# Differential gene expression of chemokines in KRAS and BRAF mutated colorectal cell lines: Role of cytokines

**Dissertation**

**Zur Erlangung des Mathematisch-Naturwissenschaftlichen Doktorgrades**

**˝Doctor rerum naturalium˝ (Dr.rer.nat)**

**der Georg-August Universität Göttingen**

**Vorgelegt von**
**Sajjad Khan**
**aus Peshawar,**
**Pakistan**

**Göttingen 2013**

**Prof. Heidi Hahn** ……..
**ÜMG Dept. Human Genetics**
**Section of Developmental Genetics**
**Heinrich Düker- Weg Straße 12, 37073 Göttingen.**

**PD. Dr.Michael Hoppert** ……..
**Allgemeine Mikrobiologie und Genetik**
**Griesbach Straße 8, 37077 Göttingen.**

**Prof. Silvio Rizzoli** ……..
**European Sciences Institute (ENI)**
**STED Microscopy of Synaptic Function**
**Griesbach Straße 5, 37077 Göttingen.**

**Prof. Ernst Wimmer** ……..
**Abtl. Entwicklungsbiolgie (GZMB)**
**STED Microscopy of Synaptic Function**
**Justus von Liebig 11, 37077 Göttingen.**

**Referent:**

**Prof. Ahmed Mansouri** ……..

**Max Planck Institute for Biophysical Chemistry**

**(Karl Friedrich Bonhoeffer Institute) am Faßberg 11, D-37077 Göttingen.**

**Korreferent:**

**Prof. Tomas Pieler** ……..

**Department of Developmental Biochemistry (GZMB)**

**Justus-von- Liebig- Weg 11, D-37077 Göttingen.**

**Tag der mündlichen Prüfung:** .....

# Declaration

I hereby declare that the PhD thesis entitled "**Differential gene expression of chemokines in KRAS and BRAF mutated colorectal cell lines: Role of cytokines**" was written independently and with no other sources and aids than quoted.

Göttingen, 2013

Sajjad Khan

# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES



# LIST OF APPENDICES

# ABBREVIATIONS

| | |
|---|---|
| 5-ASA | 5-amino salicylic acid |
| AP-1 | Activating Protein 1 |
| Akt | Protein Kinase B (PKB) |
| APC | Adenomatous Polyposis Coli |
| APC | Antigen Presenting Cells |
| ATF-2 | Activating Transcription Factor 2 |
| ATP | Adenosine Tri Phosphate |
| ACF | Aberrant Crypt Foci |
| ARMS | Amplification Refractory Mutation System |
| BRAF | Proto Oncogene B-Raf |
| CAC | Colitis Associated Cancer |
| CARD | Caspase Recruitment Domain |
| CD | Crohn's Disease |
| CD | Cluster Differentiation |
| COX | Cyclooxygenase |
| CRC | Colorectal Carcinoma |
| CSF | Colony Stimulating Factor |
| DC | Dendritic Cells |
| DNA | Deoxyribonucleic Acid |
| Dex | Dexamethasone |
| DLR | Dual Luciferase Reporter |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminetetraacetic Acid |
| EGFR | Epidermal Growth Factor Receptor |
| FCS | Fetal Calf Serum |
| GDP | g-DI-Phosphate |
| GH | Growth Hormones |
| GSK-ß | Glycogen Synthase Kinase |
| GC | Glucocorticoid |
| GM-CSF | Granulocyte Macrophage Colony Stimulating Factor |
| GR | Glucocorticoid Receptor |

| | |
|---|---|
| GRE | Glucocorticoid Receptor Responsive Element |
| GTP | Guanosine Triphosphate |
| HB-EGF | Heparin-Binding EGF-Like Growth Factor |
| HSP | Heat Shock Protein |
| IBD | Inflammatory Bowel Disease |
| iNOS | Nitric Oxide Synthases |
| IκB | Inhibitory Kappa B |
| ICAM-1 | Intercellular Adhesion Molecule-1 |
| IEC | Intestinal Epithelial Cell |
| IFNγ | Interferon Gamma |
| IGF | Insulin like Growth Factor |
| IKK | IκB kinases |
| LIFR-β | Leukemia Inhibitory Factor Receptor Beta |
| 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 |
| JAK | Janus Activated Kinases |
| LB | Luria- Bertani |
| LGSC | Low-grade serous carcinoma |
| LP | Lamina Propria |
| LPS | Lipopolysaccharide |
| KRAS | Kirsten Rat Sarcoma |
| kDa | Kilo Dalton |
| MSGA-α | Melanoma Growth Stimulating Activity, Alpha |
| MAP Kinase | Mitogen-Activated Protein Kinase |

| | |
|---|---|
| MCP-1 | Monocyte-Chemoattractant Protein 1 |
| MEK | Map Kinase Kinases |
| MHC | Major Histocompatability Complex |
| NCI | National Cancer Institute |
| NK | Natural Killer |
| NF-κB | Nuclear Factor Kappa B |
| NO | Nitric Oxide |
| NOD-2 | Nucleotide-Binding Oligomerization Domain Containing 2 |
| PDGF | Platelet-Derived Growth Factor |
| PGE2 | Prostaglandin E2 |
| PTEN | Phosphatase and Tensin Homolog |
| PI3K | Phosphatidylinositol 3-kinases |
| PBS | Phosphate Buffered Saline |
| PDTC | Pyrolidinedithiocarbamate |
| PMA | Phorbol Myristyl Acetate |
| Prl | Prolactin |
| RFLP | Restriction Fragment Length Polymorphism |
| RNI | Reactive Nitrogen Intremediates |
| ROS | Reactive Oxygen Species |
| SH2 | Src Homology 2 |
| SEM | Standard Error of Means |
| SARS | Severe Acute Respiratory Syndrome |
| TBST | Tris Buffer Saline and Tween |
| Th1 | T-helper cell 1 |
| 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 |
| STAT3 | Signal Transducer and Activator Protein 3 |
| UC | Ulcerative Colitis |

# 1. Summary

**Background:** Worldwide, colorectal cancer is the third most common malignancy. The development of the colorectal cancer (CRC) is a multistep process that involves e.g. an accumulation of mutations in tumor-growth promoting genes. In CRC, the most frequently found mutations are in the *KRAS* (30-50%) and *BRAF* (~10%) genes. Recent studies have shown that *KRAS* and *BRAF* mutations represent an important step in the development of carcinoma from the adenoma stage in colon cancer by affecting multiple pathways linked to the MAPK1, JAK-STAT, and PI3K pathways.

A role of inflammatory cells within the tumor microenvironment and tumorigenesis is well established. However, the mechanism of how inflammation promotes carcinogenesis remains unresolved.

As the survival and proliferation of tumor cells is influenced by immune cells within the tumor environment, the aim of our study was to investigate whether pro-inflammatory cytokines (TNFα, IL-1β and IFNγ) can induce pro- (CXCL1 and CXCL8) and anti-angiogenic (CXCL10) chemokines and in mutated CRC cell lines compared to wild type. Furthermore, the behaviour of these chemokines in the presence/absence of the *KRAS* by siRNA silencing in KRAS-mutated (DLD1) and wild type (Caco2) was analysed.

**Methods:** Six colonic cell lines were investigated: DLD1 (KRAS G13D), HT-29 and Colo205 (BRAF V600E) as well as the wild type (Wt) cell lines Caco-2, Colo-320 and CX-1. The presence of *KRAS* mutations was analysed in the cell lines by using specific mismatch primers to amplify genomic DNA fragments through the PCR-RFLP assay, containing the hot spots of codons G12D and G13D. The *BRAF* mutation for codon V600E was detected by real time PCR. DLD1, HT-29 and Caco-2 cell lines were treated with cytokines (TNFα 50ng, IL-1β 1ng and IFNγ 50ng) and harvested at different time points (1h-24h). *KRAS* inhibition was performed by the siRNA approach using specific nucleotide sequences in *KRAS*-mutant and wild type cell lines. Total RNA was isolated from cultured cells. Isolated RNA was converted into cDNA and further used for RT-PCR analysis. Similarly, protein was extracted from the cells to perform Western blotting.

**Results:** RT-PCR analysis in non-stimulated cells showed a low basal expression of TNFα and IL-1ß in the *KRAS* mutated (DLD1) cell line, compared to wild type (Caco2). No detection was found for IL-6 and IFNγ in any of the studied cell lines. In contrast, pro-angiogenic chemokines (CXCL1, CXCL8) showed a high constitutive expression in mutated cell lines DLD1 (KRAS),

HT-29 and Colo205 (BRAF), compared to wild type (Caco2). However, the anti-angiogenic chemokine (CXCL10) showed a high basal expression in wild type, compared to mutated cell lines.

Treatment with pro-inflammatory cytokines showed an induction of CXCL1 gene expression in mutated, and to a lesser extent in wild type cell lines at mRNA and protein level. The most pronounced and quick induction of CXCL1 gene expression was detected after TNFα stimulation in DLD1 (KRAS; 310±2.18 fold) followed by HT-29 (BRAF; 36.15±3.28 fold) compared to wild type (Caco2; 29.45±0.82 fold). Similar results were found after treatment with IL-1ß which induced the maximum gene expression of CXCL1 in HT-29 (BRAF; 46.42±5.98 fold) followed by DLD1 (KRAS; 21.19±0.37 fold); a minor but significant increase was found in Caco2 (Wt; 2.6±1.6 fold).

Likewise, CXCL8 mRNA and protein level was significantly induced by TNFα and IL-1ß in KRAS mutated cell line (DLD1) and wild type (Caco2). The maximum increase was observed in wild type (Caco2) cell line after IL-1ß treatment (806.41±19.76fold). In addition, administration of IFNγ significantly enhanced CXCL10 at mRNA and protein level in mutated cell lines HT-29 (BRAF; 15361.19±2974.33 fold) followed by DLD1 (KRAS; 597.71±64.62 fold) in comparison to wild type (Caco2; 45.75±1.44 fold).

In order to determine the factors responsible for chemokine induction in the downstream-signalling pathway of pro-inflammatory cytokines, protein expression of transcription factors (NF-κB, MAPK1 and STAT3) involved in *KRAS*-mutant (DLD1) and wild type (Caco2) cell lines were studied. An increase in protein level of NF-κB and MAPK1 was found in both, mutated and wild type cell lines after cytokine stimulation. However, p-STAT-3 was only detected in the *KRAS* mutated cell line (DLD1) after IFNγ stimulation. The protein expression of p-STAT-3 showed a time-dependent increase up to 24 h.

To understand the possible role of *KRAS* and the consequences of inhibiting its activity or expression in colorectal cancer cell lines, a *KRAS* knockdown experiment was performed in *KRAS*-mutant (DLD1) and wild type (Caco2) cell lines. *KRAS* was successfully knocked down by the siRNA technique. This down-regulation of *KRAS* showed a significant effect on chemokine gene expression: A decreased CXCL1 and CXCL10 gene expression was detected in the DLD1 (KRAS) cell line in comparison to wild type (Caco2) at 72h after *KRAS* silencing. In contrast, the specific *KRAS* inhibition resulted in an up-regulation of CXCL1 and CXCL10 and induction of the NF-κB pathway in wild type (Caco2) cell line.

To summarize, basal chemokine gene expression for pro-angiogenic chemokines was high in mutated as compared to wild type cell lines. Furthermore, cytokine treatment induces the expression of pro-angiogenic (CXCL1, CXCL8) and anti-angiogenic (CXCL10) chemokines differentially in mutated cell lines compared to wild type. The inhibition of the *KRAS* resulted in induction of chemokines gene expression through the NF-κB pathway in wild-type cell line.

**Conclusion:** This reflects the likely existence of a totally different microenvironment in tumors consistent of wild type or mutated cells. This may help to rationalize the choice of molecular targets for suitable therapeutic investigation in clinical studies.

**Key words:** KRAS, BRAF, CXCL1 (GROα), CXCL10 (IP-10), CXCL8 (IL-8), TNFα, IL-1ß, IFNγ, siRNA.

# 2. Introduction

## 2.1 Colorectal Carcinoma

Colorectal carcinoma (CRC) is considered as one of the most common lethal cancer all over the world (Weitz et al. 2005). It is globally accepted that CRC is the third most common cancer in men and regarded as second most frequent cancer in women (Armaghany et al. 2012a) The global statistics shows that it is responsible for around 529,000 deaths per year (Ferlay et al. 2007). There have been about 20% of CRC cases which have a familial basis; (Rustgi 2007a) some of them are linked with well-characterized syndromes, such as hereditary nonpolyposis colorectal cancer and familial adenomatous polyposis (FAP). CRC is developed due to the uncontrolled cell growth in the colon or rectum (Karin 2006;Yu et al. 2009). It initiates from the crypt epithelial cells that line the colon or rectum of the gastrointestinal tract (Ionov et al. 1993). The major causes which have been considered for CRC are environmental factors rather than heritable genetic changes. The most important risk factors for sporadic CRC include factors like food-borne mutagens, specific intestinal pathogens, chronic intestinal inflammation and age (Cappell 2008).

Deregulated cell cycle control is a fundamental aspect of cancer, resulting from mutation, deletion and transcriptional repression of genes (Futreal et al. 2005). A loss of methyl groups in the DNA is found very early in colorectal tumorigenesis (Fearon and Vogelstein 1990a;Vogelstein and Kinzler 2004). Familial adenomatous polyposis (FAP) which could lead to CRC, is due to inherited mutations of the adenomatous polyposis coli (APC) gene (Fearon and Vogelstein 1990a). Infact, APC gene plays an important role in colorectal carcinogenesis (Armaghany et al. 2012b). APC mutation leads to hyperproliferation and thus has helped to understand early steps in CRC carcinogenesis (De et al. 2002).

Indeed, mutations in the APC-gene trigger tumor development through the Wnt-APC-beta-catenin pathway mutation. Furthermore, loss of the TP53 tumor-suppressor gene leads to

increased cell division by loss of control the cell cycle (Shaw et al. 1991). In addition, sporadic CRCs alter the cell signalling pathways by acquiring a change in the most common genetic mutations such as KRAS, BRAF (Sheng et al. 1998;Lakatos and Lakatos 2008).

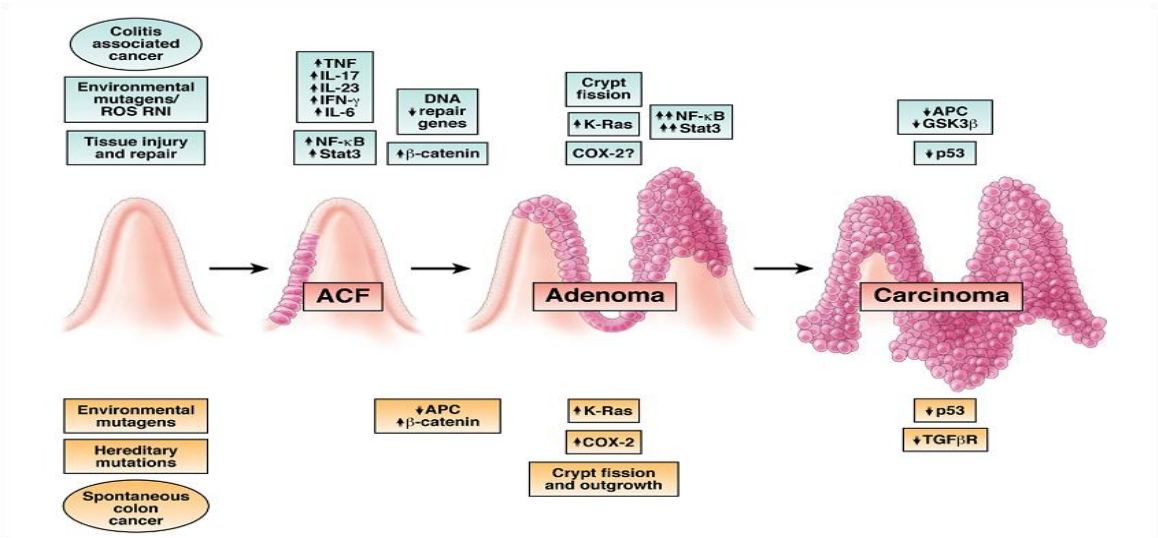



**Figure: 1** Mechanisms of colorectal cancer (CRC) and colitis-associated (CAC).Mutations in the adenomatous polyposis coli gene (APC), β-catenin, and oncogenic mutations drive the transition of single pre-neoplastic cells to aberrant crypt-foci (ACF) and then to adenoma and CRC. Chronic inflammation, which leads to colitis-associated cancer, is characterized by the production of pro-inflammatory cytokines sustaining the inflammatory and thus tumor promoting microenvironment. Adapted from: (Terzic et al. 2010)

## 2.2 KRAS & BRAF mutation in Colorectal Carcinoma

The RAS and RAF family of genes code for proteins which form part of the Ras/Raf/MEK/ERK signalling cascade within cells (Dhomen and Marais 2007). This cascade is involved in the transmission of extracellular signals which control a variety of biological processes such as cell growth, cell survival and migration (Malumbres and Barbacid 2003). Disruption of this pathway, through gain-of-function mutations in RAS and RAF family members, is well described in several different types of cancer. In CRCs, mutations are frequently found in the *KRAS* and to a lesser extent in the *BRAF* (Downward 2003;Jass 2006). In CRC, *KRAS* mutations are early events (Fearon and Vogelstein 1990b;Ilyas et al. 1999) and

are found in 30–50% of tumors (Downward 2003). KRAS mutations with different hotspots are identified in codons 12, 13, 61 and 146.

In fact, the KRAS and BRAF proteins are known to be a key downstream component of EGFR signaling, transmitting growth-promoting EGF signals from the cell surface to the transcriptional machinery in the nucleus (Hanahan and Weinberg 2000). The Ras protein family consists of *KRAS*, *H-Ras* and *N-Ras* which are small GTPase proteins and are normally present in an inactive GDP bound form. These proteins are activated through extra-cellular signals from growth factors which result in phosporylation of GDP to GTP. Furthermore, these proteins have a serine/threonine kinase domain which is activated by Ras proteins. Activated Ras-protein phosphorylates MEK (MAPK/ERK kinase) which in turn phosphorylates ERK (extracellular signal-regulated kinase). The signal is then further transmitted through phosphorylation of downstream targets such as nuclear factor (NF-κB) (Dhomen and Marais 2007).

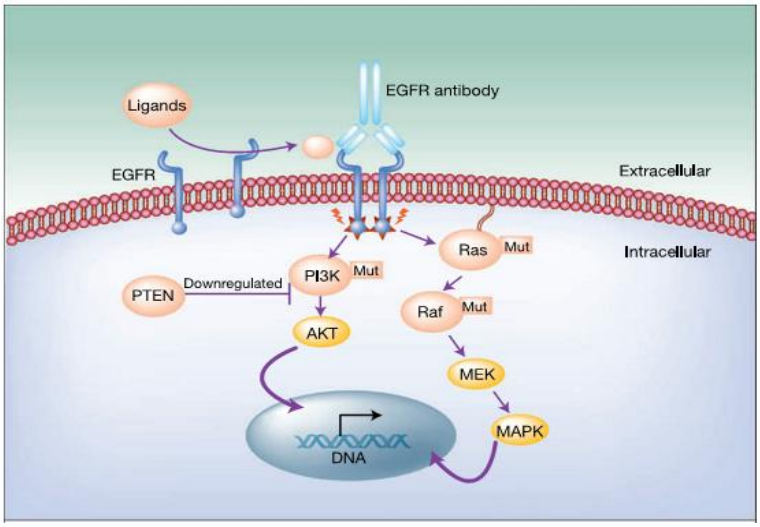



**Figure: 2** EGFR signalling pathway and candidate predictive molecular markers for the activity of EGFR antibodies in CRC. Ligand binding and therefore dimerization of the EGFR monomer and activation of the EGFR pathway is inhibited by EGFR-directed antibodies. Adapted from: Michaela S. et al Clinical Cancer Research (2009)

The hotspots for mutation in G-coupled proteins such as KRAS are evolutionarily conserved sites, and mutation results either in a protein which is irreversibly bound to GTP (for KRAS codons 12,13 and 61) (Grand and Owen 1991) or in a protein which rapidly exchanges GDP for GTP leading to enhanced signalling activity (for KRAS codon 146) (Feig and Cooper 1988). Concurrent mutations are found rarely (Davies et al. 2002a;Rajagopalan et al. 2002a;Singer et al. 2003). Both the *KRAS* and *BRAF* undergo gain of function mutations and represent different modes of action of activating the same pathway.

## 2.3 Colorectal Carcinoma and inflammation: role of cytokines

Colitis-associated cancer (CAC) is a CRC subtype which is associated with inflammatory bowel disease (IBD) (Lakatos and Lakatos 2008). IBD with colon involvement is among the top three risk conditions for CRC (Askling et al. 2001). 20% of IBD patients which develop CAC within 30 years of the onset of their disease have a mortality rate of above 50% (Lakatos and Lakatos 2008).

