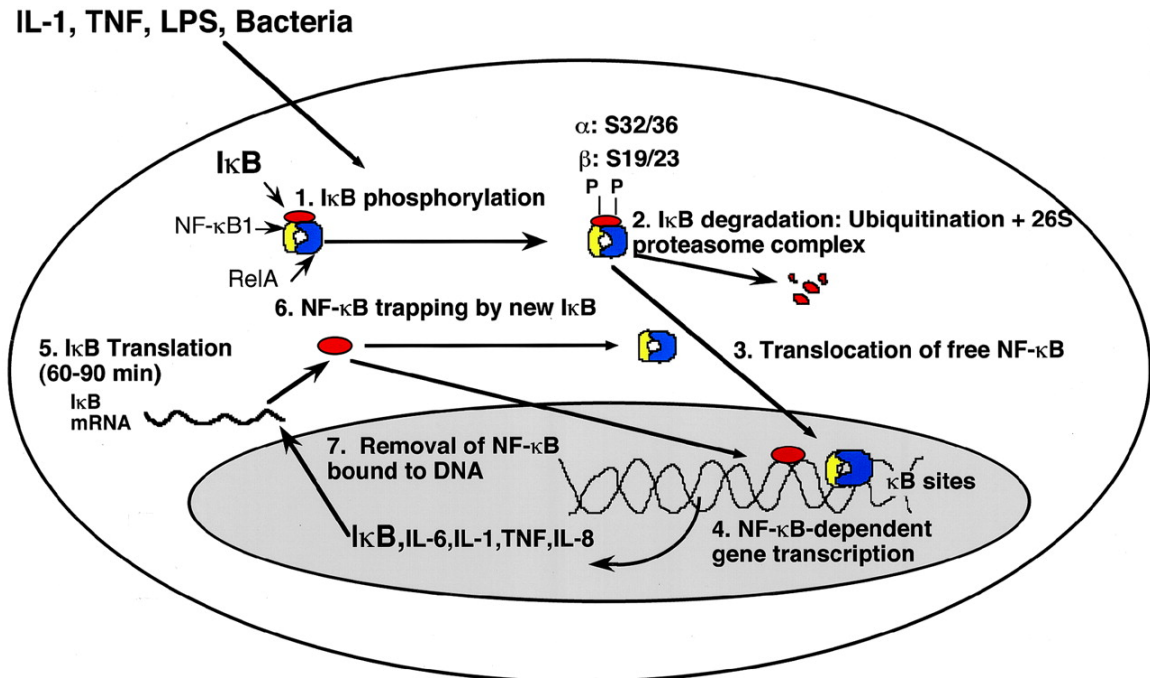
Figure 5: Mechanism of NF-κB activation and regulation by different stimuli

In normal conditions NF-κB is bound by the inhibitor protein IκB, which keeps it inactive. Appropriate Selective IκB phosphorylation by an appropriate stimuli, will result in the ubiquitination and targeted degradation of IκB by the proteasome pathway. NF-κB which is free then migrates to the nucleus by virtue of its nuclear localization signal and induces the transcription of its target genes that contains κB elements in their promoters. NF-κB is then inactivated by newly synthesized IκB both in the cytoplasm and in the nucleus. Adapted from (Jobin and Sartor, 2000)
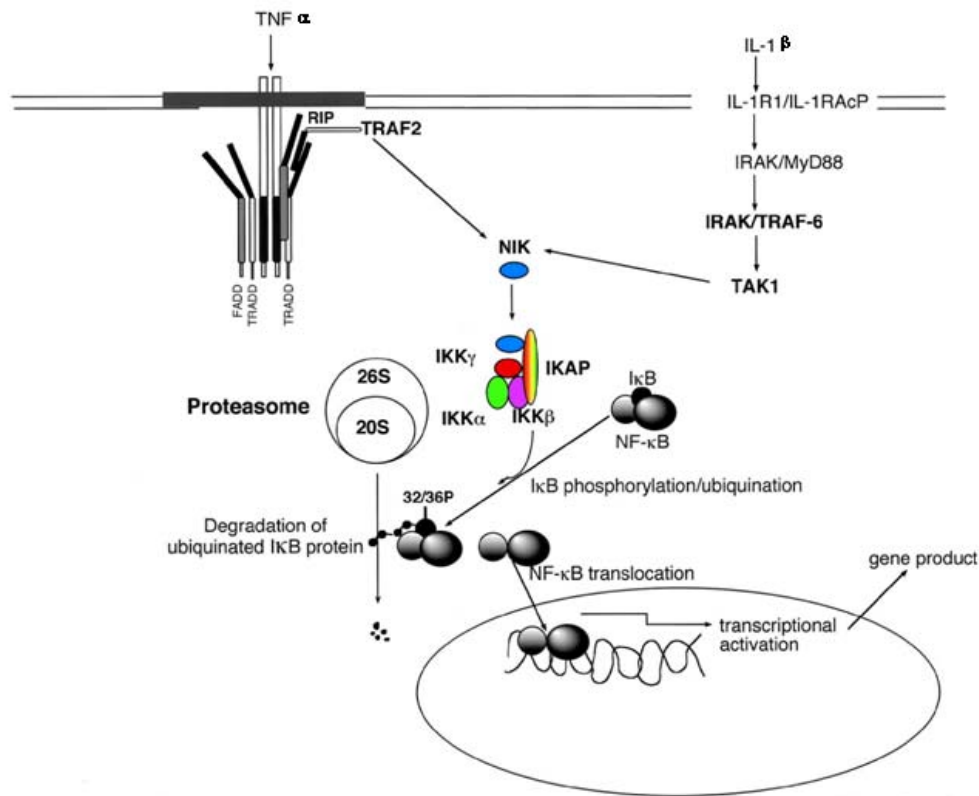
Figure 6: IL-1β and TNFα signaling mechanism through NF-κB

Upon binding of IL-1β or TNFα to their respective receptors results in the activation of a cascade of transductional signals that converge on nuclear factor-inducing kinase (NIK), which associates with the IKK complex via the action of IKAP, leading to phosphorylation of the IKKα and IKKβ. Activated IKK then phosphorylates IκB, which triggers the ubiquitination/degradation cascade and NF-κB release. Adapted from (Jobin and Sartor, 2000)

Figure 7: Regulation of IκB /NF-κB system in normal and pathogenic conditions of intestinal epithelial cells

A: In normal luminal environmenr IκB/NF-κB system in intestinal epithelial cells is relatively unresponsive to nonpathogenic bacteria or low concentrations of bacterial cell wall polymers. This low responsiveness may be a determinant of mucosal homeostasis that prevents IECs overreaction to ubiquitous luminal products. *B*: IECs when exposed to pathogenic bacteria, high concentrations of bacterial cell wall polymers and/or cytokines IκB/NF-κB system will be activated, which leads to the production of various inflammatory mediators that in turn recruit and activate inflammatory and immune effector cells. Secretion of proinflammatory cytokines by these recruited cells leads to continued activation of IEC. Adapted from (Jobin and Sartor, 2000)
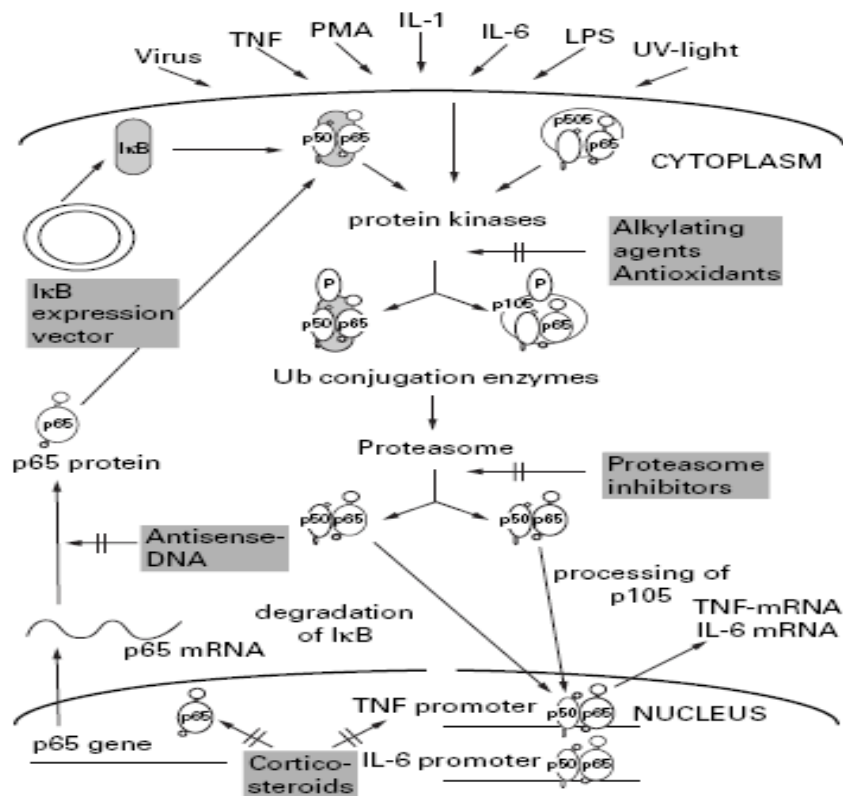
Figure 8: Targeting of the NF-κB activation pathway in intestinal inflammation

NF-κB pathway can be targeted by different agents. Of these, alkylating agents and antioxidants block protein kinases, whereas the translation of p65 can be inhibited by antisense DNA. Antiinflammatory steroids like glucocorticoids block p65 in binding to its target gene promoters. In addition, adenoviral expression vectors can deliver genes whose products can inactivate NF-κB. Adapted from (Neurath et al., 1998)

2.2. Treating IBD

2.2.1. The role of glucocorticoids (GCs)

Although the etiology of IBD is not fully understood, there has been remarkable progress in the understanding of this field in recent years. A number of drugs have been used to treat IBD, of which glucocorticoids have been the main stay of therapy. One of the major functions of GCs is to suppress the immune system by inhibiting the expression of numerous proinflammatory genes (Besedovsky et al., 1986). However, 20% of IBD patients are either resistant to the therapeutic effects of GCs or dependent on GCs (Munkholm et al., 1994). The molecular basis for the glucocorticoid resistance in other inflammatory diseases like asthma and rheumatoid arthritis has been widely assessed but the pathophysiology of glucocorticoid resistance in IBD hasn't been well studied.

The action of GCs is normally mediated through intracellular glucocorticoid receptor (GR) binding. GR belongs to the steroid family of a larger nuclear receptor family of ligand-dependent transcription factors. In normal conditions (in the absence of GC), GR is bound by an 'aporeceptor' complex that includes Hsp90, Hsp70, immunophilins and p23 and made transcriptionally inactive (Pratt et al., 2004). GRs upon activation with GCs will be translocated into the nucleus where they bind either to glucocorticoid response elements (GRE). GREs that are involved in positive gene regulation are designated as +GREs and that are involved in negative gene regulateion are called (n)GREs (Drouin et al., 1993; Lieberman and Nordeen, 1997) and intiates the target gene transcription or repression **(Fig 9)**. Once activated by GC, GR may also interact with other transcription factors like NF-κB (Nissen and Yamamoto, 2000), which is shown to be activated in macrophages and epithelial cells in biopsy specimens taken from IBD patients (Rogler et al., 1998). However, the repression of NF-κB by GR is not a universal phenomenon but depends on the cell type and state of differentiation. For instance, in rat mesangial cells the synthetic GC, dexamethasone, doesn't inhibit NF-κB activation (Auwardt et al., 1998).

There are very few studies done on the effect of GC upon intestinal epithelium. GC is known to stabilize epithelial function (Urayama et al., 1998) and restore sodium absorption in chronically inflammed epithelium (Sundaram et al., 1999). In Caco-2 cells, which has been shown to have the characteristics of normal small intestinal epithelium (Jumarie and Malo, 1991), GC is known to inhibit IL-1β mediated monocyte chemoattractive protein (Reinecker et al., 1995) and IFNγ induced nitric oxide synthesis (Chavez et al., 1999).

Several studies done in different aspects of IBD have given a little insight into the causes of GC resistance. GR and NF-κB are known to repress each other but studies done with steroid sensitive and resistant patients (Bantel et al., 2000b) have shown that NF-κB activation was found mainly in macrophages and endothelial cells in sensitive patients. Whereas 60% of steroid-resistant patients, had a staining pattern with active NF-κB predominantly localized in epithelial cells. In epithelial cells like Caco-2 and IEC-6, its has been shown that IL-1β not only counteracts GC mediated NF-κB repression but also inhibits GC driven transactivation (Raddatz et al., 2001).

2.2.2. 5-Aminosalicylic Acid (5-ASA)

5-ASA derivatives are suitable as initial monotherapy in mild to moderate CD and UC (Carter et al., 2004). 5-ASA may act by inhibition of both *in vivo* and *in vitro* activation of peripheral and intestinal lymphocytes, scavenging reactive oxygen metabolites and perhaps inhibiting the activation of the NF-κB transcription factor (Bantel et al., 2000a; Nikolaus et al., 2000). Longterm treatment with 5-ASA is effective in sustaining remission of ulcerative colitis but not in Crohn's disease.

2.2.3 Anti-TNF therapy

The anti-TNF agent infliximab has shown significant progress in therapy for patients with CD and UC (van Dullemen et al., 1995). Its efficacy suggests that TNFα, a product of activated macrophages, may have a pivotal role among the many regulatory peptides with altered expression in association with IBD (Lugering et al., 2001; Ten Hove et al., 2002). Two pivotal trials demonstrated the efficacy of infliximab in patients with Crohn's disease (Ricart and Sandborn, 1999; Targan et al., 1997). However its mechanism of action is incompletely understood. Infliximab's

therapy has its own disadvantages like patients may not remain responsive to infliximab therapy indefinitely and some patients who resumed treatment after a prolonged hiatus had a serum-sickness. Above all the therapy is quite costly.

2.2.4. Immunosuppressive and Immunoregulatory Agents

There is an increasing evidence for the usage of immunomodulatory drugs in treating patients with IBD. These drugs are used alongside with corticosteroids. These drugs include for example agents like azathioprine, methotrexate, cyclosporine etc. Methotrexate is effective in the treatment of corticoid-dependent active Crohn's disease and in maintaining remission (Feagan et al., 2000; Feagan et al., 1995). Cyclosporine is effective in the treatment of severe ulcerative colitis. Azathioprine is useful in maintaining long-term remission induced by intravenous cyclosporine in steroid-refractory severe ulcerative colitis (Fernandez-Banares et al., 1996). All these agents put patients at risk for opportunistic infections (Kashimura et al., 1998; Lichtiger et al., 1994).
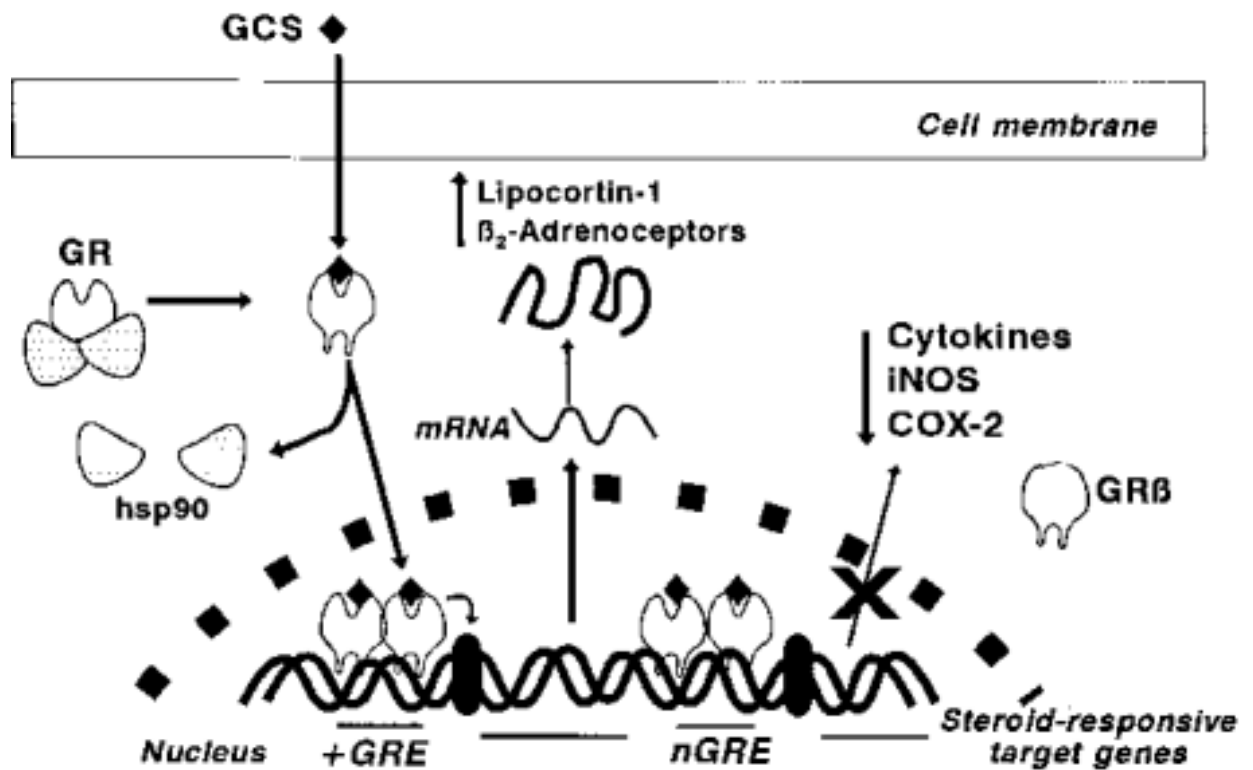
Figure 9: Classical model of glucocorticoid action

The glucocorticoid enters the cell and binds to a cytoplasmic glucocorticoid receptor (GR) that is complexed with two molecules of a 90 kDa heat shock protein (hsp90). GR translocates to the nucleus where, as a dimer, it binds to a glucocorticoid responsive elements (GRE) on the 5´-upstream promoter sequence of glucocorticoid-responsive genes. GREs may increase transcription and negative (n)GREs may decrease transcription, resulting in increased or decreased mRNA and protein synthesis. Adapted from (Barnes, 1998)

3. Aims of the Study

Little is known about the intestinal epithelial expression and secretion of CXCL10 (IP-10) involved in recruiting T-cells and monocytes. Especially, the role of IL-1β, one of the most abundant cytokines in IBD mucosa has not been established yet. Eventhough several studies were done on CXCL8 gene regulation, time kinetic regulation of CXCL8 gene in IECs by cytokines was not elucidated completely so far. We hypothesized that inflammatory cells present in the gut lumen may release certain proinflammatory cytokines like IL-1β, IFNγ and TNFα which in turn acts on IECs to secrete CXCL8 and CXCL10 via NF-κB. These chemokines in turn attract neutrophils and activated T cells into the gut lumen, thereby multiplying the inflammatory effects and subsequent tissue injury.

In the present study we aimed to evaluate

1) The expression and regulation of CXCL8/Interleukin-8 (IL-8) and CXCL10/Interferon-inducible protein 10 kda (IP-10), two of the most abundant chemokines in inflammatory bowel disease (IBD), in epithelial cell lines stimulated with proinflammatory cytokines (IL-1β, IFN γ and TNFα).

2) The role of NF-κB in cytokine mediated CXCL8 and CXCL10 gene expression in IECs.

3) The role of glucocorticoids on cytokine mediated CXCL8 and CXCL10 gene expression in IECs.
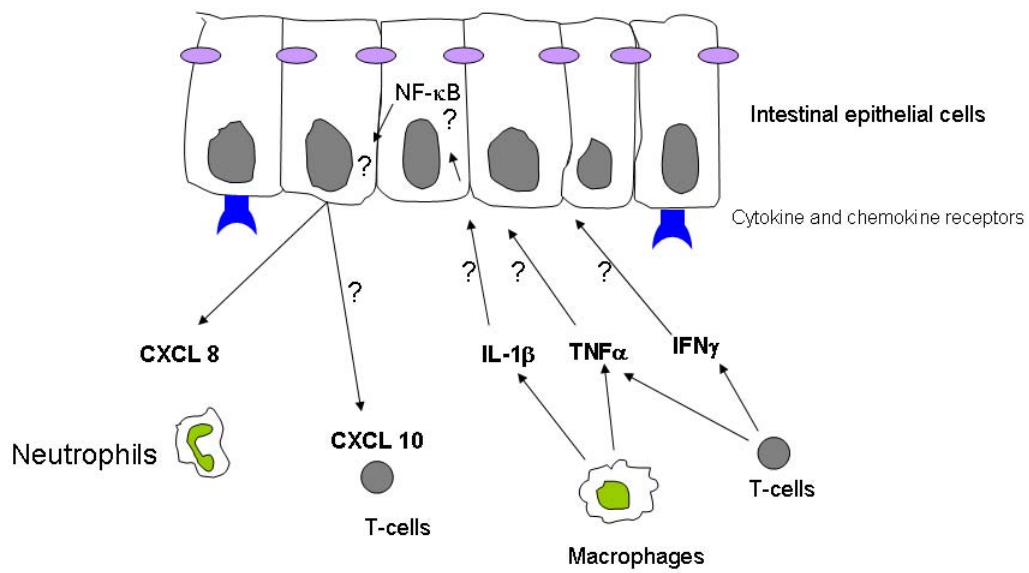
Figure 10: A hypothetical model of interaction between IECs and immune cells mediated by proinflammatory cytokines, CXCL8 and CXCL10 chemokines in the intestine

4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Laboratory devices

Centrifuge Hettich Rotixa/K (Hettich Zentrifugen)

Centrifuge Sigma 3K30 (Sigma-Aldrich Chemie GmbH, Deisenhofen)

Centrifuge Sigma 5415D (Sigma-Aldrich Chemie GmbH, Deisenhofen)

Curix 60 film processor (Agfa-Geavert)

Environmental incubator shaker (New Brunswick Scientific Co. Inc. Edison)

GelCam digital control (Phase, Luebeck)

GeneQuant RNA/DNA Calculator (Pharmacia, Freiburg)

Heraeus Laminair ®   (Heraeus Instruments GmbH, Hanau)

Heraeus Thermostat (Heraeus Instruments GmbH, Hanau)

Horizontal Gel Electrophoresis Apparatus HORIZON® 11·14 (GIBCO BRL, Grand Island)

Laboratory microscope Axiovert 25 Zeiss (Zeiss, Oberkochen)

Microplate Reader MRX Dynatech (Dynatech Technologies GmbH, Denkendorf)

Minishaker MS1 (Rettberg, Goettingen)

Power supply PowerPac 300 (Bio-Rad, Hercules)

Power supply PowerPac 200 (Bio-Rad, Hercules)

Precisious scale Sartorius (Sartorius AG, Goettingen)

Ready Gel Cell (Bio-Rad, Hercules)

Shaking platform Heidolph Polimax 2040 (Heidolph)

Stratalinker™ 180 system (Stratagene, La Jolla)

Thermomixer comfort (Eppendorf-Netheler-Hinz GmbH, Hamburg)

Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules)

Transilluminator 2020E (Stratagene, La Jolla)

Wallac 1409 liquid scintillation counter (Wallac distribution GmbH, Freiburg)

4.1.2. Tools

6 well plates	(Nunc, Rushilde)
24 well plates	(Nunc, Rushilde)
75 cm culture flasks	(Nunc, Rushilde)
Curix film cassette	(Eastman Kodak Company, Rochester)
Eppendorf tubes	(Eppendorf-Netheler-HinzGmbH, Hamburg)
Nitrocellulose membrane	(Novex, Frankfurt)
Pharmacia NICK Column	(Pharmacia Biotech)
QIAshredder	(QIAGEN GmbH, Hilden)
Tissue culture dishes (60/15 mm, Cellstar)	(Greiner GmbH, Frickenhausen)
X-OMAT autoradiography film	(Eastman Kodak Company, Rochester)

4.1.3. Cells

Caco-2, HT29 and DLD1, human colon adenocarcinoma cell lines were from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig. IEC-6 a rat normal small intestinal cell line was from American Type Culture Collection, Rockville.