IBD exists in two major forms known as 1) Crohn's disease (CD) and 2) Ulcerative colitis (UC) (Calkins and Mendeloff 1986). IBD is deemed to be a result of continuous 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 (Podolsky 2002). A failure to regulate normally protective cell-mediated immune responses in the intestinal mucosa result in sustained activation of the mucosal immune system and in uncontrolled overproduction of pro-inflammatory cytokines and mediators (Laroux. et al. 2001). Constitutive expression of pro-inflammatory cytokines and chemokines (Anisowicz et al. 1987a;Dubois et al. 1998) is hallmark of IBD (Davidson et al. 1996;McDonald et al. 1997). However, under some conditions immune cell subsets and cytokines fight to maintain dysplastic cells under control thus preventing tumor progression (Dunn et al. 2006). The role of cytokines in the mucosal

immune system has been studied intensively revealing that cytokines influence the nature of mucosal immune responses (Elson et al. 1995;Sartor 1994).

Colorectal tumors that are not associated with clinically detectable IBD, exhibit strong inflammatory infiltration and increased expression of pro-inflammatory cytokines (Atreya et al. 2008;Atreya and Neurath 2008;Clevers 2004;Waldner and Neurath 2008). Tumor cells themselves can produce cytokines, including IL-1α/β, IFNγ and TNF-α (Popivanova et al. 2008) maintaining a pro-inflammatory microenvironment. They also secrete chemokines inducing further leukocyte infiltration. Infact, there have been efforts characterizing immune-cell populations and inflammatory mediators in related murine models (Podolsky 1991). However, the distribution and type of inflammatory cells and role of their mediators is poorly described in CRC.

Indeed, inflammatory cell (leukocytes) recruitment from the blood circulation into the tissue is a crucial event in the generation and maintenance of inflammatory cell infiltrates in inflammation. Three steps of leukocyte migration, namely tethering, rolling, and adhesion and crawling are involved in heterophilic interactions between one class of molecules on the leukocyte and another class of molecules on the endothelial cell (Muller 2003;Schenkel et al. 2002). The process of leukocyte migration is also called trans-endothelial migration (TEM): diapedesis. It involves several families of cytokines (Ramadori and Armbrust 2001) and chemokines (Wasmuth et al. 2010). These mediators play a role in chronic inflammation. However, it is also unclear how immune-cell populations and inflammatory mediators ultimately promote chronic gut inflammation. Chronic inflammation is known to induce genomic mutations upon inducing DNA modifications in intestinal epithelial cells related to DNA methylation and histone modification (Colotta et al. 2009;Rustgi 2007b). Moreover, chronic inflammation results in cell proliferation, oxidative stress and ultimately in the development of dysplasia (Roessner et al. 2008). The mechanism of how chronic inflammation

develops into carcinogenesis still remains unsolved. However, it is supposed that the same genetic mutations which lead to sporadic CRC are also responsible for its development in IBD.

***Tumor Necrosis Factor***

A major role of cytokines released by epithelial and immune cells in colitis-associated cancer (CAC) is widely accepted (Popivanova et al. 2008). Tumor necrosis factor (TNF), due to its importance in mediating inflammation, shares many pro-inflammatory activities with the cytokine IL-1 (Garrity-Park et al. 2008). TNF binds to two distinct soluble receptors with different molecular masses of 55kDa (p-55) and 75kDa (p-75). Both soluble TNF receptors inhibit binding of TNF to its cellular receptors and reduce the biologic effects of TNF in a dose dependent manner (Loetscher et al. 1991). A previous study reported that single nucleotide polymorphism (SNPs) in the promoter of TNFα is associated with an increased risk for IBD which could lead to a genetic predisposition in the development of colitis-associated CRC (Garrity-Park et al. 2008)

***Interleukin-1***

Interleukin-1 is a remarkable mediator that activates many immune and inflammatory cells (Dinarello 1994). IL-1 is produced by monocytes/macrophages, neutrophils and endothelial cells (Stevens et al. 1992). It consists of IL-1α and IL-1β that bind to two types of IL-1 receptors on their target cells, named IL-1 receptor type I and IL-1 receptor type II. IL-1 receptor type I is responsible for the signal transduction. Effects of IL-1 are controlled by the IL-1 receptor antagonist, which is produced virtually by the same cells as IL-1 itself and competitively counteracts the actions of IL-1 (Dinarello and Thompson 1991). Previous studies have shown that the expression of IL-1 is increased in inflammatory lesions of patients with IBD (Mahida et al. 1989;Rogler and Andus 1998a). IL-1 receptor antagonist is increased as well, but not to the same extent as IL-1. This results in a local imbalance between IL-1 and IL-1 receptor antagonist in the inflamed mucosa (Rogler and Andus 1998a;Andus et al. 1997;Casini-

Raggi et al. 1995). It is known that the macrophages are the main producer of IL-1 in the inflamed mucosa. However, IL-1 receptor antagonist seems to be an important source in the intestinal epithelial cells (Rogler and Andus 1998a).

***Interferons***

Type I interferons comprise two different groups of proteins. The first group is interferon α (IFNα) that comprises a family of about 20 structurally related polypeptides of approximately 18-kDa. The interferons are mainly produced by mononuclear phagocytes (Breese et al. 1993). Type II interferon, also called IFNγ or immune IFN, is a homo dimeric glycoprotein containing 21-to 24-kDa subunits. It is produced by Th1 lymphocytes and NK cells. IFNγ binds to a receptor different from that utilized by type I interferons. It is anti-viral and anti-proliferative and one of the most potent activators of mononuclear phagocytes, increases MHC class I and II expression, promotes T and B cell differentiation, and activates neutrophils, NK cells, and vascular endothelial cells (Langaas et al. 2001) . In 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).

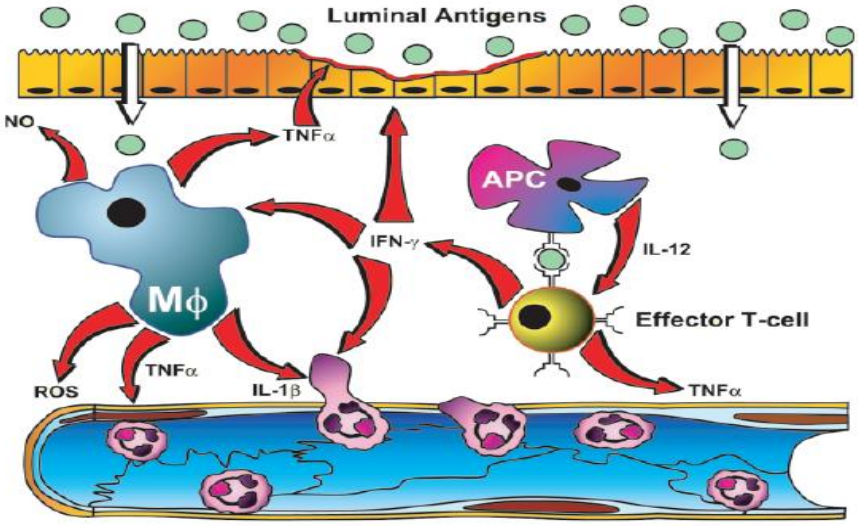



**Figure 3: Intestinal immune response to enteric antigens.** Effector $CD4^+$ T cells produce T helper-1 (Th1)-type cytokines in response to processed and presented antigen by antigen-presenting cells (APCs). These cytokines may affect the gut epithelium directly and/or

activate resident macrophages (MΦ) to release large amounts of pro-inflammatory mediators: cytokines as well as reactive metabolites of oxygen (ROS) and nitric oxide (NO). The net result is the recruitment of additional leukocytes and subsequent tissue injury. Modified from (Laroux et al. 2001).

## 2.4 Role of Chemokines in Colorectal Carcinoma

Chemo-attractant cytokines play a key role in the modulation of the immune system (Aggarwal and Pocsik 1992). They can induce or increase the synthesis of many structural and secretory proteins, (Malik et al. 2011;Ramadori and Christ 1999;Ramadori et al. 2008) including chemokines. Furthermore, they also help in the infiltrating process of inflammatory cells, recruited from the blood by chemokine mediators released at the site of damage (Malik et al. 2010). In turn, inflammatory cells are responsible for production of cytokines (IL-6, IL-1β, TNF-α and IFN-γ), which are released into the blood (Malik et al. 2011;Ramadori and Christ 1999;Ramadori and Armbrust 2001) and can act distantly on intact organs (Malik et al. 2011).

Chemokines are thought to be responsible for recruiting inflammatory cells. They are actively involved in inflammation, tissue repair, development of fibrosis and tumor (Malik et al. 2010;Marra 2002;Owen et al. 1997). Chemokines, comprise a set of low-molecular weight cytokines (7-10 kDa), which play a key role in directing migration and activation of leukocytes in the inflammatory processes (Macdermott 1999). Based on their primary structure, chemokines are distinguished as C, CC, CXC or CX3C where "X" represents a non conserved amino acids substitution. Apart, from 'C' –subgroups, all chemokines consists of a four cystine residue motif which is linked by disulfide bonds in the conserved region for the formation of the ß-sheet structures (Fernandez and Lolis 2002). The chemokine nomenclature named the receptor 'R' like in CXCR1 and the respective ligand 'L' as in CXCL8. The chemokines are secreted by both, the stromal and malignant part of the cancer tissue. Functionally, the chemokine ligand and receptor pairs can exert a direct effect on tumor proliferation and survival (Boimel et al. 2012;Erreni et al. 2009). It is possible that the chemokines from stromal

cells may influence the survival of malignant cells by binding to the functional receptors acquired on the cancerous cells, which increases metastasis in a chemokine rich environment (Balkwill 2012;Mantovani et al. 2010).

Several studies have been published to elucidate the dual role of chemokines in promotion and inhibition of angiogenesis (Kiefer and Siekmann 2011;Keeley et al. 2008). The CXC chemokines which regulate angiogenesis CXCL1 and CXCL8 have strong effect in tumor angiogenesis, on the other side, CXCL10 inhibits neo-vascularisation (Belperio et al. 2000;Moore et al. 1998;Rossi and Zlotnik 2000;Wang et al. 1998).

***CXCL1/GROα***

CXCL1 belongs to the CXC chemokine family which was previously named KC, GRO1, GROα, and MSGA-α. This chemokine is secreted by melanoma cells in human, has mutagenic properties and is implicated in pathogenesis (Anisowicz et al. 1987b;Richmond and Thomas 1988). Moreover, it is highly expressed by macrophages, neutrophils and epithelial cells (Becker et al. 1994;Iida and Grotendorst 1990) and has a role in the neutrophil chemo-attractant activity (Moser et al. 1990;Schumacher et al. 1992). CXCL1 exerts its effects by signaling through the chemokine receptor CXCR2 (Tsai et al. 2002). Previously *in vitro* and *in vivo* studies have shown that exogenous PGE2 (Prostaglandin E2) induces CXCL1 expression and release from CRC cells. The induction of CXCL1 by PGE2 is dose dependent and activates the epidermal growth factor receptor (EGFR)-mitogen activated protein kinase (MAPK) pathway (Wang et al. 2006).

***CXCL 8/ IL 8 Interleukin-8***

CXCL8 is a soluble small peptide of 8-10kDa (Baggiolini et al. 1992). Myriad cell types secrete CXCL8 chemokine including intestinal epithelial cells. Its two commonly known homologous receptors CXCR1 and CXCR2 binds to its ligand CXCL8 with high affinity (Fusunyan et al. 1998;Kim et al. 2001;Holmes et al. 2009;Murphy et al. 2000). CXCL8 is

considered to be one of the prototype among the chemokines that has chemotactic activities, is secreted by monocytes and macrophages and assists in the directive migration of neutrophils, basophils and T-lymphocytes (Baggiolini et al. 1992;Rossi and Zlotnik 2000). It has an active role in the recruitment and transmigration in the inflammation locus (Struyf et al. 2005). CXCL8 induces neutrophils to secrete lysosomal enzymes, change shape and adhesion to endothelial cells (Hoch et al. 1996). Prior studies have shown that TNF and IL-1 induce CXCL8 expression in human monocytes and lymphocytes (Chaly et al. 2000). The exact role and actual mechanisms of CXCL8 mRNA and protein expression as well as the effect of different cytokines on the expression and secretion of CXCL8 in colorectal carcinoma is still unclear.

***CXCL10(IP-10/) Interferon-gamma activated protein***

CXCL10 which represents CXC chemokines is the human interferon inducible protein 10 (Liu et al. 2011b). CXCL10 is originally known as an interferon (IFN) inducible gene and due to its high affinity receptor CXCR3 (Dwinell et al. 2001). CXCL10 is constitutively produced by intestinal epithelial cells after stimulation with IFNγ (Gasperini et al. 1999). It is mostly expressed through activation of $CD4^+$ memory T cells that produce a Th1 cell pattern of cytokines (Loetscher et al. 1991;Qin et al. 1998). Previous studies examined that stimulation with pro-inflammatory cytokines enhances the expression of CXCL10 in intestinal epithelial cells *in vitro* (Dwinell et al. 2001). It is also reported that CXCL10 is responsible for the inhibition of cell proliferation and induces apoptosis in endothelial cells (Loetscher et al. 1991;Feldman et al. 2006). Basically CXCL10 shows a minor production in the colon, however, in abnormal conditions a significant enhancement of the expression is detected in the colonic lamina propria of biopsied specimen of ulcerative colitis (UC) patients (Uguccioni et al. 1999).

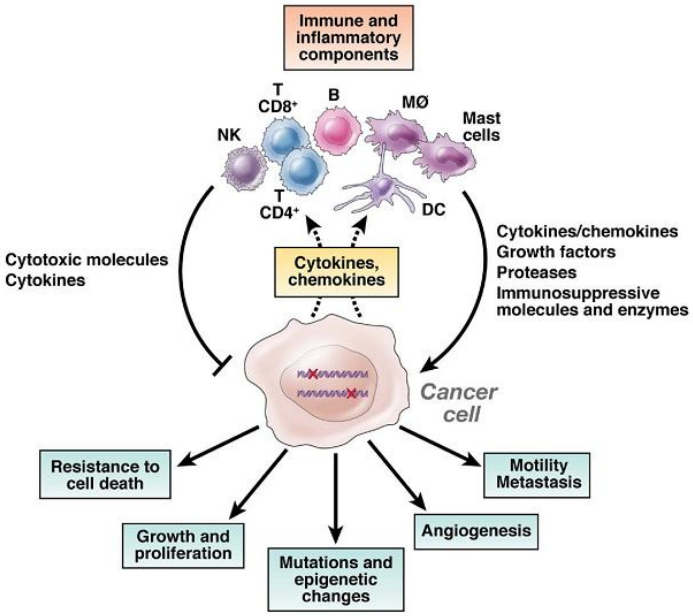



**Figure 4:** Immunosurveillance and inflammation in colorectal cancer (CRC) and colitis associated cancer (CAC). Innate and adaptive immune cells promote tumor development. Inflammation can promote colitis-induced tumorigenesis. In sporadic CRC, the balance of immune cells may be shifted towards tumor-promoting inflammation. DC, dendritic cells; NK, natural killer cells. Adapted from: (Terzic et al. 2010).

## 2.5 Role of Transcription Factors in colorectal carcinoma

The identification of signalling pathways within tumor cells that eventually induce tumor growth or suppress anti-tumor immunity is a significant challenge (Bollrath and Greten 2009). To understand these complex interactions could help to identify targets for the cancer treatment and prevention. A large percentage of CRCs and cell lines reveal a constitutive activation of transcription factors that are important components of multiple inflammatory pathways, namely nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) (Sakamoto et al. 2009;Yu et al. 2009).

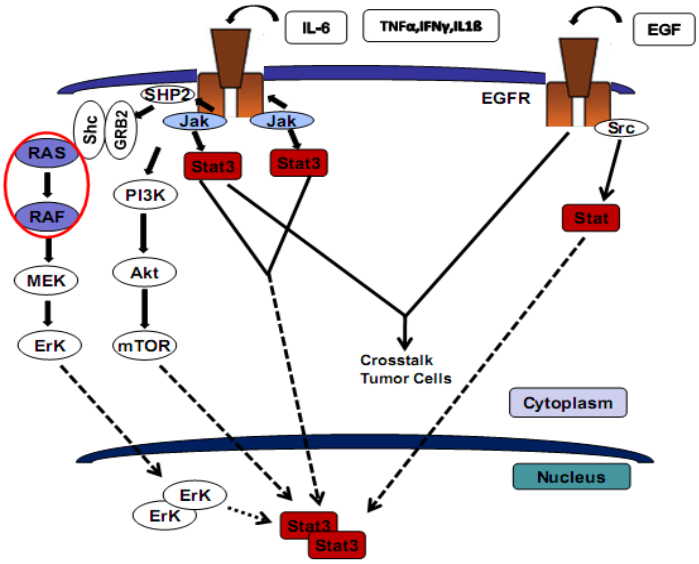



**Figure 5:** JAK and STAT are critical components of many cytokine receptor systems. Jaks and Stats are critical components of many cytokine receptor systems, regulating growth, survival, differentiation and pathogen resistance. An example of these pathways is shown for the IL-6 (or gp130) family of receptors, which co-regulate B cell differentiation, plasma cytogenesis and the acute phase reaction. Cytokine binding induces receptor dimerization, activating the associated Jaks, which phosphorylate themselves and the receptor. The phosphorylated sites on the receptor and Jak serve as docking sites for the SH2-containing Stats, such as Stat3, and for SH2-containing proteins and adaptors that link the receptor to MAP kinase, PI3K/Akt, and other cellular pathways. Adapted from: Cell Signalling Technology (2010)

NF-κB occurs as a heterodimer which consists of five subunit members: p105/p50, p100/p52, RelA (p65), c-Rel, and RelB (Ruben et al. 1992). In the classical NF-κB pathway, these family subunit members are passive in the cytoplasm and bound to specific inhibitors like κB (IκB) proteins. It is reported that the classic mechanisms of NF-κB activation occur in colitis associated cancer (CAC) and colorectal carcinoma (CRC) and include signalling by pattern recognition receptors and by tumor-promoting cytokines like TNF, IL-1 and IL-17 (Karin 2006). NF-κB transcriptionally activates many genes involved in cell function which are responsible in early inflammatory responses comprising CXCL8, iNOS, TNF-α, IL-1β, IL-6, COX-2(Baldwin, Jr. 1996;Grilli and Memo 1999). Some studies have also revealed that NF-κB

could be a main regulator in chemokine gene expression and a key regulator in neutrophil migration when intestinal epithelial cells were stimulated with *Bacteroides fragilis* enterotoxin (Kim et al. 2002).

Several oncogene products that can activate NF-κB have been identified such as *ras* (Finco and Baldwin, Jr. 1993). It is reported that oncogenic RAS increases NF-κB transcriptional activity through RAF dependent and RAF-independent mitogen-activated protein kinase signaling pathways (Norris and Baldwin, Jr. 1999). In colorectal carcinoma and in particular Ulcerative Colitis and Crohn's disease the enhancement of NFκB activation prevail in IECs together with macrophages (Andresen et al. 2005;Jobin et al. 1997;Rogler and Andus 1998b).

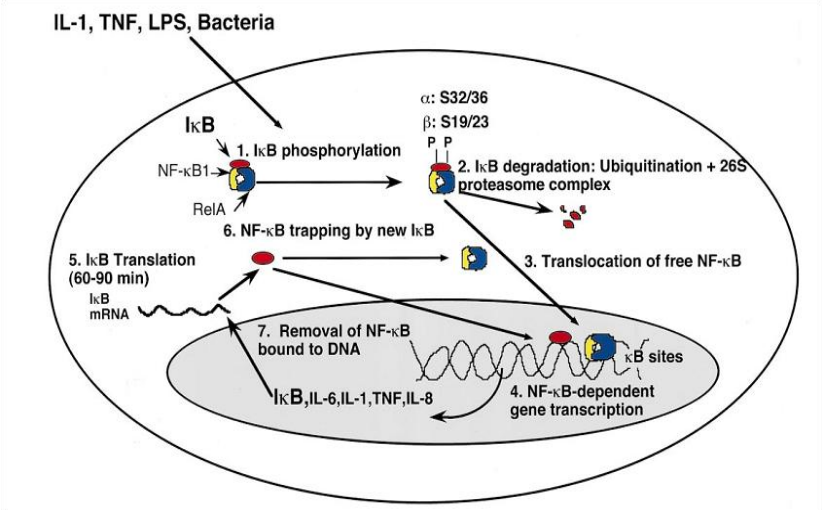



**Figure 6:** Mechanism of NF-κB activation and regulation. NF-κB is kept latent in the cytoplasm by the inhibitor protein IκB. IκB phosphorylation leads to ubiquitination and degradation by the proteasome pathway. Free RelA (p65) migrates to the nucleaus and by virtue of its nuclear localization activates transcription of multiple kB-dependent genes. IL-interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide. Adapted from: (Jobin et al. 1997).