4.1.4. Chemicals

1, 4-dithiothreitol /DTT	(GIBCO BRL, Grand Island)
2'-deoxynucleoside5'-triphosphates/dNTPMix	(Roche Molecular Biochemicals, Mannheim)
2-mercaptoethanol	(Merck KGaA, Darmstadt)
2-propanol	(Merck KGaA, Darmstadt)
[α-^{32}P]-labelled deoxy-cytidine-triphosphate (specific activity 3,000 Ci/mmol)	(Amersham Biosciences, Freiburg)
Acrylamide	(Sigma-Aldrich, Deisenhofen)
Agarose	(GIBCO BRL, Grand Island)
Albumin / from bovine serum, fatty acid free/	(Roche Molecular Biochemicals, Mannheim)
Albumin /from bovine serum/	(Bio-Rad, Hercules)
Aminoacetic acid /glycin/	(Sigma-Aldrich, Deisenhofen)
Ammonium persulfate	(Sigma-Aldrich, Deisenhofen)
Ampicillin	(Sigma-Aldrich, Deisenhofen)

Bacto-agar	(DIFCO, Detroit)
Bacto-tryptone	(DIFCO, Detroit)
Bacto-yeast extract	(DIFCO, Detroit)
Bis-acrylamide	(Sigma-Aldrich, Deisenhofen)
Dexamethasone	(Sigma-Aldrich, Deisenhofen)
Dimethyl Sulphoxide /DMSO/	(Sigma-Aldrich, Deisenhofen)
Di-Sodium hydrogen phosphate /Na2HPO4/	(Merck KGaA, Darmstadt)
Dulbecco's minimal essential medium /DMEM/	(BioWhittaker, Verniers)
Eagle's minimal essential medium /EMEM/	(BioWhittaker, Verniers)
Ethanol absolut	(Merck KGaA, Darmstadt)
Ethidium bromide	(Sigma-Aldrich, Deisenhofen)
Ethylenediaminetetraacetic acid /EDTA/	(Sigma-Aldrich, Deisenhofen)
Ethyleneglycol-bis(β-aminoethylether)N,N,N',N'-tetraacetic acid /EGTA/	(Sigma-Aldrich, Deisenhofen)
Fetal calf serum	(GIBCO BRL Grand Island)
Fish sperm DNA	(Roche Molecular Biochemicals, Mannheim)
FuGENE TM 6 Transfection Reagent	(Roche Molecular Biochemicals, Mannheim)
Gelatine (from bovine skin)	(Sigma-Aldrich, Deisenhofen)
Glucose	(Sigma-Aldrich, Deisenhofen)
Glycerin	(Sigma-Aldrich, Deisenhofen)
Glycerol	(Merck KGaA, Darmstadt)
Hydrochloric acid /HCl	(Merck KGaA, Darmstadt)
Insulin /from porcine pancreas	(Sigma-Aldrich, Deisenhofen)
Interferon γ/human	(Roche Molecular Biochemicals, Mannheim)
Interleukin-1β /human	(Roche Molecular Biochemicals, Mannheim)
Magnesium sulphate /MgSO4	(Merck KGaA, Darmstadt)
Methanol	(Merck KGaA, Darmstadt)
Moloney Murine Leukemia Virus Reverse Transcriptase /M-MLV-RT/	(Roche Molecular Biochemicals, Mannheim)
N-2 (hydroxyethyl) piperazine-N'-	

(2-ethanesulfonic acid)/HEPES/	(Sigma-Aldrich, Deisenhofen)
Non essential amino acids	(BioWhittaker, Verniers)
Nonidet NP-40	(Sigma-Aldrich, Deisenhofen)
PD 98059	(Calbiochem, San Diego)
Penicillin/Streptomycin	(GIBCO BRL Grand Island)
Phenylmethansulfonyl fluoride /PMSF/	(Sigma-Aldrich, Deisenhofen)
Potassium chloride /KCl/	(Merck KGaA, Darmstadt)
Potassium dihydrogen phosphate /KH2PO4/	(Merck KGaA, Darmstadt)
Primer for cDNA Synthesis /p(dT)15/	(Roche Molecular Biochemicals, Mannheim)
Pyrolidinedithiocarbamate/PDTC	(Sigma-Aldrich, Deisenhofen)
RPMI-1640	(BioWhittaker, Verniers)
SB 205380 (p38MAPK inhibitor)	(Calbiochem, San Diego)
Scintillation fluid	(Hirschmann, Eberstadt)
Sodium acetate	(Sigma-Aldrich, Deisenhofen)
Sodium citrate	(Sigma-Aldrich, Deisenhofen)
Sodium chloride (NaCl)	(Merck KGaA, Darmstadt)
Sodium dodecyl sulphate (SDS)	(Sigma-Aldrich, Deisenhofen)
Sodium hydroxide solution (NaOH)	(Merck KGaA, Darmstadt)
Sodium pyruvate	(GIBCO BRL Grand Island)
Tumor Necrosis Factor α(human)	(Roche Molecular Biochemicals, Mannheim)
TEMED/N,N,N',N',-Tetramethyl ethylenediamine	(Sigma-Aldrich, Deisenhofen)
Trypsin/EDTA	(BioWhittaker, Verniers)
QuikHyb® Hybridization Solution	(Stratagene, Heidelberg)

4.1.5. Solutions

1.5 % agarose gel:

        agarose 1.5 % (w/v)

        ethidium bromide 0.4 µg/ml

        ----

        in TBE buffer

Extraction Buffer for protein isolation:

        HEPES pH 7.9 20 mM

        KCl 10 mM

        EDTA 0.1 mM

        DTT 1 mM

        PMSF 0.5 mM

        ----

        in distilled water

Extraction buffer with NaCl for protein isolation:

        HEPES pH 7.9 20 mM

        NaCl 0.4 M

        EDTA 0.1 mM

        DTT 1 mM

        PMSF 0.5 mM

        In distilled water

Luria-Bertani agar plates:

        bacto-agar 1.5 % (w/v)

        ampicillin 50 µg/ml

        ----

        in LB medium

Luria-Bertani /LB/ medium:

        bacto-tryptone 1.0 % (w/v)

        bacto-yeast extract 0.5 % (w/v)

        NaCl 1.0 % (w/v)

        ----

        pH 7.0

        in distilled water

Phosphate buffered saline /PBS/ solution:

        NaCl 0.13 M

        KCl 2.68 mM

        Na_2HPO_4 10.2 mM

        KH_2PO_4 1.5 Mm

        ----

        in distilled water

SOC medium:

        bacto-tryptone 2.0 %

        bacto-yeast extract 0.5 %

        NaCl 10 mM

        KCl 2.5 mM

        $MgSO_4$ 10 mM

        glucose 20 mM

        ----

        in distilled water

TBE-buffer (10X):

        Tris base 0.1 M

        Boric acid 0.1 M

        EDTA pH 8 1 mM

        ----

        in distilled water

10x running buffer for RNA gel :

MOPS	0.2 M
Sodium acetate	0.05M
EDTA	0.01M

Set pH 7. Prepared in RNase-free water, sterile filtered and stored in dark at 4°C.

Sample buffer for RNA :

Deionized formamide	500 µl
37% formaldehyde	169 µl
10x running buffer	100 µl

The solution was dispensed into 1 ml aliquots and stored at –20°C for 2-3 months.

Deionized formamide:

Formamide	100 ml
Mixed Bed Resin AG 501-X8 (D)	10 gms

The mixture was stirred for 30 min in the dark at RT, afterwards it was sterile filtered, dispensed into 50 ml aliquots and stored in the dark at –20°C.

Loading buffer:

10x running buffer	5 ml
Ficoll 400	1.5 gm
Bromophenol blue	10mg

In distilled water and stored for 2-3 months at –20°C.

20X SSC:

$$NaCl \qquad 350.6 \text{ gms}$$

Sodium citrate.$2H_2O$ 176.4 gms

In 2 litres of distilled water, autoclaved and stores at RT

10x TE:

Tris-HCl, pH 7.4 0.1M

EDTA   10mM

Set pH 7.6. Prepared in distilled water, sterile filtered and stored at RT.

4.1.6. Kits

Dual-Luciferase [®] Reporter Assay system   (Promega, Madison)

EndoFree Plasmid Maxi Kit   (QUIAGEN GmbH, Hilden)

RNeasy Mini Kit   (QUIAGEN GmbH, Hilden)

SEAP Reporter Gene Assay Kit   (Roche Molecular Biochemicals, Mannheim)

4.1.7. Primers for real-time PCR

Human β-Actin (Invitrogen GmbH, Karlsruhe)

Forward 5'-CTG GCA CCC AGC ACA ATG-3'

Reverse 5'-CCG ATC CAC ACG GAG TAC TTG-3'

Human CXCL8 (Invitrogen GmbH, Karlsruhe)

Forward 5'-ATG ACT TCC AAG CTG GCC G-3'

Reverse 5'-GCT GCA GAA ATC AGG AAG GC-3'

Human CXCL10 (Invitrogen GmbH, Karlsruhe)

Forward 5'-CCA GAA TCG AAG GCC ATC AA-3'

Reverse 5' -CAT TTC CTT GCT AAC TGC TTT CAG-3'

Human TNFR 2 (Invitrogen GmbH, Karlsruhe)

Forward 5'-AGG CAG GCC ACC ATA TTC AGT-3'

Reverse 5' -TGG CTT TGT CGT TGG CTT G-3'

Rat β-Actin (MWG-BIOTECH GmbH, Edersberg)

Forward 5'-ACC ACC ATG TAC CCA GGC ATT-3'

Reverse 5'-CCA CAC AGA GTA CTT GCG CTC A-3'

Rat CINC-1 (MWG-BIOTECH GmbH, Edersberg)

Forward 5'-CCC CCA TGG TTC AGA AGA TTG-3'

Reverse 5'-TTG TCA GAA GCC AGC GTT CAC-3'

4.1.8. Oligo nucleotides for Gel Shift Assay

NF-kB Oligo (Promega, Madison)

 5'-AGT TGA GGG GAC TTT CCC AGG C-3'

 3'-TCA ACT CCC CTG AAA GGG TCC G-5'

4.1.9. Anti body for Supershift Assay

Anti-p65 antibody (SC-8008X)   (Santa Cruz Biotechnology, Santa Cruz, CA)

4.1.10. Plasmid vectors

pBluescript® SK +/- phagemid Vector   (Stratagene, La Jolla, CA)

pGRE-SEAP Vector   (CLONTECH Laboratories, Heidelberg)

pNF-κB-SEAP Vector   (CLONTECH Laboratories, Heidelberg)

pRShGRα Vector (Hollenberg, 1985)   (American Type Culture Collection, Rockville)

pTGL-IP10 vector   (Majumder et al., 1998)

pTGL-IP10 κB2 Mut vector   (Majumder et al., 1998)

pUHC13-3 IL-8 vector   (Holtmann et al., 1999)

pRL-TK vector   (Promega, Madison)

4. 2. Methods

4.2.1. Cell culture conditions and Stimulation

Caco-2 cells were grown in EMEM medium containing 20% FCS supplemented with 100 U/ml each penicillin and streptomycin and 1% non essential amino acids at 37°C and 5% CO_2. HT29 and DLD1 cells were grown in RPMI medium containing 10% FCS and 100 U/ml penicillin and streptomycin at 37°C and 5% CO_2. IEC-6 cells were cultured in combination of Dulbecco`s MEM and RPMI 1640 (1:1) containing 10 % FCS, 1 % sodium pyruvate, 100 U/ml penicillin and 100 U/ml streptomycin at 37°C under an atmosphere of 5 % CO2. 10^{-8} M insulin was freshly added to the cells every day.

For cytokine stimulation, intestinal epithelial cells were plated into 6-well plates at a density of 5×10^5 cells per well and grown till they reached 70-80% confluence. These cells were then stimulated with IL-1β (0.1ng/ml, 0.5ng/ml, 1 ng/ml, 2ng/ml, 10ng/ml and 50ng/ml), TNFα (2ng/ml, 10ng/ml and 50ng/ml) and IFNγ (2ng/ml, 5ng/ml, 10ng/ml and 50ng/ml) based on the type of experiments.

4.2.2. Transformation of E. coli

Transformation of competent bacteria was performed by the TA cloning kit according to manufacturer's protocol. 2 µl of 0.5 M β-mercaptoethanol was pipetted into a 50 µl vial of melted One Shot INVαF′ competent cells and mixed by stirring gently with the pipette tip. 500 ng of plasmid DNA was added directly to the competent cells, mixed then incubated on ice for 30 minutes. Heat shock was performed for 30 seconds in 42°C water bath, then the cells were placed on ice for 2 minutes. 450 µl of SOC medium was added into the cells at room temperature and the vial was shaken horizontally at 37°C for one hour at 225 rpm in a rotary-shaking incubator. Thereafter the vial with transformed bacteria was placed on ice. 100 µl of transformed bacteria was spread on LB agar plate containing 50 µg/ml of ampicillin. The agar plate was incubated at 37°C for 18 hours.

4.2.3. Purification of plasmid DNA

Purification of plasmid DNA from transformed bacteria was performed with EndoFree Plasmid Maxi Kit according to manufacturer's protocol. A single colony of transformed bacteria was picked from the streaked LB agar plate and a 2 ml starter culture of ampicillin containing LB medium was inoculated. The bacterial culture was incubated for 12 hours at 37°C with vigorous shaking at 300 rpm. Thereafter the starter culture was diluted to 1:1000 and bacteria were grown at 37°C for 16 hours with shaking at 300 rpm.

The bacterial cells were harvested by centrifugation at 6000 g for 15 minutes at 4°C. The medium was removed and the pellet was resuspended in 10 ml buffer P1. The cells were lysed by adding 10 ml buffer P2 and the lysate was incubated at room temperature for 5 minutes. Genomic DNA, proteins and cell debris was precipitated by 10 ml chilled buffer P3 and filtered by QIAfilter Cartridge. The filtered lysate was first incubated on ice for 30 minutes after adding 2.5 ml buffer ER and then applied to the anion-exchange resin containing QIAGEN-tip followed by two washes with 30 ml buffer QC. Plasmid DNA was eluted with 15 ml buffer QN and precipitated by adding 10.5 ml room-temperature isopropanol. The mixture was centrifuged immediately at 15000 g for 30 minutes at 4°C. After centrifugation the DNA pellet was washed with 70 % ethanol, pelleted at 15000 g for 10 minutes. Thereafter the supernatant was disturbed and the pellet was air-dried. Finally DNA was redissolved in 200-500 µl buffer TE and its concentration was determined by spectrophotometry measuring the absorbance at 260 and 280 nm.

4.2.4. Transient transfection of Caco-2, HT29 and DLD1 cells

Caco-2, HT29 and DLD1 cells were plated in 24 well plates, 24 hours before transfection, at a density of 50,000/well in 1 ml medium. After 24 hours the cells were transfected using the non liposomal formulation FuGENE. On the day of transfection fresh medium was added. FuGENE was added to the plasmid DNA at a ratio of 3µl/µg DNA. FuGENE was prediluted in 100µl serum free medium and added drop wise to the concentrated plasmid DNA. Then the FUGENE and plasmid DNA mixture in the serum free medium was incubated for 15-25 minutes at room temperature, then pipetted into the wells containing 900µl medium.

In transactivation experiments, 200 ng of respective reporter gene plasmids per each well was added to the cells based on the kind of experiments done. 24 hours post transfection, cells were treated with the respective stimulants (IL-1β, TNFα and IFNγ etc). In all the studies control cells were treated with appropriate amount of vehicle (PBS or ethanol).

4.2.5. Luciferase Reorter Gene Assay

Luciferase assays were performed by using promega DLR^{TM} (Dual-Luciferase® Assay System). Firefly and renilla luciferases, because of their distinct evolutionary origins, have dissimilar enzyme structures and substrate requirements. These differences make it possible to selectively discriminate between their respective bioluminescent reactions. Thus, using the DLR^{TM} assay systems, the luminescence from the firefly luciferase reaction may be quenched while simultaneously activating the luminescent reaction of renilla luciferase.

The luciferase assay activity was determined according to protocol of DLR^{TM} assay systems Kit. After the stimulation time, growth medium from cultured cells was removed and rinsed with 1X PBS twice. Then the remaining PBS was completely removed and 100μl of 1X passive lysis buffer was added into culture vessel. Then the plates were gently rocked for 15 minutes at room temperature and the lysate was transferred into an eppendorf tube and stored at -70°C or used immediately for further assay. 20μl of passive buffer lysate was mixed with 100μl of LARII in a 96 well plate and firefly luciferase activity was measured in a luminometer. Then 100μl of Stop and Glo® reagent was added to the wells and renilla luciferase activity was measured again in the luminometer. Values of firefly luciferase were normalized with renilla luciferase values.

4.2.6. SEAP Reporter Gene Assay

The quantification of alkaline phosphatase secreted by transfected cells is based on the phosphorylation of CSPD [3-(4-metoxyspiro [1, 2-dioxetane-3, 2' (5'-chloro)-tricyclo (3.3.1.1) decane]-4-yl) phenyl-phosphate] by alkaline phosphatase. The resulting unstable dioxetane anion decomposes and emits light with its maximum activity at 477 nm. The alkaline phosphatase activity was determined according to the manufaturer's protocol. 50μl aliquot of culture supernatant was centrifuged to pellet

any debris then diluted 1:4 with dilution buffer followed by incubation for 30 minutes at 65°C to heat-inactivate the endogenous alkaline phosphatase activity. Thereafter samples were pelleted again, then 100µl of supernatant was transferred to a tube and the same amount of inactivation buffer was added. After 5 minutes incubation 100µl of substrate reagent was pipetted into the tube. The sample was rocked gently for 10 minutes. The chemiluminescent signal was counted in a liquid scintillation counter.

4.2.7. RNA isolation

Caco-2, HT29 and DLD1 cells were cultured in 6 well tissue culture plates at a density of 500000/well in 2 ml medium. 24 hours after seeding cells, they were incubated with different concentrations of IL-1β, TNFα, IFNγ and /or Dexamethasone etc.

Total RNA was isolated by using Qiagen RNeasy mini kit. The cells were first washed with 3 ml PBS then lysed in 350µl highly denaturing guanidinium isothiocyanate-containing buffer (Buffer RLT). RNases were inactivated by adding β-mercaptoethanol to the buffer previously (10µl/1ml Buffer RLT). The lysate was pipetted directly onto a QIAshredder column sitting in the 2 ml collection tube and centrifuged for 2 minutes at maximum speed to homogenize. 350µl of 70% ethanol was added to the homogenized lysate to provide appropriate binding conditions and mixed well by pipetting. The lysate was applied to an RNeasy spin column and centrifuged for 1 minute at 10000 g. The membrane-bound RNA was washed first by pipetting 700µl washing buffer (Buffer RW1) then by adding 500µl ethanol containing buffer RPE onto the column and centrifuging for 1 minute at 10000 g. The RNeasy membrane was dried by 500µl buffer RPE centrifuging for 2 minutes at maximum speed and the column was transferred to a 1.5 ml Eppendorf tube. 30µl RNase-free water was added to the membrane and the RNA was eluted by centrifuging for 1 minute at 10000 g. The total RNA was stored at –80°C. The concentration of total RNA was determined by spectrophotometry measuring the absorbance at 260 and 280 nm. (An absorbance of 1 unit at 260 nm corresponds to 40 µg RNA per ml. This relationship is valid for measurements of water-diluted RNA). RNA was subsequently used for real-time PCR and Northern blot analysis.

4.2.8. Real-Time PCR

1μg of total RNA was reverse transcribed to complementary DNA (cDNA) in a final volume of 40μl containing 400U M-MLV reverse transcriptase, 50mM Tris HCl pH 8.3, 75mM KCl, 3mM MgCl2, 10mM DTT, 1.6nM (dT)15-primer and 0.5mM dNTP for 1 hour at 37°C. To determine the mRNA expression of CXCL8 and CXCL10, real-time PCR was carried out using gene-specific primers in an ABI Prism 7000 sequence detection system. PCR reaction was set up with Sybr® Green PCR Master mix containing 0.3μmol/L primers each and 1μl of RT-product in 25μl volume. A two-step amplification protocol was chosen consisting of initial denaturation at 95°C for 10 minutes followed by 45 cycles with 15 seconds denaturation at 95°C and 30 seconds annealing/extension at 60°C. Finally a dissociation protocol was performed to control specificity of amplification products. Relative expression of chemokines was then calculated using the comparative threshold-cycle (CT) method. The amount of target mRNA in each sample was normalized to the amount of β-actin mRNA designated as calibrator, to give Δ CT (CT β-actin–CT chemokines). The relative expression of chemokines was calculated as the chemokine/β-actin ratio = 2 Δ CT. mRNA expression is presented as fold increase calculated in relation to unstimulated cells after normalization to β-actin or 2 Δ CT values were compared in the case of quantification of basal level mRNA expression in different cell lines .

4.2.9. Preparation of Nuclear extract

Caco-2 cells were grown for 24 hours, pre-treated 1 hour with BAY 11-7082 (10μM), an irreversible inhibitor of IkappaB-alpha phosphorylation and then stimulated with respective cytokines for 30 minutes. Cells were washed with 10ml cold PBS twice, harvested, centrifuged at 1000g for 5 minutes and resuspended in 1 ml of cold PBS. Centrifuged briefly, excess PBS was removed and the pellet was resuspended in ice cold extraction buffer by gentle pippeting. Cells were then kept on ice for 15 minutes and 30μl of 10% Nonidet NP-40 was added and vortexed briefly for 10 seconds. The lysed cell suspension was centrifuged for 30 seconds and the supernatant was discarded. The pellet was resuspended in 50μl extraction buffer containing 400mM NaCl and agitated vigorously for 15 minutes at 4°C. Then the cell suspension was

centrifuged for 5 minutes at 4°C and the supernatant containing the nuclear extract was collected and stored at -80°C until further use.

4.2.10. Electrophoretic mobility shift assay (EMSA) and Super shift assay

For binding reactions, 5 µg of nuclear extracts were incubated in 20µl reaction mixture containing 40mM HEPES pH 7.5, 50mM NaCl, 1mM EDTA, 1mM DithiotreHSCl, 1mM PMSF, 2µl (1,28 µg/µl) PCR amplified coding sequence of pBlue-script as half of the reaction (10µl) and the rest being nuclear extract plus other components. Consensus NF-κB oligonucleotide duplex probes (Promega) were end labelled with T4 polynucleotide kinase and [γ^{32}P] ATP (1×10^4 cpm).

Labelling of NF-κB oligonucleotide:

NF-κB oligo	2 µl
T4 polynucleotide Kinase Buffer 10X	1 µl
(γ-32P) ATP 10mCi/ml	1 µl
Nuclease-Free water	5 µl
T4 polynucleotide Kinase (10 units/ml)	1 µl

Total volume	10 µl

The mixture was incubated at 37 °C for 10 minutes and the reaction was stopped with 0.2M EDTA. Synthesized labelled oligonucleotides were separated from non-incorporated nucleotides by gel filtration on Sephadex® G-50 using Pharmacia NICK Column. Then labelled NF-κB oligonucleotide were added to reaction mixtures and incubated overnight at 4°C. Reaction products were analysed by non-denaturing electrophoresis in a 5% polycralamide gel with 0.5X TBE buffer at room temperature.

Gel preparation and electrophoresis:

Inorder to make sure that after electrophoresis the gel remains attached to the bigger plate the smaller glass plate was cleaned with Silicon towards the innerside, before preparing the gel.

10X TBE	2.5 ml
2%Bis-acrylamide	3.33ml
40% Acrylamide	5ml
87% Glycerin	1.5ml
Water	37.7ml
Ammonium per sulphate	375μl
TEMED	25μl

Total volume	50ml

Once the gel was poured on to the glass plates, it was left at room temperature for 2-3 hours to get polymerised. After polymerisation the gel was pre run for 30 minutes at 168 volts. Then the samples were added with 10X loading buffer and loaded in to the wells of the gel and run at room temperature for approximately 1-1.5 hours. Gels were then wraped in saran wrap and exposed directly to X-ray films at $-70^{o}C$ for autoradiography. For competition unlabeled oligonucleotides were added in molar excess at room temperature for 15 min before adding radiolabeled probe. In super shift experiments, 3 μl anti-p65 anti-body (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with nuclear extracts overnight at $4^{o}C$.

4.2.11 Northern Blotting

Under RNase-free conditions, total RNA was separated in a denaturing formaldehyde/ agarose gel, blotted by capillary transfer to a nylon membrane and fixed by UV cross-linking. Specific transcripts were identified by autoradiography after hybridization with a specific radiolabelled cDNA probe.

Preparation of RNA Samples

10 µg of total RNA were mixed with 7.5 µl of sample buffer and denatured by heating at 65°C for 10 minutes. The samples were briefly cooled down on ice and centrifuged (10,000 rpm, 1 minute, RT) in an Eppendorf bench-top centrifuge. Finally, each sample was mixed with 3µl of loading buffer, centrifuged (10,000 rpm, 1 minute, RT) and loaded onto the gel.

Formaldehyde/Agarose Gel Electrophoresis of RNA

For preparation of 1% denaturing formaldehyde/agarose gel, 1 gram of agarose was mixed with 72.2 ml of RNase-free water (Ampuwa®), dissolved by microwaving and then slightly cooled down to approximately 70°C. Subsequently, 10ml of 10x running buffer and 16.7 ml of 37% formaldehyde were added. To visualize RNA bands in the gel, in particular 28S and 18S rRNA bands, 8µl of ethidium bromide (10mg/ml) was added as well. The solution was mixed, poured onto the casting plate and left for polymerization under the fume hood. Thereafter, prepared RNA samples were loaded onto the gel, and the electrophoresis was performed at a constant voltage of 80 V. After electrophoresis, equal loading and integrity of RNA were evaluated by ultraviolet shadowing in the UV transilluminator, the gel was then photographed and immediately subjected to RNA transfer.

RNA Transfer to Nylon Membrane

Size-fractionated RNA samples were transferred to nylon membranes using capillary transfer system. A plastic tray was filled with 500ml of 20x SSC buffer and covered with a glass plate. Whatman 3MM filter paper was soaked in 20x SSC buffer and draped over the glass plate with edges submerging in the buffer. Air bubbles between the Whatman paper and glass plate were squeezed out by rolling a glass pipette over surface. The gel was carefully placed on the filter and covered with 2x SSC buffer. Bubbles were carefully removed. Four strips of parafilm were placed in contact with the gel edges to prevent direct absorbtion of buffer by paper towels. A wetted piece of nylon membrane was delicately placed on the exposed gel surface, smoothed out and covered with two additional sheets of Whatman 3MM paper wetted in 2x SSC buffer. Finally, paper towels were stacked on top of Whatman papers to a height of

approximately 8 cm, covered with another glass plate and pressed with a 1 kg blotting weight. The transfer was carried out overnight. After transfer, RNA was cross-linked to the membrane by ultraviolet radiations in the Stratalinker™ 180 system.

DNA Labelling by Nick Translation Method

DNA labelling by nick translation method was performed using Nick Translation System kit according to manufacturer's protocol. Nick translation requires the activity of two different enzymes. DNase I is used to cleave (nick) phosphodiester bonds at random sites in both strands of a double-stranded target DNA. *E. coli* DNA polymerase I is necessary for integration of deoxynucleotides to the 3′-hydroxyl termini created by DNase I. In addition to its polymerizing activity, DNA polymerase I carries a 5′→3′ exonucleolytic activity that removes nucleotides from the 5′ side of the nick. The simultaneous elimination of nucleotides from the 5′ side and the addition of radiolabelled nucleotides to the 3′ side results in movement of the nick (nick translation) along the DNA, which becomes labelled to high specific activity (Kelly et al., 1970). The reaction was carried out at low temperature (15°C).

In sterile 1.5 ml tube placed on ice, nuclease free H_2O was added to the solution containing 1 μg of template DNA to the volume of 35μl. Consequently, the following reagents were added to the DNA in the indicated order:

5 μl dNTP mix (mixture of dATP, dTTP and dGTP),

5 μl [γ-^{32}P]-dATP (3,000 Ci/mmol, 50 μCi),

5 μl Pol I/DNase I mix.

The components were mixed gently but thoroughly and centrifuged briefly in an Eppendorf bench-top centrifuge. The mixture was incubated at 15°C for 60 minutes. Finally, 5μl of stop buffer was added to stop the reaction.

Purification of Labelled DNA

Synthesized labelled cDNA probe was separated from non-incorporated nucleotides by gel filtration on Sephadex® G-50 using Pharmacia NICK Column (Pharmacia Biotech).

A column was opened according to the manufacturer's instructions and equilibrated with 3 ml of 1x TE buffer, pH 8.0. After the entire volume of buffer had entered the gel, random priming reaction mixture was applied onto the column, followed by addition of 400 μl of 1x TE buffer. The flow-through was collected in the tube placed under the column and kept for further measurement of radioactivity. A new reaction tube was placed under the column and the purified labelled DNA was eluted with 400 μl of 1x TE. Finally, the β-radioactivity in the obtained sample was measured, and labelled DNA was either immediately used for hybridization or stored at −20°C for further experiments.

Measurement of β –Radioactivity

After purification, the radioactivity of labelled cDNA samples was measured using Wallac 1409 liquid scintillation β-counter (EG&G, Turku, Finland). 2μl aliquots from the flow-through and elution fractions were transferred to screw-lid plastic tubes containing 5 ml of scintillation liquid, mixed by inverting and subjected to measurement of β-radioactivity. The latter was expressed in counts per minute (cpm). The activity value in flow-through fraction was used to evaluate the efficiency of radioactive nucleotide incorporation. For effective labelling this value should not exceed 10% of the radioactivity value of the cDNA sample.

Hybridization of RNA with Radiolabelled cDNA Probe

After cross-linking, the nylon membrane was rinsed with RNase-free water to remove traces of agarose. Afterwards, the membrane was placed into the hybridization tube, and any bubbles between the membrane and internal wall of the tube were carefully squeezed out. To prevent unspecific binding, the membrane was first prehybridized in QuikHyb® hybridization solution at 68°C for 2 hours. Radiolabelled probe (1.5×10^6-3 $\times10^6$ cpm/ml) was mixed with a double volume of fish sperm DNA and denatured at 95°C for 5 min. After cooling down on ice, the DNA probe was applied into QuikHyb® solution inside the hybridization tube. The tube was placed back in the hybridization oven, and hybridization was carried out at 68°C for 2 h. After hybridization, the membrane was washed once in 2x SSC/0.1% SDS for 10 min at RT, then twice in 0.1x SSC/0.1% SDS for 10 min at 55^0C (CXCL8 cDNA), 45^0C

(CXCL10 cDNA) and, finally, twice in 2x SSC/0.1% SDS for 10 min at RT. To visualize 28S rRNA, the membrane was prehybridized in QuikHyb$^{®}$ hybridization solution at 42°C for 2 hours followed by overnight hybridization at 42°C with the labelled oligonucleotide specific for 28S rRNA and washed three times in 2x SSC/0.1% SDS for 10 min at 37^{0}C. After washing, membranes were wrapped in a saran wrap, placed in X-ray film cassette and autoradiographed during various exposure times. Each membrane was reprobed up to 3 times. Typically, first hybridizations were performed to visualize low abundance transcripts followed by sequential hybridizations with cDNAs complementary to more abundant mRNAs. When reprobing, the membrane was unwrapped, immediately placed in warm (60-80^{0}C) 1x TE buffer and incubated with slight shaking until overall radioactivity (checked by Geiger hand counter) on the membrane surface disappeared. The membrane was then placed in a new hybridization tube and subjected to the next hybridisation.

4.3. Statastical Analysis

Student's *t*-test was used to evaluate differences between the sample of interest and its respective control. Means ± standard error of the mean (SEM) are indicated; $p < 0.05$ was considered statistically significant.

5. Results

5.1. Role of cytokines in inducing CXCL8 and CXCL10 gene expression in intestinal epithelial cells (IECs).

5.1.1. Differential expression of CXCL8 and CXCL10 mRNA in IECs under basal conditions.

It has been known that IECs depending on their origin and maturity may have a different and distinct pattern of chemokine/cytokine expression (Yang et al., 1997). So in the present study, the differences in basal mRNA expression of CXCL8 and CXCL10 in three different IECs like Caco-2, HT29 and DLD1 cells were studied first. Real-time PCR experiments using gene specific primers for CXCL8 and CXCL10 genes revealed that, the basal mRNA expression of CXCL8 normalised to β actin expression was highest in HT29 followed by DLD1 and Caco-2. In contrast, CXCL10 basal mRNA expression was highest in Caco-2 followed by HT29 and DLD1 (**Fig 11**).
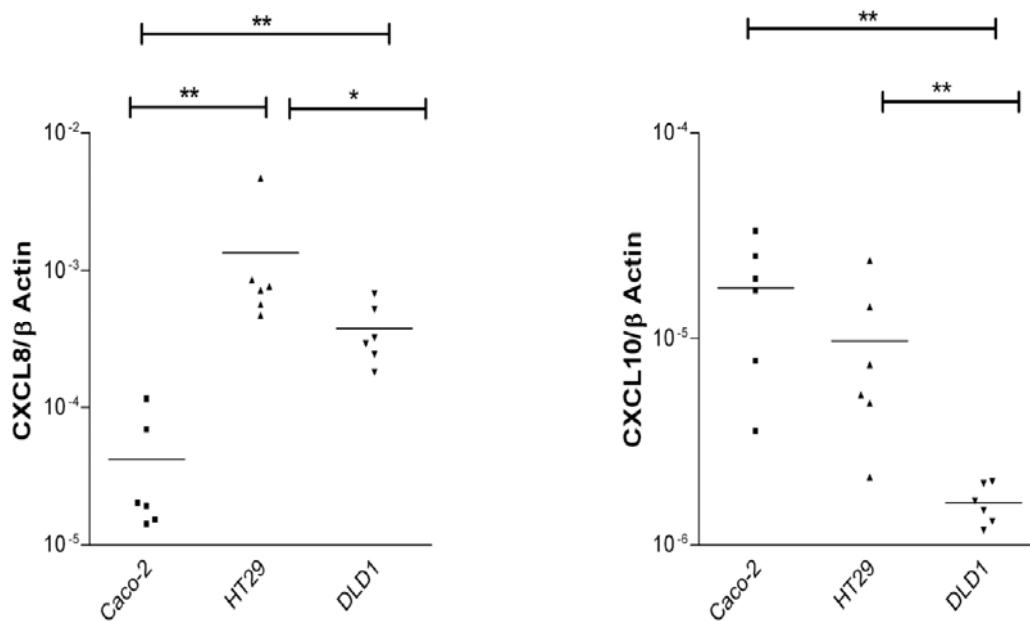
Figure 11: Comparison of basal level expression of CXCL8 and CXCL10 mRNA in IECs

5×10^5 cells were plated into 6 well plates and grown for 24 hours. The cells were harvested, total RNA was isolated and first strand cDNA was prepared from 1 μg of total RNA. Real-Time PCRs were performed to check the basal level expression of CXCL8 and CXCL10 using gene specific primers in human IECs. Chemokine expression was normalized to β actin expression measured in the same sample as an internal control. Data represent the means ± SEM of 6 independent experiments. ** corresponds to $P < 0.005$, * corresponds to $P < 0.05$.

5.1.2. IL-1β, TNFα and IFNγ dose dependent influence on CXCL8 and CXCL10 mRNA expression in IECs.

Dose response experiments showed a dose dependent increase of CXCL8 mRNA by TNFα in Caco-2, HT29 and DLD1cells by 4 hours after stimulation with cytokines. TNFα (2 ng/ml, 10 ng/ml and 50 ng/ml) induced the strongest expression of CXCL8 mRNA in HT29 cells (25.7 ± 3.2, 71.65 ± 10.8 and 116.76 ± 13.74 fold increase) **(Fig 12A)**. IFNγ was ineffective in inducing the CXCL8 expression in all the cell lines used. As CXCL8 expression was already maximally stimulated by 2 ng/ml IL-1β **(Fig 12A)**, stimulation of Caco-2 cells was performed with lower doses of IL-1β (0.1 ng/ml, 0.5 ng/ml and 1 ng/ml) and for a shorter time (1 hour), resulting in a 56.6 ± 31.3, 173.4 ± 74.5 and 266.2 ± 84.3 fold increase. CXCL8 mRNA was dose dependently increased to 2 ± 0.3, 5.2 ± 1.1 and 11.8 ± 1.19 fold increase by 2 ng/ml, 10 ng/ml and 50 ng/ml of TNFα respectively **(Fig 12)**. Preliminary data on time kinetics of CXCL8 mRNA revealed that CXCL8 mRNA is highly induced 1 hour after cytokine stimulation. So we checked the low dose effect of IL-1β and TNFα on CXCL8 mRNA expression at 1 hour in Caco-2.

TNFα and IFNγ dose dependently increased CXCL10 mRNA expression in Caco-2, HT29 and DLD1 cells, IFNγ being the major inducer of CXCL10 mRNA in DLD1 and HT29 cells. In HT29 cells, TNFα (2, 10 and 50 ng/ml) was also potent in inducing CXCL10 mRNA to 30.2 ± 8.6, 92.6 ± 23.8 and 171.64 ± 43.21 folds. IFNγ led to a 22.08 ± 8.2, 187.6 ± 29.5 and 216 ± 40.5 fold increase at concentrations of 2ng/ml, 10ng/ml and 50ng/ml, respectively. In DLD1 cells, IFNγ (2, 10 and 50 ng/ml) and but not IL-1β or TNFα significantly induced CXCL10 mRNA (96.6 ± 45.3, 183.8 ± 42.5 and 187.8 ± 30.2 fold increase) **(Fig 12B)**. As in case of CXCL8 mRNA, incubation with IL-1β (2-50 ng/ml) also resulted in a maximum stimulation of CXCL10 in Caco-2, HT29 and DLD1 cells. Therefore, Caco-2 cells were stimulated with lower doses of IL-1β (0.1ng/ml, 0.5ng/ml and 1ng/ml) for 4 hours. IL-1β at a concentration of 0.5ng/ml (96.9 ± 5.9 fold increase) was more potent in inducing CXCL10 mRNA than TNFα 50ng/ml (25.12 ± 6.8 fold increase) and IFNγ 50 ng/ml (18.32 ± 6.31 fold increase) **(Fig 13)**.

A)
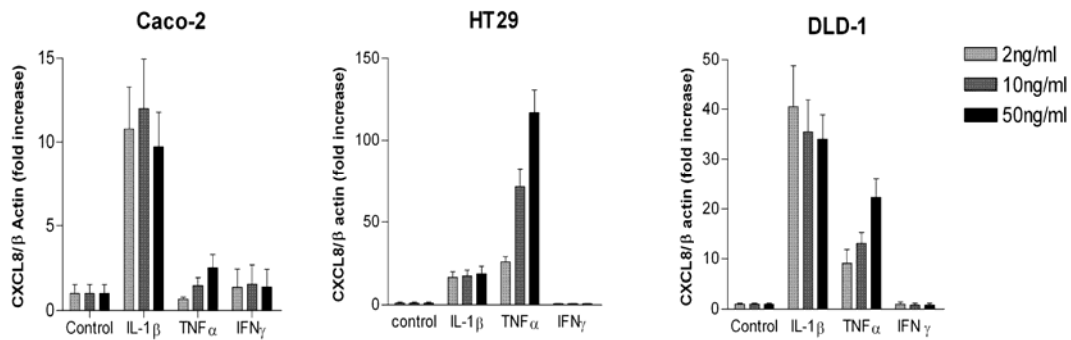
B)

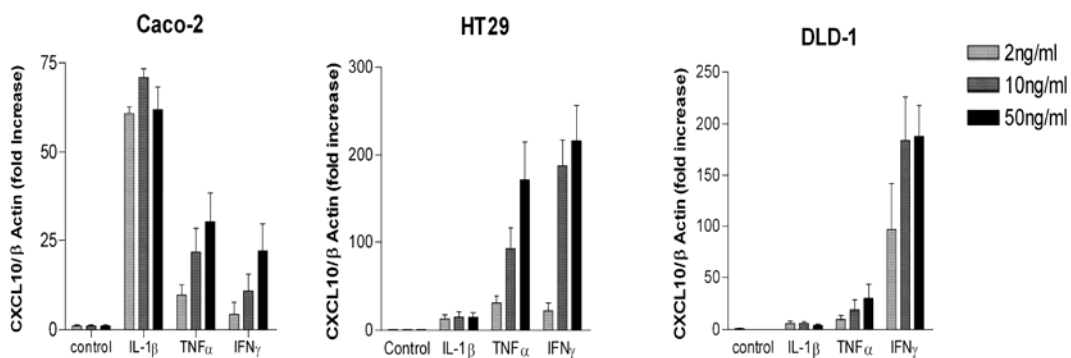**Figure 12: Dose dependent induction of CXCL8 and CXCL10 mRNA expression by cytokines**

5×10^5 cells were plated into 6 well plates, grown for 24 hours and then stimulated with IL-1β, TNFα and IFNγ at an increasing doses of 2ng/ml, 10ng/ml and 50ng/ml. The cells were harvested after 4 hours, total RNA was isolated and first strand cDNA was prepared from 1 μg of total RNA. Real-time PCR was performed for CXCL8, CXCL10 and β actin genes. β actin was used as an internal control. Data represent the means of 4 individual experiments ± SEM.
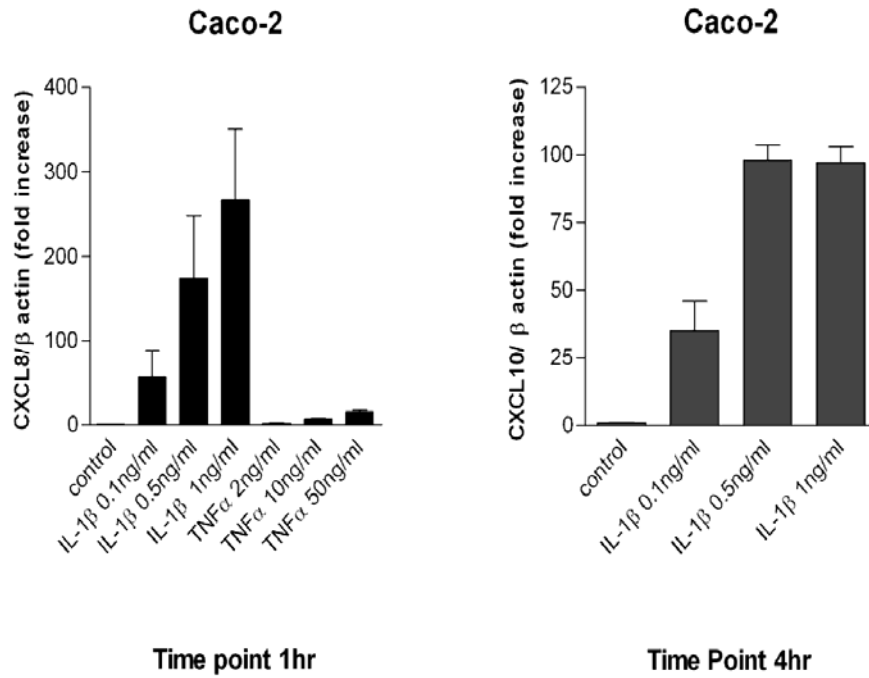
Figure 13: Dose dependent expression of CXCL8 mRNA at 1hour and CXCL10 mRNA at 4 hours

5×10^5 Caco-2 cells were plated into 6 well plates, grown for 24 hours and then stimulated with IL-1β and TNFα at an increasing doses as shown in the figure. The cells were harvested after 1 hour and 4 hours, total RNA was isolated and first strand cDNA was prepared from 1μg of total RNA. Real-time PCR was performed for CXCL8, CXCL10 and β-actin was used as an internal control. Data represent the means of 3 individual experiments ± SEM.

5.1.3. Regulation of CXCL8 mRNA expression and protein secretion in IECs by cytokines: Time kinetic experiments.

Time kinetics of CXCL8 mRNA and protein secretion, were carried out to find out how quickly IECs respond to cytokines **(Fig 14)**. In all cell lines, CXCL8 mRNA was inducible as early as 40 minutes and the expression reached the maximum, 1 hour after stimulation with IL-1β (1ng/ml) and TNFα (50ng/ml), while IFNγ (50ng/ml) was ineffective. In Caco-2 **(Fig 14A)** and DLD1 cells **(Fig 14C)**, IL-1β was able to induce CXCL8 mRNA within 40 minutes, 15.94 ± 7.4 (Caco-2) and 30.99 ±13.19 (DLD1) fold increase and TNFα led to 3.07 ±1.71 (Caco-2) and 24.72 ± 9.73 (DLD1) fold increase respectively. In HT29 cells, TNFα was able to induce CXCL8 mRNA within 20 minutes (2.2 ± 1.1 fold increase) and the combination of TNFα+IFNγ synergistically induced CXCL8 mRNA expression (3.93 ± 2.8 fold increase). In both Caco-2 and DLD1 cells, the combination of IL-1β+TNFα prolonged the effect on CXCL8 mRNA expression with an increased expression after 1 hour when compared to IL-1β alone. The same combination didn't have any effect in HT29 cells. In contrast, in HT29 the combination of TNFα+IFNγ had an inhibitory effect at 1 hour and then a synergistic effect after 2 hours onwards when compared with TNFα alone **(Fig 14B)**.

In Caco-2 cells, IL-1β led to 1.86 fold increase in CXCL8 protein concentration (0.491±0.063 ng/ml vs 0.263±0.008 ng/ml) after 3 hours and reached to a maximum of 2.22 fold increase (0.585±0.096 ng/ml vs 0.263±0.008 ng/ml) after 24 hours, when compared to the control. The combination of IL-1β+TNFα had a small synergistic effect inducing CXCL8 protein secretion to 0.703±0.131 ng/ml after 24 hours **(Fig 15)**. In HT29 cells, TNFα was the potent inducer of CXCL8 protein, increasing CXCL8 concentration from 0.654.4±0.091 ng/ml at baseline to 30.205±1.727 ng/ml after 24 hours. IL-1β was less potent, increasing CXCL8 protein concentration to 4.805±0.450 ng/ml after 24 hours. The combination of IL-1β+TNFα and IL-1β+IFNγ didn't have a significant effect on CXCL8 protein secretion when compared to the respective cytokines alone. HT29 when stimulated with TNFα+IFNγ had 15.485±0.700 ng/ml and 34.965±1.139 ng/ml secretion of CXCL8 at 12 hours and 24

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hours respectively, which was not significantly different from stimulation with TNFα alone (13.849±0.603 ng/ml and 30.206±1.728 ng/ml).

A)
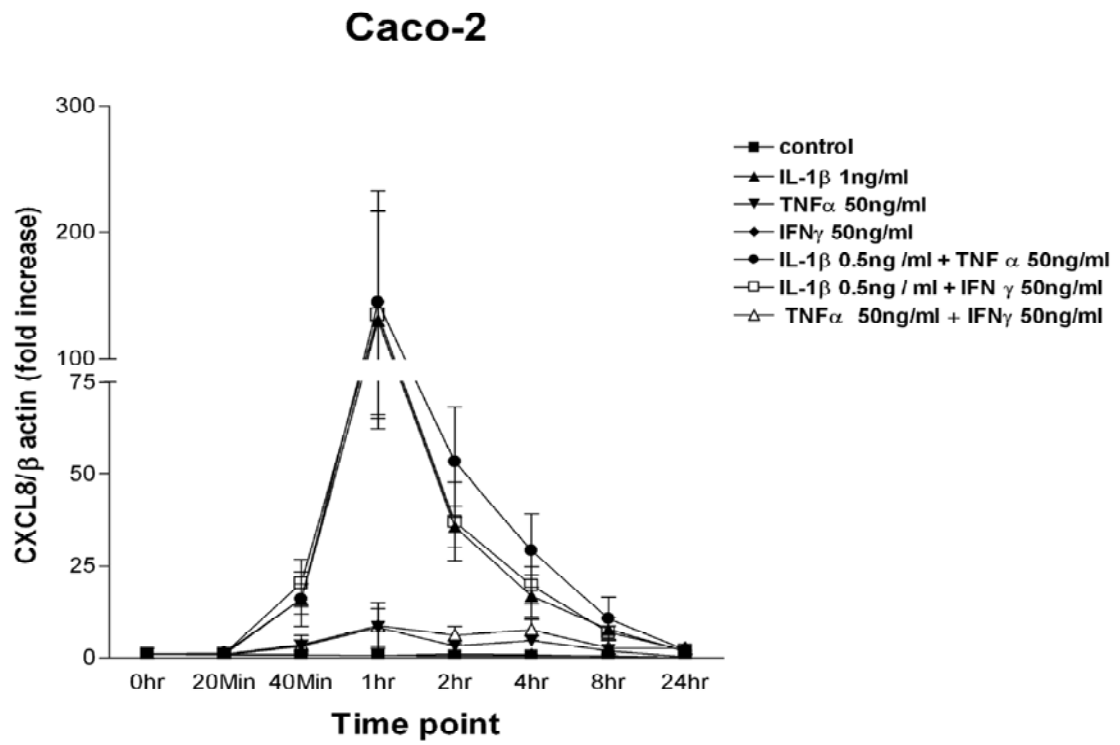
B)
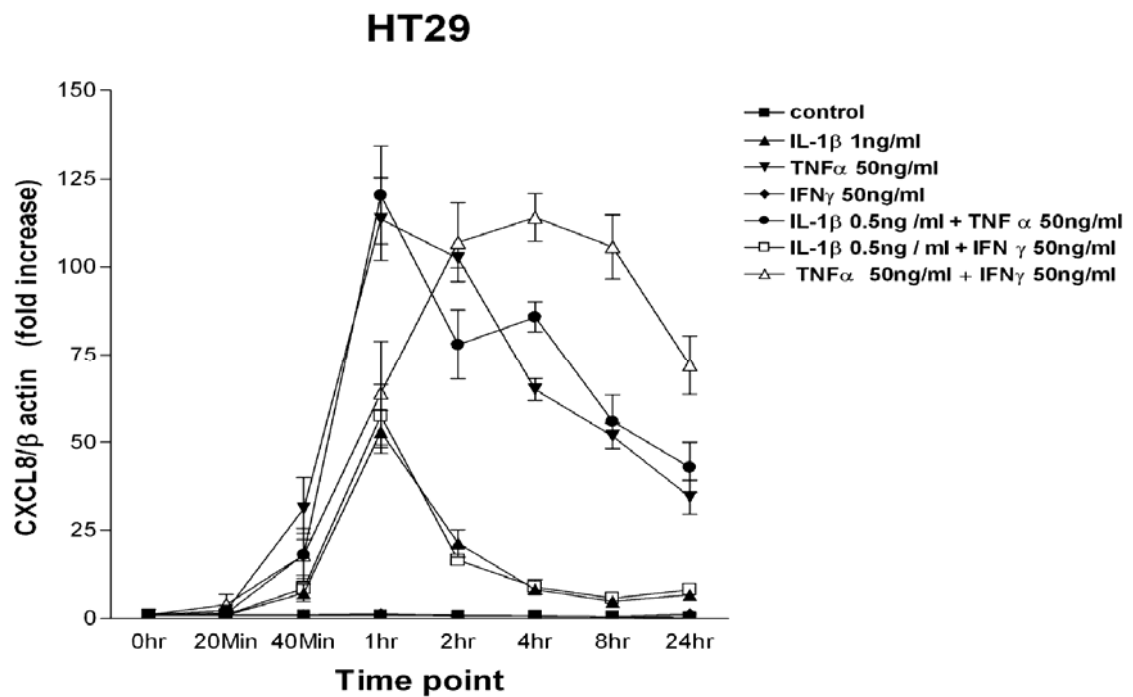
Time kinetics of CXCL8 mRNA

C)
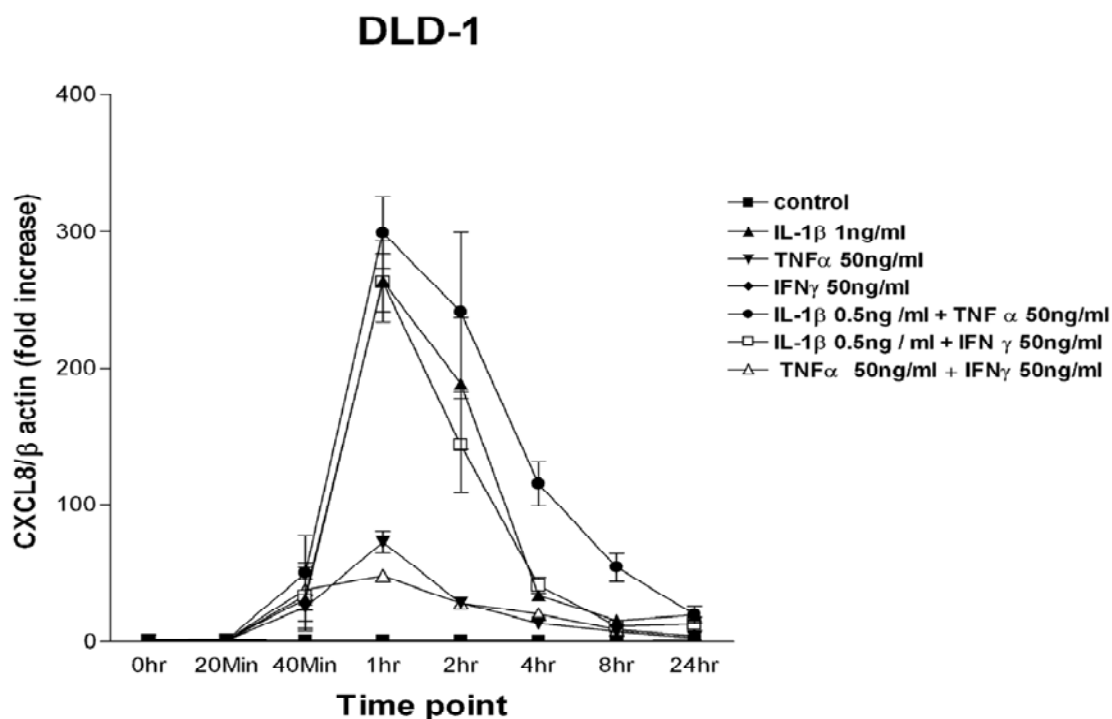
DLD-1

Figure 14: Timekinetics of CXCL8 mRNA expression in Caco-2, HT29 and DLD1 cells stimulated with cytokines

5×10^5 cells were plated into 6 well plates and grown for 24 hours and then stimulated with IL-1β, TNFα and IFNγ as indicated. The cells were harvested at respective time intervals, total RNA from the cells was isolated and first strand cDNA was prepared from 1μg of total RNA. Real-time PCR was performed for CXCL8 gene with gene specific primers and β actin was used as an internal control. Data are shown as the fold increase compared to the baseline controls, represent the mean of 3-5 experiments ± SEM.
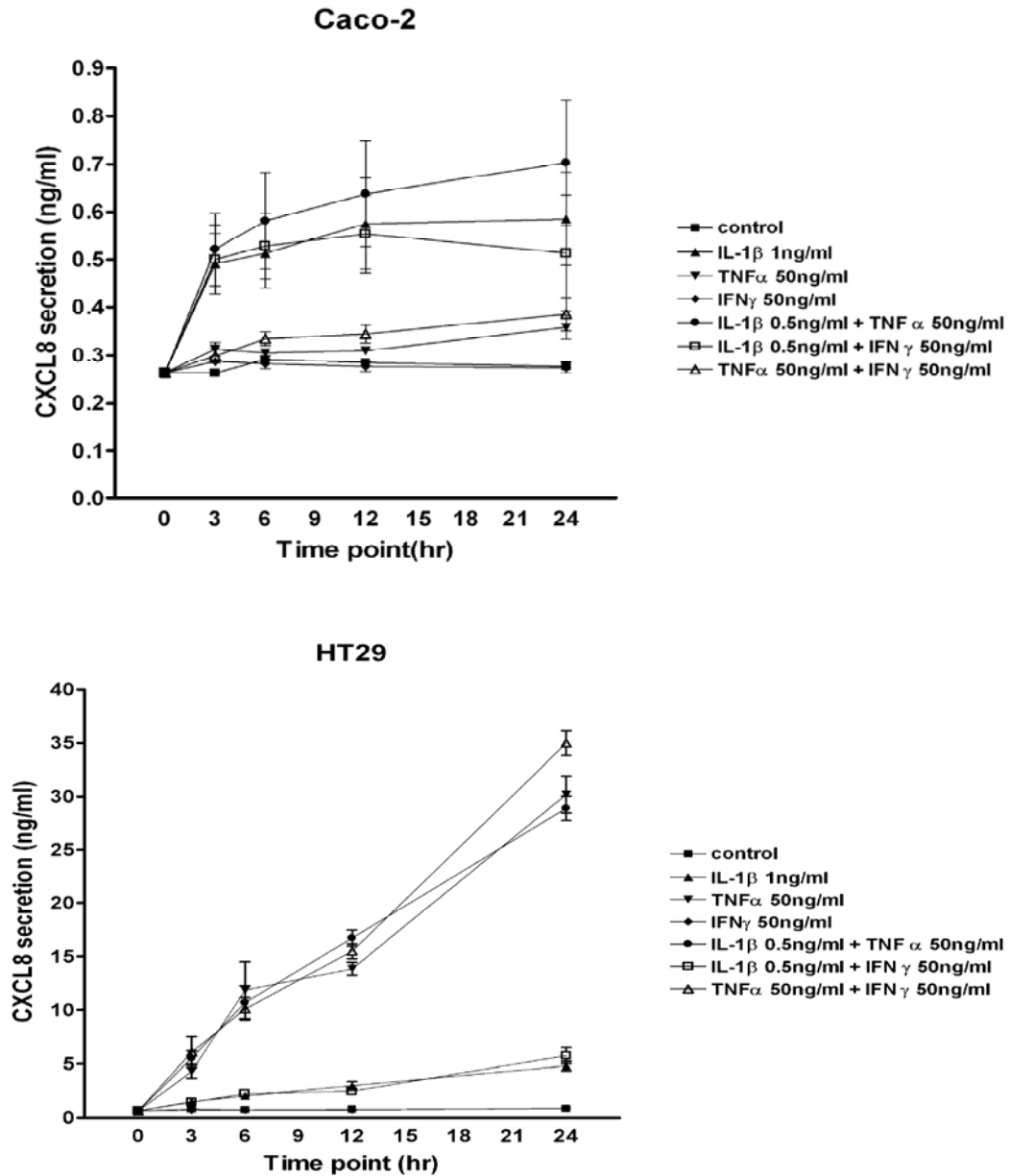
Figure 15: Time kinetics of CXCL8 protein secretion in Caco-2 and HT29 cells stimulated with cytokines

5×10^5 cells were plated into 6 well plates and grown for 24 hours and then stimulated with IL-1β, TNFα and IFNγ as indicated. Cell supernatants were collected at respective time points and ELISA for CXCL8 protein was performed. Data represent the means of 3-5 experiments ± SEM done in duplicates.