Another important transcription factor in inflammatory pathways is STAT3 (Darnell, Jr. 2007;Schindler 2002). It is activated through phosphorylation of tyrosine 705, in response to various cytokines and growth factors including interferons, epidermal growth factor,

Interleukin-6 and hepatocyte growth factor. For STAT3 a constitutive activation in primary human colorectal carcinoma cells and established human colorectal cancer cell lines has been shown (Corvinus et al. 2005;Kusaba et al. 2005;Ma et al. 2004). Constitutive STAT3 activation in colorectal cancer cells is associated with invasion, survival, and growth of colorectal cancer cells and colorectal tumor model in mice *in vivo* (Corvinus et al. 2005;Lin et al. 2005;Tsareva et al. 2007;Xiong et al. 2008). In fact, STAT3 was shown to have an oncogenic or tumor suppressor role depending on the mutational background of the tumor. Moreover, its role as a tumor suppressor gene has also been reported (de la Iglesia et al. 2008). STAT3 is a vital oncogenic pathway activator in colorectal cancer and can serve as a potential therapeutic target for colorectal carcinoma (Lin et al. 2011).

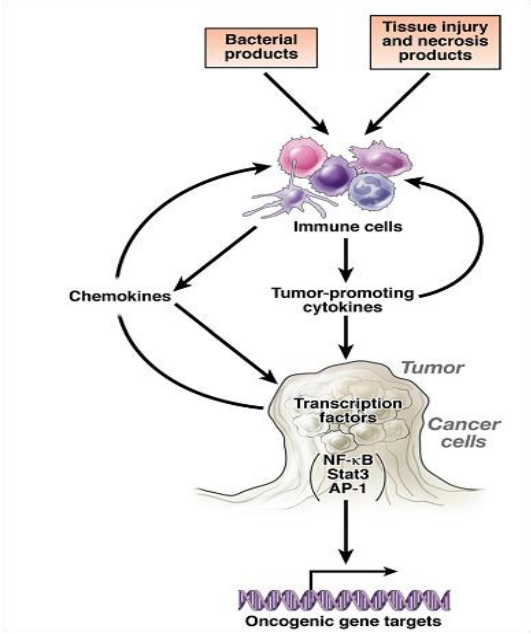



**Figure 7:** Signal transducers and activators of transcription (STAT) are activated by cytokine and growth factor receptors. Involvement of cytokines and transcription factors in tumor promotion: Cytokines and growth factors in the tumor microenvironment activate transcription factors such as NF-κB, STAT3, and AP-1, which regulate genes that control numerous processes such as cell proliferation, tumor growth, and resistance to cell death, angiogenesis and tumor progression and invasion. Adapted from: (Terzic et al. 2010).

## 2.6 Treatment selection in Colorectal Carcinoma

The four main types of treatment for colorectal cancer are surgery, radiation therapy, chemotherapy and targeted therapies (like monoclonal antibodies).

KRAS gene somatic mutations at codon 12 and 13 occur with an incidence of 30 to 40% in CRC (Downward 2003). KRAS is the central mediator of epidermal growth factor receptor (EGFR) signalling, it is reasonable to suggest that the KRAS mutational status might modify the response of a tumor to anti-EGFR therapy (Feng et al. 2007). Cetuximab (Erbitux) and Panitumumab (Vectibix), monoclonal antibodies directed against EGFR, are commonly used to treat metastatic CRC. They inhibit the signal from the EGFR and block downstream effects including BRAF and KRAS-mediated events. Whenever KRAS and BRAF mutations exist, the RAS-RAF-MEK-ERK signaling pathway remains in the "on" state leading to continuous cell division and proliferation (Cunningham et al. 2004;Zhang et al. 2006). Tumors with mutant KRAS genes may not respond to Cetuximab but they are still receptive to chemotherapy and research is in progress to develop drugs which will boost the efficacy of chemotherapy for these tumor types.

Anti-TNF therapy is a new class of biological therapy (Garces et al. 2012) that inhibits TNF released by macrophages which augments rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) such as Crohn's disease and ulcerative colitis (Atzeni et al. 2011). Three types of anti-TNF therapy are used so far: etanercept (Enbrel®), infliximab (Remicade®) and adalimumab (Humira®). Many studies revealed that infliximab has made a significant progress in therapy for patients with Crohn's disease (van Dullemen et al. 1995). The efficiency of infliximab suggests that TNF, a product of activated macrophages, could have a major role among the many regulatory peptides with altered expression in association with IBD (Lugering et al. 2001;Ten et al. 2002).

## 2.7 Aims of study

A major contributor to the tumor microenvironment is inflammation and inflammatory mediators (especially the cytokines IL-1β, IFNγ and TNFα). There have been studies about the effect of cytokines on chemokine gene expression and cross talk between EGFR and chemokines. However, little is known about the effect of these mediators on gene expression of chemokines (CXCL1, CXCL8 and CXCL10) in the presence/absence of KRAS mutation.

Based on these studies, we hypothesized that stromal cells of the tumor microenvironment may release pro-inflammatory cytokines (IL-1β, IFNγ, and TNFα) and EGF which in turn acts on CRC-cells to secrete chemokines (CXCL1, CXCL8 and CXCL10). These chemokines in turn attract inflammatory cells which act on the tumor cell and its microenvironment, thereby multiplying the inflammatory effects and subsequent tumor initiation and promotion.

The inhibition of these pro-inflammatory and angiogenic pathways may lead to new insights into new drug development discoveries for the KRAS and BRAF mutated patients who are irresponsive to anti EGFR therapies. This study is based on the findings that KRAS and BRAF mutations in CRCs are considered mutually exclusive in the activation of the RAS/RAF/MEK/ERK pathway. In view of this knowledge, six CRC cell lines were investigated for their mutation and regulation of pro- (CXCL1 & CXCL8) and anti-angiogenic (CXCL10) chemokine gene expression. By adding pro-inflammatory cytokines to the medium, a different microenvironment was created. Moreover, it was also the aim of study to investigate the transcriptional factors which are involved in the regulation of these chemokines in CRC mutated and non-mutated cell lines after administration of pro-inflammatory cytokines (TNFα, IFNγ and IL-1ß).

Aims of the project:

1. To validate the previously published KRAS (12, 13, 61, 146), BRAF (V600E) and NRAS (12, 61) mutations in the six CRC cell lines.

2. To investigate the cytokine (TNFα, IFNγ and IL1ß) / chemokine (CXCL1, CXCL8 & CXCL10) profile in view of the different mutations of the CRC cell lines.

3. To understand the effect of cytokines (TNFα, IFNγ and IL1ß) on pro- and anti-angiogenic chemokines in CRC mutated and wild type cell lines.

4. To explore the role of these chemokines and their signalling pathway by knocking down the KRAS-gene in mutant (DLD1) and wild type (Caco2) colorectal cancer cell lines.

# 3. MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1. Laboratory devices

Applied Bio-System PRISM® 7000 Sequence Detection System (ABI, USA)

Centrifuge Hettich Rotixa/K (Hettich Zentrifugen)

DNA Engine System 9600 (Gene Amp Perkin Elmer)

Electro blotting apparatus, type Mini Trans-BlotR, Bio-Rad /Munich, Germany

Electrophoresis apparatus, type Mini-ProteanR III, Bio-Rad /Munich, Germany

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)

Ice machine, Ziegra /Isernhagen, Germany

Minishaker Bioshaker BD (BioRad,Goettingen)

Microscope Leica, DMIL (Oberkochen, Germany)

Microwave oven, Siemens /Germany

pH-Meter 761 Calimatic, Knick (Berlin, Germany)

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)

StepOnePlus$^{TM}$Real-Time PCR System Applied Biosystems (ABI, USA)

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)

Video Densitometer (Herlob, Wiesloch, Germany)

Water bath 1083, GFL (Burgwedel, Germany)

X-ray film cassettes 10×18, (Siemens /Germany)

X-ray film-developing machine SRX-101A, Konica Europe (Hohenbrunn, Germany)

### 3.1.2. Tools

| | |
|---|---|
| 6 well plates | (Nunc, Rushilde) |
| 24 well plates | (Nunc, Rushilde) |
| 75 cm culture flasks | (Nunc, Rushilde) |
| Siemens film cassette | (Siemen, Germany) |
| 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) |

### 3.1.3. Cells

Caco2, HT-29, DLD1 Colo-320, Colo-205 and CX-1 were from human colon adenocarcinoma (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig). Some cell lines were cultured and used for positive control i.e. EGI-1, SW-948 and ML-2.

### 3.1.4. 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) |
| 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) |
| Ampuwa water | (Fresenius /Bad Homburg, Germany) |
| Bis-acrylamide | (Sigma-Aldrich, Deisenhofen) |
| BstX1 Enzyme | (New England Bio-Labs) |

| | |
|---|---|
| β-Glycero Phosphate | (Sigma-Aldrich, Deisenhofen) |
| Dimethyl Sulphoxide /DMSO/ | (Sigma-Aldrich, Deisenhofen) |
| Di-Sodium hydrogen phosphate /Na2HPO4/ | (Merck KGaA, Darmstadt) |
| Eagle's minimal essential medium /EMEM/ | (BioWhittaker, Verniers) |
| Ethanol absolut | (Merck KGaA, Darmstadt) |
| Ethidium bromide | (Sigma-Aldrich, Deisenhofen) |
| Ethylenediaminetetraacetic acid /EDTA/ | (Sigma-Aldrich, Deisenhofen) |
| Edetate disodium $Na_2$ EDTA | (Sigma-Aldrich, Deisenhofen) |
| Ethyleneglycol-bis(β-aminoethylether)N,N,N',N'-tetraacetic acid /EGTA/ | (Sigma-Aldrich, Deisenhofen) |
| Fetal calf serum (FCS) | (GIBCO BRL Grand Island) |
| Hydrochloric acid /HCl | (Merck KGaA, Darmstadt) |
| Interferon γ/human | (Roche Molecular Biochemicals, Mannheim) |
| Interleukin-1β/human | (Roche Molecular Biochemicals, Mannheim) |
| Leupeptin | (Sigma-Aldrich, Deisenhofen) |
| Magnesium sulphate /$MgSO_4$ | (Merck KGaA, Darmstadt) |
| Magnesium Chloride /$MgCl_2$ | (Merck KGaA, Darmstadt) |
| Methanol | (Merck KGaA, Darmstadt) |
| Moloney Murine Leukemia Virus Reverse Transcriptase /M-MLV-RT/ | (Roche Molecular Biochemicals, Mannheim) |
| Sodium orthovanadate/ $Na_3VO_4$ | (Sigma-Aldrich, Deisenhofen) |
| Sodium Chloride/NaCl | (Sigma-Aldrich, Deisenhofen) |
| N-2 (hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)/HEPES/ | (Sigma-Aldrich, Deisenhofen) |
| Nonidet NP-40 | (Sigma-Aldrich, Deisenhofen) |
| Penicillin/Streptomycin | (GIBCO BRL Grand Island) |
| Phenylmethansulfonyl fluoride /PMSF/ | (Sigma-Aldrich, Deisenhofen) |
| Phosphate buffered saline /PBS | (Bio-Chrome AG, Berlin) |
| 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) |
| 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) |
| Sodium Pyrophosphate tetra basic dehydrate | (Sigma-Aldrich, Deisenhofen) |
| Tumor Necrosis Factor α(human) | (Roche Molecular Biochemicals, Mannheim) |
| TEMED/N, N, N', N',-Tetramethyl ethylenediamine | (Sigma-Aldrich, Deisenhofen) |
| Trypsin/EDTA | (BioWhittaker, Verniers) |
| Triton X-100 4-(1, 1, 3, 3-Tetramethylbutyl) phenyl-polyethylene glycol *t*-Octylphenoxypolyethoxyethanol Polyethylene glycol *tert*-octylphenyl ether | (Sigma-Aldrich, Deisenhofen) |
| Trypan blue | (Biochrom /Berlin) |
| Tween 20 | (Bio-Rad /Munich) |
| Xcm1 Enzyme | (New England BioLabs) |
| QuikHyb® Hybridization Solution | (Stratagene, Heidelberg) |

### 3.1.5. Solutions

**Restriction Digest Buffer 2 (Xcm1):**

**1X NEBuffer 2:**

| | |
|---|---|
| Tris-HCl | 50 mM |
| NaCl | 100 mM |
| $MgCl_2$ | 10 mM |
| DTT | 1 mM |
| pH | 7.9 @ 25°C |

**Restriction Digest Buffer 3 (BstXI):**

**1X NEBuffer 3:**

| | |
|---|---|
| Tris-HCl | 50 mM |

| | |
|---|---|
| NaCl | 100 mM |
| $MgCl_2$ | 10 mM |
| DTT | 1 mM |
| pH | 7.9 @ 25°C |

**Agarose gel 1.5 % (In TBE buffer):**

| | |
|---|---|
| Agarose | 1.5 % (w/v) |
| Ethidium bromide | 0.4 µg/ml |

**Extraction Buffer for protein isolation (In Distilled $H_2O$):**

| | |
|---|---|
| HEPES | pH 7.9 20 mM |
| KCl | 10 mM |
| EDTA | 0.1 mM |
| DTT | 1mM |
| PMSF | 0.5 mM |

**Extraction buffer with NaCl for protein isolation (In Distilled $H_2O$):**

| | |
|---|---|
| HEPES | pH 7.9 20 mM |
| NaCl | 0.4 M |
| EDTA | 0.1 mM |
| DTT | 1mM |
| PMSF | 0.5 mM |

**Triton X-100 Lysis Buffer (Cell Signalling):**

| | |
|---|---|
| Tris-HCl | pH 7.5 |
| NaCl | 150mM |
| $Na_2$ EDTA | 1 mM |
| EDTA | 1mM |
| Triton | 1% |
| Sodium Pyrophosphate | 2.5mM |
| β-Glycero Phosphate | 1mM |
| $Na_3VO_4$ | 1mM |
| Leupeptin | 1µg/ml |

**TBE-buffer (10X) (In Distilled $H_2O$):**

| | |
|---|---|
| Tris base | 0.1 M |
| Boric acid | 0.1 M |

| | |
|---|---|
| EDTA pH 8 | 1 mM |

**Loading buffer (In distilled water and stored for 2-3 months at –20°C):**

| | |
|---|---|
| Running buffer | 5 ml |
| 2ß- Mercaptoethanol | 40µl |
| Bromophenol blue | 10mg |

**Blocking buffer (In TBS-T)**

| | |
|---|---|
| Dried Milk | 5% |

**Stripping Buffer (In distilled $H_2O$)**

| | |
|---|---|
| Tris-HCL (pH 7) | 50mM |
| SDS | 2% |
| DTT | 50mM |

### 3.1.6. Kits

| | |
|---|---|
| RNeasy Mini Kit | (QIAGEN GmbH, Hilden) |
| DNA/RNA All Prep Mini Kit | (QIAGEN GmbH, Hilden) |

## 3.2. Methods

### 3.2.1 Cell culture conditions and Stimulation.

Caco-2 and CX-1 cells were grown in DMEM medium containing 20% FCS for Caco2 and 10% for CX-1 supplemented with 100 U/ml each penicillin and streptomycin and 1% non essential amino acids at 37°C and 5% $CO_2$. HT-29, Colo-205, Colo-320 and DLD1 were grown in RPMI medium containing 10% FCS and 100U/ml penicillin and streptomycin at 37$^o$C and 5% $CO_2$. Intestinal Epithelial Cells (IECs) 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 % $CO_2$. For cytokine stimulation, IECs were plated into 6-well plates at a density of $5\times10^5$ cells per well and grown till they reached 70-80% confluence. These cells were then stimulated with IL-1β (1 ng/ml), TNFα (50ng/ml) and IFNγ (50ng/ml) based on the type of experiments.

### 3.2.2 Primers for real-time PCR.

**Table1: List of Human Primers Sequences used for Real Time PCRs.**

| Primer | 5' →3' Forward | 5' →3' Reverse |
|---|---|---|
| *House Keeping* | | |
| Human β-Actin | CTG GCACCCAGCACAATG | CCGATCCACACGGAGTACTTG |
| *Chemokines* | | |
| Human CXCL1 (GROα) | GTGTGAACGTGAAGTCCCCC | GCTGCAGAAATCAGGAAGGC |
| Human CXCL8 (IL-8) | ATGACTTCCAAGCTGGCCG | GCTGCAGAAATCAGGAAGGC |
| Human CXCL10 (IP-10) | CCAGAATCGAAGGCCATCAA | CATTTCCTTGCTAACTGCTTTCAG |
| *Cytokines* | | |
| Human TNFα | CCCAGGCAGTCAGATCATCTTC | AGCTGCCCCTCAGCTTGA |
| Human IFNγ | CCAACGCAAAGCAATACATGA | TTTTCGCTTCCCTGTTTTAGC |
| Human IL-1ß | AATTTGAGTCTGCCCAGTTCCC | AGTCAGTTATATCCTGGCCGCC |
| *Receptors* | | |
| Human TNFα Rec1 | AGGAAGAACCAGTACCGGCAT | TCTGTTTCTCCTGGCAGGAGA |
| Human IL1ß Rec1 | TGTTCAGGAGCTGAAGCCCAT | AATTCACACAGCAGGACAG |
| Human IFNγ Rec1 | AAGAGCCGTTGTCTCCAGCAA | TAAAGCGATGCTGCCAGGTTC |
| *Oncogenes* | | |
| Human KRAS Codon 12 & 13 | RAS A (Forward): 5' ACTGAATATAAACTTGTGGTCCATGGAGCT 3' | |
| | RAS B (Reverse): 5' TTATCTGTATCAAAGAATGGTCCTGCACCA 3' | |
| | RAS C (Reverse): 5' GGATGGTCCTCCACCAGTAATATGGATATT 3' | |
| Human BRAF V600E | A-Allele (Forward): 5' AAAAATAGGTGATTTTGGTCTAGCTACTGTA 3' | |
| | T-Allele (Forward): 5' AAAAATAGGTGATTTTGGTCTAGCTACTGT 3' | |
| | (Reverse): 5' ACACTGATTTTTGTGAATACTGGGAACT 3' | |
| *Transcription Factors* | | |
| Human NF-kB | GCAAAATCCTGACCTGGTGT | GCTCGTCCTCTGTGAACTCC |
| Human STAT3 | CTGGCCTTTGGTGTGTTGAAAT | AAGGCACCCACACAAACAAC |
| Human MAPK1 | CCAGACCATGATCACACACAGG | TGTGATGGGGATCCAAGAAT |
| *KRAS siRNA sequence* | | |
| Sequence 1 | GCAAGUAGUAAU UGAUGGA | UCCAUCAAUUACUACUUGC |
| Sequence 2 | ACAGGCUCAGGACUUAGCA | UGCUAAGUCCUGAGCCUGU |
| Sequence 3 | GCAAGAAGUUAUGGAAUUCUA | GAAUUCCAUAACUUCUUGCUC |

### 3.2.3. Purification of genomic DNA

DNA isolation was performed using the Qiagen DNA/RNA all Prep Mini Kit according to the manufacturer's recommendations (Qiagen, Hilden,Germany). Colorectal Carcinoma cells ($5x10^4$) were washed and resuspended with 3ml 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 2ml 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 amount of isolated DNA was determined Spectrophotometrically (GeneQuantII; Pharmacia, Freiburg, Germany).

### 3.2.4 Anti bodies for Western Blot Analysis

**Table2: List of Human Antibodies used for Western Blot Analysis.**

| *Primary Antibody* | *Origin* | *Dilutions PrimaryAb* | *Provider* |
|---|---|---|---|
| β-Actin | Mouse | 1:5000 | Sigma Aldrich |
| CXCL1/GROα | Goat | 1:500 | R&D System |
| CXCL10/ IP-10 | Goat | 1:500 | R&D System |
| CXCL8/ IL-8 | Goat | 1:500 | R&D System |
| NF-κB (p65) | Rabbit | 1:1000 | Santa Cruz Biotechnology |
| MAPK1 (ERK1/2) | Rabbit | 1:1000 | Cell Signalling |
| IκBα | Rabbit | 1:10000 | Abcam |
| KRAS | Rabbit | 1:1000 | ABBIO Technology |
| p-STAT3 | Mouse | 1:1000 | Cell Signalling |

### 3.2.5. KRAS Mutation

KRAS Mutations at codons 12 and 13 were confirmed in all six cell lines by a highly sensitive PCR-RFLP assay **(Fig. 7).** 10ng of DNA were used as template for the first PCR, which consisted volume of containing Taq DNA polymerase, deoxynucleotide triphosphates , reaction buffer Buffer including $MgCl_2$; Invitrogen), and the oligo-nucleotide primers Ras A and Ras B (3μM each for the first PCR). The first PCR generated an amplicon of 166-bp length. For amplification, a DNA Engine System 9600 (Gene Amp Perkin Elmer) thermocycler was used. Cycling conditions of the first PCR were as follows: initial denaturation (5 min at 95°C), followed by 30 cycles of denaturation (1 min at 94°C), annealing (50sec at 50°C), and elongation (50sec at 72°C). After the last cycle, a final extension (10 min at 72°C) was added, and thereafter, the samples were kept at 4°C. The Ras A (sense) primer is designed to introduce

a restriction site for BstXI and XcmI into the wild-type amplicon. Because of this altered sequence, BstXI restricted the resulting amplicon if the first two bases of codon 12 were wild type. Similarly, XcmI cut the amplicon only, if the first two bases of codon 13 were wild type **(Table. 2 & Figure. 8)**.