5.1.4. Regulation of CXCL10 mRNA expression and protein secretion in IECs by cytokines: Time kinetic experiments.

In case of CXCL10, each cell line had a different time course of expression when stimulated with cytokines. In Caco-2 cells stimulated with IL-1β (1ng/ml) or TNFα (50ng/ml), CXCL10 mRNA was already induced by 40 minutes (7.09 ± 4.53 fold increase by IL-1β and 2.35 ± 0.48 fold increase by TNFα), while IFNγ (50ng/ml) had a delayed effect showing a first increase of CXCL10 mRNA expression after 2 hours (3.52±1.26 fold increase) **(Fig 16A)**. IL-1β showed a maximum induction after 4 hours (39.71±9.34 fold increase), whereas TNFα (21.98±5.45 fold increase). IFNγ stimulation took 8 hours to induce maximum CXCL10 mRNA expression (40.28±11.72 fold increase). The combination of IL-1β+IFNγ had a strong and rapid synergistic effect which induced CXCL10 mRNA already after 20 minutes (5.02±2.07 fold increase) and reached the maximum induction after 4 hours (503.81 ± 78.77 fold increase). The combination of TNFα+IFNγ had a delayed but strong synergistic effect on CXCL10 mRNA expression starting at 40 minutes (3.70±1 fold increase) and reaching a maximum after 24 hours (1964.41±1214.11 fold increase).

In HT29 and DLD1 cells, the time kinetics deviated from that in Caco-2 cells, CXCL10 mRNA induction being delayed until 1 hour irrespective of the kind of cytokine stimulation. In HT29 cells **(Fig 16B)**, IL-1β, TNFα and IFNγ led to delayed induction of CXCL10 mRNA. IL-1β and TNFα showed a clear induction of CXCL10 mRNA by 2 hours (2.14±0.89 and 17.86±7.04 fold increase) and IFNγ by 1 hour (2.56±1.42 fold increase). TNFα and IFN γ once started had a continuous stimulatory effect on CXCL10 mRNA, whereas IL-1β in comparison had a low stimulatory effect reaching the maximum after 4 hours. In DLD1 cells **(Fig 16C)**, IL-1β, TNFα and IFNγ had a delayed induction of CXCL10 mRNA starting at 1 hour (3.07±0.65, 2.82±0.88 and 1.88±0.47 fold increase respectively). The stimulatory effects of IL-1β (7.29±1.75 fold increase) and TNFα (25.60±3.91 fold increase) reached to maximum after 4 hours, whereas IFNγ had a continous stimulatory effect on CXCL10 mRNA upto 24 hours. In HT29 cells, the combination of IL-1β+IFN γ led to a delayed and less pronounced induction when compared to TNFα+IFNγ. The combination of

IL-1β+IFNγ was more potent than TNFα+IFNγ at 1 hour in DLD1 cells (538.55±170.73 fold increase vs 142.65±43.92 fold increase). IL-1β+TNFα in combination didn't have any synergistic effect on CXCL10 mRNA.

Under basal conditions Caco-2 cells secreted 0.012±0.003 ng/ml of CXCL10 protein. Treatment with IL-1β, TNFα and IFNγ led to a time dependent increase of CXCL10 protein, with IL-1β being the most potent stimulus during the first 6 hours (0.113±0.021ng/ml) **(Fig 17)**. Of importance, after 12 hours CXCL10 protein secretion was stimulated more strongly by IFNγ than IL-1β (0.281±0.048ng/ml vs 0.135± 0.022 ng/ml). Coincubation of Caco-2 cells with respective cytokines revealed a synergistic effect of IL-1β+IFNγ and TNFα+IFNγ on CXCL10 protein secretion, reaching levels of 3.354±0.690 and 7.808.1±0.173 ng/ml after 24 hours. The combination of IL-1β+TNFα didn't have any synergistic effect on CXCL10 secretion when compared to the respective cytokines alone. These time kinetic studies revealed that in Caco-2, IL-1β alone or in combination with IFNγ induced the secretion of CXCL10 very early, but the combination of TNFα+IFNγ induced a delayed but strongest synergistic secretion of CXCL10 protein. In HT29 cultures, CXCL10 secretion was not detectable under basal condition. TNFα and IFNγ led to an induction of CXCL10 protein secretion within 3 hours (0.0172±0.0045 and 0.011±0.0013ng/ml), whereas IL-1β didn't induce the secretion of CXCL10. All these cytokines (IL-1β, TNFα and IFNγ) led to a time dependent increase of CXCL10 concentrations in HT29 supernatants showing that IFNγ was the strongest inducer of CXCL10 secretion when compared to other two cytokines in this cell line **(Fig 17)**. The combination of TNFα+IFNγ and IL-1β+IFNγ increased CXCL10 protein secretion to 1.822±0.109 ng/ml and 0.410±0.027 ng/ml within 3 hours and reached to a maximum of 492.728±83.776 ng/ml and 87.158±7.913 ng/ml after 24 hours, respectively.

A)

Caco-2
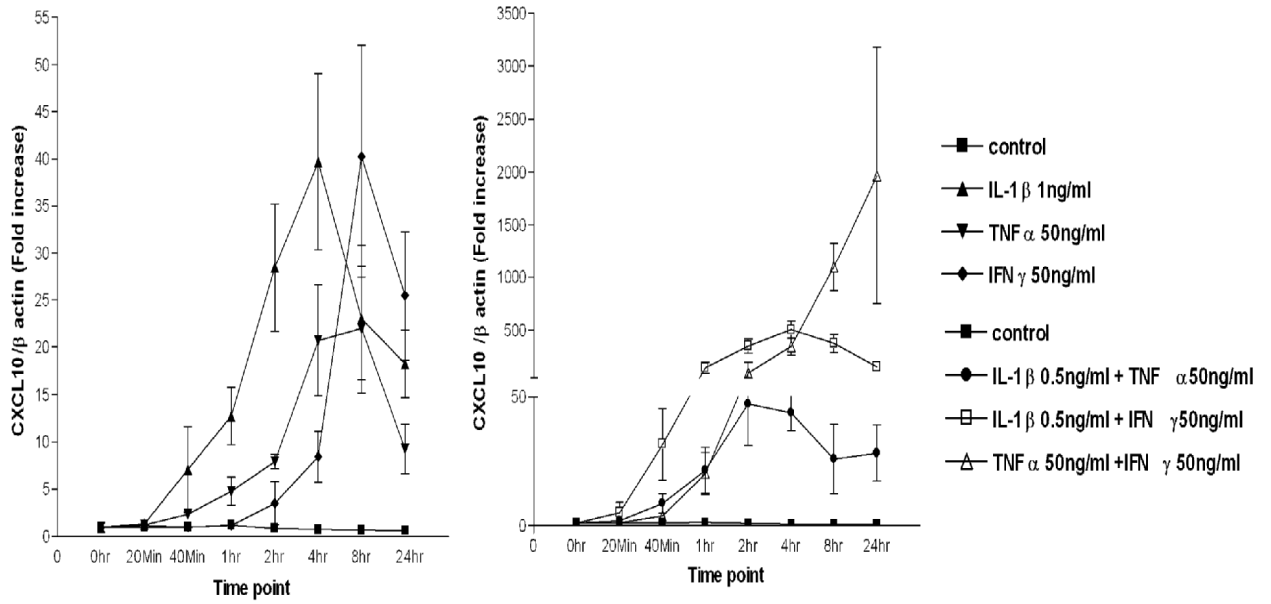
B)

HT29
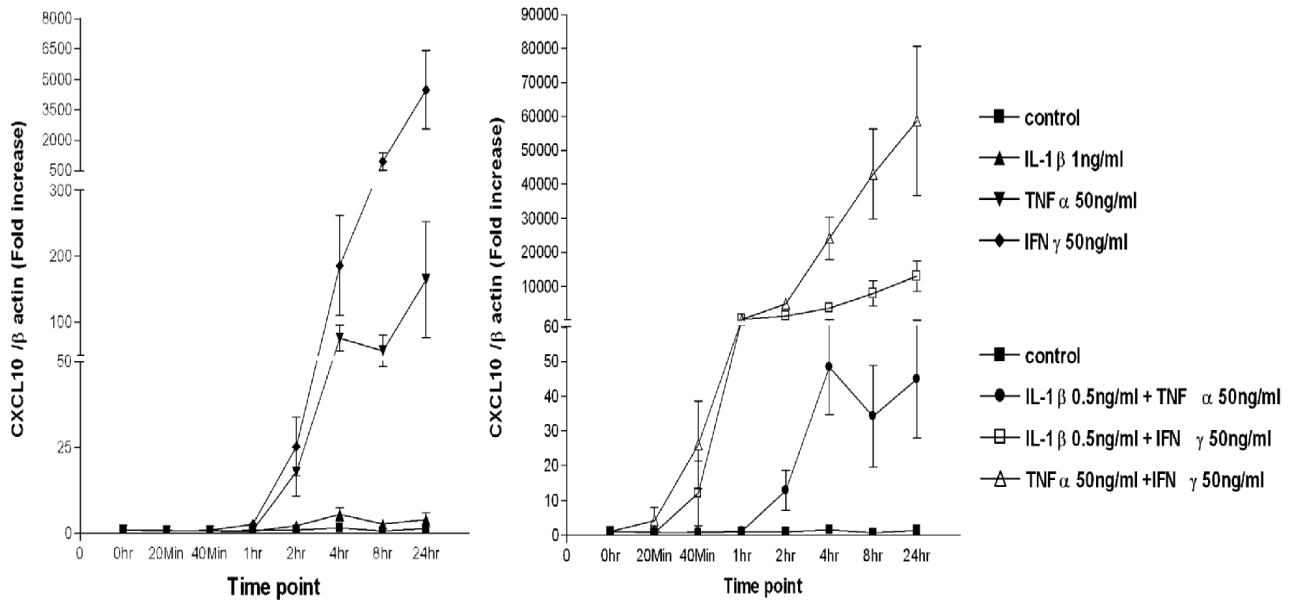
Time Kinetics of CXCL10 mRNA

C)
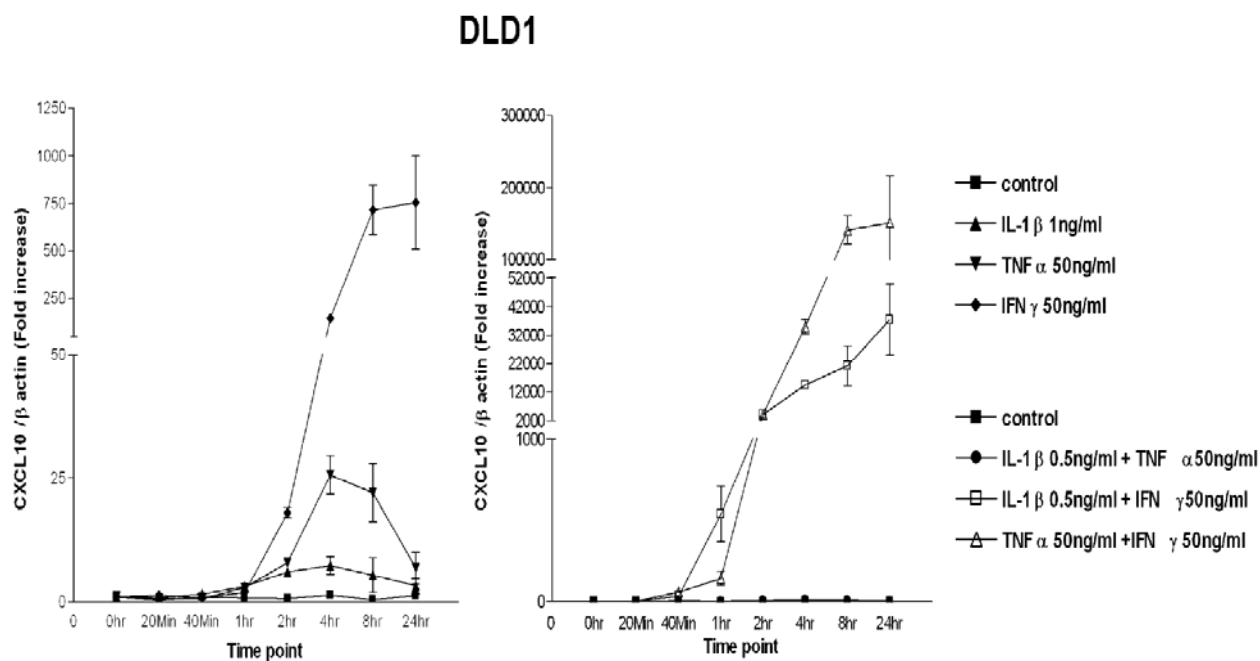
Figure 16: Time kinetics of CXCL10 mRNA expression in Caco-2, HT29 and DLD1 cells stimulated with cytokines

5×10^5 cells were plated into 6 well plates, grown for 24 hours and then stimulated with IL-1β, TNFα and IFNγ as indicated. The cells were harvested at respective time intervals, total RNA from the cells was isolated, first strand cDNA was prepared from 1μg of total RNA. Real-time PCR was performed using gene specific primers for CXCL10 and β actin. CXCL10 expression was normalized to β-actin expression. Data are shown as the fold increase compared to the baseline controls and represent the means of 3-5 experiments ± SEM
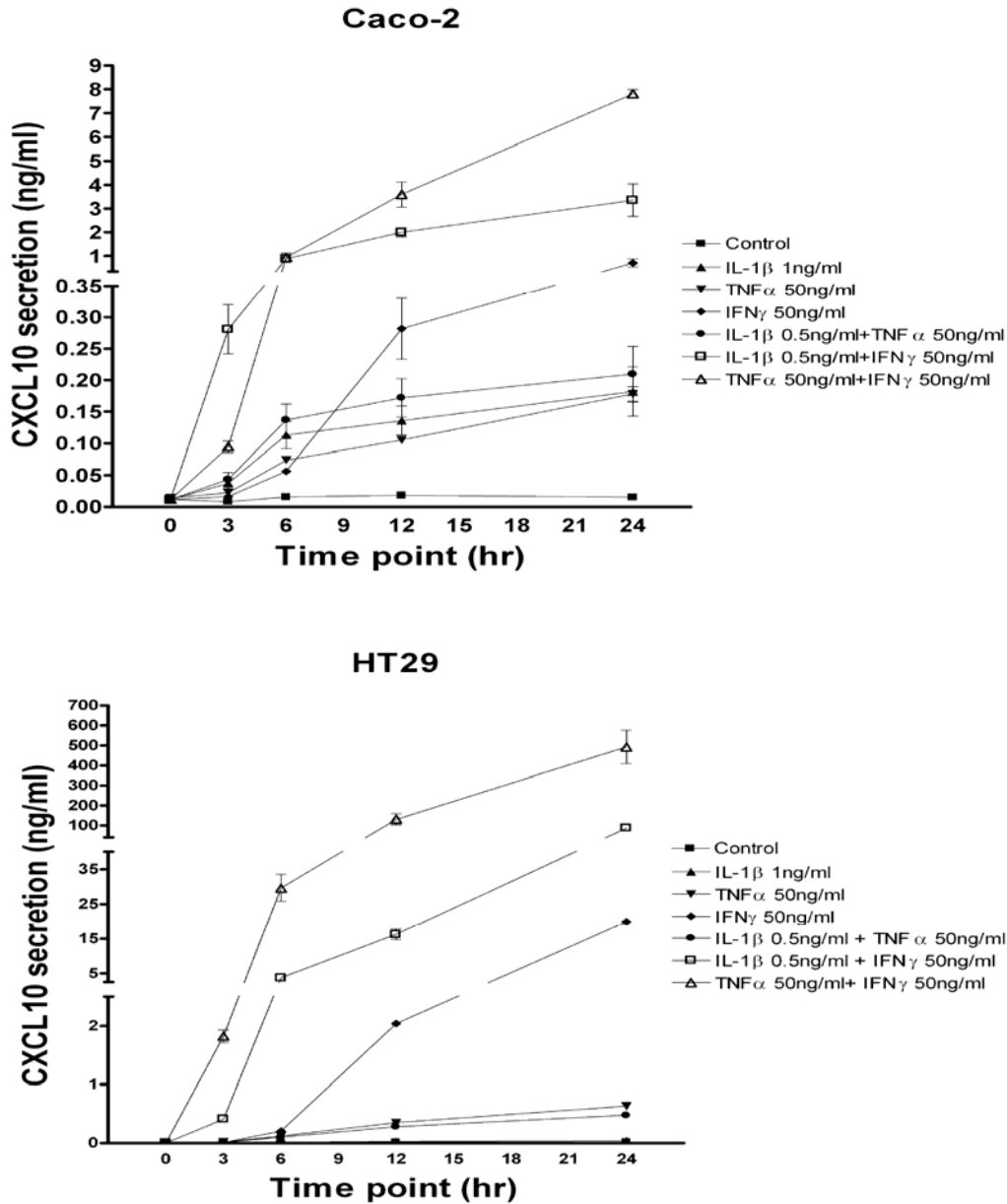
Figure 17: Timekinetics of CXCL10 protein secretion in Caco-2, HT29 and DLD1 cells stimulated with cytokines

5×10^5 cells were plated into 6 well plates and grown for 24 hours and then stimulated with IL-1β, TNFα and IFNγ as indicated. Cell supernatants were collected at respective time points and ELISA for CXCL10 protein was performed. Data represent means of 3-5 experiments ± SEM done in duplicates for each sample.

5.1.5. Confirmation of the real-time PCR results by Northern blotting.

The real time PCR results could be confirmed by Northern blots for both CXCL8 and CXCL10 mRNA in Caco-2 and HT29 cells **(Fig 18)**. However, in case of CXCL10 mRNA we couldn't detect the expression induced by IL-1β (1 ng/ml), TNFα (50 ng/ml) and IFNγ (50 ng/ml) in Caco-2 stimulated for 4 hours and in HT29 stimulated with IL-1β (1 ng/ml) and TNFα (50 ng/ml) for 8 hours. This might be most probably because of low expression level of CXCL10 mRNA when compared to CXCL8 mRNA. The quantitative relationship of CXCL8 and CXCL10 mRNA expression in Caco-2 cells is shown in Table 1.
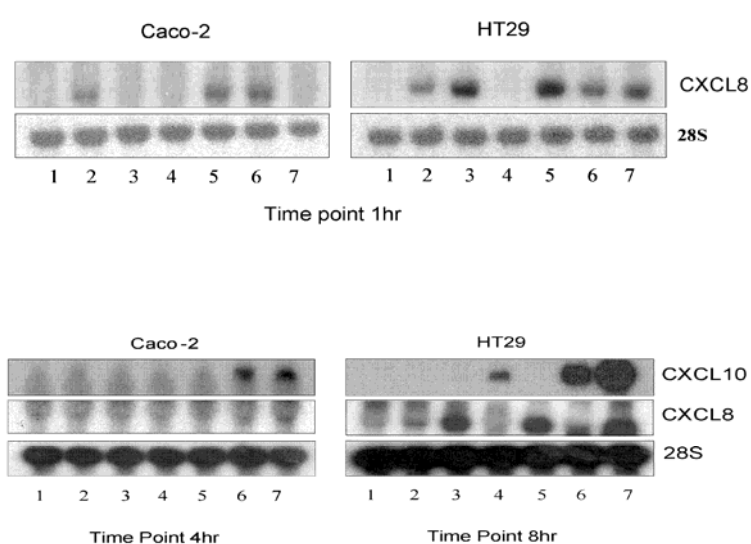
Figure 18: Northern blot autoradiographs showing CXCL8 and CXCL10 mRNA expression in Caco-2 and HT29 cells

Caco-2 and HT29 cells were stimulated with Control lane 1, IL-1β 1ng/ml lane 2, TNFα 50ng/ml lane 3, IFNγ 50ng/ml lane 4, IL-1β 0.5ng/ml+TNFα 50ng/ml lane 5, IL-1β 0.5ng/ml+IFNγ 50ng/ml lane 6 and TNFα 50ng/ml+IFNγ 50ng/ml lane 7 at respective time intervals. Total RNA was isolated and 10μg were separated by 1% agarose gel electrophoresis, blotted and probed with CXCL8 and CXCL10 cDNA respectively. Equal loadings of RNA was demonstrated after stripping and rehybridizing of membranes with an oligonucleotide complementary to 28s RNA.

Caco-2 4hr	IL-1β (1ng/ml)	TNFα (50ng/ml)	IFNγ (50ng/ml)
CXCL8	$6.609×10^{-4} ± 2.26×10^{-4}$	$1.794×10^{-4} ± 5.7×10^{-5}$	$3.094×10^{-5} ± 1×10^{-5}$
CXCL10	$3.579×10^{-4} ± 8.4×10^{-5}$	$1.864×10^{-4} ± 5.3×10^{-5}$	$7.624×10^{-5} ± 2.435×10^{-5}$

Table 1: Quantitative relationship of CXCL8 and CXCL10 mRNA in Caco-2

Mean CXCL8 and CXCL10 mRNA levels (2 ᴬCT values) obtained from real-time PCR experiments and normalized to β-actin, in Caco-2 treated with respective cytokines for 4 hours. Data represent the means of 4 individual experiments ± SEM.

5.1.6. Synergistic induction of TNFR 2 (Tumor necrosis factor receptor 2) by IL-1β, TNFα and IFNγ in HT29 cells.

In order to investigate the cause for synergistic effects of TNFα+IFNγ on CXCL8 mRNA in HT29, we evaluated the expression of TNF receptors in HT29 cell line (**Fig 19**). The combination of TNFα+IFNγ induced an early TNFR 2 mRNA expression by 2 hours (4.61±1.49 fold increase) while the combination of IL-1β+IFNγ was less efficient (2.44±0.67 fold increase). IFNγ alone led to the induction of TNFR 2 mRNA expression after 4 hours (4.07±1.29 fold increase). There was the strongest response to TNFα+IFNγ combination (434±18.3 fold increase) after 24 hours. The combination of IL-1β+IFNγ also led to a synergistic induction of TNFR 2 in HT29 (67.49±13.27 fold increase) after 24 hours. IFNγ alone induced TNFR 2 expression reached to maximum after 24 hours (38±1.33 fold increase).
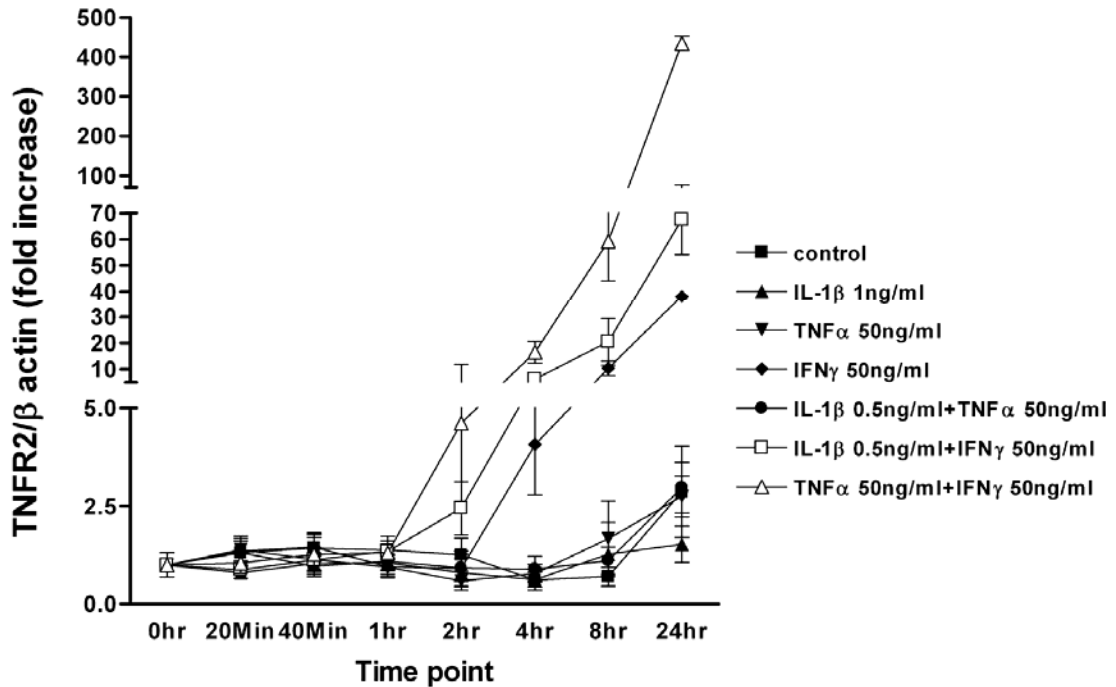
Figure 19: Time kinetics of TNFR 2 mRNA expression in HT29 cells stimulated with cytokines

5×10^5 HT29 cells were plated into 6 well plates, grown for 24 hours and then stimulated with IL-1β, TNFα and IFNγ as indicated. The cells were harvested at respective time intervals, total RNA from the cells was isolated and first strand cDNA was prepared from 1μg of total RNA. Real-time PCR was performed using gene specific primers for TNFR 2 and β actin. TNFR 2 expression was normalized to β-actin expression. Data are shown as the fold increase compared to the baseline controls, representing the means of 3-5 experiments ± SEM.

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5.1.7. Promoter analysis of CXCL8 and CXCL10 genes.

To further elucidate the intracellular steps involved in cytokine induced CXCL8 and CXCL10 gene expression, we performed transient tansfection assays either with CXCL8 or CXCL10 gene promoter containing plasmid, that harbor luciferase reporter gene downstream to the promoter.

5.1.7. a. Activation of CXCL8 gene promoter by cytokines in IECs.