For the first PCR restriction, 10µl from the first PCR reaction were digested with five units of either BstXI or XcmI in a total volume of 20µl, according to the manufacturer's recommendations (New England Biolabs, Schwalbach, Germany). 2.5µl of this first digest were used as template for the second PCR, in which primer Ras C (antisense) was used instead of Ras B ,thus creating a restriction site in both mutant and wild-type amplicons for enzymes BstXI or XcmI. The PCR was run under the same conditions as the first PCR for 32 cycles. For the second restriction, 10µl of the second PCR were digested with five units of either BstXI or XcmI in a volume of 20µl. 10µl of the product were run on a 4% Agarose Gel (Roth,Karlsruhe, Germany) with ethidium bromide for 1 min, and analyzed under UV light by a video densitometer (Herolab, Wiesloch, Germany). The DNA bands were visualised for the codon 12 and 13 with positive controls according to the base pairs of the specific positive controls band. Amplicons of mutated DNA were cut only once into fragments of 134 and 18bp, whereas amplicons of wild-type DNA were cut twice into bands of 106, 28 and 18bp length. A positive control DNA (5µg) from MDA-MB-231(For Codon 13) (Health Protection Agency Culture Collections UK) and positive cell lines EGi-I (For Codon 12) was used as a standard to control the efficacy of the restriction enzymes. Autoclaved, double deionized water was used as negative control.

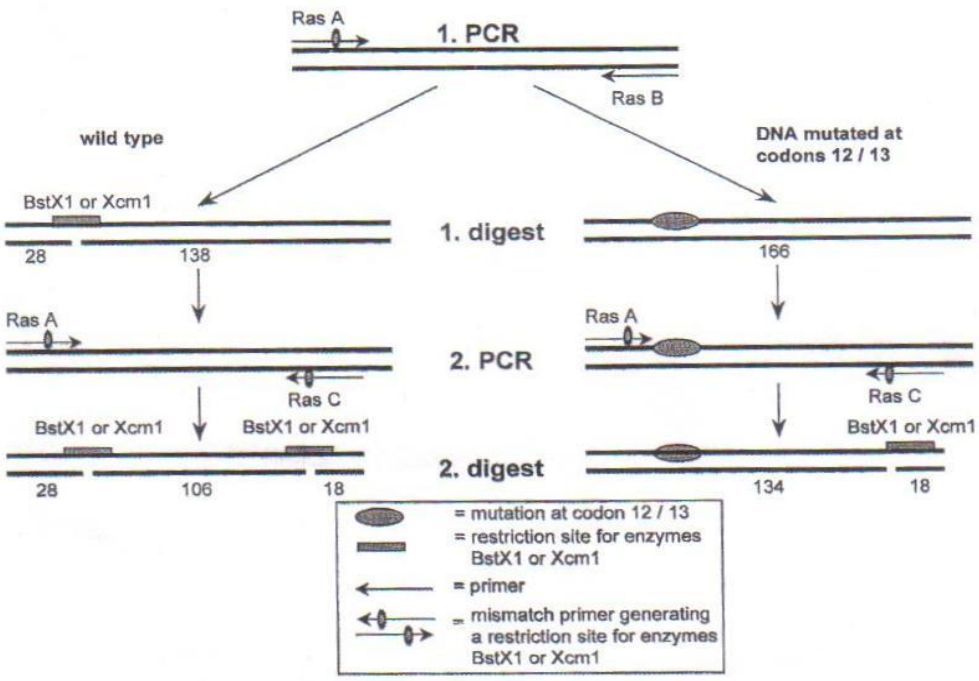



**Figure 8:** Restriction Length Polymorphism RFLP PCR Schematic two step assay for KRAS detection at codon G12D and G13D with restriction enzymes *BstXI* or *XcmI.* Source: (Schimanski et al. 1999)

**Table3: Reaction Setup and Running Protocol of RFLP PCR with two Restriction Enzymes (BstX1 & Xcm1).**

## KRAS Mutation Codon 12 & 13

| PCR 1 (25µl) | |
|---|---|
| **Component** | **Volume** |
| dNTPs 10mM | 0.5µl |
| Ras A 3µM | 5.0µl |
| Ras B 3µM | 5.0µl |
| $MgCL_2$ Inclusive 10x PCR Buffer | 2.5µl |
| Taq-Polymerase | 0.25µl |
| DNA | 2.5µl |
| $H_2O$ | 8.5µl |

| Digest 1 (BstX1) | |
|---|---|
| PCR Product 1 | 10µl |
| 10x PCR NEBuffer 3 | 2.0µl |
| Enzyme BstX1 (5U) | 0.5µl |
| $H_2O$ | 7.5µl |
| **Digest 1 (Xcm1)** | |
| PCR Product 1 | 10µl |
| 10x PCR NEBuffer 2 | 2.0µl |
| Enzyme Xcm1 (5U) | 1µl |
| $H_2O$ | 7.0µl |

| Temperatures °C | Time | | Time | | Time | Cycle |
|---|---|---|---|---|---|---|
| 94°C | 5Min | | | | | 1x |
| 94°C | 1Min | 50°C | 50sec | 72°C | 50sec | 30x |
| 94°C | 1Min | 50°C | 50sec | 72°C | 10Min | 1x |
| 4°C | Hold | | | | | |

In the table above the reaction setup, running protocol and digestion is shown for PCR1 and Digestion 1. The PCR2 set up and digestion2 is conducted in the same manner except the changes in Forward Ras A is used with Reverse Ras C with the same volume and in the running protocol the $2^{nd}$,$3^{rd}$ and $4^{th}$ step was done with 32 cycles.

### 3.2.6. BRAF Mutation

Braf Mutations at codons V600E were confirmed in all six cell lines by Real Time ARMS PCR method (Table. 5). For each PCR, 1 µL template DNA( 6.4ng/µl) was mixed with 5 µL (2× SYBR Green) PCR master mix and 3µM each of the forward (A or T) and reverse primers in a final volume of 10 µL. One was the standard protocol of the ABI Prism 7000 Sequence Detection System, 2min at 95°C annealing temperature, 15s at 95°C with 30s at 60°C for 45

cycles, and followed by the thermal denaturing step to generate the dissociation curves to verify amplification specificity (Table. 5).

For the ARMS PCR assay an additional standard curve, serial 10-fold dilutions (range 2:0–1:1024) of the positive control (Colo-205) mixed into negative control (A212XI) were constructed from each dilution used to generate standard comparison curves against which to compare the unknown cancer cell lines. Each reaction contained in a 10µl reaction volume as stated above. Reactions were run for 45 cycles (95°C for 15s initiation, then 60°C 1min, 95°C 15s) on a 96-well plate using an Applied Biosystems 7700 Sequence detector (Applied Biosystems, CA, USA). All samples were run in duplicate, and were run concurrently on the same plate. Each plate also included multiple water blanks as an additional negative control (Table. 5).The same method is used for the confirmation of single nucleotide for KRAS A61T and A146T (Sequence not mentioned).

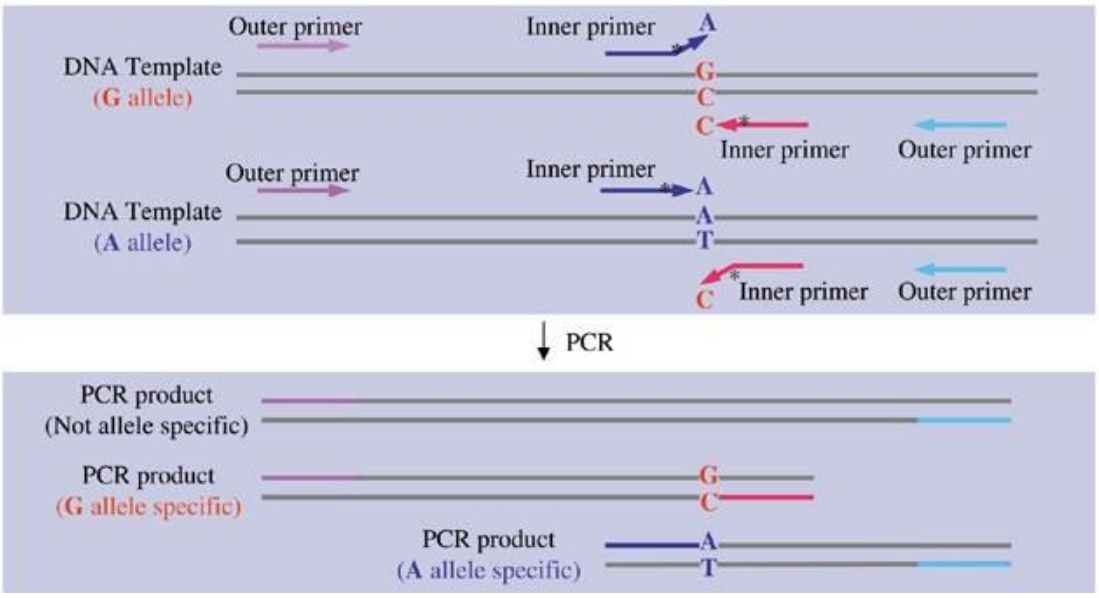



**Figure 9:** Schematic presentation of the tetra- Primer ARMS-PCR method. The single nucleotide polymorphism used here as an example is a G→A substitution, but the method can be used to type other types of single base substitutions. Adapted from: (Ye et al. 2001)

**Table4: Reaction Setup and Running Protocol of ARMS PCR with Standard Curve.**

# ARMS PCR Protocol

| PCR Estimation A Allele (10µl) | |
|---|---|
| Component | Volume |
| SYBER Green q PCR super Mix 2x | 5µl |
| ROX Reference Dye 50x | 0.2µl |
| Primer A Forward 3µM | 5.0µl |
| Primer A Reverse 3µM | 2.5µl |
| DNA (6.5ng) | 1.0µl |
| $H_2O$ (Ampuwa) | 1.8µl |

| PCR Estimation T Allele (10µl) | |
|---|---|
| Component | Volume |
| SYBER Green q PCR super Mix 2x | 5µl |
| ROX Reference Dye 50x | 0.2µl |
| Primer T Forward 3µM | 5.0µl |
| Primer A Reverse 3µM | 2.5µl |
| DNA (6.5ng) | 1.0µl |
| $H_2O$ (Ampuwa) | 1.8µl |

| Standard Curve | | |
|---|---|---|
| Dilution | | Quantity |
| Concentrated | 6.4ng (Any positive Control Cell line DNA for the specified hot spot) | 2 |
| 1:2 | 1 Part (positive Control)+ 1 Part (Negative Control) | 1 |
| 1:4 | 1 Part (positive Control)+ 3 Part (Negative Control) | 0.5 |
| 1:16 | 1 Part (positive Control)+ 1 Part (Negative Control) | 0.125 |
| 1.64 | 1 Part (positive Control)+ 1 Part (Negative Control) | 0.03 |
| 1:256 | 1 Part (positive Control)+ 1 Part (Negative Control) | 0.008 |
| 1:1024 | 1 Part (positive Control)+ 1 Part (Negative Control) | 0.002 |

## Thermophil (Step one plus)

| Temperatures °C | Time | | Time | Cycle |
|---|---|---|---|---|
| 50°C | 2Min | | | 1x |
| 95°C | 2Min | | | 1x |
| 95°C | 15Sec | 60°C | 1Min | 45x |

### 3.2.7. RNA isolation

Colo-205, Colo-320, CX-1, Caco-2, HT-29 and DLD1 cells were cultured in 6 well tissue culture plates at a density of $5x10^5$/well in 2 ml medium. 24 hours after seeding cells, they were incubated with different concentrations of IL-1β, TNFα, and IFNγ. Total RNA was isolated by using Qiagen RNeasy mini kit. The cells were first washed with 3ml PBS then lysed in 350µl highly denaturing guanidinium isothiocyanate-containing buffer (Buffer RLT). RNases were inactivated by adding β-mercaptoethanol to the buffer (10µl/1ml Buffer RLT). The lysate was

pipetted directly onto a QIAshredder column sitting in the 2ml 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 rpm. The membrane-bound RNA was washed first by pipetting 500 µl washing buffer (Buffer RW1) than by adding 500 µl ethanol containing Buffer RPE onto the column and centrifuging for 1 min at 10000 rpm. The RNeasy membrane was dried by 500 µl Buffer RPE centrifuging for 2 min 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 min at 10000 rpm. 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).

### 3.2.8. Reverse Transcription & Real-Time PCR

RNA was reverse transcribed to copy-DNA (cDNA) in a final volume of 40µl containing 400U M-MLV reverse transcriptase, 50mM Tris HCl pH 8.3, 75mM KCl, 3mM $MgCl_2$, 10mM DTT, 1.6nM (dT)15-primer and 0.5mM dNTP for 1 hour at 37°C. To determine the mRNA expression of cytokines and their receptors, chemokines, oncogenes and transcription factors, 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 0.3µM primers each and 1µl (7-10ng) 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 specific genes was then calculated using the comparative threshold-

cycle (CT) method. The amount of target mRNA in each sample was set in relation to the amount of β-actin mRNA designated as calibrator, to give ΔCT (CT gene - CT **β**-actin). The relative expression of gene was calculated as the chemokine/**β**-actin ratio = $2^{-\Delta}$CT. mRNA gene expression is presented as fold change calculated normalized to β-actin or $2^{-\Delta\Delta CT}$ values were compared in the case of quantification of basal level gene expression in cell lines.

### 3.2.9. Preparation of Cell Lysates Protein

$5\times10^5$ cells per dish was homogenized with an Ultra-turrax TP 18/10, three times for 10s each, in 10 vol 50 mM TRIS-HCl buffer, pH 7.4, containing 150 mM NaCl, 1 mM EDTA solutions, 1%Triton X-100, 1 mM phenylmethane sulfonyl-fluoride (PMSF), 1 mM benzamidine, 1 mg/ml leupeptin, 10 mM chymostatin, 1 mg/ml antipain, and 1 mg/ml pepstatin A. The entire procedure was carried out at 4°C. Crude homogenates were passed five times through a 22-G needle attached to a syringe and centrifuged for 5 min at 10,000rpm, 4°C. The protein concentration was determined in supernatants by using the BCA (bicinchoninic acid) protein assay reagent kit (Pierce, Bonn, Germany). Aliquots of the homogenates were stored at −20°C until further used for Western blot analysis and measurement.

### 3.2.10. Western Blotting

20μg from the total protein lysate were loaded in a 4-12% Nu-PAGE Bis-Tris (Invitrogen) gel and separated after 2h electrophoresis at 80V. After the transfer in a semidry apparatus at 30V for 1.5h, the membranes were blocked in 5% milk, and blotted with primary antibodies overnight at 4°C. The secondary antibodies were horse reddish peroxidase conjugated goat anti-rabbit and goat anti-mouse immunoglobulins (DAKO) diluted at 1:1000. Membranes were developed with ECL chemiluminescence Kit (Amersham). ß-actin was analyzed as an internal control.

### 3.2.11. RNA interference

All the synthetic siRNAs were designed by Qiagen. siRNA transfections were performed in 24-well plates. Transfection parameters were optimized for each cell line prior to validation according to the instructions given in the HiPerFect Transfection Reagent handbook. Optimized parameters were 20nM siRNA in combination with (1.5µl, 3µl, 4.5µl) HiPerFect (Qiagen) for Caco2 & DLD1 cell lines. Colorectal cells $4x10^4$ (Caco2 & DLD1) were plated in 24-well plates prior to transfection the 70% density was monitored. Briefly, for triplicate transfections, siRNA and HiPerFect were diluted in 100µl DMEM (Gibco, Grand Island, NY) without serum and incubated for 10 min at room temperature. After cell culture medium removal, 500µl fresh medium and 100µl transfection complexes were added per well. Cells were incubated for 48h and 72h before analyzing the degree of knockdown. Transfection performance was monitored using a validated MAPK1 siRNA (MAPK1 control siRNA; Qiagen). RNA isolation RNA isolation and purification was performed using the RNeasy protocol (Qiagen), according to the manufacturer's guidelines. RNA was eluted in 100µl of RNase-free water (Qiagen).

Generally, at least five independent transfections were carried out per siRNA with three replicates each. This procedure was repeated once, resulting in sixteen knockdown values for each siRNA. Transfection performance was verified by analyzing the degree of silencing obtained with the positive control siRNA (targeting MAPK1) which was transfected in parallel on each plate. Plates displaying lower positive control knockdown efficiencies were not analyzed. A single PCR was performed for analysis of target and reference gene expression for each siRNA transfection. Samples of untransfected cells were analyzed in duplicate for both genes. Knockdown values were calculated by the $^{-\Delta}CT$ method and β-Actin was used as an internal standard for normalization. Knockdown values were calculated with reference to transfected cells with scrambled siRNA.

For Western Blot Analysis 40µg from the total protein lysate were loaded in a 4-12% Nu-PAGE Bis-Tris (Invitrogen) gel and separated after 2h electrophoresis at 80V. After the transfer in a semidry apparatus at 30V for 1.5h, the membranes were blocked in 5% milk, and blotted with KRAS primary antibodies overnight at 4°C. The secondary antibodies were horse reddish peroxidase conjugated goat anti-rabbit and goat anti-mouse immunoglobulins (DAKO) diluted at 1:1000. Membranes were developed with ECL chemiluminescence Kit (Amersham). ß-actin was analyzed as an internal control.

### 3.2.12. Statistical Analysis

The data were analyzed using Prism Graph pad 5 software (San Diego, USA). All experimental errors are shown as SEM. Statistical significance was calculated by Student´s T test and one way ANOVA test. Significance was accepted at $P < 0.05$.

# 4. RESULTS

## 4.1 KRAS Mutation in Cell lines

The known mutations of KRAS were confirmed for the six cell lines (Caco-2, CX-1, Colo-320, DLD1, HT-29 and Colo-205). Specific mismatch primers were used to amplify genomic DNA fragments through PCR-RFLP assay containing the hot spots of codons G12D and G13D. As shown in **Figure 10A** the positive controls for KRAS G12D and KRAS G13D are identified by a band at 134bp whereas the wild type shows a band at 106bp. The detection limit of this assay is based on the binding of mismatch primers generating a restriction factor site for BstXI (For Codon G12D) and XcmI (For Codon G13D). The amplicons of mutated DNA (Codon 12 & 13) were cut only once into fragments of 134 and 18bp, whereas amplicons of wild type DNA were cut twice into bands of 106bp, 28bp and 18bp length. The cell line DLD1 showed a band at 134bp indicating a KRAS G13D mutation **(Figure 10B)** in comparison to the wild type cell lines, which showed a band at 106bp. The other cell lines Caco2, CX-1, Colo-205 and HT-29 were found to be wild type after repetitive experiments for the KRAS mutation.

The KRAS codons A61T and A146V, other hotspots for KRAS mutations, were detected using real time PCR. Specific hot spot ARMS primers were designed for the two codons and all the six cell lines did not show these KRAS mutations with their respective positive control cell lines **(Table.5)**.

The Ras protein family N-Ras is a small GTPase protein which is normally present in an inactive GDP bound form (Dhomen and Marais 2007). These proteins can be activated by extracellular signals (e.g. through growth factor receptors) which result in an exchange of GDP for GTP (Fearon and Vogelstein 1990a). The hot spot detection via ARMS primer for the N-Ras mutations G12C and Q61R was performed for the six cell lines and none of the cell line was mutated **(Table 5)**.

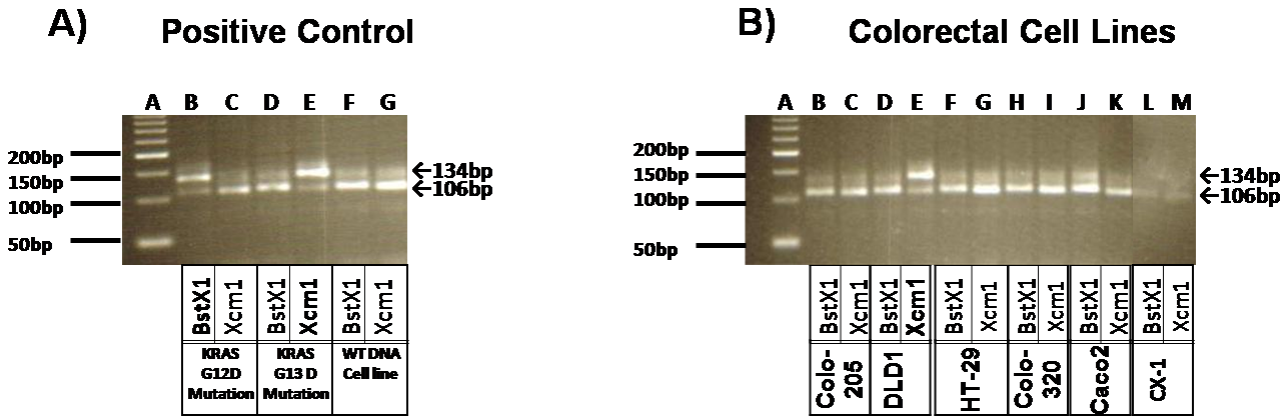



**Figure 10:** RFLP PCR electrophoresis for KRAS Codon 12 & 13 mutation analysis for six cell lines. The experiment was performed using genomic DNA (10ng) with two restriction enzymes BstX1 (Codon G12D) and Xcm1 (Codon G13D) for all the CRC cell lines. (**A)** Lane **A:** DNA ladder. The lane **B** and **C** shows the G12D positive DNA fragment digested either with BstX1 (positive control for G12D) or Xcm1 (positive control for G13D). The lane **D** and **E** shows the G13D positive DNA fragment digested either with BstX1 (positive control for G12D) or Xcm1 (positive control for G13D). Lane **F** and **G** shows the wild type DNA fragment digested either with BstX1 (positive control for G12D) or Xcm1 (positive control for G13D). Positivity resulted in a band at 134bp (fragment cut only at control cleavage site). **(B)** Colorectal Cell lines: Lane A; DNA Ladder; Lanes B-M fragments obtained after PCR and digestion with either BstX1 or Xcm1. Lane E confirms G13D mutation in the DLD1 cell line at base pair 134.