The effect of cytokines on CXCL8 promoter activity was investigated using the plasmid pUHC13-3 IL-8. This plasmid is a kind gift from Dr. Michael Kracht (Hannover medical school, Germany) the details of which can be find in the reference (Holtmann et al., 1999). As in the case of CXCL8 mRNA **(Fig 12A)** dose dependent effect of IL-1β on CXCL8 promoter was also not seen with the concentrations 2ng/ml, 10ng/ml and 50ng/ml. TNFα dose dependently induced CXCL8 promoter activity in the three cell lines we used for the study. IFNγ (50ng/ml) was ineffective in activating CXCL8 promoter activity, in all the three cell lines used. IL-1β (1ng/ml) led to a maximum of 5.37±0.93, 3.09±0.82 and 3.45±0.32 fold increase of CXCL8 promoter activity in Caco-2, HT29 and DLD1cells, respectively. TNFα (50ng/ml) led to a maximum of 2.12±0.77, 7.13±3.06 and 3.76±1.1 fold increase of the CXCL8 promoter activity in Caco-2, HT29 and DLD1 cells, respectively **(Fig 20)**. These data could show that the effect of cytokines on CXCL8 gene expression was indeed at the level of transcription, except in HT29 cells, where the combination of IL-1β+TNFα (25.93 ± 4.34 fold increase) had a stronger synergistic effect than the combination of TNFα+IFNγ (10.4 ± 0.44 fold increase) on CXCL8 promoter activity but not on mRNA expression **(Fig 14B)**.
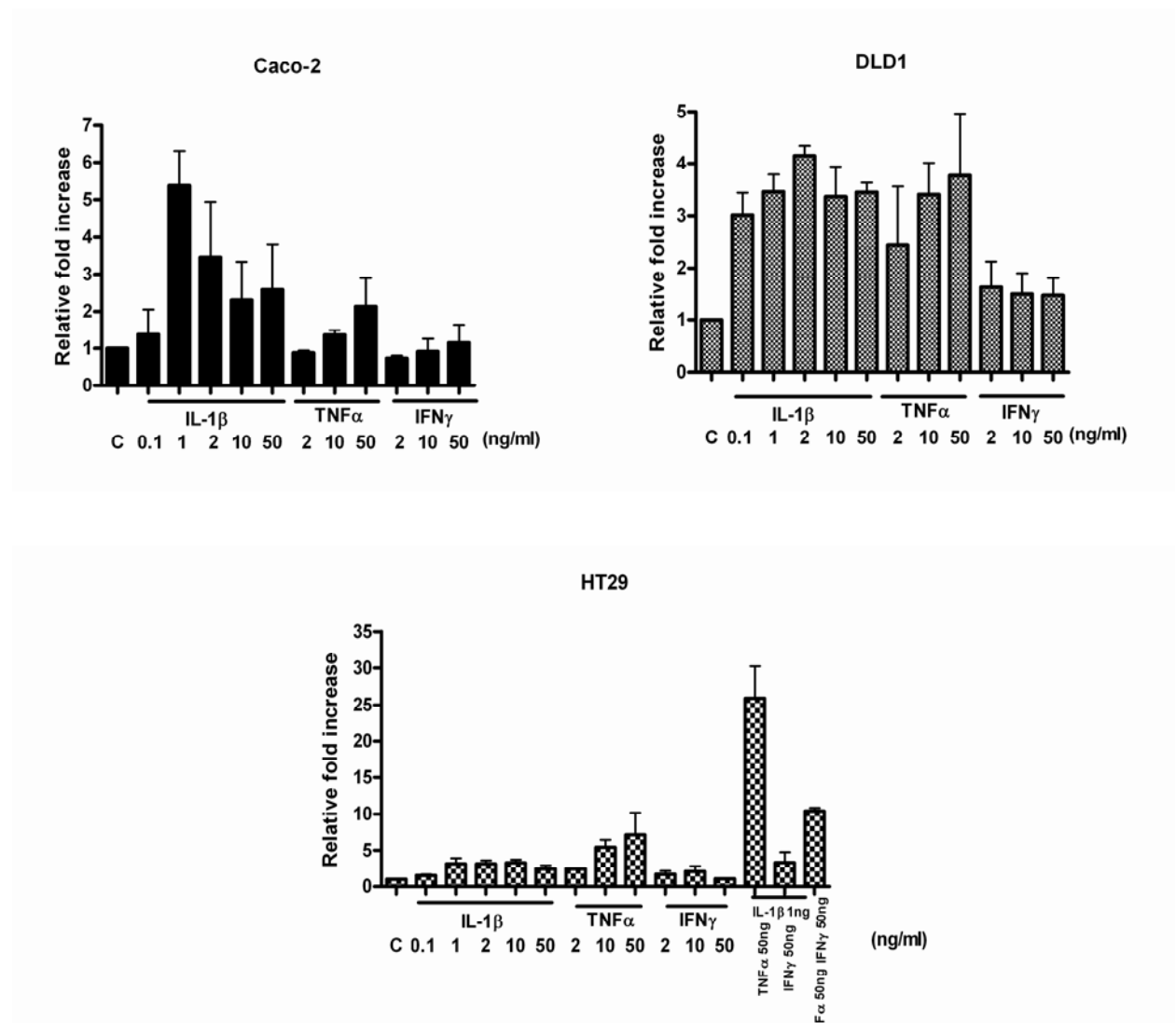
Figure 20: CXCL8 gene promoter analysis in IECs stimulated with cytokines

The CXCL8 gene promoter containing reporter plasmid, pUHC13-3 IL8 and pRL-TK (renilla) plasmids were transiently transfected into Caco-2, HT29 and DLD1 cells. 24 hours after transfection cells were stimulated with cytokines for 24 hours, harvested and promoter activity was monitored by luciferase assay. Data were normalized with renilla luciferase values and included as fold increase vs control. Data represent the means ± SEM of 3-5 independent experiments done in duplicates for each sample.

5.1.7. b. Activation of CXCL10 gene promoter by cytokines in IECs.

TGL-IP10, a luciferase reporter plasmid containing 435 bp of human CXCL10 promoter sequence upstream of the transcriptional start site (Majumder et al., 1998), was used to evaluate the transcriptional role of IL-1β, TNFα and IFNγ alone or in combination with each other in the regulation of CXCL10 gene expression. This plasmid is a kind gift from Dr.Richard M Ranshoff (Cleveland clinic, Ohio) the details of which can be find in the reference (Majumder et al., 1998). When TGL-IP10 transfected Caco-2 cells were stimulated with the single cytokines, IL-1β (1ng/ml) led to a stronger induction of promoter activity than TNFα (50ng/ml) and IFNγ (50ng/ml) (6.53 ± 1.65 fold increase, 2.76±0.53 and 3.14±1.2 fold increase). As in the case of CXCL10 mRNA dose dependent effect of IL-1β on CXCL10 promoter activity was not seen when 2ng/ml, 10ng/ml and 50ng/ml of IL-1β was used to stimulate the cells. The Combination of IL-1β+IFNγ led to a stronger synergistic induction (26.18±11.68) than the combination of TNFα+IFNγ (13.97±3.25 fold increase). In HT29 cells, stimulated with single cytokines, IFNγ (50ng/ml) led to a maximum induction of promoter activity (6.52±0.58 fold increase), followed by 50ng/ml TNFα (4.44±0.16 fold increase) and 1ng/ml IL-1β (1.43±0.05 fold increase). The combination of IL-1β+IFNγ synergistically led to 12.76±3.21 fold increase and TNFα+IFNγ led to 26.70±7.5 fold increase in CXCL10 promoter activity. In the case of DLD1 cells, IFNγ (50ng/ml) led to a maximum induction of (8.06±0.51 fold increase) CXCL10 promoter activity followed by TNFα (50ng/ml) induction (3.59±0.08 fold increase) and IL-1β (1ng/ml) induction (2.32±0.12 fold increase). The combination of IL-1β+IFNγ led to 40.72±12.19 fold increase and TNFα+IFNγ led to 20.56 ± 6.27 fold increase in CXCL10 promoter activity. In all the three cell lines the combination of IL-1β+TNFα had no synergistic effect **(Fig 21)**.
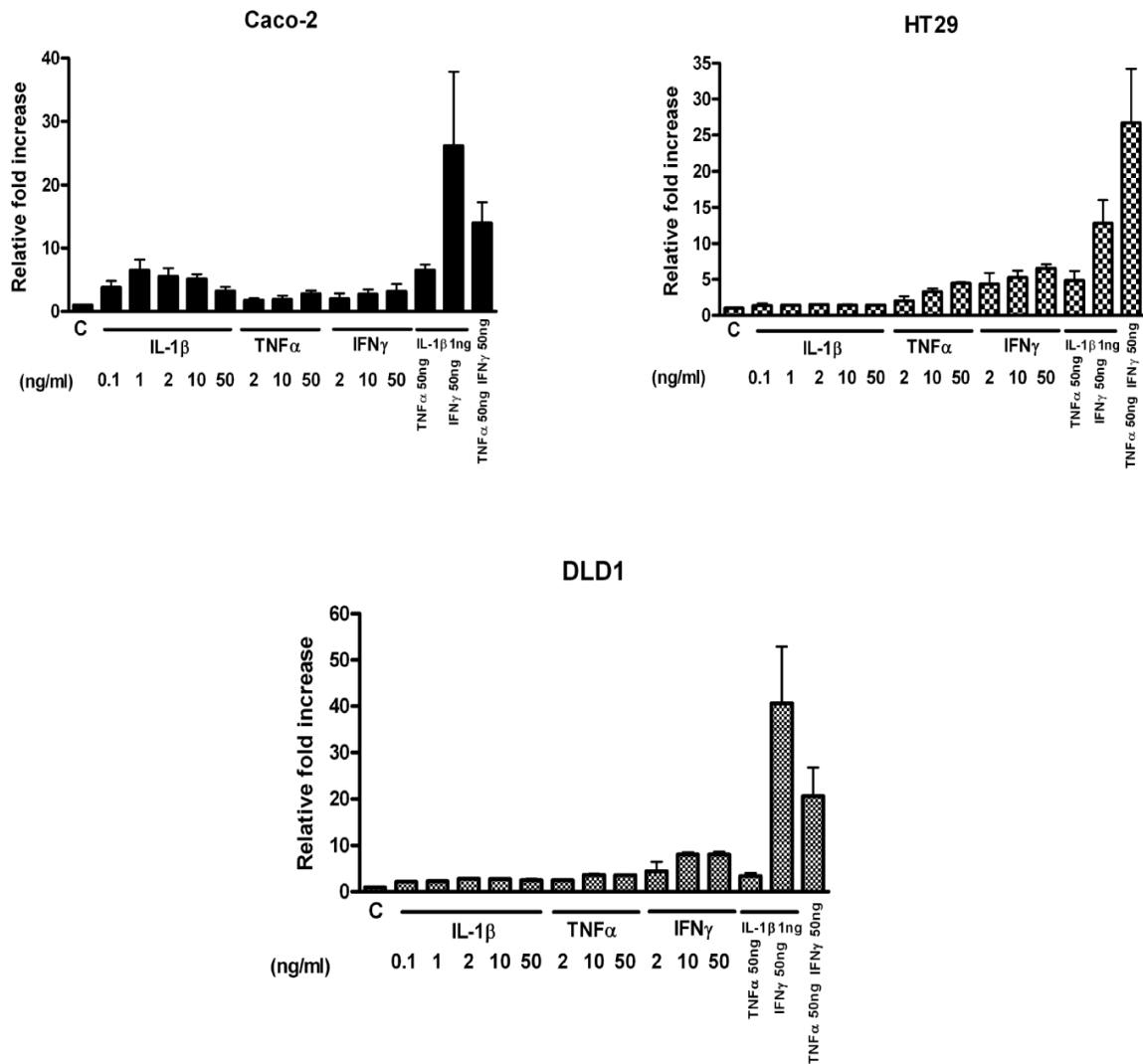
Figure 21: CXCL10 gene promoter analysis in IECs stimulated with cytokines

The CXCL10 promoter containing reporter construct, pTGL-IP10 and pRL-TK (renilla) plasmids were transiently transfected into Caco-2, HT29 and DLD1 cells. 24 hours after transfection cells were stimulated with cytokines as indicated for 24 hours, harvested and promoter activity was monitored by luciferase assay. Data were normalized with renilla luciferase values and included as fold increase vs control Data represent the means ± SEM of 3-5 independent experiments done in duplicates for each sample.

5.1.8. Evaluation of the role of NF-κB in cytokine induced CXCL10 gene expression in IECs.

To evaluate the possible role of NF-κB in cytokine induced CXCL10 gene expression, experiments with a specific NF-κB inhibitor were performed. The effect of BAY 11-7082 on NF-κB inhibition was evaluated by reporter assays. BAY11-7082 was able to dose dependently inhibit IL-1β induced pNF-κB-SEAP gene reporter activity in Caco-2 **(Fig 22)**. Based on this experiment we could determine that 10 μM of BAY11-7082 was sufficient to inhibit the NF-κB activation.

The induction of CXCL10 gene expression by cytokines was inhibited by addition of the NF-κB specific inhibitor BAY11-7082 in Caco-2, HT29 and DLD1 cells with a varying degree of inhibition in individual cell line. Inhibition of NF-κB led to almost complete inhibition (approximately 90-95% inhibition) of CXCL10 mRNA induction and protein secretion induced by cytokines in Caco-2 and DLD1 cells **(Fig 23 and 24)**. In DLD1 cells, IL-1β (1ng/ml) didn't have any inducing effect on CXCL10 secretion. In HT29, inhibition of NF-κB had approximately 60-75% inhibitory effect on cytokine induced CXCL10 mRNA expression and secretion **(Fig 23 and 24).**

To further evaluate the role of NF-κB, transient transfections were done with a CXCL10 promoter construct bearing a mutated NFkB-binding site (TGL-IP10 κB2 Mut). In Caco-2 cells, TGL-IP10 κB2 Mut was not responsive to any of the cytokines and their combinations, suggesting that CXCL10 gene induction is strongly dependent on NF-κB in this cell line **(Fig 25)**. In HT29 and DLD1 cells the combination of TNFα+IFNγ led to 6.49±2.13 and 4.87±1.43 fold increase and the combination of IL-1β+IFNγ led to 2.26±0.63 and 4.75±0.53 fold increase of the TGL-IP10 κB2 Mut promoter activity **(Fig 25)**, revealing that a smaller portion of CXCL10 mRNA might be upregulated independently from NF-κB.

In order to confirm further our results from promoter and inhibitor studies, we did Gel-shift and super shift assays to show that NF-κB is activated by cytokines and that the activated NF-κB complex contains the NF-κB functional subunit p65. In the nuclear extracts of Caco-2 cells, we could clearly see a shift in the samples treated with IL-1β, TNFα alone or the combinations of IL-1β+IFNγ and TNFα+IFNγ. The protein-DNA complex was efficiently competed by unlabelled κB mutant oligo.

There was no shift in the samples treated with the NF-κB inhibitor BAY11-7082, stating that the shift was indeed because of NF-κB binding. Furthermore antibody super shift assay using anti-p65 antibody had clearly shown that the complex contains NF-κB functional sub unit, p65 **(Fig 26)**.

Caco-2 6hr

IL-1β 10ng/ml	-	+	-	-	-	+	+	+
BAY11-7082 (μM)	-	-	1	10	100	1	10	100
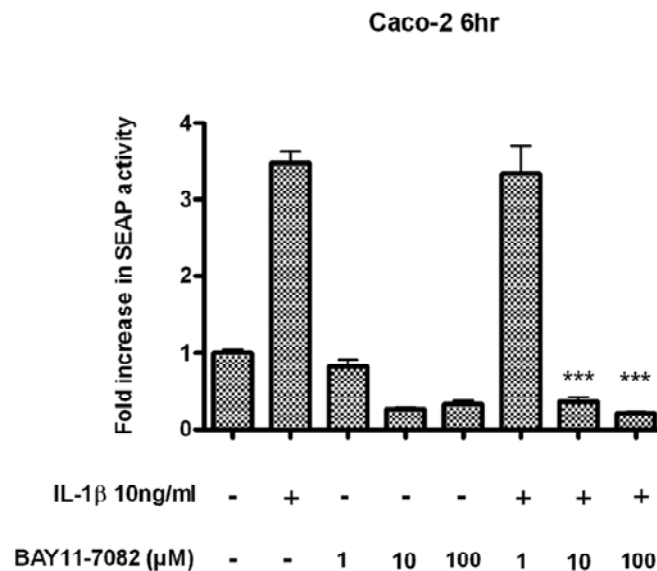
Figure 22: Dose dependent effect of the NF-κB inhibitor BAY11-7082 on IL-1β mediated pNF-κB-SEAP reporter gene activity in Caco-2 cell line

Caco-2 cells were transfected with pNF-kB–SEAP plasmid and 24 hours after transfection cells were stimulated with IL-1β in presence (pretreatment for 1 hour) or absence of the NF-κB inhibitor BAY11-7082 as indicated. Cell supernatant were collected after 6 hours and SEAP assays were performed Data presented are the means ± SEM of 4 independent experiments done in duplicates for each sample. *** corresponds to $p < 0.0005$ (compared to IL-1β stimulation alone).
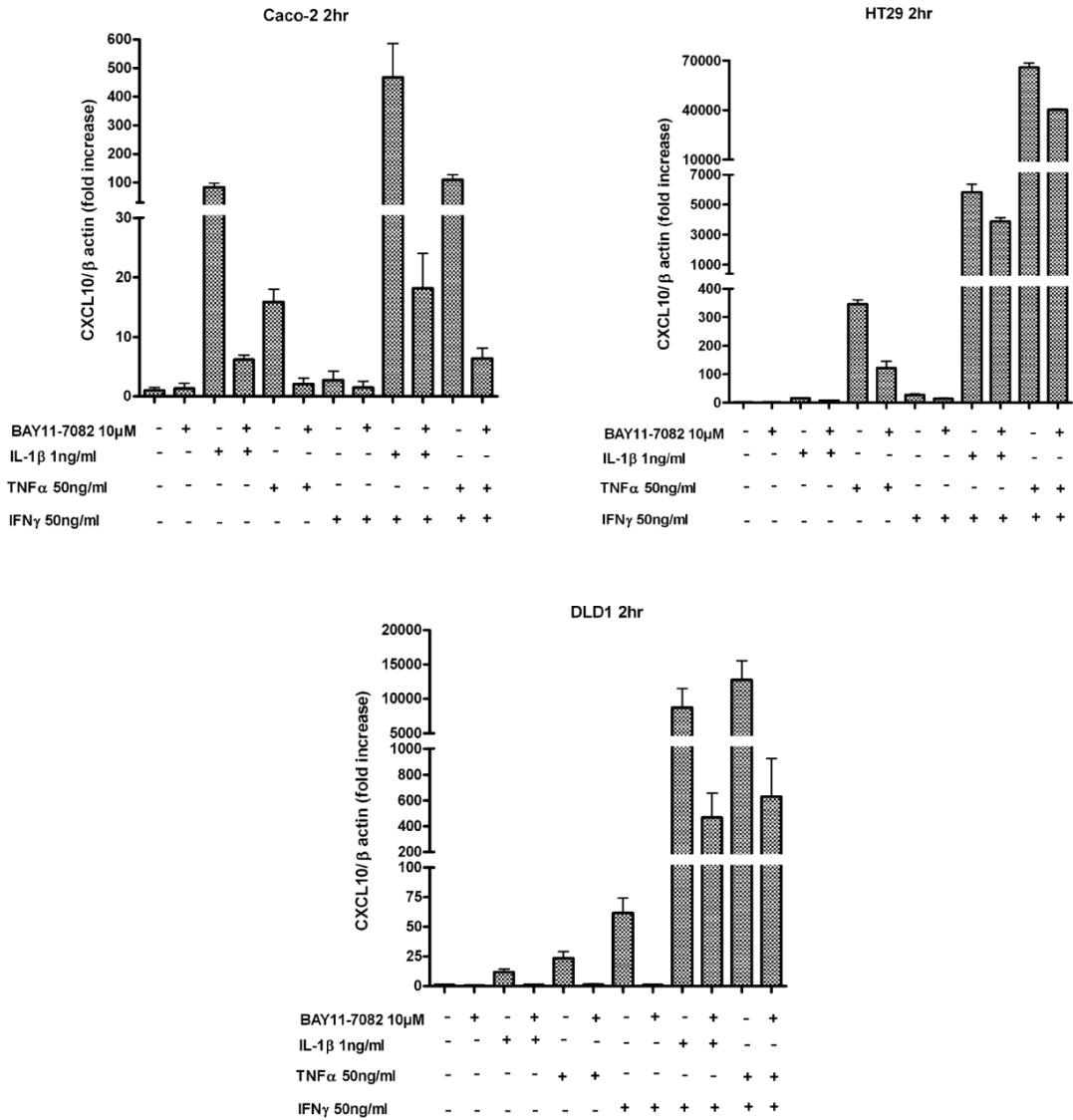
Figure 23: Role of NF-κB in cytokine induced CXCL10 mRNA expression by IECs

5×10^5 cells were plated in 6 well plates and grown for 24 hours. Then cells were pretreated with BAY 11-7082 (10μM) for 1 hour and stimulated with the respective cytokines for 2 hours. Total RNA was isolated, first strand cDNA was prepared from 1μg of total RNA. Real-time PCR was performed using gene specific primers for CXCL10 and β actin. CXCL10 expression was normalized to β-actin expression. Data represent the means ± SEM of 3-5 independent experiments.
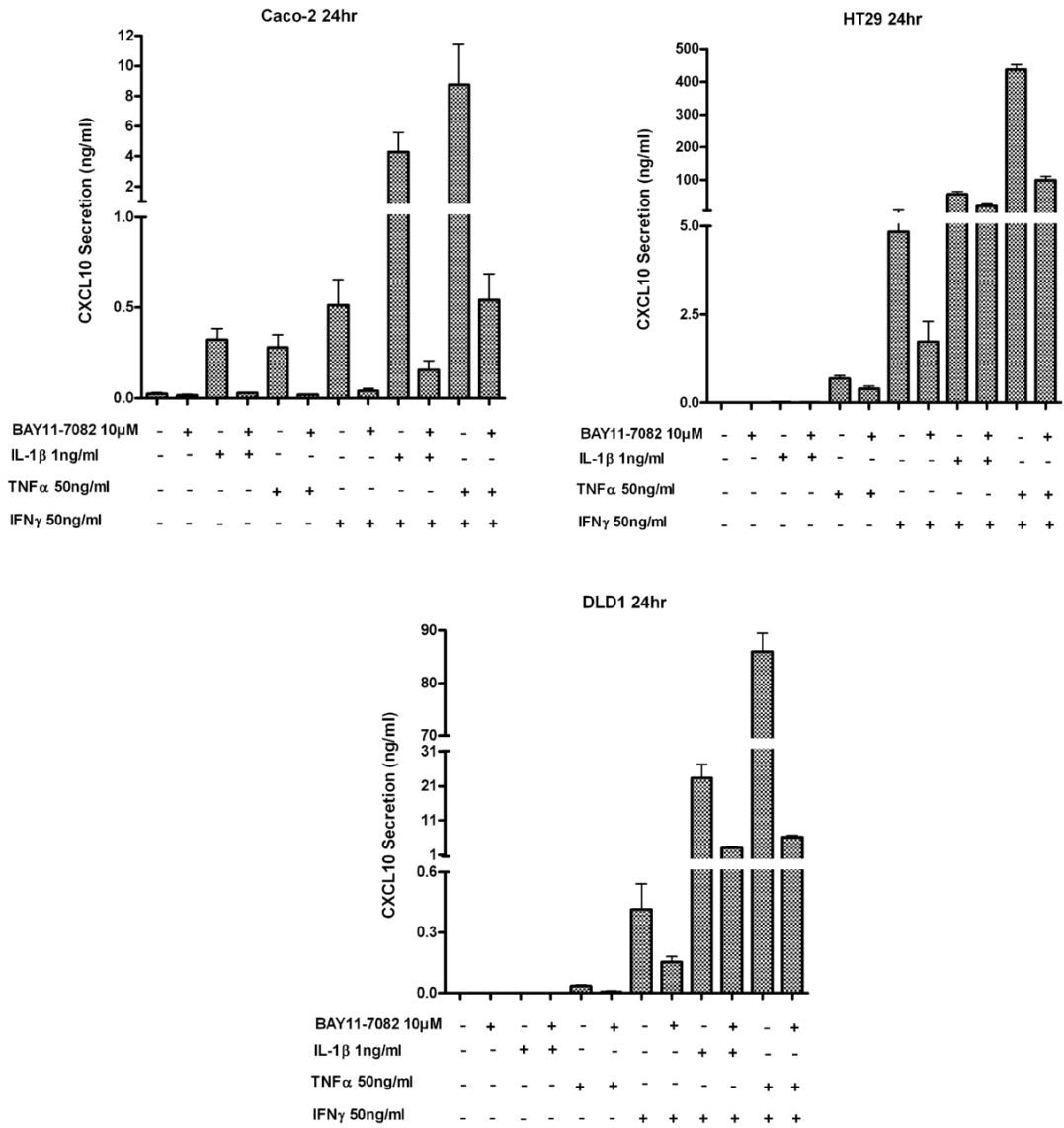
Figure 24: Role of NF-κB in cytokine induced CXCL10 protein secretion by IECs

5×10^5 cells were plated in 6 well plates and grown for 24 hours. Then the cells were pretreated with BAY 11-7082 (10μM) for 1 hour and stimulated with respective cytokines for 24 hours. Cell supernatants were collected and stored at -70°C before CXCL10 protein was measured by ELISA. Data represent the means ± SEM of 3-5 independent experiments done in duplicates for each sample.
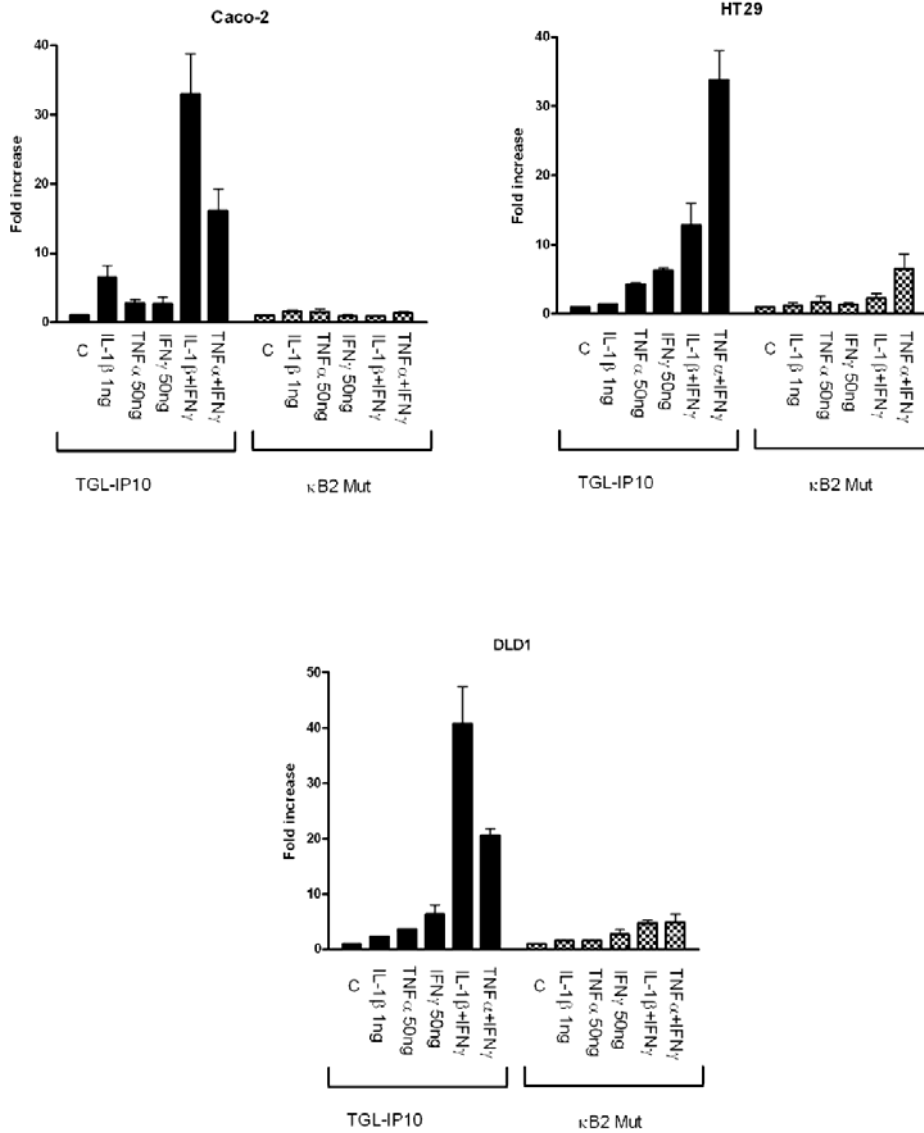
Figure 25: Role of NF-κB binding site in cytokine mediated CXCL10 promoter activation in IECs