### 4.2 BRAF Mutation in Cell lines

BRAF is a serine/threonine kinase that belongs to the RAS/RAF/MEK/ERK/MAPK pathway, which is involved in the transduction of mitogenic signals from the cell membrane to the nucleus. RAS is inactive when bound to GDP, but if activated it promotes phosphorylation and activation of BRAF and further activation of the pathway signal (Davies et al. 2002a).

Genomic DNA (10ng) extracted from the human CRC cell lines was amplified as described in the methods. Two allele-specific amplicons were generated using two pairs of primers, one pair producing an amplicon representing the A (mutated) allele and the other T (wild type) allele. The x-axis in the (**Fig.11)**, representing the ΔRn (normalized reporter) which is obtained after the normalization of Rn by subtracting the baseline. The x-axis **(Fig. 11)** shows the $C_T$ values the fractional cycle number at which the fluorescence passes the threshold. In a real time PCR

assay a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e the lower the Ct level the greater the amount of target nucleic acid in the sample). The genotyping primer mix contains two labeled probes homologous to the two genotypes (A or T). During real-time PCR amplification of the target DNA, the probes will compete for binding across the variant region. The probe that is 100% homologous to the DNA binding site will preferentially bind and give a fluorescent signal as PCR proceeds. It follows that the wild type sequence will give a strong amplification plot through one channel whilst giving a very weak signal through the alternative channel. Homozygous variant samples will give an exactly inverse result. Heterozygous samples contain both probes binding sites on each of the two alleles and therefore give an intermediate signal through both channels.

All the cell lines were tested for the Braf mutations. Braf mutation (heterozygote) at hotspot V600E was confirmed in the two cell lines HT-29 and Colo-205 **(Table.5)**. The other four cell lines were wild type for the Braf mutation.

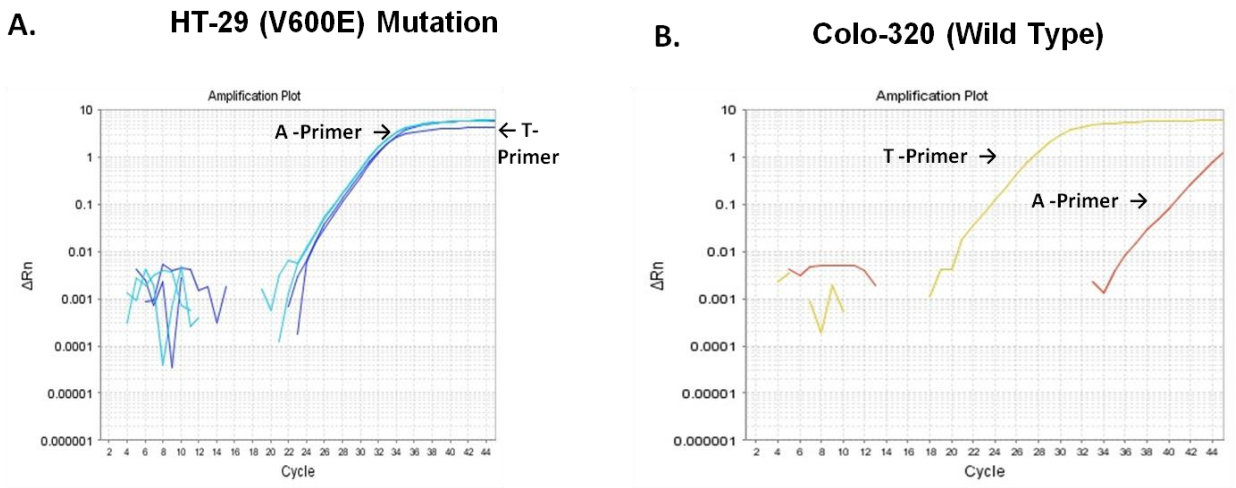



**Figure 11: Real Time® PCR assays for *BRAF* V600E mutation detection. (Fig11A.)** The plot confirms the heterozygotic mutant allele in HT-29 cell line. **(B)** Negative control cell line

that indicates the primer A and T for the wild type cell lines at distant position resembles the wild type.

**Table5: List of Mutations of KRAS, NRAS and BRAF for different hotspots in six colorectal cell lines.**

| Colo-Epithelial Cell Lines | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Caco-2* | *DLD-1* | *HT-29* | *CX-1* | *Colo-320* | *Colo-205* |
| **K-Ras** | | | | | | | |
| | ***Codon G12D*** | WT | WT | WT | WT | WT | WT |
| | ***Codon G13D*** | WT | **Mutated** | WT | WT | WT | WT |
| | ***Codon A61T*** | WT | WT | WT | WT | WT | WT |
| | ***Codon A146T*** | WT | WT | WT | WT | WT | WT |
| **N-Ras** | | | | | | | |
| | ***G12C*** | WT | WT | WT | WT | WT | WT |
| | ***Q61R*** | WT | WT | WT | WT | WT | WT |
| **Braf** | | | | | | | |
| | ***V600E*** | WT | WT | **Mutated** | WT | WT | **Mutated** |

**Table6: List of Mutations for different hotspots in six colorectal cell lines.**

| Cell Lines | Gene | AA Mutation | Zygosity |
|---|---|---|---|
| **Caco2** | *APC* | p.Q1367 (Substitution-Nonsense) | Homozygous |
| | *CTNNB1* | p.G245A (Substitution-Missense) | Unknown |
| | *SMAD4* | p.D351H (Substitution-Missense) | Unknown |
| | | | |
| **DLD-1** | *APC* | p.I1417 (Deletion-Frameshift) | Unknown |
| | *KRAS* | p.G13D (Substitution-Missense) | Heterozygous |
| | *SMO* | p.1918A (Substitution-Missense) | Unknown |
| | | | |
| **HT-29** | *APC* | p.E853 (Substitution-Nonsense) | Unknown |
| | *APC* | p.T1556 (Insertion-Frameshift) | Unknown |
| | *PIK3CA* | p.P449T (Substitution-Missense) | Heterozygous |
| | *SMAD4* | p.Q311 (Substitution-Nonsense) | Homozygous |
| | *TP-53* | p.R273H (Substitution-Missense) | Unknown |
| | *BRAF* | **p.V600E (Substitution-Missense)** | **Heterozygous** |
| | | | |
| **CX-1** | *APC* | p.E853 (Substitution-Nonsense) | Unknown |
| | *APC* | p.T1556 (Insertion-Frameshift) | Unknown |
| | | | |
| **Colo -320-HSR** | *APC* | p.S811 (Substitution-Nonsense) | Homozygous |
| | *TP-53* | p.R248W (Substitution-Missense) | Unknown |
| | | | |
| **Colo 205** | *APC* | p.T1556 (Insertion-Frameshift) | Homozygous |
| | *BRAF* | **p.V600E (Substitution-Missense)** | **Heterozygous** |
| | *SMAD4* | p.? (Deletion) | Homozygous |
| | *TP-53* | p.Y103 L (Complex-Deletion Inframe) | Homozygous |

Source: Atlas of Genetics and Cytogenetics (www.sanger.ac.uk)
The Roche Cancer Genome Database (www.rcgdb.bioinf.uni-sb.de)

## 4.3 Basal changes in mRNA expression of acute phase cytokines in Intestinal Epithelial Cell lines (IECs)

The gene expression of major cytokines (TNFα, IL-1ß and IFNγ) was studied at basal level in five CRC cell lines. We excluded the CX-1 (non-adherent) cell line in the following experiments and continued with two wild type cell lines i.e Caco2 and Colo-320. Previously, it

has been published that IECs depending on their origin and maturity may have a different and distinct pattern of chemokine/cytokine expression (Yang et al. 1997). Using gene specific primers the real time PCR data showed that the basal mRNA expression of TNFα normalised to ß-actin expression was highest in Caco2 (Wt) followed by HT-29 (BRAF) and the lowest expression was observed in the DLD1(KRAS) cell line **(Figure 12A; *p*<0.05)**. The highest IL-1ß expression was observed in both the BRAF mutated cell lines HT-29 and Colo-205, followed by the two wild type Colo-320 and Caco2 respectively. The lowest expression for IL-1ß was found in DLD1 (KRAS) **(Figure 12B; *p*<0.05)**. The overall analysis of the basal level indicates that expression was very low in the KRAS mutated cell line DLD1 for the pro-inflammatory cytokines (TNFα and IL-1ß). Moreover, IFNγ and IL-6 have shown no expression for any of the cell lines in the experiment **(Figure 12C& D)**.

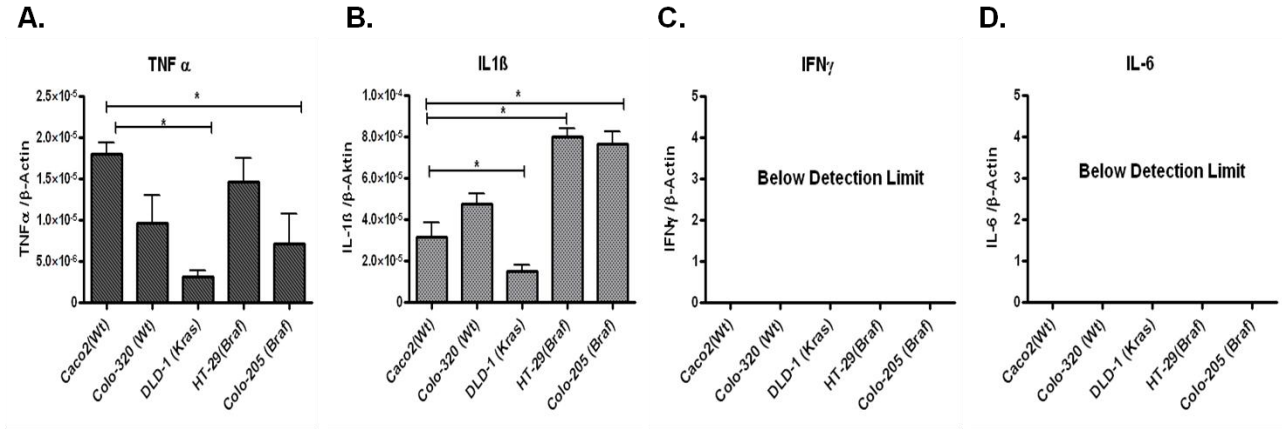



**Figure 12**: Basal mRNA expression of acute-phase cytokines (TNF-α, IL1-β & IFNγ) in Intestinal Epithelial Cells. $5\times10^5$ cells were plated into 6 well plates and grown for 24 hours. The cells were harvested, total RNA was isolated and first strand cDNA was prepared from 1 µg of total RNA. Ct values were normalized to ß-actin as a housekeeping gene. The results were compared with the fold changes of Caco2 mRNA expression, taken as a control. Results represent mean value ± S.E.M. ($^*p \leq 0.05$, $^{**}p \leq 0.01$ analyzed by one way ANOVA, n=4).

## 4.4 Basal changes in mRNA expression of acute phase cytokines receptors in Intestinal Epithelial Cell lines (IECs)

The basal level of cytokine receptor mRNA expression in the five cell lines revealed, that Colo-205 and HT-29 (BRAF mutated) have shown the highest expression for TNFα Rec1 **(Figure 13A; *p*<0.05)**. IL-1ß Rec was found in Caco2 (Wt) followed by DLD1 (KRAS) and Colo-205 (Braf) **(Figure 13B; *p*<0.05)**. Even though IFNγ did not show any expression for the five cell lines at the basal level, IFNγ Rec1 has shown a relatively high mRNA expression for the cell lines, also in comparison with TNFα Rec1 and IL-1ß Rec 1. IFNγ Rec1 resulted in maximum expression for the Colo-205 and HT-29 (BRAF) followed by Caco2 (Wt) **(Figure 13C; *p*<0.05).**

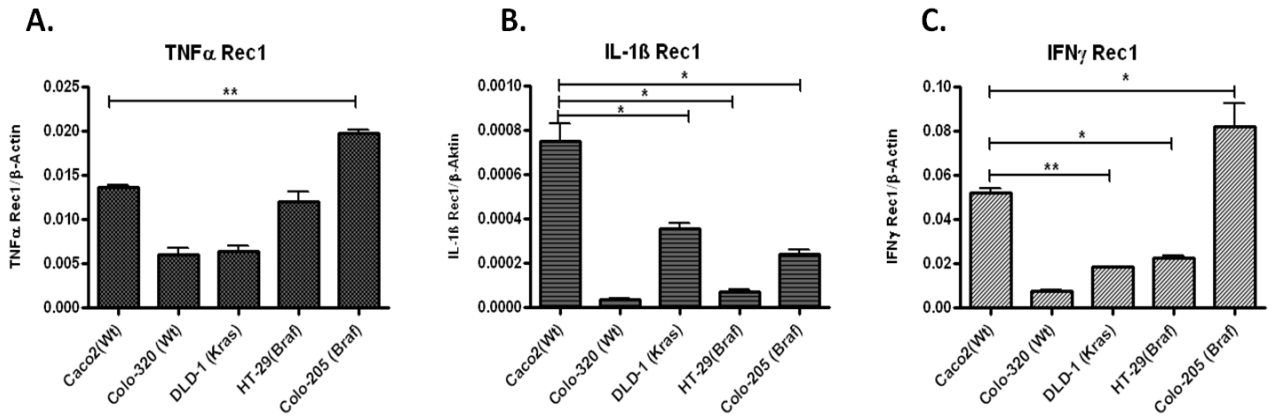



**Figure 13**: Basal mRNA expression of cytokine receptors (TNF-α Rec1, IL1-β and IFNγ Rec1) in IECs. $5\times10^5$ cells were plated into 6 well plates and grown for 24 hours. The cells were harvested, total RNA was isolated and first strand cDNA was prepared from 1 µg of total RNA. Ct values were normalized with ß-actin as a housekeeping gene. The results were compared with the fold changes of Caco2 mRNA expression, taken as a control. Results represent mean value ± S.E.M. ($^*p \leq 0.05$, $^{**}p \leq 0.01$ analyzed by one way ANOVA, n=4).

## 4.5 Differential Basal mRNA expression of pro inflammatory chemokines (CXCL1, CXCL8 and CXCL10) in Colorectal Cell lines (CRC)

The differences in the basal level of mRNA expression of chemokines were studied in five different cell lines. The mRNA expression of CXCL1 and CXCL8 was significantly higher in the mutated cell lines HT-29 (Braf) followed by Colo-205 (BRAF) and DLD1 (KRAS) **(Figure 14A&B; $p<0.05$).** However, the expression was low in the wild type cell lines Caco2 and Colo-320. In contrast CXCL10 mRNA was significantly increased in the wild type cell lines Caco2 and Colo-320. It was found that CXCL10 mRNA expression was lowest in the mutated cell lines (HT-29, DLD1 and Colo-205) **(Figure 14C; $p<0.05$)**.

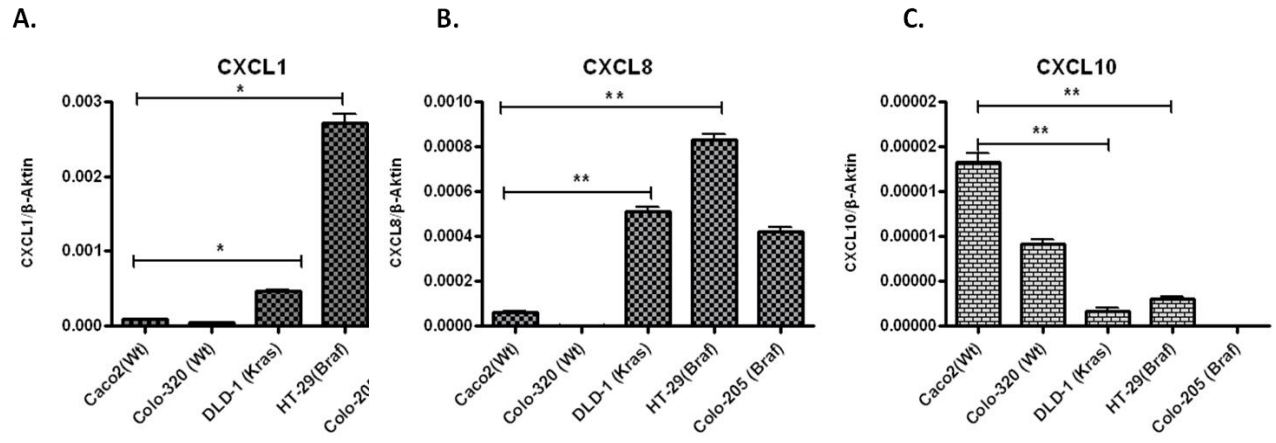



**Figure 14**: Basal mRNA expression of chemokines (CXCL1, CXCL8, and CXCL10) in IECs. $5\times10^5$ cells were plated into 6 well plates and grown for 24 hours. The cells were harvested, total RNA was isolated and first strand cDNA was prepared from 1 µg of total RNA. Ct values were normalized with ß-actin as a housekeeping gene. The results were compared with the fold changes of Caco2 mRNA expression, taken as a control. Results represent mean value ± S.E.M. ($^*p \leq 0.05$, $^{**}p \leq 0.01$ analyzed by one way ANOVA, n=4).

## 4.6 Selection of cell lines for the cytokine stimulation.

Three cell lines were chosen for the cytokine stimulation; Caco2 (Wt), DLD1 (KRAS) and HT-29 (BRAF). As a focus of our study was to investigate the cytokine / chemokine profile in view of the *KRAS* and *BRAF* mutations of the CRC cell lines, the three above stated cell-lines were

selected. Caco2 is wild type for *KRAS* and *BRAF* and therefore served as control. It has a high basal mRNA expression level for the TNFα, TNFα Rec1, IL-1ß Rec1, and IFNγ Rec1 by comparison to CX-1and colo-320. HT-29 (BRAF) was selected instead of Colo-205 (BRAF) because of its highly mutational status, growth properties (adherent) and its basal mRNA expression for TNFα and IL-1ß. DLD1 selection was based on its mutational status of KRAS codon G13D (Table.6).

The two cell lines CX-1 and Colo-320 have other mutations in the APC (both) and TP-53 (colo-320) gene (Table.6).

## 4.7 Changes in the mRNA expression of CXCL1 in colorectal cancer cell lines Caco2 (Wt), DLD1 (KRAS) and HT-29 (BRAF) by cytokines (TNFα, IL-1ß and IFNγ).

### 4.7.1 Regulation of CXCL1 mRNA expression by cytokines.

Three cell lines with two different mutations DLD1 (KRAS), HT-29 (BRAF) and Caco-2 (Wild type) were examined for the time kinetics of CXCL1 mRNA expression and protein secretion. The cytokines were administered at the following concentrations: IL-1β [1ng/ml], TNFα [50ng/ml] and IFNγ [50ng/ml] were administered to IECs.

Under control conditions **CXCL1 mRNA** expression did not change over the time. CXCL1 mRNA was inducible early at 1h after stimulation with TNFα in DLD1 (KRAS), (310±2.18fold) followed by HT-29 (BRAF; 36.15±3.28fold) whereas no change was detected in CXCL1 mRNA expression in the Caco2 cell line. The induction by TNFα of CXCL1 in HT-29 was milder as compared to DLD1 but lasted until 8h after stimulation, while in DLD1 it lasted only until 2h at high levels **(Figure 15B; *p*<0.05).** IL-1ß induced gene expression of CXCL1 in HT-29 (BRAF; 46.42±5.98 fold) was highest, followed by DLD1 (KRAS; 21.19±0.37fold) however, in Caco2 (Wt) IL-1ß did not effect the CXCL1 gene expression

**(Figure 15C; *p*<0.05)**. IFNγ stimulation showed a delayed increase of CXCL1 gene expression in Caco2 (Wt; 346.84±23.01fold) which was highest at 24h, followed by HT-29 (BRAF; 14.43±.50fold) at 8h **(Figure 15D; *p*<0.05).**

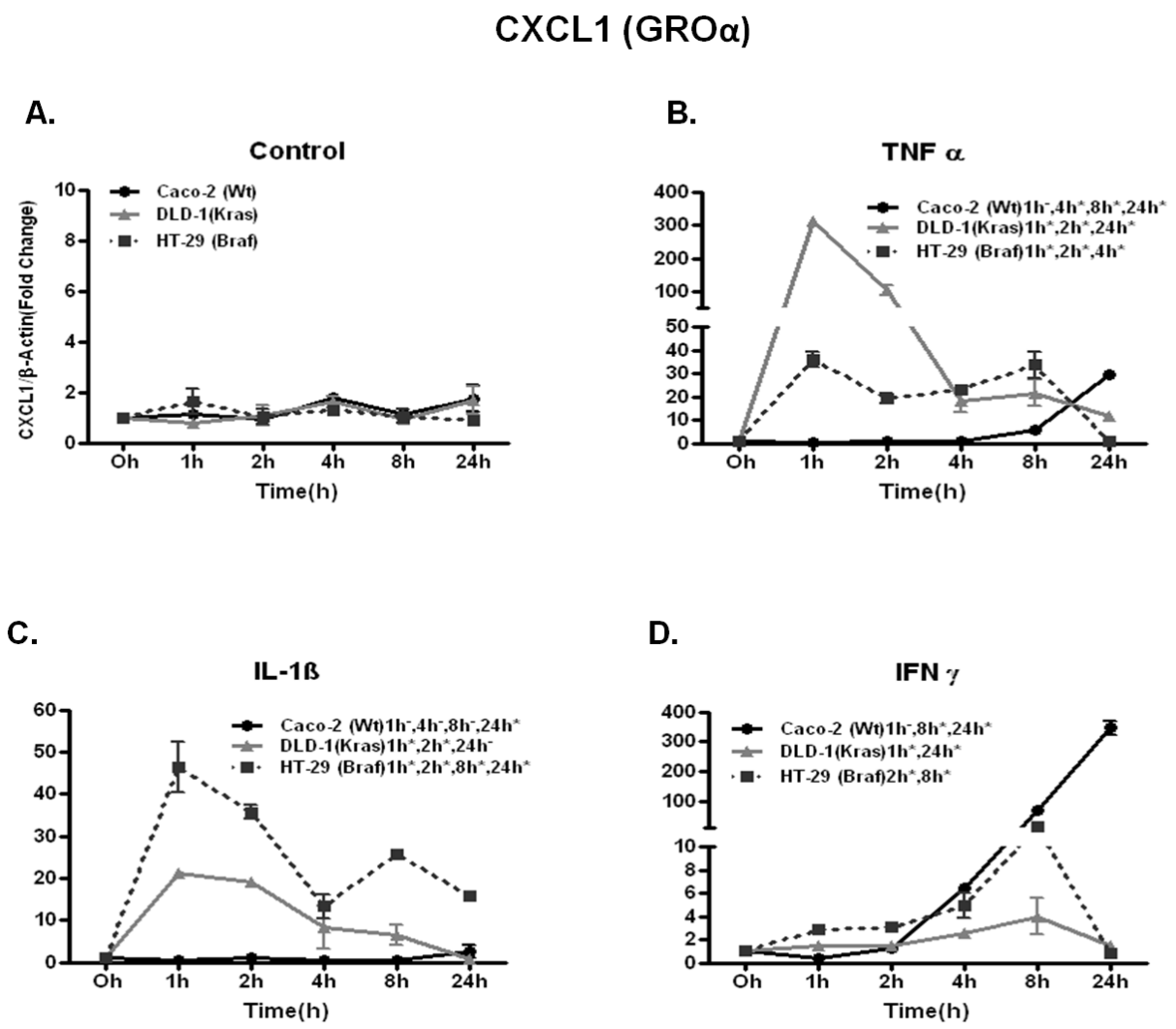



**Figure 15**: **(A, B, C, and D).** Regulation of CXCL1 mRNA expression by cytokine in Intestinal Epithelial Cells. $5\times10^5$ cells were plated into 6 well plates and grown for 24 hours and then stimulated with TNFα (50ng), IL-1ß (1ng), and IFNγ (50ng). The cells were harvested, total RNA was isolated and first strand cDNA was prepared from 1 µg of total RNA. Ct values were normalized with ß-actin as a housekeeping gene. Results represent mean value ± S.E.M. (*$p\leq0.05$, **$p\leq0.01$ analyzed by one way ANOVA, n=3).

### 4.7.2 Changes in the protein expression of CXCL1 in colorectal cancer cell lines Caco2 (Wt), DLD1 (KRAS) and HT-29 (BRAF) by cytokines (TNFα, IL-1ß and IFNγ).

The effect of cytokine stimulation on CXCL1 was further analysed at protein level by Western blot **(Figure 16A, B & C)** in Caco-2 (Wt), DLD1 (KRAS) and HT-29 (BRAF) cell lines. Western blot analysis was performed by using anti-CXCL1 antibody to confirm the changes occurring at mRNA level and to document the protein expression of CXCL1 chemokine in IECs over the time.

*TNF-α stimulation*

The CXCL1 protein secretion under control conditions did not vary to a greater extent in the analysed cell lines. Similar to what was observed at mRNA level, DLD1 showed a significant and early increase at 1h (1126±130-percent) and a maximum at 2h (1236±151-percent) after TNFα stimulation compared to the baseline conditions. In contrary to mRNA level, an increase in CXCL1 was detected with a maximum at 8h (847±180-percent) in the Caco2 cell line in comparison to their controls. The HT-29 cell line showed an increase at 2h and 8h followed by decrease at 24h as compared to respective controls. The data demonstrates that TNFα at protein level also showed significant increase in KRAS mutated cell line (DLD1).

*IL-1ß stimulation*

Similar to the mRNA expression, IL-1ß induced a significant protein level of CXCL1 in all the studied cell lines. Among them, an increase in Caco2 was the most pronounced at 2h (837±108-percent) and 8h (827±160-percent) compared to untreated cells. However, a statistically significant expression was detected in all studied time points as was also observed for HT-29 and DLD1 after IL-1ß stimulation.

*IFNγ stimulation*

Likewise regarding mRNA expression, a clear gradual increase for CXCL1 in Caco2 was observed after IFNγ stimulation. This increase was at its maximum by 8h (1371±293-percent) in Caco2. HT-29 (BRAF) and DLD1 (KRAS) also showed an increase with a maximum at 2h after IFNγ stimulation. (**Figure 16A, B&C).**

Taken together, a significant increased protein level of CXCL1 was observed by treatment of cytokines (TNF-α, IL1-β & IFNγ) in all studied cell lines [(Caco-2(Wt), DLD1(KRAS) and HT-29 (BRAF)]. However, we could observe a difference among some cytokines treatments and cell lines between the mRNA and protein expression. It might be due to the secretory nature of proteins which makes it difficult to compare CXCL1 protein expression to mRNA expression in mutated and wild type cell lines, as the proteins might be released into the supernatant.

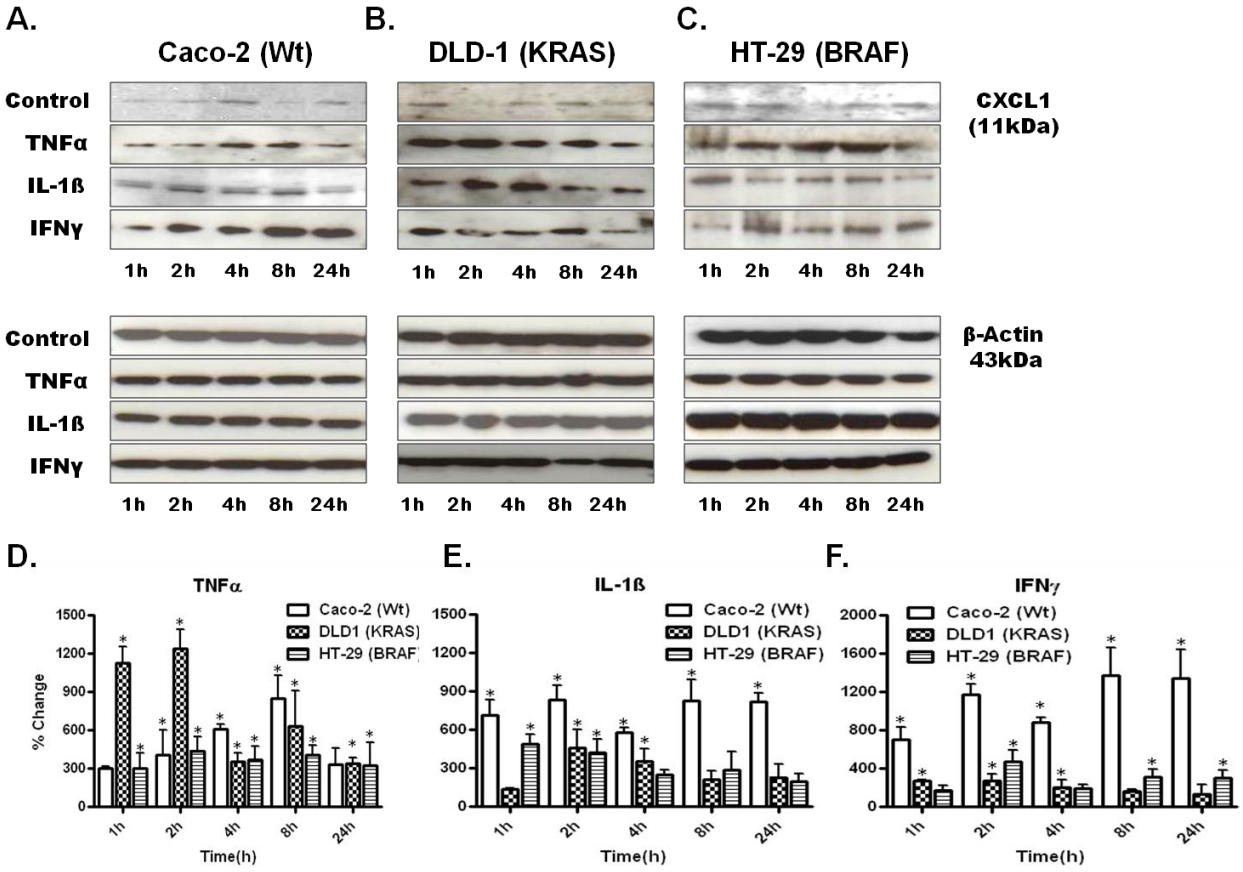



**Figure 16: (A, B, C)** shows Caco-2(Wt), DLD1(KRAS) and HT-29 (BRAF) western blot analysis. The cytokines IL-1β (1ng/ml), TNFα (50ng/ml) and IFNγ (50ng/ml) were stimulated to the cells and the total cell lysates was isolated and 20µg were separated by 15-20% NuPAGE Bis-Tris gel electrophoresis, blotted and probed with **CXCL1** antibody. ß-Actin (43Kda) was analyzed as an internal control. **Figure16 (D, E, F)** represents the desitometric analysis of western blots of three independent experiments of **CXCL1 (11kDa)**. The graphs shows percentage changes of protein amount in comparison with their respective controls. Densitometry was performed using Image J software. Results represent mean value ± S.E.M. ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$ analyzed by one way ANOVA, n=3).

## 4.8 Changes in the mRNA expression of CXCL8 in colorectal cancer cell lines Caco2 (Wt), DLD1 (KRAS) and HT-29 (BRAF) by cytokines (TNFα, IL-1ß and IFNγ).

### 4.8.1 Regulation of CXCL8 mRNA expression by cytokines.

Due to its chemo-attractive function, CXCL8 is also known to increase angiogenesis in many carcinomas (Fujimoto et al. 2002;Inoue et al. 2000;Smith et al. 1994;Yoneda et al. 1998). The CXCL8 mRNA gene expression showed that no induction was observed under control conditions in all three cell lines **(Figure 17A, *p*<0.05)**. In accordance to mRNA data, TNFα stimulation strongly induced CXCL8 in the mutated cell lines compared to control. A strong and constant up-regulation was observed in HT-29 (BRAF; 165$\pm$30.33fold) after TNFα treatment that persists until 24h. Accordingly, the maximum expression after TNFα stimulation at 1h in DLD1(KRAS; 80.31$\pm$4.77fold) and Caco-2(Wt; 45.43$\pm$1.43fold) was increased **(Figure 17B; *p*<0.05)**. IL-1ß treatment showed significant up-regulation of CXCL8 with a maximum expression by 1h in Caco2 (Wt; 806.41±19.76 fold) followed by DLD1 (KRAS; 353.22±40.63 fold) and HT-29 (BRAF; 41.51±0.72 fold) **(Figure 17C; *p*<0.05).** IFNγ was ineffective in inducing the CXCL8 expression at early time, however, a mild and late increase was observed in HT-29 (BRAF; 5.70±1.18) fold **(Figure 17D; *p*<0.05)**.

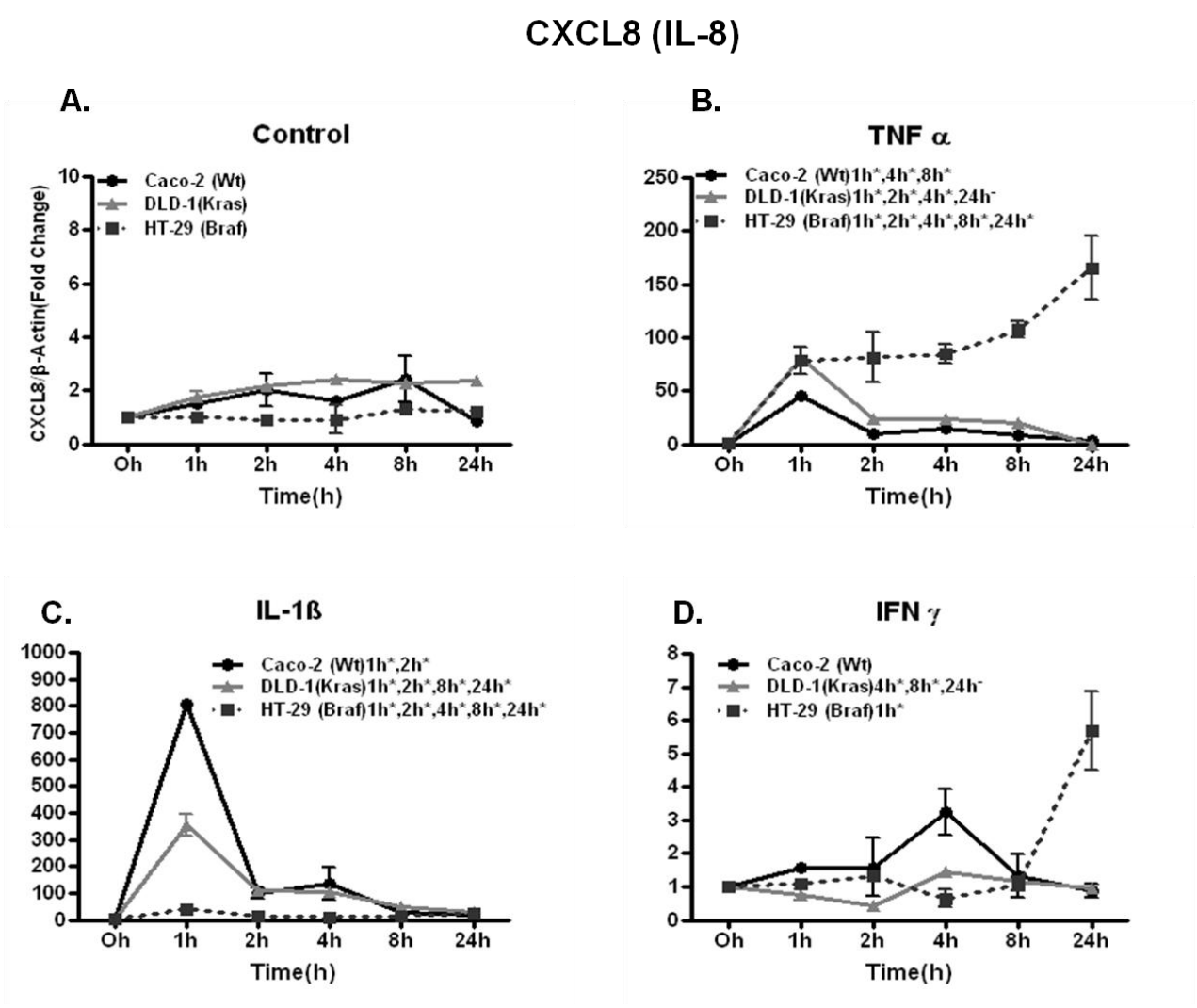



**Figure 17**: (**A, B, C, and D)** Time Kinetics of **CXCL8** mRNA expression in Intestinal Epithelial Cells. $5\times10^5$ cells were plated into 6 well plates and grown for 24 hours and then stimulated with TNFα (50ng), IL-1ß (1ng), and IFNγ (50ng). The cells were harvested, total RNA was isolated and first strand cDNA was prepared from 1 µg of total RNA. Ct values were normalized with ß-actin as a housekeeping gene. Results represent mean value ± S.E.M. ($^*p\leq0.05$, $^{**}p\leq0.01$ analyzed by one way ANOVA, n=3).

### 4.8.2 Changes in the Protein expression of CXCL8 in colorectal cancer cell lines Caco2 (Wt), DLD1 (KRAS) and HT-29 (BRAF) by cytokines (TNFα, IL-1ß and IFNγ).

Western blot analysis was performed for the protein CXCL8 (11Kda) in Caco-2 (Wt), DLD1 (KRAS) and HT-29 (BRAF). Similar to mRNA expression under control condition, there were no significant changes observed at protein level.

After TNFα stimulation, the quantification of CXCL8 protein expression indicated an early gradual increase in DLD1 that lasts till 24h (1240±300-percent). Caco2 also showed an increase at 4h (843±97-percent) and 8h (828±107-percent) respectively. HT-29 showed a gradual mild increase due to TNFα stimulation at protein level in comparison with other two cell lines (**Figure 18D)**.

IL-1β stimulation revealed an early increase at 1h in DLD1 and this increase had its maximum at 8h (1020±326-percent). A mild but significant increase in Caco-2 was also revealed due to the IL-1ß stimulation that lasts until 24h, however the changes in HT-29 were non-significant compared to the untreated controls (**Figure 18E)**.

As was observed in DLD1, IFNγ stimulation, showed an early increase of CXCL8 with a maximum at 4h (1189±211-percent) which persisted until 24h (unlike for mRNA expression), whereas Caco2 and HT-29 showed a mild increase at protein level compared to controls (**Figure 18F).**

Taken together, at protein level CXCL8 data demonstrated that due to cytokines stimulation the KRAS mutated cell line DLD1 showed the most prominent and continuous changes compared to the other cell lines.

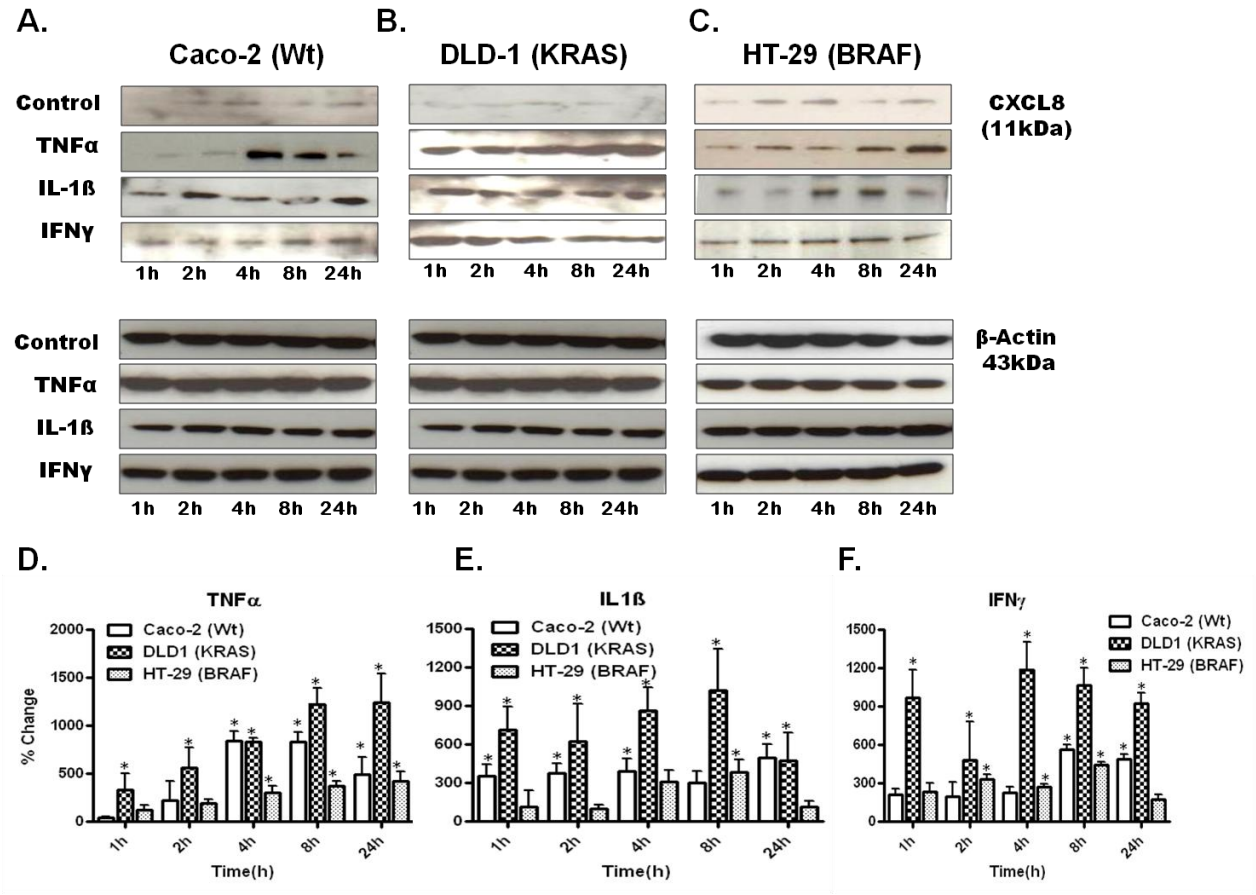



**Figure 18: (A, B, C)** shows Caco-2(Wt), DLD1(KRAS) and HT-29 (BRAF) western blot analysis. The cytokines IL-1β (1ng/ml), TNFα (50ng/ml) and IFNγ (50ng/ml) were stimulated to the cells and the total cell lysates was isolated and 20μg were separated by 15-20% NuPAGE Bis-Tris gel electrophoresis, blotted and probed with CXCL8 antibody. ß-actin (43Kda) was analyzed as an internal control. **Figure18 (D, E, F)** represents the desitometric analysis of western blots of three independent experiments of CXCL8 (11kDa). The graphs shows percentage changes of protein amount in comparison with their respective controls. Densitometry was performed using Image J software. Results represent mean value ± S.E.M. (*$p \leq 0.05$, **$p \leq 0.01$ analyzed by one way ANOVA, n=3).