TGL-IP10, TGL-IP10-κB2 Mut and pRL-TK (renilla) plasmids were transiently transfected into Caco-2, HT29 and DLD1 cells. 24 hours after transfection, cells were stimulated with cytokines for 24 hours and promoter activity was monitored by luciferase assay. Data were normalized with renilla luciferase values and included as fold increase vs control. Data presented are the means ± SEM of 4 independent experiments done in duplicates for each sample.
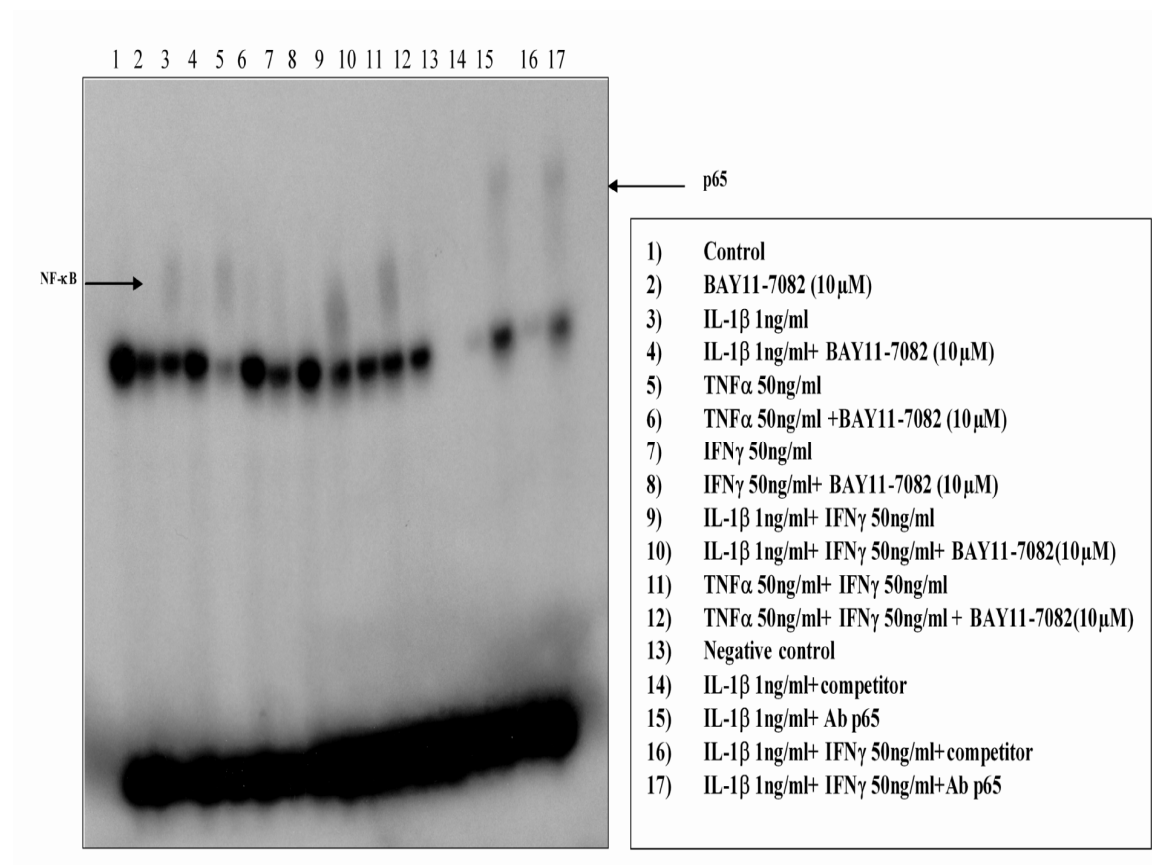
Figure 26: Gel-Shift and Supershift assays for cytokine stimulated NF-κB binding to consensus κB oligonucleotides in Caco-2 cells.

5×10^5 Caco-2 cells were plated in 6 well plates and grown for 24 hours, before pre-treating with BAY11-7082 (10μM) for 1 hour and stimulation with respective cytokines for 30 minutes. Cells were then harvested and nuclear extracts were prepared. 5 μg of nuclear extracts were used for gel shifts with a consensus κB oligonucleotide. Mutant oligonucleotides were used for competition. Super shifts were performed with an anti-p65 antibody. Similar results were obtained in two separate experiments.

5.2. Evaluating the influence of NF-κB inhibitors on IL-1β induced CXCL8 gene expression.

There are several inhibitors that are known to inhibit NF-κB activation with more or less specifity. Therefore effects of a non specific inhibitor (PDTC) and a specific inhibitor of NF-κB (BAY11-7082) on IL-1β induced CXCL8 gene expression in IECs was evaluated.

5.2.1. PDTC and BAY11-7082 dose depent inhibition of IL-1β mediated pNFκB-SEAP reporter gene activity in Caco-2 cells.

In order to find whether PDTC and BAY11-7082 were functional in inhibiting NF-κB we did reporter assays by using pNFκB-SEAP reporter which harbors κB elements. Caco-2 cells transfected with pNFκB-SEAP were pretreated with PDTC (0.2, 2 and 20μg/ml) and BAY11-7082 (1μM, 10μM and 100μM) for 1 hour and then stimulated with IL-1β (1ng/ml) for 6 hours. IL-1β treatment resulted in 4.01±0.416 fold increase of the reporter gene activity. This induction was dose dependently inhibited by PDTC. This showed that PDTC was indeed active in inhibiting NF-κB activation **(Fig 27)**. BAY11-7082 also dose dependently inhibited IL-1β induced pNFκB-SEAP reporter gene activity in Caco-2 cells **(Fig 22)**.
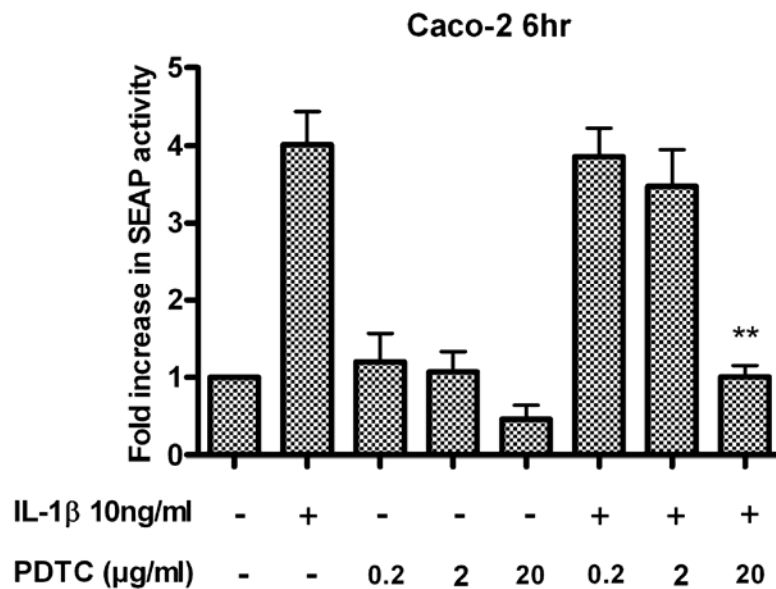
Figure 27: Dose dependent effect of PDTC on IL-1β mediated pNF-κB-SEAP reporter gene activity in Caco-2 cells

Caco-2 cells were transiently transfected with pNF-κB-SEAP plasmid. 24 hours after transfection, cells were pre-treated for 1 hour with increasing concentrations of PDTC (0.2μg, 2μg and 20 μg per ml of medium). After 1 hour, cells were treated with IL-1β and PBS in the control sample, respectively. 6 hours after stimulation, cell supernatants were collected and SEAP assays were performed. Data shown represent means ± SEM of 4 individual experiments done in duplicates for each sample. ** corresponds to p<0.005 (Compared to IL-1β stimulation alone)

5.2.2. PDTC dose dependent enhancement of IL-1β mediated CXCL8 mRNA expression and protein secretion in Caco-2 cells.

Caco-2 cells were pretreated with 0.2, 2 and 20μg/ml of PDTC for 1 hour and then stimulated with IL-1β (1ng/ml) for 1 hour and 24 hours. Real time PCR was done with CXCL8 gene specific primers to check the CXCL8 mRNA expression. PDTC a known inhibitor of NF-κB was expected to inhibit IL-1β induced CXCL8 mRNA, as CXCL8 expression is regulated by NF-κB. Surprisingly IL-1β induced CXCL8 mRNA was enhanced by PDTC in a dose dependent manner. IL-1β induced 117±9.1 fold increase of CXCL8 mRNA which was enhanced to 150 ± 21.6 and 262 ± 62.35 fold increase in the presence of PDTC 2μg/ml and 20μg/ml, respectively. This observation was also confirmed at protein level using ELISA experiments. In Caco-2 cells, IL-1β alone induced 356±6 pg/ml of CXCL8 protein secretion, whereas in the presence of PDTC (0.2 μg/ml, 2 μg/ml and 20 μg/ml) and stimulated with IL-1β lead to 532±43 pg/ml, 872.25±116pg/ml and 995±19.5 pg/ml of CXCL8 secretion **(Fig 28)**.

5.2.3. BAY11-7082 dose dependent inhibition of IL-1β mediated CXCL8 mRNA expression and protein secretion in Caco-2 cells.

As it is known that PDTC inhibits NFκB in a non specific manner we aimed to characterize the role of NFκB in the transcription of CXCL8 gene by using BAY11-7082 a specific inhibitor of IκB phosphorylation. In Caco-2 cells, BAY11-7082 (10μM) dose dependently inhibited IL-1β mediated CXCL8 mRNA expression and protein secretion **(Fig 29)**. IL-1β induced CXCL8 mRNA to 120.85±33.7 fold increase after 1 hour. In the presence of BAY11-7082 at concentrations of 1, 10 and 100μM, IL-1β induced CXCL8 mRNA was inhibited to 119.02±31.35, 8.19±3.73 and 0.97±0.15 respectively. CXCL8 secretion induced by IL-1β reached to 522.49±46.68 pg/ml after 24 hours. In the presence of BAY11-7082 (1, 10 and 100 μM) IL-1β induced CXCL8 protein secretion was inhibited to 429.17±17.09 pg/ml, 328.98±21.81pg/ml and 244.84±5.67 pg/ml.
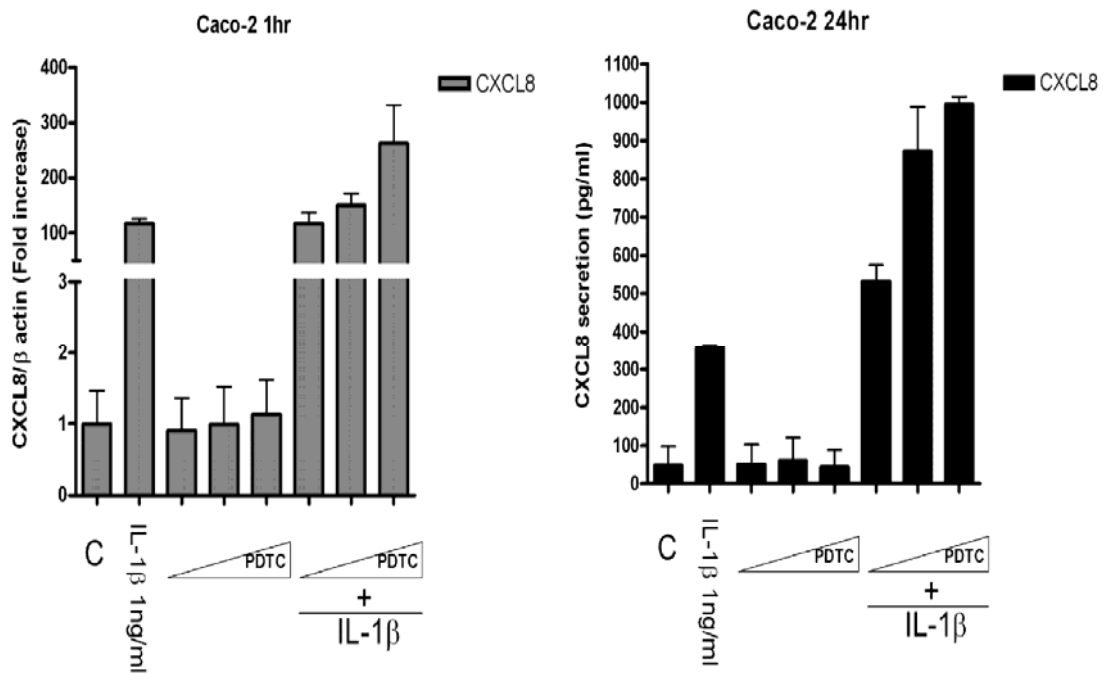
Figure 28: Dose dependent effect of PDTC on IL-1β induced CXCL8 mRNA expression and protein secretion in Caco-2 cells

5×10^5 Caco-2 cells were plated in 6 well plates and grown for 24 hours. Then the cells were pre-treated with 0.2μg, 2μg and 20μg PDTC per ml of culture medium for 1 hour before stimulating with IL-1β for 1 hour. After 1 hour, cells were harvested and total RNA was isolated. cDNA was prepared using 1μg of total RNA. Real-time PCR was performed using CXCL8 gene specific primers and β-actin, which was used as an internal control. After 24 hours cell supernatants were collected and ELISA for CXCL8 protein was performed. Data shown represent the mean of 4 individual experiments ± SEM. For ELISA cells were plated in duplicates for each sample.
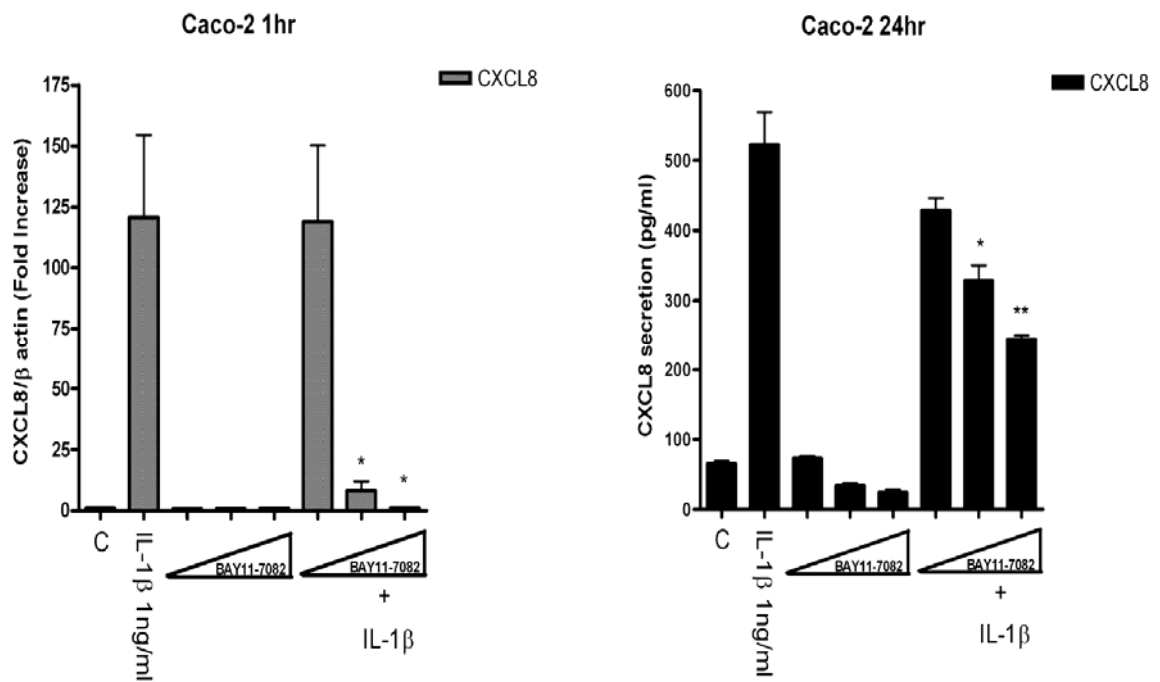
Figure 29: Dose dependent effect of BAY11-7082 on IL-1β induced CXCL8 mRNA expression and protein secretion in Caco-2 cells

5×10^5 Caco-2 cells were plated in 6 well plates and grown for 24 hours. Then the cells were pre-treated with 1μM, 10μM and 100μM of BAY 11-7082 for 1 hour before stimulating with IL-1β for 1 hour (mRNA expression) and 24 hours (protein expression). After 1 hour cells were harvested and total RNA was isolated. cDNA was prepared using 1μg of total RNA. Real-time PCR was performed using CXCL8 gene specific primers and β-actin was used as an internal control. After 24 hours cell supernatants were collected and ELISA for CXCL8 protein was performed. Data shown represent the mean of 4 individual experiments ± SEM. For ELISA cells were plated in duplicates for each sample. * corresponds to $p < 0.05$, ** corresponds to $p < 0.005$ (Compared to IL-1β stimulation alone)

5.2.4. Comparision of the effects of the NF-κB inhibitor PDTC and MAPKinase inhibitors SB203580 and PD 98059 on IL-1β mediated CXCL8 mRNA expression and protein secretion in Caco-2 and HT29 cells.

As IL-1β mediated CXCL8 gene expression is also known to be regulated by MAPKinases via NF-κB, we next sought to examine the effect of the MAPK inhibitors SB203580 (p38 MAPK inhibitor) and PD 98059 (MEK inhibitor) on CXCL8 gene expression induced by IL-1β 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 (10μM) led to a significant reduction in IL-1β induced CXCL8 mRNA expression and protein secretion. In Caco-2, IL-1β (1ng/ml) induced CXCL8 mRNA to19.75±2.52 fold increase which was reduced to 7.52±0.77 fold increase in the presence of SB203580. IL-1β induced CXCL8 secretion in Caco-2 was reduced from 286.79±32.99 pg/ml to 55.50±34.09 pg/ml in the presence of SB203580 (10μM). Pretreatment of Caco-2 cells with both PDTC (20μg/ml) and SB203580 (10μM) couldn't inhibit IL-1β induced CXCL8 mRNA expression and protein secretion. However PDTC mediated enhancement of IL-1β induced CXCL8 mRNA expression and protein secretion was inhibited by SB203580 **(Fig 30)**. We then wondered whether this enhancement effect of PDTC is a 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 case of HT29 cells, PDTC couldn't inhibit IL-1β induced CXCL8 gene expression but it didn't enhance the CXCL8 expression like it was in the case of Caco-2 cells. In HT29 cells, CXCL8 was induced to 11.49±2.39 fold increase 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 secretion in HT29 cells was reduced from 5163.30±777.04 pg/ml to 1157.72±179.59 pg/ml and 1718.86±166.67 in the presence of SB203580 and PD98059 respectively **(Fig 31)**. We used a single time point (4 hours) to check the mRNA expression of both CXCL8 and CXCL10 genes in the same sample to maintain consistency in the treatment conditions.

Figure 30: Comparison of the effects of the NF-κB inhibitor PDTC and MAPKinase inhibitors SB203580 and PD 98059 on IL-1β mediated CXCL8 mRNA expression and protein secretion in Caco-2 cells

5×10^5 Caco-2 cells were plated in 6 well plates and grown for 24 hours. Then the cells were pretreated with 20μg/ml of PDTC, 10μM of SB 203580 and 10μM of PD 98059 for 1 hour before stimulating with IL-1β for 4 hours (mRNA expression) and 24 hours (protein secretion). After 4 hours cells were harvested and total RNA was isolated. cDNA was prepared using 1μg of total RNA and real-time PCR was performed using CXCL8 gene specific primers and β-actin was used as an internal control. After 24 hours cell supernatants were collected and ELISA for CXCL8 protein was performed. Data shown represent the mean of 4 individual experiments ± SEM. For ELISA, cells were plated in duplicates for each sample.
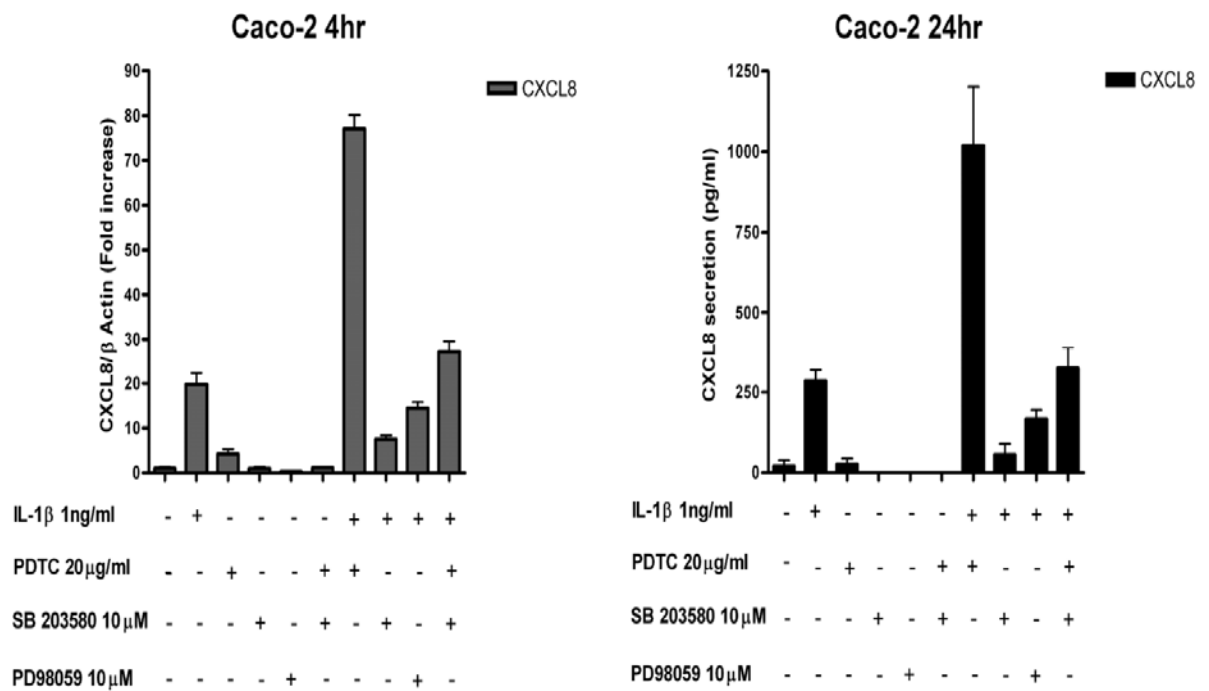
Figure 31: Comparison of the effects of the NF-κB inhibitor PDTC and MAPKinase inhibitors SB203580 and PD 98059 on IL-1β mediated CXCL8 mRNA expression and protein secretion in HT29 cells

5×10^5 HT29 cells were plated in 6 well plates and grown for 24 hours. Then the cells were pre-treated with 20μg/ml of PDTC, 10μM of SB 203580 and 10μM of PD 98059 for 1 hour before stimulating with IL-1β for 4 hours (mRNA expression) and 24 hours (protein secretion). After 4 hours cells were harvested and total RNA was isolated. cDNA was prepared using 1μg of total RNA and real-time PCR was performed using CXCL8 gene specific primers and β-actin was used as an internal control. After 24 hours cell supernatants were collected and ELISA for CXCL8 protein was performed. Data shown represent the mean of 4 individual experiments ± SEM. For ELISA, cells were plated in duplicates for each sample.
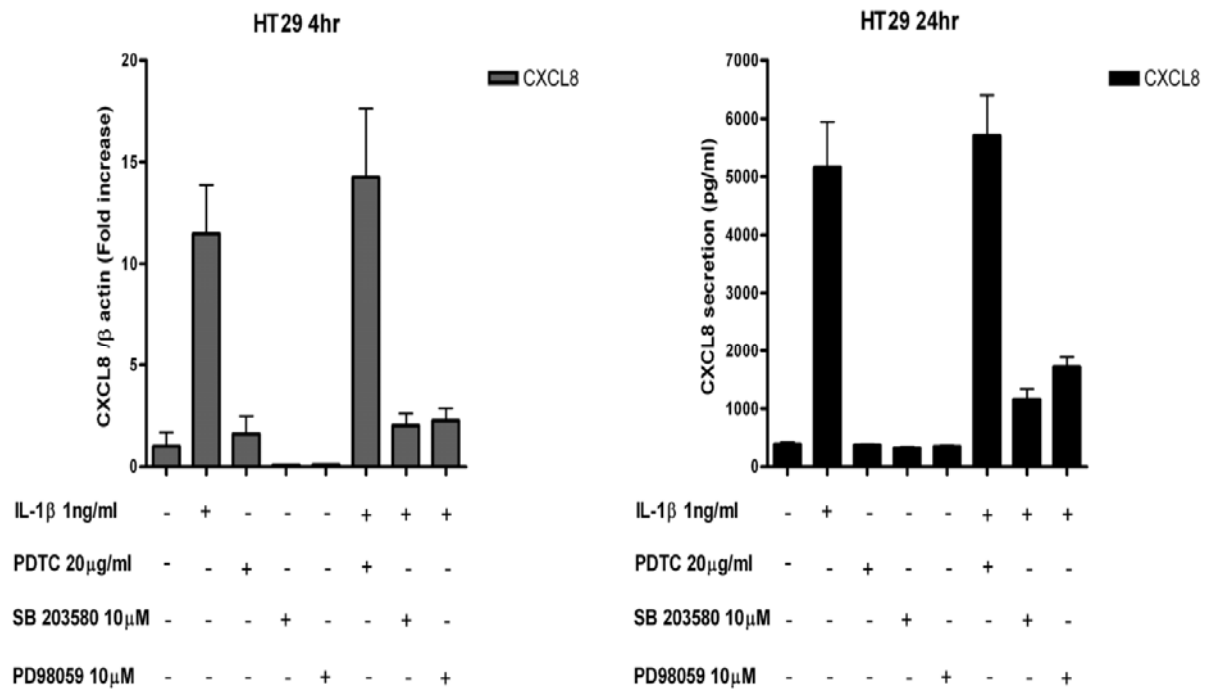
5.2.5. Comparision of the effects of the NF-κB inhibitor PDTC and MAPKinase inhibitors SB203580 and PD 98059 on IL-1β mediated CXCL10 mRNA expression and protein secretion in Caco-2 and HT29 cells.