## 4.9 Changes in the mRNA expression of CXCL10 in colorectal cancer cell lines Caco2 (Wt), DLD1 (KRAS) and HT-29 (BRAF) by cytokines (TNFα, IL- 1ß and IFNγ).

### 4.9.1 Regulation of CXCL10 mRNA expression by cytokines.

CXCL10 has shown different expression after cytokine stimulation in each cell line. The mRNA expression under control conditions showed no difference in the cell lines. TNFα induced the maximum CXCL10 gene expression in HT-29 (BRAF; 163.14±0.1fold) after 8h.

However, in DLD1 (KRAS) and Caco2 (Wt) cell lines a low CXCL10 mRNA expression was observed **(Figure 19B; *p*<0.05).**

IL-1ß treatment showed a maximum CXCL10 gene expression in HT-29 (BRAF) (49.72±6.25fold) followed by Caco2 (Wt) (36.86±5.13fold) with a maximum expression after 2h, whereas DLD1 showed mild changes compared to non-stimulated controls **(Figure 19C; *p*<0.05).**

In our experiment IFNγ significantly enhanced CXCL10 mRNA expression in mutated cell lines HT-29 (BRAF; 15361.19±2974.33fold) followed by DLD1 (KRAS; 597.71±64.62 fold) in contrast to the wild type cell line Caco2 (Wt; 45.75±1.44fold) **(Figure 19D; *p*<0.05).**

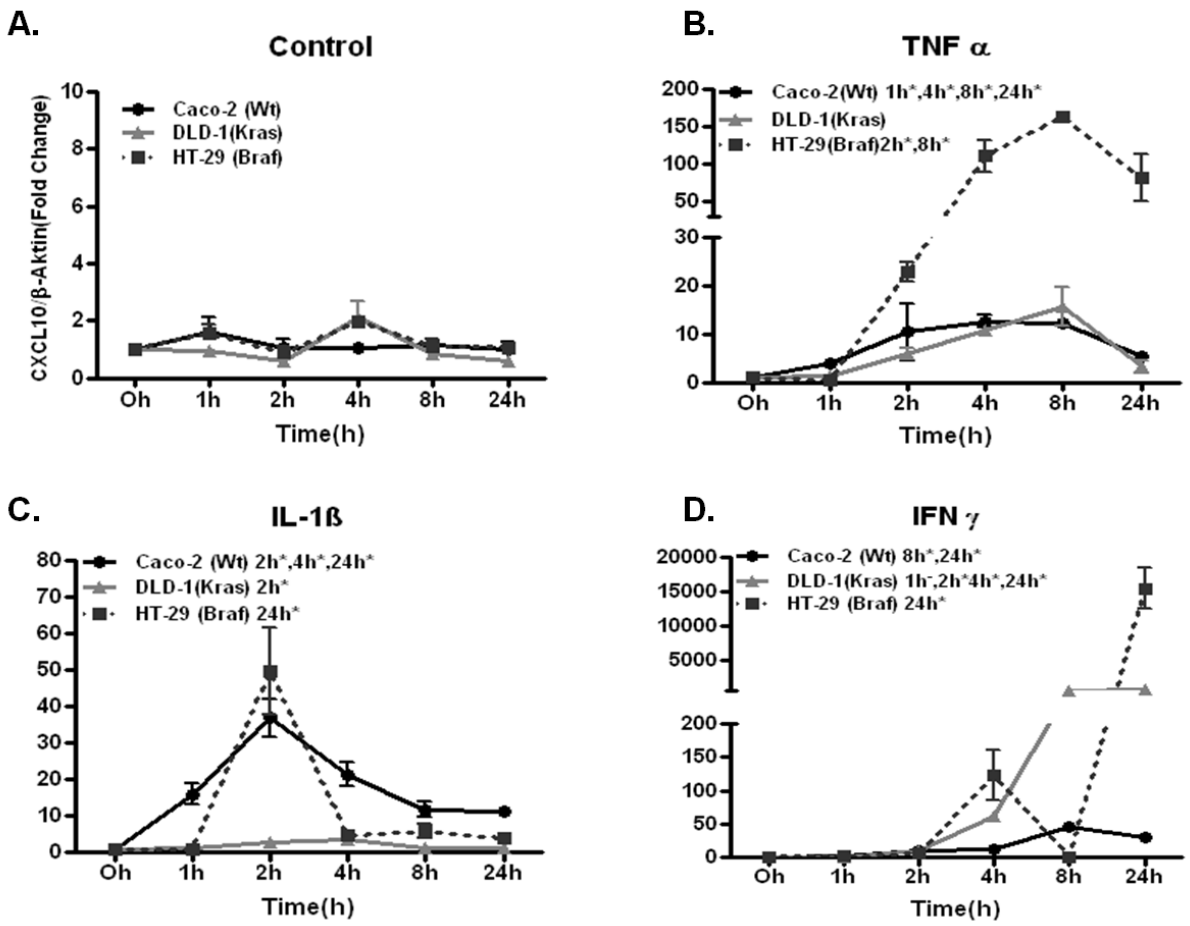



**Figure 19**: (**A, B, C, and D)** Time Kinetics of **CXCL10** mRNA expression in IECs. $5\times10^5$ cells were plated into 6 well plates and grown for 24 hours and then stimulated with TNFα (50ng), IL-1ß (1ng), and IFNγ (50ng). The cells were harvested, total RNA was isolated and first strand cDNA was prepared from 1 µg of total RNA. Ct values were normalized with ß-Actin as a housekeeping gene. Results represent mean value ± S.E.M. ($^*p\leq0.05$, $^{**}p\leq0.01$ analyzed by one way ANOVA, n=3).

### 4.9.2 Changes in the Protein expression of CXCL10 in colorectal cancer cell lines Caco2 (Wt), DLD1 (KRAS) and HT-29 (BRAF) by cytokines (TNFα, IL-1ß and IFNγ).

The Western blot analysis of CXCL10 revealed weak expression for all the cell lines under control conditions.

TNFα stimulation showed no significant changes in any of the three cell lines.

HT-29 showed a highly significant increase due to IL-1ß after one hour and reaching at its maximum by 24h (1525±125-percent).

Also IFNγ stimulation increased CXCL10 expression in HT-29 at early 1h (1358±81-percent) and the same expression was found throughout the study **(Figure 20 D, E, F)**.

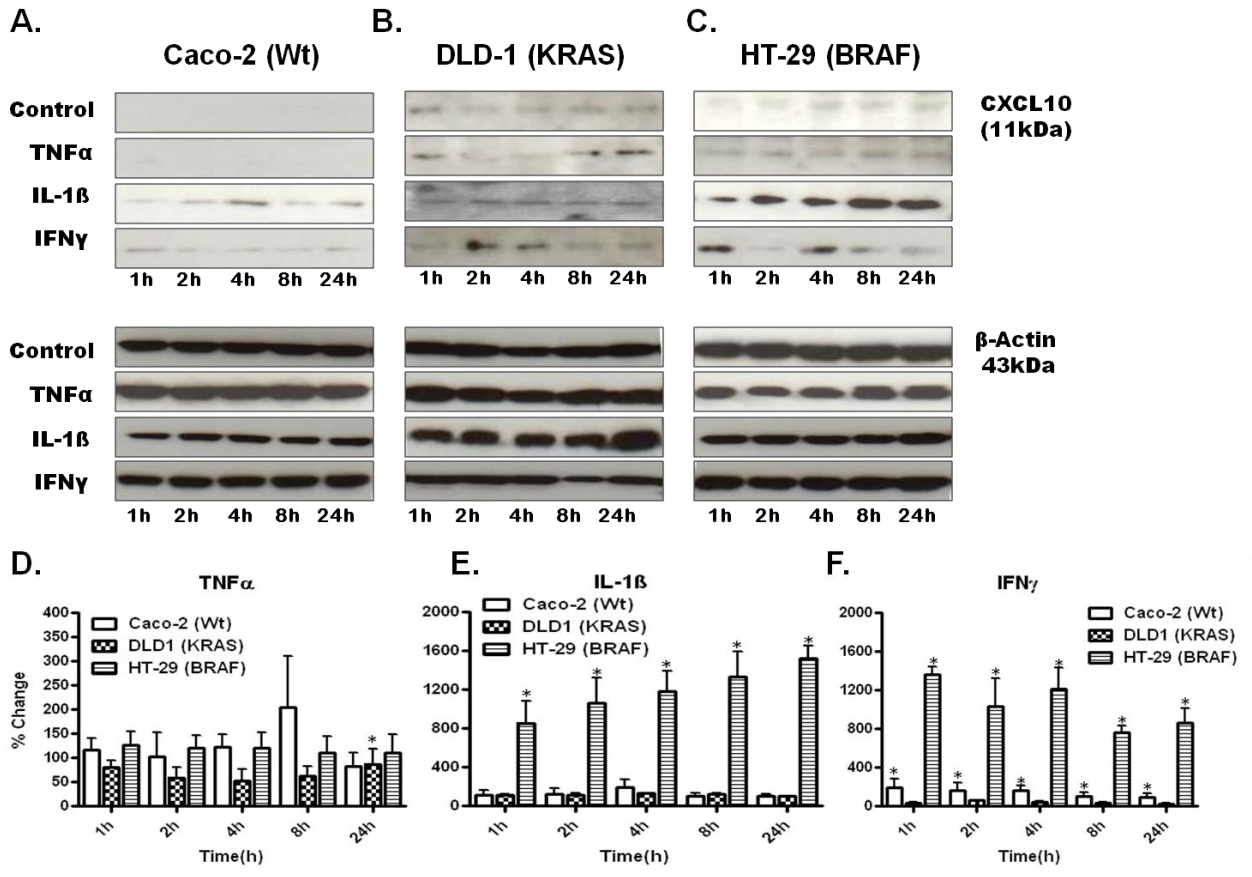



**Figure 20: (A, B, C)** shows Caco-2(Wt), DLD1(KRAS) and HT-29 (BRAF) western blot analysis. The cytokines IL-1β (1ng/ml), TNFα (50ng/ml) and IFNγ (50ng/ml) were stimulated to the cells and the total cell lysates was isolated and 20μg were separated by 15-20% NuPAGE Bis-Tris gel electrophoresis, blotted and probed with CXCL10 antibody. ß-actin (43Kda) was analyzed as an internal control. **Figure 20 (D, E, F)** represents the densitometric analysis of western blots of three independent experiments of CXCL10 (11kDa). The graphs shows percentage changes of protein amount in comparison with their respective controls. Densitometry was performed using Image J software. Results represent mean value ± S.E.M. (*$p \leq 0.05$, **$p \leq 0.01$ analyzed by one way ANOVA, n=3).

## 4.10 Transcription Factors Mitogen Activated Protein Kinases (MAPK1), Nuclear Factor of kappa B (NFκB) and Signal Transducer and Activator of Transcription (STAT3) Phosphorylation at base level in Intestinal Epithelial Cells (IECs).

### 4.10.1 Basal mRNA expression of MAPK1 in DLD1 and Caco2:

Mitogen-activated protein kinases (MAPKs) play a pivotal role in the mitogenic signal transduction pathway and are essential components of the MAPK cascade, which includes MEK (also known as MAP kinase), Raf-1, and Ras (Oka et al. 1995). In this study, we examined whether constitutive activation of the MAPK cascade was associated with the carcinogenesis of human colorectal carcinomas compared to controls.The basal mRNA expression was studied first. The gene specific primers for MAPK1 revealed that the basal mRNA expression was higher in DLD1(KRAS) compared to Caco2 (Wt) **(Figure 21A; *p*<0.05).**

### 4.10.2 Basal mRNA expression of NF-κB in DLD1 and Caco2:

To evaluate the possible role of NF-κB in cytokine induced chemokine gene expression and transcription factors detected against the activated proteins in the intestinal epithelial cell lines, experiments with a gene specific NF-κB primer were performed. Most tumor-promoting cytokines are activated via NF-κB or activate NF-κB signalling in pre-malignant cells and immune/inflammatory cells (Karin 2006). The basal mRNA expression was studied in the two colorectal cell lines. The gene specific primers for NF-κB revealed that the basal mRNA expression of NF-κB was highest in DLD1(KRAS) in comparison with Caco2 (Wt) **(Figure 21B; *p*<0.05).**

### 4.10.3 Basal mRNA expression of STAT3 in DLD1 and Caco2:

STAT3 basal mRNA expression was analyzed for the two colorectal cell lines. STAT3 is frequently activated in many types of human solid and blood cancers and contributes to

progression of those cancers (Buettner et al. 2002;Turkson and Jove 2000). The STAT3 pathway is also frequently constitutively activated in CRC and is considered to play an important role in colorectal carcinogenesis (Corvinus et al. 2005;Kusaba et al. 2005;Lin et al. 2005;Ma et al. 2004;Tsareva et al. 2007;Xiong et al. 2008). To study the effect of the STAT3 pathway in IECs, we performed real time-PCR for the two colorectal cancer cell lines by using the gene specific oligo-nucleotides. The basal mRNA expression of STAT3 in two different cell lines (Caco2 and DLD1) revealed that a mild increase of STAT-3 in the mutated cell line DLD1 (KRAS) compared to Caco2 **(Figure 21C; *p*<0.05).**

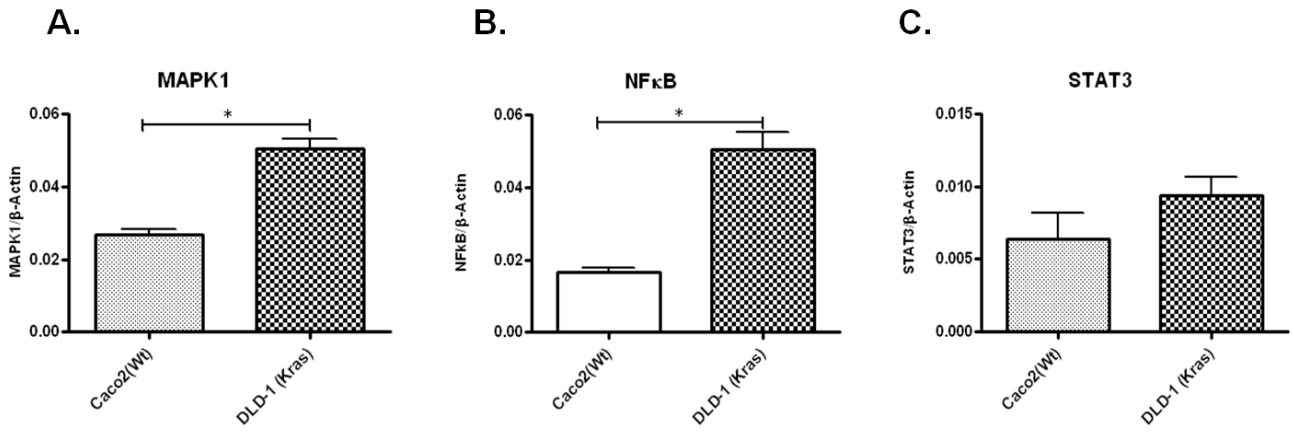



**Figure 21:** **(A).** Basal mRNA expression Transcriptional factor MAPK1. **(B).** Basal mRNA expression Transcriptional factor NF-κB. **(C).** Basal mRNA expression Transcriptional factor STAT-3. $5\times10^5$ cells were plated into 6 well plates and grown for 24 hours. Ct values were normalized with ß-actin as a housekeeping gene. The results were compared with the Caco2 taken as a control. Results represent mean value ± S.E.M. ($^*p\leq0.05$, $^{**}p\leq0.01$ analyzed by one way ANOVA, n=4).

## 4.11 Changes in the Protein expression of MAPK1 in colorectal cancer cell lines Caco2 (Wt) and DLD1 (KRAS) by cytokines (TNFα, IL-1ß and IFNγ).

Western blot analysis was performed to demonstrate the changes in p-MAPK1 protein expression in Caco2 (Wt) and DLD1 (KRAS) cell lines. It was possible to detect the p-MAPK1 protein in both cell lines by using antibody against p-MAPK1 without any stimulation (controls). An increased protein level of the p-MAPK1 was observed after different cytokine

stimulation. A significant increase in p-MAPK1 at protein level was detected with a maximum at 8h (541.04±101.17-percent) after TNFα stimulation in the DLD1 cell line. A similar pattern was also seen in Caco2 with a maximum expression at 8h (448.57±32.20-percent) **(Figure 22C)**. Similarly, IL-1ß stimulation also showed an increase of p-MAPK1 protein expression in DLD1 and Caco2 cell lines. The desitometric analysis of DLD1 showed a maximum increase at 24h (898±252-percent) after IL-1ß stimulation as compared to Caco2 **(Figure 22D)**. IFNγ stimulation showed continuous increase at p-MAPK1 protein level expression in DLD1 (KRAS) and a maximum increase was detected at 24h (1230±158-percent) as compared to Caco2 cell line **(Figure 22E)**. Taken together, the DLD1 (KRAS) cell line showed a higher MAPK1 induction after cytokines stimulation compared to Caco-2 (Wt).