After we confirmed that the effect of PDTC on CXCL8 expression was cell line dependent, we next evaluated whether the enhancement of IL-1β induced CXCL8 by PDTC is specific to CXCL8 gene or not. For this reason, CXCL10 mRNA expression and protein secretion induced by IL-1β in Caco-2 **(Fig 32)** and HT29 **(Fig 33)** were analysed in the presence of inhibitors. In Caco-2, IL-1β (1ng/ml) induced 55.89±4.84 fold increase of CXCL10 mRNA, which was reduced to 45.42±4.66, 9.51±0.72, 17.03±3.21 and 9.94±1.30 fold increase by PDTC (20μg/ml), SB203580 (10μM), PD98059 (10μM) and SB203580 (10μM)+PDTC (20μg/ml) respectively. IL-1β induced CXCL10 secretion in Caco-2 was reduced from 220.66±13.86 pg/ml to 134.33±28.42 pg/ml, 84.46±16.82 pg/ml,150.66±6.06 pg/ml and 93.21±23.21 pg/ml in the presence of PDTC, SB203580, PD98059 and SB203580+PDTC respectively. In contrast to Caco-2 cells, CXCL10 mRNA expression and protein secretion induced by IL-1β was inhibited to a significant level by PDTC in HT29 cells. In HT29, IL-1β induced 11.49±2.39 fold increase of CXCL10 mRNA and 86.88±26.83 pg/ml of CXCL10 protein secretion. In the presence of the inhibitors PDTC, SB203580 and PD98059, IL-1β induced CXCL10 mRNA was reduced to 5.12±1.50, 10.76±1.15 and 46.12±10.97 folds respectively. Surprisingly PD98059 alone and in the presence of IL-1β lead to upregulation of CXCL10 mRNA but this effect was not seen at the level of CXCL10 protein secretion. In the presence of PDTC, SB203580 and PD98059, IL-1β induced CXCL l0 protein secretion was reduced to 34.93±10.83 pg/ml, 8.70±4.38 pg/ml and 14.54±14.54 pg/ml in HT29 cell line.
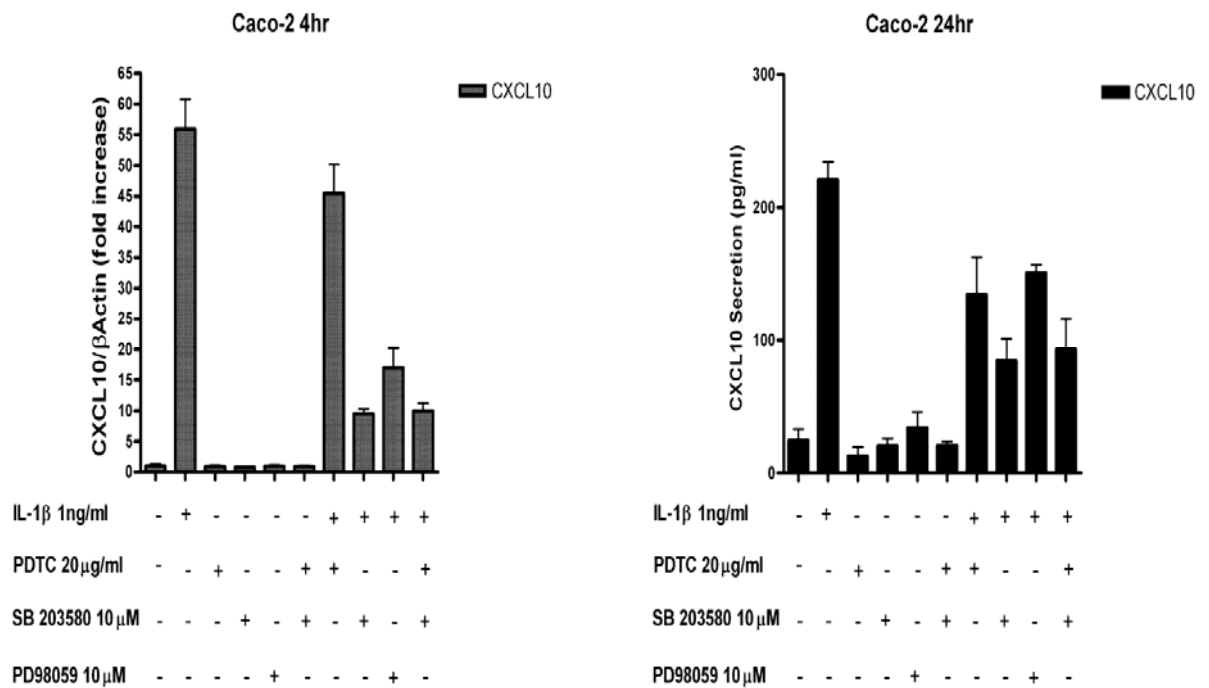
Figure 32: Comparison of the effects of the NF-κB inhibitor PDTC and MAPKinase inhibitors SB203580 and PD 98059 on IL-1β mediated CXCL10 mRNA expression and protein secretion in Caco-2 cells

5×10^5 Caco-2 cells were plated in 6 well plates and grown for 24 hours. Then the cells were pre-treated with 20μg/ml of PDTC, 10μM of SB 203580 and 10μM of PD 98059 for 1 hour before stimulating with IL-1β for 4 hours (mRNA expression) and 24 hours (protein secretion). After 4 hours cells were harvested and total RNA was isolated. cDNA was prepared using 1μg of total RNA and real-time PCR was performed using CXCL10 gene specific primers and β-actin was used as an internal control. After 24 hours cell supernatants were collected and ELISA for CXCL10 protein was performed. Data shown represent the mean of 4 individual experiments ± SEM. For ELISA, cells were plated in duplicates for each sample.

Figure 33: Comparision of the effects of the NF-κB inhibitor PDTC and MAPKinase inhibitors SB203580 and PD 98059 on IL-1β mediated CXCL10 mRNA expression and protein secretion in HT29 cells

5×10^5 HT29 cells were plated in 6 well plates and grown for 24 hours. Then the cells were pre-treated with 20μg/ml of PDTC, 10μM of SB 203580 and 10μM of PD 98059 for 1 hour before stimulating with IL-1β for 4 hours (mRNA expression) and 24 hours (protein secretion). After 4 hours cells were harvested and total RNA was isolated. cDNA was prepared using 1μg of total RNA and real-time PCR was performed using CXCL10 gene specific primers and β-actin was used as an internal control. After 24 hours cell supernatants were collected and ELISA for CXCL10 protein was performed. Data shown represent the mean of 4 individual experiments ± SEM. For ELISA cells were plated in duplicates for each sample.
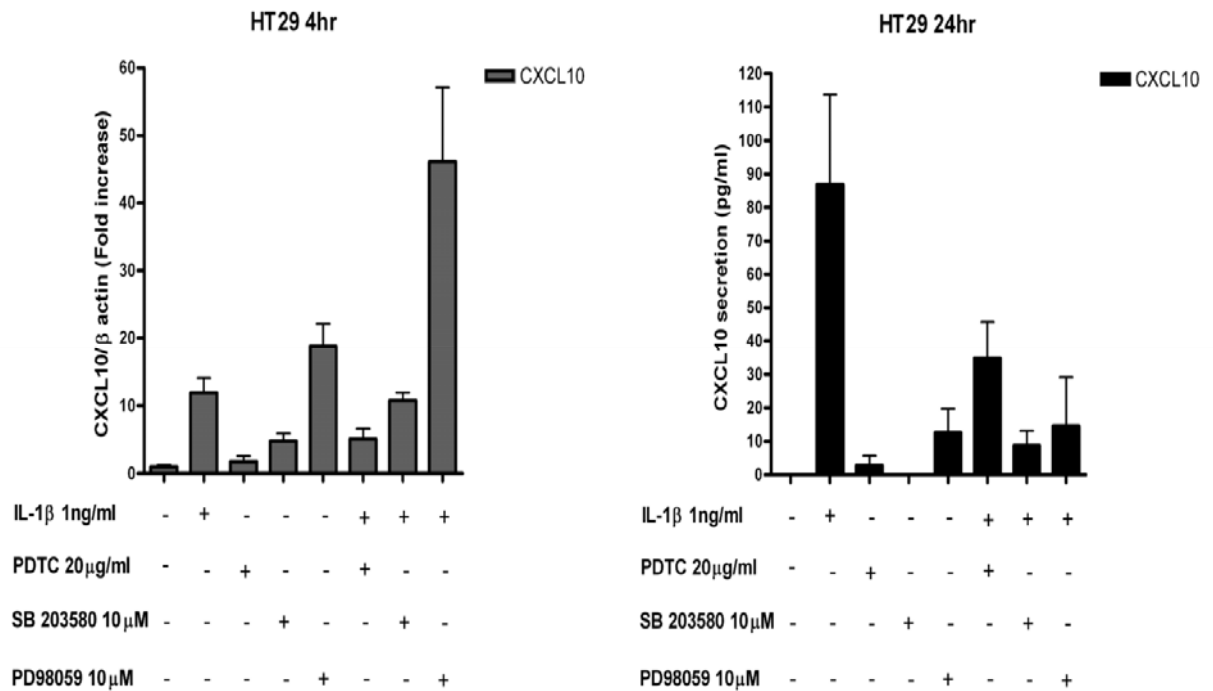
5.3. Effect of Glucocorticoids on Cytokines induced CXCL8 and CXCL10 gene expression in Human IECs.

Previously, Raddatz *et al.,* showed that in Caco-2 cells dexamethasone had a weak and neglectable influence on IL-1β induced, NF-κB activation (Raddatz et al., 2001). They also showed that in IEC-6 cells dexamethasone could inhibit the NF-κB activation induced by IL-1β. For that reason we compared the role of dexamethasone on cytokine induced CXCL8 and CXCL10 gene expression in human IECs and CINC-1 mRNA expression in rat intestinal epithelial cells.

5.3.1. Basal level expression of glucocorticoid receptor alpha (GRα) mRNA in human IECs

In the present study, the differences in basal level GRα mRNA expression in Caco-2, HT29 and DLD1 cells were studied first. Real-time PCR experiments using gene specific primers for GRα revealed that, the basal level GRα mRNA expression was approximately 1000 fold higher in Caco-2 compared to HT29 and DLD1 **(Fig. 34.)**.
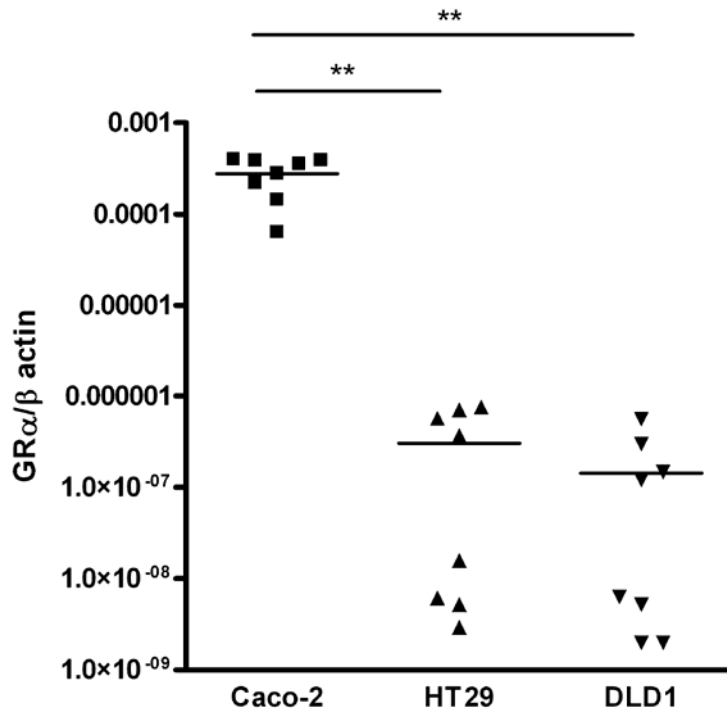
Figure 34: Comparison of basal level GRα mRNA expression in Caco-2, HT29 and DLD1 cells

5×10^5 cells were plated in six well plates and grown for 24 hours and harvested, RNA was isolated and reverse transcribed to cDNA. Real-time PCRs were performed with gene specific primers for GRα. β-actin was used as an internal control and GRα/β-actin ratio was calculated. Data shown represent the mean of transformed CT values (2Δ CT) of 4 independent experiments done in duplicates ± SEM. ** corresponds to P< 0.005.

5.3.2. Effect of dexamethasone on cytokine mediated CXCL8 and CXCL10 mRNA expression in Caco-2.

In the view of previous data (Raddatz et al., 2001), the effect of dexamethasone on IL-1β, TNFα and IFNγ induced CXCL8/CXCL10 mRNA expression in Caco-2 was investigated **(Fig 35)**. Caco-2 cells were pretreated with 1μM dexamethasone and then stimulated with, IL-1β (1ng/ml) and TNFα (10ng/ml) for 1 hour in case of CXCL8 gene or for 4 hours with IL-1β (1ng/ml), TNFα (10ng/ml) and IFNγ (5ng/ml) in the case of CXCL10 gene. Real-time PCR results showed that dexamethasone reduced IL-1β induced CXCL8 mRNA to 82.7%±0.5% and TNFα induced CXCL8 mRNA to 88.5%±14.1% when compared to cells stimulated with the same cytokines in the absence of dexamethasone (100%). IL-1β and TNFα induced CXCL10 mRNA expression was reduced to 81.3%±9.4% and 81%±15% in the presence of dexamethasone. In the presence of dexamethasone, IFNγ induced CXCL10 mRNA even tended to increase to 108%±16% when compared with cells stimulated with the same cytokines in the absence of dexamethasone (100%).

5.3.3. Effect of dexamethasone on cytokine mediated cytokine induced neutrophil chemoattractant-1 (CINC-1) mRNA expression in rat intestinal epithelial cell line, IEC-6.

Previous study showed that dexamethasone inhibited IL-1β and TNFα induced CINC-1 (homologue of human GRO alpha) gene expression in rat kidney epithelial cell line (Ohtsuka et al., 1996). So we checked the effect of dexamethasone on IL-1β and TNFα induced CINC-1 mRNA expression in rat intestinal epithelial cell line, IEC-6. When IEC-6 was stimulated with IL-1β (1ng/ml) and TNFα (10ng/ml) with and without dexamethasone for 4 hours, real-time PCR revealed that in presence of dexamethasone IL-1β induced CINC-1 mRNA expression was reduced to 49.9%±11.4% and TNFα induced CINC-1 mRNA expression was reduced to 30.7%±2.3% when compared to cells stimulated with the same cytokines in the absence of dexamethasone (100%), showing a clear inhibition of IL-1β and TNFα induced CINC-1 mRNA expression by dexamethasone **(Fig 36)**.
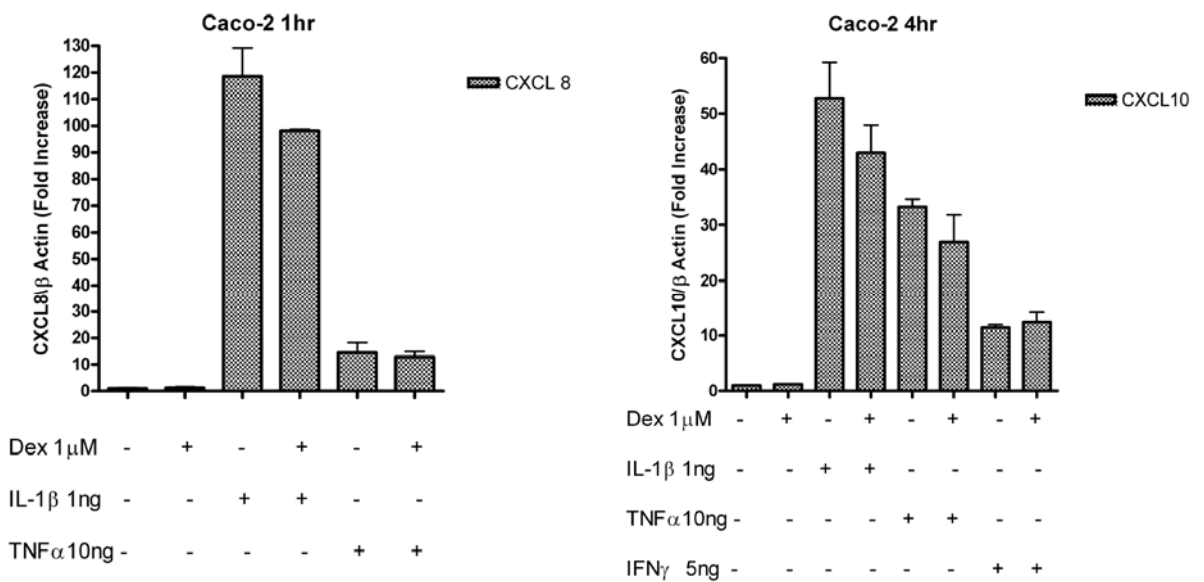
Figure 35: Effect of dexamethasone on cytokine mediated CXCL8 and CXCL10 mRNA expression in Caco-2 cells

5×10^5 Caco-2 cells were plated into 6 well plates and grown for 24 hours. Then cells were pretreated with dexamethasone (1µM) for 1 hour before stimulating them with IL-1β (1ng), TNFα (10ng) and IFNγ (5ng) as indicated. Cells were harvested at 1 hour and 4 hours, total RNA was isolated, first strand cDNA was prepared from 1µg of total RNA and real-time PCR using gene specific primers was performed for CXCL8, CXCL10 and β-actin, which was used as an internal control. Data are shown as the fold increase compared to the baseline controls and represent the means of 3 individual experiments ± SEM.
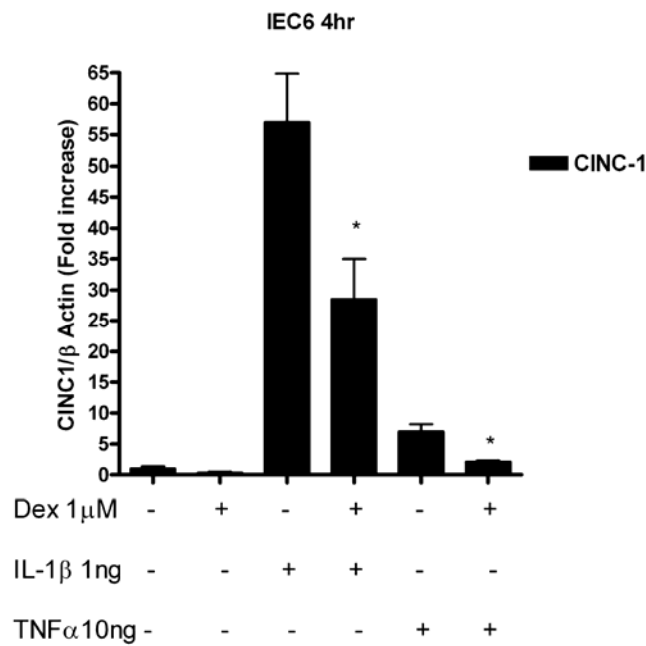
Figure 36: Effect of dexamethasone on cytokine mediated CINC-1 mRNA expression in IEC-6 cells

5×10^5 IEC-6 cells were plated into 6 well plates and grown for 24 hours Then cells were pretreated with dexamethasone (1µM) for 1 hour before stimulating them with either IL-1β (1ng) or TNFα (10ng) for 4 hours. Cells were harvested after 4 hours and total RNA was isolated. First strand cDNA was prepared from 1µg of total RNA and real-time PCR using gene specific primers was performed for CINC-1and rat β-actin, which was used as an internal control. Data are shown as the fold increase compared to the baseline controls, represent the mean of 4 individual experiments ± SEM. * Corresponds to $p < 0.05$ (Compared to their respetive cytokine stimulation alone)

5.3.4. Effect of Dexamethasone on cytokine mediated CXCL8 and CXCL10 Protein secretion in Caco-2 and HT29 cells.

ELISA for CXCL8 and CXCL10 proteins were done from cell culture supernatants of Caco-2 and HT29 treated with IL-1β (1ng/ml), TNFα (10ng/ml) and IFNγ (5ng/ml) in the presence or absence of dexamethasone, for 24 hours. ELISA results showed that dexamethasone was ineffective in inhibiting cytokine induced CXCL8 **(Fig 37)** and CXCL10 **(Fig 38)** protein secretion in both, Caco-2 and HT29 cells. In the presence of dexamethasone IL-1β induced 88.03%±19.7% (Caco-2) and 95.6%±6.5% (HT29), whereas TNFα induced 83.8%±21.2% (Caco-2) and 90.9%±3.1% (HT29) CXCL8 secretion when compared to cells stimulated with the same cytokines in the absence of dexamethasone (100%). In case of CXCL10 protein, IL-1β induced 92.6%±17.2% (Caco-2) and 109.5%±3.7% (HT29), whereas TNFα induced 108.3%±35.6% (Caco-2) and 78.5%±5.9% (HT29), IFNγ induced 121.9%±15.9% (Caco-2) and 94.9%±16.5% (HT29) of CXCL10 protein secretion in the presence of dexamethasone when compared to cells stimulated with the same cytokines in the absence of dexamethasone (100%).
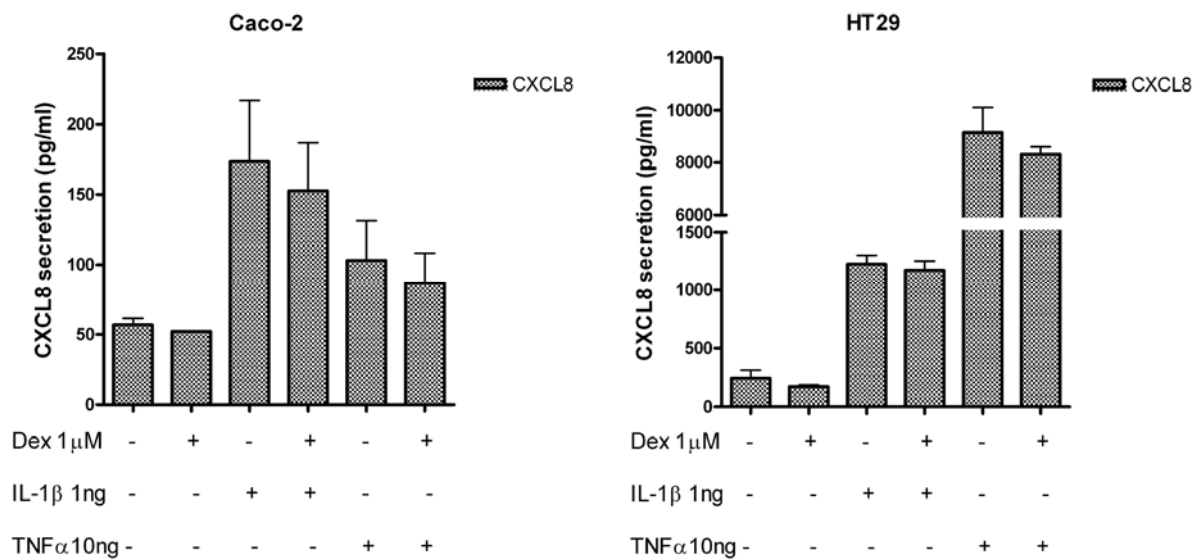
Figure 37: Effect of dexamethasone on cytokine mediated CXCL8 protein secretion in Caco-2 and HT29 cells

5×10^5 cells were plated into 6 well plates and grown for 24 hours. Then cells were pretreated with dexamethasone (1μM) for 1 hour before stimulating them with either IL-1β (1ng) or TNFα (10ng) for 24 hours. Cell supernatants were collected after 24 hours and ELISA for CXCL8 protein was performed. Data shown represent the means of 3 individual experiments ± SEM done in duplicates for each sample.
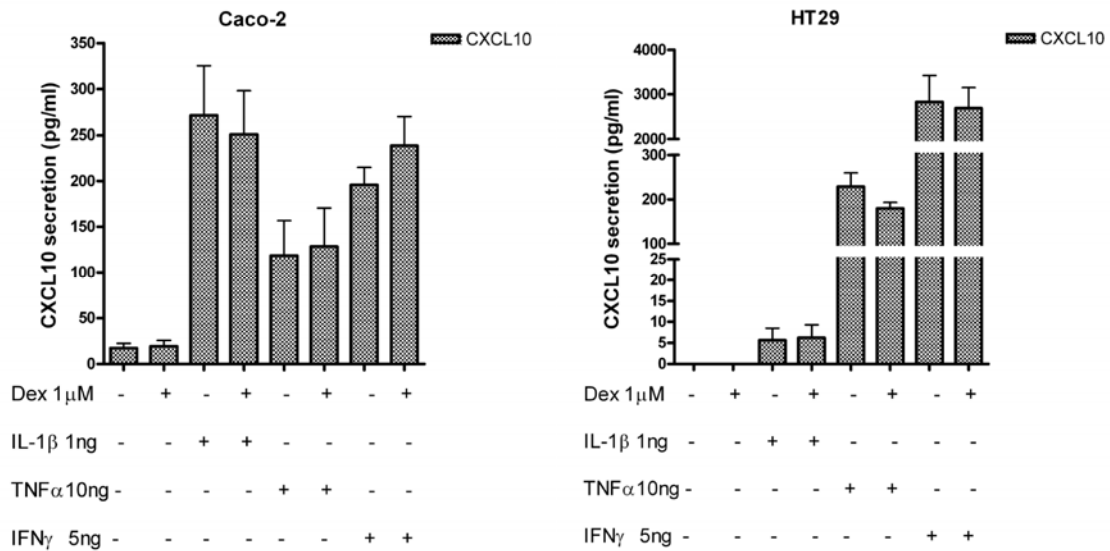
Figure 38: Effect of dexamethasone on cytokine mediated CXCL10 protein secretion in Caco-2 and HT29 cells

5×10^5 cells were plated into 6 well plates and grown for 24 hours. Then cells were pretreated with dexamethasone (1μM) for 1 hour before stimulating them with IL-1β (1ng), TNFα (10ng) and IFNγ (5ng) for 24 hours. Cell supernatants were collected after 24 hours and ELISA for CXCL10 protein was performed. Data shown represent the mean of 3 individual experiments ± SEM done in duplicates for each sample.

6. Discussion

In the present study, cytokine mediated CXCL8 and CXCL10 gene expression and the signalling mechanisms involved were studied in intestinal epithelial cells. The crypt-villus axis of the intestinal mucosa is composed of a dynamic cell population in perpetual transition from a proliferative, undifferentiated stage to mature surface villus epithelial cells. The migration from the crypt base to the surface of the colon is accompanied by cellular differentiation that leads to important morphological and functional changes. The cell lines used in this study have similar characteristics to some cells of the crypt-villus axis. Caco-2 cells were previously described to possess characteristics of normal small intestinal epithelium (Jumarie and Malo, 1991) whereas HT29 and DLD1 cells were known to possess the characteristics of surface villus cells (Panja et al., 1998). In the present study it was shown that proinflammatory cytokines which may be produced by submucosal immune cells can differentially and coordinately induce the gene expression of inflammatory chemokines CXCL8 and CXCL10 in intestinal epithelial cells.

Real-time PCR experiments in Caco-2, HT29 and DLD1 cells showed that, they differentially expressed basal levels of CXCL8 and CXCL10 mRNA **(Fig 11)**. We found that CXCL8 mRNA expression is the highest in HT29 cells followed by DLD1 and Caco-2 cells, whereas in the case of CXCL10, its basal mRNA expression was the highest in Caco-2 cells followed by HT29 and DLD1 cells. Studies performed in normal and IBD patients had shown that, intestinal epithelial cells from healthy subjects also produce chemokines basally, suggesting that the differences in the basal expression of CXCL8 and CXCL10 mRNA in the cell lines used in this study may mimic *in vivo* conditions in normal intestinal epithelium (Banks et al., 2003). This differential expression of CXCL8 and CXCL10 genes may be due to the differences in the constitutive activation of transcription factors like nuclear factor kappa B (NF-κB), signal transducer and activator protein 1 (STAT1), and interferon regulatory factor 1 (IRF-1) in a cell type specific manner. NF-κB is known to be constitutively activated in some colorectal cancers and also in IBD patients (Lind et al., 2001; Neurath et al., 1998). STAT1 is known to be constitutively activated in certain cells (Kirito et al., 2002) and in pathological conditions like acute myelogenous leukemia

(Spiekermann et al., 2001) and in airway epithelial cells of asthma patients (Sampath et al., 1999). IRF-1 was shown to be constitutively expressed and spatially regulated in the mouse lens (Li et al., 1999) but such studies in IBD patients or in human intestinal epithelial cells are still lacking.

Dose dependency studies were done to find out the responsiveness of Caco-2, HT29 and DLD1 cells to cytokines in inducing CXCL8 and CXCL10 gene expression. Based on the previous studies done in HT29 cells and other IECs (Eckmann et al., 1993; Schuerer-Maly et al., 1994; Yang et al., 1997), we selected the 4 hours time point for performing dose dependency experiments. Caco-2, HT29 and DLD1 cells, when stimulated with TNFα showed a dose dependent induction of both CXCL8 and CXCL10 mRNA expression levels. IFNγ was ineffective in inducing CXCL8 mRNA expression but dose dependently induced CXCL10 mRNA expression in all three cell lines. IL-1β didn't show a dose dependent effect although it induced both CXCL8 and CXCL10 mRNA expression **(Fig 12)**. This could be explained by the possible occupance of all the available IL-1β receptors on the cells. Caco-2 cells, when treated with lower doses of IL-1β (0.1ng, 0.5ng and 1ng) showed a dose dependent induction of CXCL8 and CXCL10 mRNA expression supporting the above observation **(Fig 13)**.

The CXCL8 gene expression in all three cell lines followed a time course that is grossly similar to that of other NF-kB dependent genes, being characterized by an immediate increase of mRNA within minutes and an early maximum at approximately 1 hour followed by a rapid decrease **(Fig 14)**. IFNγ alone didn't have any effect on CXCL8 mRNA expression. While in Caco-2 and DLD-1 cells, IL-1β was the most potent inducer of CXCL8 mRNA, TNFα led to the strongest induction in HT-29 cells. Previously, Bocker *et al.,* had shown that, cellular differentiation of HT29 cells selectively impairs the IL-1β signalling pathway, inhibiting both NF-kB and JNK activity in response to IL-1β (Bocker et al., 2000). This possibility can be ruled out as we used undifferentiated HT29 cells rather than methotrexate differentiated HT29 cells. The authors also demonstrated a rapid degradation of IL-1 receptor-associated kinase (IRAK) protein in Caco-2 cells and altered low IRAK degradation in HT29 cells, irrespective of their differentiation state.

Therefore a possible explanation, for this relative low responsiveness to IL-1β, could be a relative low expression of IL-1β receptors in HT29 cell line compared to that of the other two cell lines. In fact, Asit Panja *et al.*, could previously show that Caco-2, HT29 and DLD1 cells have a different expression of IL-1β and TNFα receptors (Panja et al., 1998). They demonstrated that IL-1β receptors are most abundantly expressed in Caco-2 and DLD1 cells than HT29 cells, whereas TNFα receptors are expressed most abundantly on HT29 cells followed by Caco-2 and DLD1 cells. This suggests that the differential regulation of CXCL8 gene expression by TNF-α and IL-1β may depend on the number of available receptors and also on the cell type studied. Also it might probably be relative to the type and number of downstream signalling molecules found in a particular cell type as discussed earlier (Bocker et al., 2000).

The combination of TNFα+IL1β was not more potent in inducing CXCL8 mRNA in Caco-2 and DLD1 cells than the single cytokines alone, suggesting that the single cytokine incubation might have led to a full activation of available intracellular signalling molecules and/or transcription factors (e.g.NFκB). However, the combination of TNFα+IFNγ in HT29 cells led to a later maximum but a prolonged upregulation of CXCL8 mRNA compared to TNFα alone **(Fig 14B)**. As CXCL8 gene is not known to possess IFNγ responsible elements in its promoter regions (Holtmann et al., 1999), we hypothesized an IFNγ dependent upregulation of signalling molecules or transcription factors involved in TNFα signalling. Indeed, previous studies in other cell models (Bebo and Linthicum, 1995; Carrel et al., 1995; Ruggiero et al., 1986) have shown an effect of IFNγ on TNF receptor expression. In fact, we could also show that IFNγ induced the expression of TNFR 2 mRNA in HT29 cells, supporting the most probable explanation for this synergism **(Fig 19).**

We found that unstimulated Caco-2 and HT29 cells basally secreted CXCL8 protein. CXCL8 gene is constitutively upregulated in a variety of human cancers such as melanoma, lung, gastric, prostate and bladder cancers (Inoue et al., ; Kitadai et al., 1998; Luca et al., 1997; Schadendorf et al., 1993). Interestingly, upregulation of CXCL8 has been recently linked to β-catenin activation based on microarray analysis of differentially expressed genes between normal and neoplastic colon (Peifer and

Polakis, 2000). Therefore, the basal CXCL8 protein secretion in the current cell lines may be the result of a constitutively active β-catenin signalling pathway. In Caco-2 cells, when stimulated with cytokines, maximum CXCL8 protein was secreted within 3 hours followed by a slight increase after 24 hours. In contrast, HT29 cells were able to produce increasing amounts of CXCL8 protein with time **(Fig 15)**. Transient transfections with a CXCL8 promoter containing plasmid had revealed a bell-shape effect of IL-1β on this promoter activity in all three cell lines used. TNFα led to a stronger CXCL8 promoter activity when compared to IL-1β in HT29 cells but not in Caco-2 and DLD1 cells which was similar to the CXCL8 mRNA induction in the same cell lines. As there wasn't any synergistic effect of cytokine combinations, experiments with combinations of cytokines for the transient transfection studies in Caco-2 and DLD1 cells were not performed. However in HT29 cells, the effect of the combination of cytokines on CXCL8 promoter activity was investigated **(Fig 20)**. The combination of TNFα+IFNγ led to a synergistic induction of the promoter activity similar to the case of CXCL8 mRNA levels **(Fig 14B)**. In addition, we detected a strong synergistic effect of IL-1β+TNFα, but we were unable to detect this synergism either at the level of CXCL8 mRNA induction or protein secretion, suggesting a rate limiting step beyond transcription for example mRNA processing etc.

Dwinell *et al.,* (Dwinell et al., 2001) have previously shown that the stimulation of IECs with TNFα or IL-1α strongly potentiated IFNγ induced CXCL10 gene expression. However, it is well known that among IL-1α and IL-1β, only the latter is secreted and has an active role in inflammation. Though the synergistic effects of TNFα+IFNγ on CXCL10 gene regulation and the signalling mechanisms involved were studied in astrocytes (Majumder et al., 1998) and on iNOS expression in IECs and macrophages (Saura et al., 1999) had been studied in detail, the synergistic action of these cytokines on CXCL10 expression and the signalling mechanisms involved have not been evaluated in IECs. Especially the role of IL-1β alone and its synergism with both TNFα and IFNγ on CXCL10 gene expression in IECs has been given no attention so far.

In light of the findings that IECs may express elevated levels of CXCL10 (Dwinell et al., 2001) and its expression is increased in the mucosa of patients with IBD (Uguccioni et al., 1999), we assessed whether the stimulation of IECs in culture by inflammatory mediators could induce CXCL10 gene expression. All three intestinal epithelial cell lines used in this study expressed CXCL10 mRNA in a concentration dependent manner when stimulated with IFNγ and TNFα alone **(Fig 12B)**. These data support a vast body of evidence that demonstrate that IFNγ can induce CXCL10 gene expression in a variety of cell types including granulocytes, T lymphocytes, bronchial epithelial cells and keratinocytes (Hardaker et al., 2004; Kanda and Watanabe, 2002), while TNFα is a potent inducer for CXCL10 in hepatocytes (Narumi et al., 2000). Of note, in addition to these data, we could also show that Caco-2 cells stimulated with IL-1β upregulated CXCL10 mRNA to a significant level, whereas in HT29 and DLD1, IL-1β led to a weaker induction of CXCL10 mRNA when compared to the induction by TNFα and IFNγ **(Fig 12B)**.

A number of authors have reported functional synergy between TNFα and IFNγ in promoting gene expression for proteins such as RANTES, IRF-1, MIG, and ICAM-1 (Czech et al., 1993; Lee et al., 2000; Ohmori et al., 1997; Pine, 1997) in a variety of cell types. Previously, it has been shown that this synergy depends on the coexistence of TNFα-responsive NF-κB binding sites and IFNγ-responsive STAT binding elements within the promoters of the genes of interest (Lee et al., 2000; Ohmori et al., 1997). In the present study, when IFNγ+TNFα were combined, the accumulation of CXCL10 mRNA and protein was elevated compared to cells stimulated with either cytokine alone, implying an additive effect on transcription of the CXCL10 gene **(Fig 16 and 17)**. A synergistic effect also on CXCL10 expression was demonstrated by these two cytokines in astrocytes and this synergy has been shown to be at least in part dependent on NF-κB activation and binding to a NF-κB binding site within the CXCL10 promoter (Majumder et al., 1998).

A synergism of IL-1β with IFNγ in IECs has not been characterised so far. Recently, Takami *et al.* showed the synergistic induction of hepatocyte growth factor in human skin fibroblasts by IL-1β+IFNγ (Takami et al., 2005). Here we report for the first

time, that in intestinal epithelial cell lines, IL-1β in combination with IFNγ, synergistically induced CXCL10 gene expression. In Caco-2 cells, the combination led to earlier synergistic induction of both CXCL10 mRNA and protein than the combination of IFNγ+TNFα (**Fig 16A**). This early induction of CXCL10 mRNA and protein by IL-1β+IFNγ in Caco-2 cells might be due to the early convergence of signalling pathways that activates transcription factors like NF-κB, IRF-1 and STAT1 etc or factors that mediate these transcription factor interactions. CBP, a transcriptional coactivator was shown to co-operate with IFN-γ/STAT1 and TNFα/NF-κB induced transcriptional synergistic activation of CXCL9 promoter, providing a stable scaffold for RNA polymerase II (Hiroi and Ohmori, 2003). They demonstrated that the STAT1/NF-κB-dependent transcriptional synergy could result from the enhanced recruitment of RNA polymerase II complex to the promoter via simultaneous interaction of CBP with STAT1 and NF-κB. Based on this, we hypothesized that the early induction of CXCL10 in Caco-2 by the combination of IL-1β+IFNγ might result in the faster recruitment of CBP and RNA polymerase II whereas this could be delayed in the case of IFNγ+TNFα combination.

Majumder *et al.,* had previously shown an important role of p48/STAT-1 and NF-kB proteins in the synergistic action of TNFα+IFNγ on human CXCL10 promoter in astrocytes (Majumder et al., 1998). In order to find out the signalling mechanism involved, we first explored the role of NF-κB in the regulation of CXCL10 gene in intestinal cell lines. It's a known fact that the activation of IL-1β receptor I by IL-1β activates at least two intracellular pathways; MAP kinases and the NF-κB pathway (Karin and Greten, 2005). NF-κB, one of the major transcriptional regulators of many genes, is known to be highly activated in IBD. A number of studies had revealed that cytokines like IL-1β, TNFα, enteroinvasive bacteria and the bacterial cell wall product LPS induce the expression of several genes through the transcription factor NF-κB in IECs (Elewaut et al., 1999; Kim et al., 2004; Kim et al., 2002; Vallee et al., 2004). So we aimed at evaluating the role of NF-κB in cytokine mediated CXCL10 gene expression in IECs.

In our study, NF-κB inhibition with BAY11-7082 clearly inhibited both CXCL10 mRNA and protein induced by single cytokine alone or the synergistic combinations used, in Caco-2, HT29 and DLD1 cells **(Fig 23 and 24)**. Transient transfection experiments with a κB2 mutant of CXCL10 gene promoter in the three cell lines used showed that binding of NF-κB to the CXCL10 promoter plays a major role in its gene regulation at the level of transcription **(Fig 25)**. Gel-shift experiments in Caco-2 had confirmed that NF-κB is activated upon treating Caco-2 cells with either IL-1β or TNFα. NF-κB was also activated in Caco-2 treated with the combinations of IL-1β+IFNγ and TNFα+IFNγ. Super shift experiments in Caco-2 revealed that the DNA-protein complex formed after treating the cells with IL-1β and the combination, IL-1β+ IFNγ, contains the functional p65 sub unit of NF-κB in the complex **(Fig 26)**. Therefore we could conclude that NF-κB plays a crucial role in CXCL10 gene expression induced by cytokines in IECs. Interestingly, IFNγ induced CXCL10 mRNA and protein was inhibited upon NF-kB inhibition, though we couldn't find any activation of NF-κB by IFNγ in gel shift experiments. Previously, Hiroi M *et al.,* showed that a co-operation between IFNγ induced STAT1 and the constitutive or inducible NF-κB is necessary for the transcriptional activity of IFNγ inducible genes like MIG and CXCL10 (Hiroi and Ohmori, 2003). Consequently a similar mechanism for IFNγ induced CXCL10 gene regulation in IECs can be assumed.

Taken together, our data suggest that while IFNγ and IL-1β may work individually through disparate signalling pathways to stimulate CXCL10 gene regulation, coordinately these cytokines appear to enhance transcription of the CXCL10 gene in NF-κB dependent manner. The results support the hypothesis that the synergistic effect of IL-1β+IFNγ depends on simultaneous activation of STAT1 and NF-κB and their binding to ISRE and κB elements within the CXCL10 promoter region. Further experiments will have to be carried out to find out the role of other transcriptional factors like STAT1 and IRF-1 in CXCL10 expression by IECs in order to gain a complete understanding of CXCL10 gene regulation in IECs. Although it is evident that a variety of cell types, especially inflammatory cells, secrete CXCL10, we have shown that intestinal epithelial cells can serve as a source of CXCL10 during intestinal inflammation. Also our data indicate that IL-1β, TNFα and IFNγ, cytokines

associated with the pathogenesis of IBD, alone and in combination, regulates CXCL10 gene expression in IECs. The fact that IL-1β via activation of NFκB mimics the effect of TNFα in augmenting CXCL10 expression might be of importance in patients with IBD, who do not respond to an anti-TNFα antibody therapy. In those cases an additional inhibition of IL-1β would be desirable as a future therapy option in treating IBD.

Previous studies showed that NF-κB activation in intestinal epithelial cells is a major regulator of cytokine/chemokine gene expression (Elewaut et al., 1999). Inhibiting NF-κB has a pharmacological importance in treating IBD. Several inhibitors are present which inhibit NF-κB activation. Some of them are non specific and some are specific inhibitors. Here we evaluated the role of a non specific inhibitor of NF-κB, PDTC and a specific inhibitor BAY-11 7082 in IL-1β mediated CXCL8 gene expression in human colonic epithelial cell lines.

In our study, both PDTC and BAY11-7082 dose dependently inhibited IL-1β induced NF-κB reporter gene activity in Caco-2 cells revealing their inhibitory effect on NF-κB activation **(Fig 27 and 22)**. Further, treatment of Caco-2 cells with PDTC in a dose dependent manner, resulted in an enhancement of IL-1β induced CXCL8 mRNA expression rather than its inhibition **(Fig 28)**, which is not in agreement with previous studies that showed IL-1β induced CXCL8 gene via NF-κB in Caco-2 (Jobin et al., 1997). ELISA experiments also showed that the effect was persistent at the level of protein synthesis and secretion. In contrast IL-1β induced CXCL8 expression and secretion was dose dependently inhibited by BAY11-7082 **(Fig 29)**. One can speculate that PDTC mediated enhancement of IL-1β induced CXCL8 expression and secretion could possibly be due to the activation of other signaling pathways.

Previous studies with PDTC had revealed that PDTC is not only involved in inhibiting NF-κB but also activates other signaling pathways. In rat mesangial cells PDTC induced gene expression of stromelysin through a tyrosine kinase mediated activation of the transcription factor AP-1 (Yokoo and Kitamura, 1996). ICAM-1 expression was up-regulated by PDTC in human endothelial cells via activation of the AP-1 pathway (Munoz et al., 1996). In vascular smooth muscle cells PDTC induced

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G1 phase cell cycle arrest, partially by activating p38 MAPK (Moon et al., 2004). Since it was shown that IL-1β induced CXCL8 gene expression was regulated by p38 MAPK with its effect on CXCL8 promoter in Caco-2 (Parhar et al., 2003), we therefore aimed to check the role of p38 MAPK in IL-1β induced CXCL8 gene expression.

We compared the effects of PDTC, the p38 MAPK inhibitor SB205380 and MEK inhibitor PD98059 on IL-1β induced CXCL8 expression in Caco-2. Inhibition of p38 MAPK led to an inhibition of IL-1β induced CXCL8 expression and secretion in Caco-2, in accordance with the previous published data (Parhar et al., 2003). The combination of PDTC and SB203580 clearly inhibited PDTC mediated enhancement of IL-1β induced CXCL8 expression but couldn't inhibit IL-1β induced CXCL8 expression (**Fig 30)**, revealing a role of PDTC at least in partly activating the p38 MAPK pathway and thereby activating the CXCL8 gene expression. This might be explained by the presence of single AP-1 binding site besides the two NF-κB binding sites in CXCL8 promoter (Holtmann et al., 1999). Previously, it was shown that IL-1β mediated activation of p38 MAPK leads to AP-1 activation (Jung et al., 2002). In addition to activation of transcription, post transcriptional mechanisms contribute to the induction of CXCL8 gene expression (Chaudhary and Avioli, 1996; Stoeckle, 1991; Villarete and Remick, 1996). Holtman *et al.*, had shown that the p38 MAP kinase pathway contributes to induction of CXCL8 synthesis by stabilizing its mRNA (Holtmann et al., 1999). So the activation of p38 MAPK by PDTC may be an additional reason for PDTC enhancement of IL-1β induced CXCL8 gene expression in Caco-2 cells.

As we showed that intestinal epithelial cells respond differentially to cytokines, we hypothesized that the enhancing effect of PDTC was cell line dependent, so we checked the effects of PDTC, SB203580 and PD98059 on IL-1β induced CXCL8 gene expression also in HT29 cells **(Fig 31)**. In HT29 cells, PDTC either didn't inhibit or enhance the IL-1β induced CXCL8 expression and secretion whereas SB203580 and PD98059 had an inhibitory effect, suggesting that the enhancing effect of PDTC in IL-1β induced CXCL8 gene expression was cell line dependent.

As we showed that, IL-1β induced CXCL10 gene expression in Caco-2 cells and that this induction was mediated by NF-κB. We raised the question whether the enhancing effect of PDTC on IL-1β induced CXCL8 gene expression was restricted to CXCL8 gene or not. Therefore we checked the role of PDTC, SB203580 and PD98059 on IL-1β induced CXCL10 gene expression in Caco-2 and HT29 cells (**Fig 32 and 33**). Both real-time PCR and ELISA experiments revealed that PDTC inhibited IL-1β induced CXCL10 gene expression in both Caco-2 and HT29 with varying degree of inhibitory effects. 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 stabilize 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. This to our knowledge is the first report revealing the role of MAPKs in CXCL10 gene expression induced by IL-1β in IECs. Our results proved that PDTC dose dependently enhanced IL-1β induced CXCL8 gene expression and this effect was cell line specific. Further experiments, like western blots for different signaling proteins that are involved in NF-κB pathways and MAP kinase pathways and promoter analysis studies of CXCL8 gene, are required to explain PDTC enhancement of IL-1β induced CXCL8 gene expression in Caco-2, which will be of pharmacological importance in treating IBD.

Many studies carried out during the past decades have exposed the broad spectrum of actions of glucocorticoids (GCs) on the immune system, e.g. the down regulation of cytokines, chemokines and adhesion molecules etc. Glucocorticoids are valuable anti-inflammatory drugs in several diseases, such as asthma, rheumatoid arthritis and inflammatory bowel disease, in which multiple inflammatory genes are over expressed. In the present study we therefore evaluated the effect of glucocorticoids that are activated by dexamethasone on cytokine induced CXCL8 and CXCL10 gene expression in human intestinal epithelial cell lines, the two chemokines believed to have a crucial role in IBD.

Raddatz *et al*., previously showed glucocorticoid receptor (GR) protein expression in different types of immune cells in inflammatory bowel disease including the nucleus

of intestinal epithelial cells, suggesting that these cells are also targets of GC therapy. They could also find a nuclear GR localisation, even in controls without GC treatment (Raddatz et al., 2004). Our experiments in the untreated IECs showed a difference in the basal level GRα mRNA expression **(Fig 34)**. This differential expression in may be explained with different differentiation status of the cell lines we used. This may reflect the situation *in vivo*, since there is a different GR expression in colonic crypts being highest on the surface and absent in the deep parts of the crypts. While in the small intestine there is an equal distribution of GR in all epithelial cells except goblet cells (Raddatz et al., 2004). Although Caco-2 cells express approximately 1000 fold more GRα than HT29 and DLD1 cells, dexamethasone couldn't significantly inhibit both CXCL8 and CXCL10 mRNA expression induced by IL-1β, TNFα and IFNγ in Caco-2 **(Fig 35)**.

Previously, it has been shown that in Caco-2 cells, dexamethasone induced GR transactivation (Raddatz et al., 2001). This showed that at least the transactivation mediated by dexamethasone is functional in Caco-2 cells, making a general damage of the GR activation pathway unlikely. The same authors also demonstrated that IL-1β counteracted GC effects on NF-κB repression and also inhibited GC driven transactivation both in rat and human intestinal epithelial cell lines, IEC-6 and Caco-2. Another study by Ohtsuka *et al.,* showed that IL-1β and TNFα induced CINC-1 gene expression could be inhibited by dexamethasone in the normal rat kidney epithelial cell line NRK-52E (Ohtsuka et al., 1996).

Based on these data we checked the role of GCs on IL-1β and TNFα induced CINC-1 expression in the rat intestinal epithelial cell line IEC-6. In the IEC-6 cell line, dexamethasone clearly inhibited IL-1β and TNFα induced CINC-1 (homologue of human GRO chemokine) mRNA expression **(Fig 36)**. This suggests that the failure of dexamethasone to inhibit cytokine mediated CXCL8 and CXCL10 mRNA expression in Caco-2 was probably being a matter of the quantity of available GR being lower when compared to IEC-6. This could be supported by the finding that over expression of GRα in Caco-2 was previously shown to restore NF-κB inhibition by dexamethasone (Raddatz et al., 2001). ELISA experiments in Caco-2 and HT29 **(Fig 38)** revealed that dexamethasone didn't have a significant inhibitiory effect on

cytokine induced CXCL8 and CXCL10 protein secretion in these cell lines, excluding the possibility of dexamethasone involvement on the post-transcriptional modifications of these proteins. Our results concluded that dexamethasone is ineffective in inhibiting cytokine mediated CXCL8 and CXCL10 gene expression in Caco-2 and HT29 cells. We predict a similar kind of response *in vivo* for dexamethasone in GC-resistance patients with IBD.

7. Conclusions

Our results could confirm the hypothesis that, proinflammatory cytokines IL-1β and TNFα quickly induced both CXCL8 mRNA expression and protein secretion in IECs, while IL-1β alone and/or in combination with IFNγ induced an early and strong CXCL10 mRNA expression and protein secretion in Caco-2 cell line through the transcription factor NF-κB. CXCL10 gene regulation can be synergistically induced by the combinations of IL-1β+IFNγ and TNFα+IFNγ in IECs via NF-κB pathway. Therefore a specific inhibition of NF-κB is a desirable goal in treating IBD. Ineffectiveness of glucocorticoids to inhibit cytokine mediated CXCL8 and CXCL10 gene expression, underlines the concept that IEC may play an important role in the outcome of GC-resistance in IBD patients
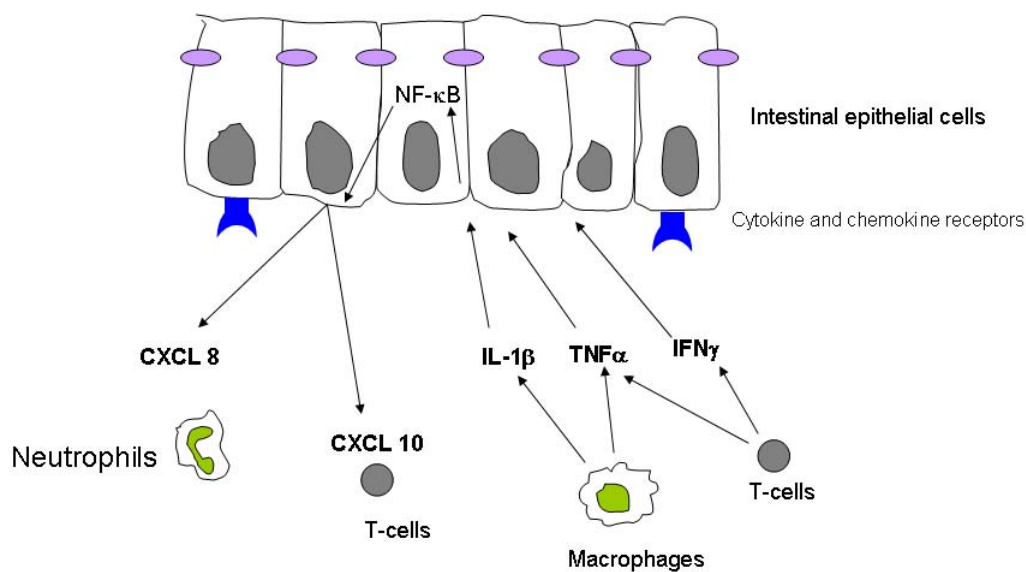
Figure 39: Role of proinflammatory cytokines, CXCL8 and CXCL10 chemokines in the interaction between IECs and immune cells in intestine

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9. Acknowledgements.

Curriculum Vitae

I was born on 8th July 1979 as the first child of Yeruva Nagireddy and Yeruva padmavathi in Nellore, Andhra pradesh, India.

From 1984 till 1994, I had my elementary education in Sangamithra High Scool in Nellore, India. In 1994 I got a secondary school leaving certificate. In 1994, I started studying higher secondary in Sangamithra Jr. College, Nellore, India and successfully compeleted in1996.

During 1997-2000, I studied Genetics, Botany and Chemistry in P.B.Siddartha College of Arts and Science, Vijayawada affiliated to Nagarjuna University, India, to obtain my Bachelor of Science degree. At Devi Ahilya University, Indore, India, I specialized my Master's degree in Life Sciences, with a dissertation entitled 'Studying the interactions of XRCC1 and DNA Ligase III BRCT domains using Yeast –Two Hybrid System', during 2000-2002.

In 2002, I joined as a Junior research fellow in the department of Microbiology and Cell biology in Indian Institute of Sciences, Bangalore, India and carried out projects entitled "Characterization of AP-2 target genes" and "Screening for the proteins which can interact with N-terminus BRCA1 protein using Yeast-Two Hybrid from human mammary gland cDNA library".

In March 2004, I had started my Ph.D thesis presented here with the title 'Role of intestinal epithelium in inflammatory bowel disease: effect of cytokines and glucocorticoids on CXCL8 and CXCL10 gene expression and NF-κB signalling in intestinal epithelial cell lines' in the department of Internal medicine, University Clinic, Georg-August Universität Göttingen, Germany under the guidance of Dr. Dirk Raddatz.