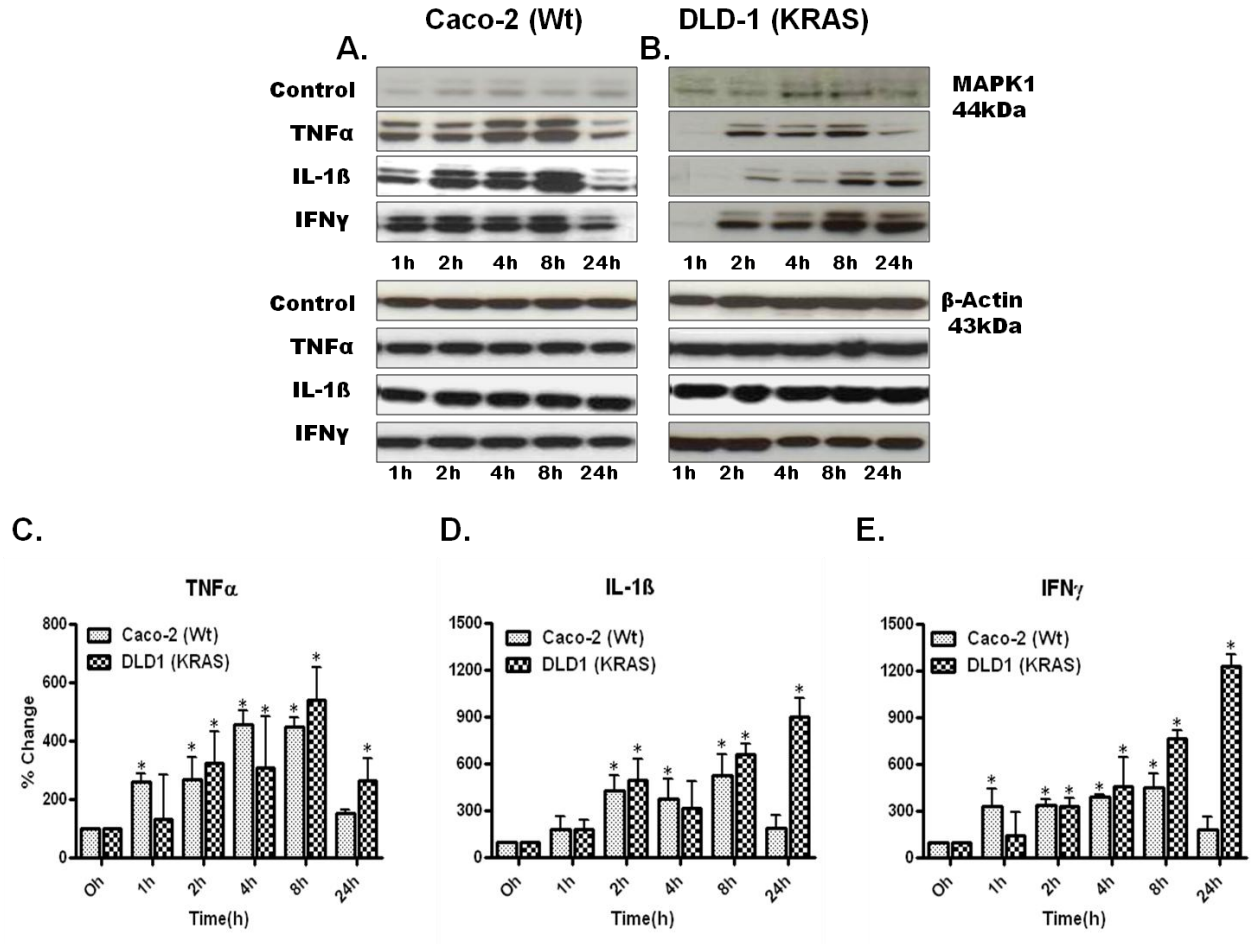



**Figure 22: (A, B)** shows Caco-2(Wt) and DLD1(KRAS) western blot analysis. The cytokines IL-1β (1ng/ml), TNFα (50ng/ml) and IFNγ (50ng/ml) were stimulated to the cells and the total cell lysates was isolated and 20μg were separated by 15-20% NuPAGE Bis-Tris gel electrophoresis, blotted and probed with MAPK1 antibody. ß-actin (43Kda) was analyzed as an internal control.

**Figure 22 (C, D, E)** represents the desitometric analysis of western blots of three independent experiments of MAPK1 (44kDa). The graphs shows percentage changes of protein amount in comparison with their respective controls. Densitometry was performed using Image J software. Results represent mean value ± S.E.M. ($^{*}p \leq 0.05$, $^{**}p \leq 0.01$ analyzed by one way ANOVA, n=3).

## 4.12 Changes in the Protein expression of NF-κB in colorectal cancer cell lines Caco2 (Wt) and DLD1 (KRAS) by cytokines (TNFα, IL-1ß and IFNγ).

By using antibody against NF-κB in Western blot, it was possible to detect the NF-κB protein in both cell lines without any stimulation (controls). A dramatic increase was detected in protein level of NF-κB in both DLD1 and Caco-2 after different cytokine stimulation. After

TNFα stimulation, a significant increase in NF-κB at protein level was detected with a maximum at 8h (658±16.01-percent) in the Caco2 cell line. A similar pattern was also seen in DLD1 with a maximum expression at 4h (415.77±42.75-percent) **(Figure 23C)**. Likewise, IL-1ß stimulation also showed an increase of NF-κB protein expression in Caco2 and DLD1cell lines.

After IL-1ß stimulation, the Western blot analysis of Caco2 showed a maximum increase at 2h (782±46-percent) as compared to DLD1 **(Figure 23D)**.

IFNγ treatment showed an increase in NF-κB protein level expression in Caco2 (Wt) cell line, and a maximum increase was detected at 2h (1179±157.25-percent) as compared to DLD1 (KRAS) cell line **(Figure 23E)**.

The desitometric analysis showed an increase at protein level by the treatments (TNFα, IL1ß and IFNγ) stimulation in the Caco2 and DLD1 cell line throughout the experiment **(Figure 23C, D & E; *p*<0.05).** These data suggest that Caco-2 (Wt) cell line showed a higher NF-κB induction after cytokine stimulation compared to DLD1 (KRAS) cell line.

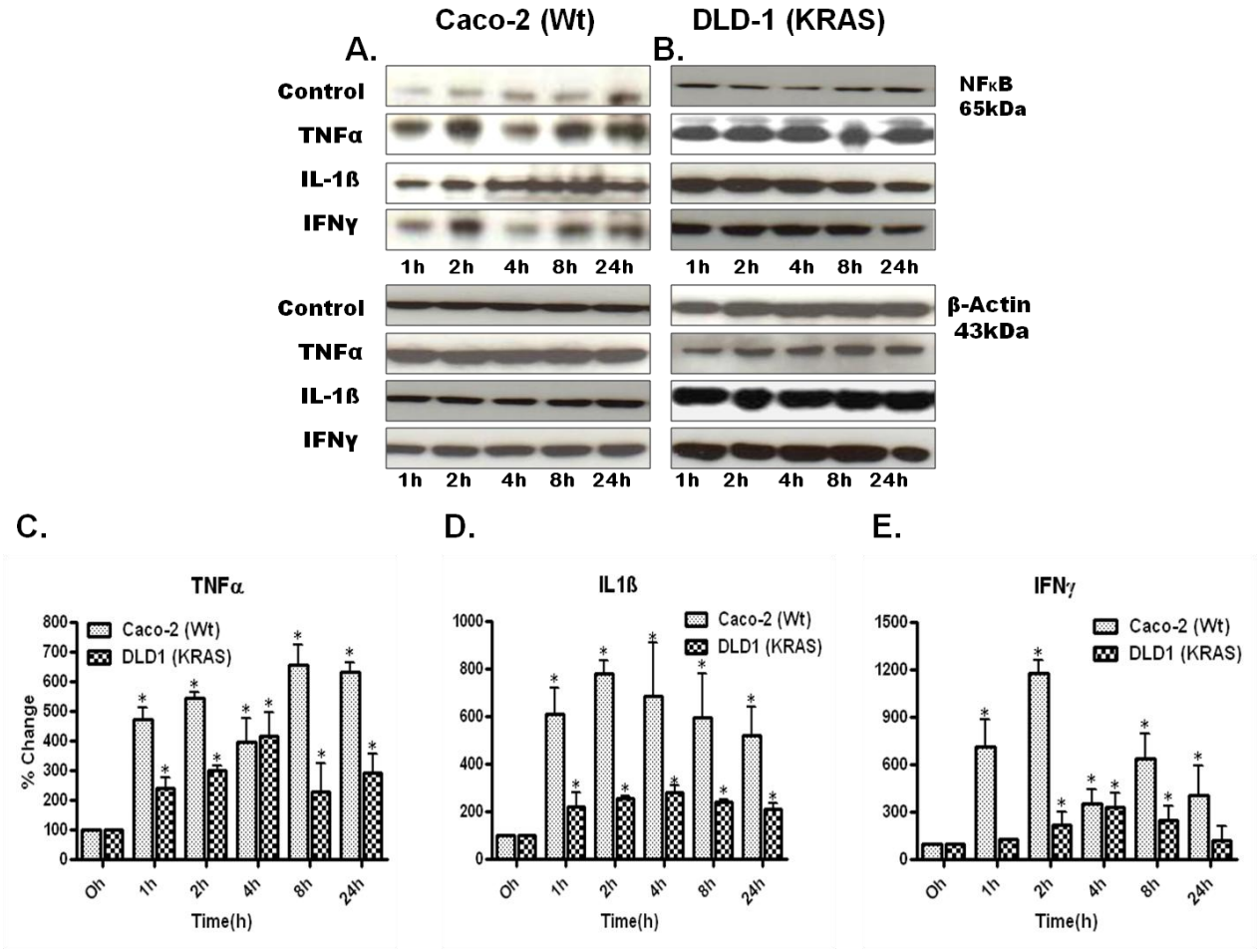



**Figure 23: (A, B)** shows Caco-2(Wt) and DLD1(KRAS) western blot analysis. The cytokines IL-1β (1ng/ml), TNFα (50ng/ml) and IFNγ (50ng/ml) were stimulated to the cells and the total cell lysates was isolated and 20μg were separated by 15-20% NuPAGE Bis-Tris gel electrophoresis, blotted and probed with IκBα antibody. ß-actin (43Kda) was analyzed as an internal control. **Figure 23 (C, D, E)** represents the densitometric analysis of western blots of three independent experiments of NFκB (65kDa). The graphs shows percentage changes of protein amount in comparison with their respective controls. Densitometry was performed using Image J software. Results represent mean value ± S.E.M. (*$p \leq 0.05$, **$p \leq 0.01$ analyzed by one way ANOVA, n=3).

### 4.13 Changes in the Protein expression of p-STAT3 in colorectal cancer cell lines Caco2 (Wt) and DLD1 (KRAS) by cytokines (TNFα, IL-1ß and IFNγ).

Western blot analysis was performed to demonstrate the changes in p-STAT3 protein expression in Caco2 (Wt) and DLD1 (KRAS) cell lines after treatment with inflammatory cytokines. It was not possible to detect a basal protein expression for the p-STAT3 in Caco2 and DLD1 cell lines. Western blot analysis showed fully diminished expression in the cell lines

under control conditions. However, our Western blot results revealed a strong induction of p-STAT3 after IFNγ stimulation in the DLD1 cell line. The p-STAT3 started to increase at 1h (1304±389.19-percent) and a pronounced increase was measured at 4h (2597±350 percent) which lasted till 24h (739±199percent) **(Figure 24B & E)**.

Taken together, it clearly indicates the possible role of p-STAT3 after IFNγ stimulation in KRAS mutated cell line (DLD1). In contrast, TNFα and IL-1ß showed no detection of p-STAT3 in DLD1. Similarly, no visible band was observed in Caco2 by treatment of any cytokines **(Figure 24A,C & D)**.

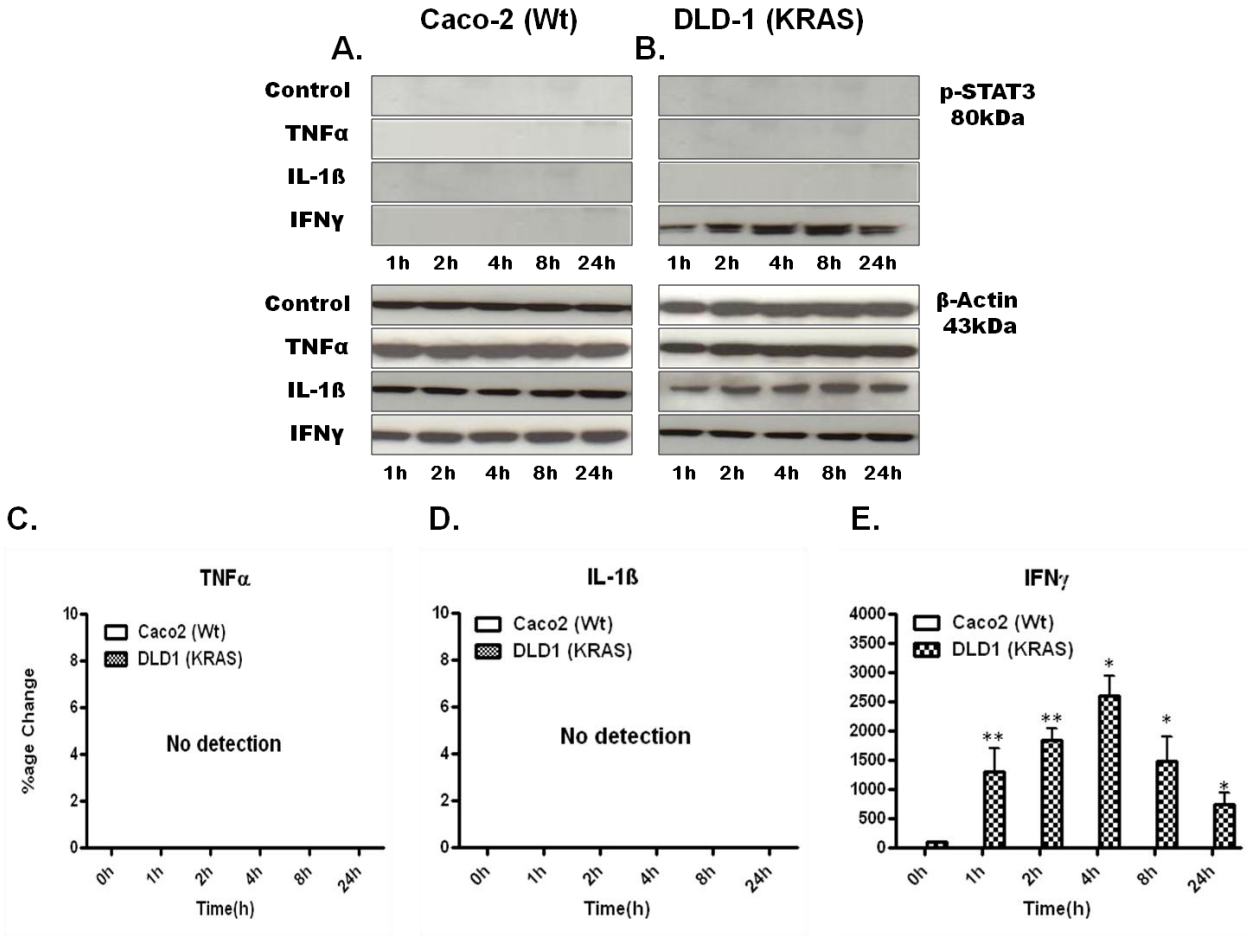



**Figure 24: (A) (B)** Shows Caco-2(Wt) and DLD1(KRAS) western blot analysis. The cytokines IL-1β (1ng/ml), TNFα (50ng/ml) and IFNγ (50ng/ml) were stimulated to the cells and the total cell lysates was isolated and 20μg were separated by 15-20% NuPAGE Bis-Tris gel electrophoresis, blotted and probed with p-STAT3 antibody. ß-actin (43Kda) was analyzed as an internal control. **(C) (D) and (E) r**epresents the desitometric analysis of western blots of three independent experiments of p-STAT3 (80kDa) in DLD1 cell lines.

The graphs shows percentage changes of protein amount in comparison with their respective controls. Densitometry was performed using Image J software. Results represent mean value ± S.E.M. (*$p \leq 0.05$, **$p \leq 0.01$ analyzed by one way ANOVA, n=3).

## 4.14 Knockdown of KRAS Expression in DLD1(KRAS) and Caco-2(Wt).

### 4.14.1 KRAS knockdown mRNA and protein expression in DLD1 and Caco2:

For down-regulation of KRAS expression in DLD1 (KRAS) and Caco-2 (Wt) cells, KRAS-specific siRNA (Qiagen) was used in transfection studies. Two human colorectal carcinoma cell lines DLD1 and Caco2 were chosen to examine the different effects of KRAS knockdown in a wild type compared to a KRAS mutated cell line. We tried to keep the siRNA concentration as low as possible for each cell line, and performed several experimental combinations of siRNA transfection **(Table.7).** All experiments were carried out with high perfect reagent according to the Qiagen protocol. The initial experiments were started with 1nM siRNA and 2µM (1.5µl) high perfect reagent. The experiments were performed in three replicates for both cell lines. Knockdown of KRAS was determined by real time PCR and at protein level through Western blot. Finally $5x10^4$ cells were plated in 24 wells with a concentration of 20nM siRNA (6µl) and 10nM High perfect (12µl) which was considered to be a combination which resulted in an acceptable KRAS knock down after 48h and 72h incubation time for the following experiments **(Figure 25 A, B).** Scrambled siRNA was taken as a control.

**Table7: Different conditions tested to achieve optimal siRNA knock down.**

| *Culture Format* | *Transfection Time (h)* | | | *Volume (µl)* | *siRNA (ng)* | | | | *High Perfect (µl)* | | | *Final Volume of diluted siRNA (µl)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 24 Well plate | 24 | 48 | 72 | 500 | 7.5 (1nM) | 37.5 (5nM) | 75 (10nM) | 150 (20nM) | 1.5 | 3 | 5 | 100 |

In both cell lines, DLD1 and Caco2, the total KRAS knockdown expression was studied after 48h and 72h and compared to the scrambled siRNA. To quantitatively determine the down regulation of KRAS expression, both the cell lines were measured by Real Time PCR at mRNA

level. The results revealed that siRNAs down-regulated KRAS mRNA expression after 48h in DLD1 (25%) and Caco2 (20%), respectively. Whereas after 72h incubation siRNA significantly reduced KRAS mRNA expression to approximately DLD1 (55%) and Caco2 (58%), respectively **(Figure 25A&B; *p*<0.05).**

However, at protein level the results showed a striking difference as compared to mRNA results. By using antibody against KRAS, Western blots analysis of three independent experiments revealed a significant reduction in KRAS protein expression in DLD1 cell line. The most pronounced inhibition was observed at 48h (67% KRAS knockdown) and 72h (85% KRAS knockdown) after siRNA transfection.

Similar results were also detected in the Caco2 cell line with a maximum at 48h (67% KRAS knockdown) and after 72h (62% KRAS knockdown) after KRAS transient transfection **(Figure 25C,D,E&F; *p*<0.05).**

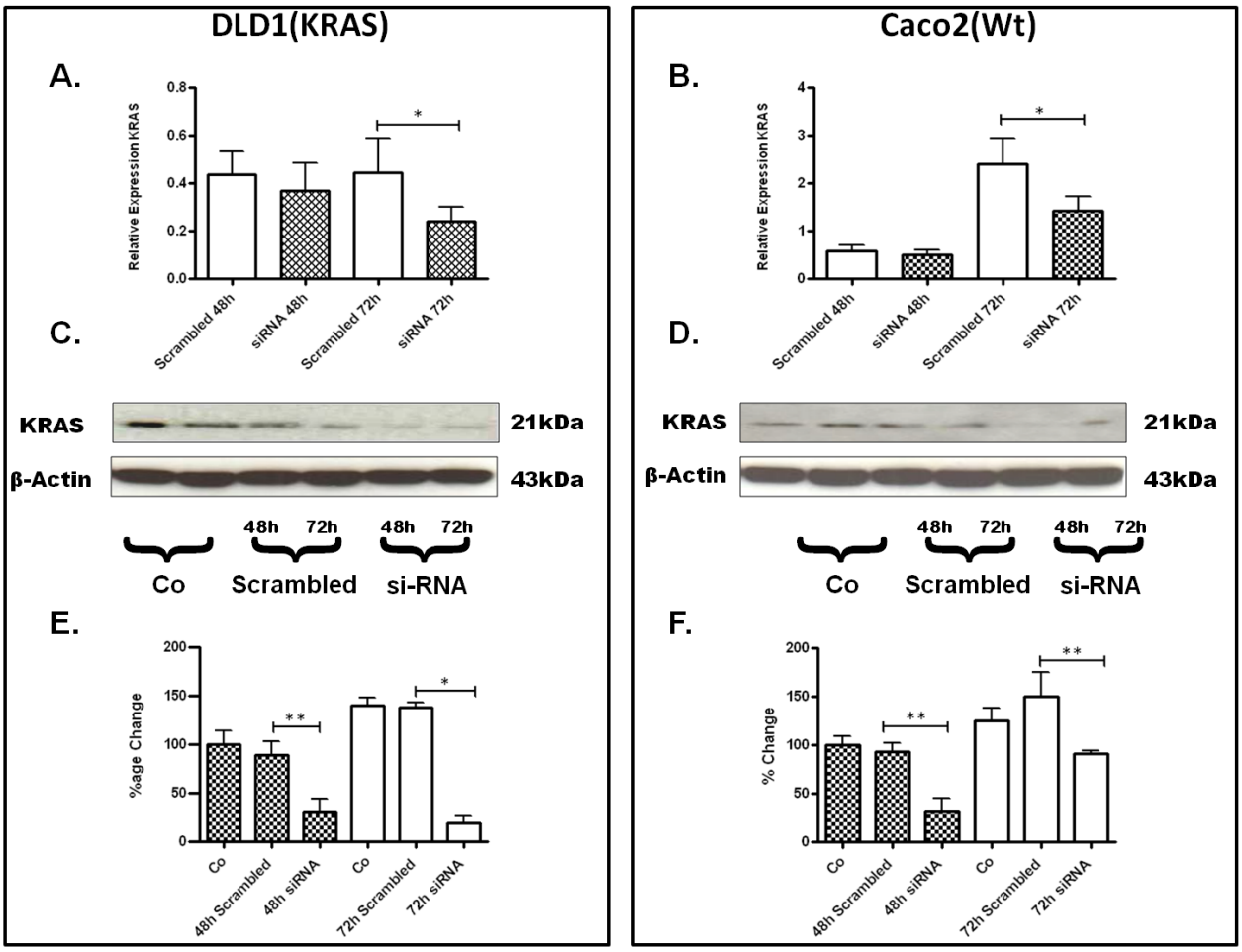



**Figure 25**: **(A&B)** Shows transient Transfection of KRAS siRNA in DLD1 and Caco2 Cell lines at mRNA level. The data is the representation of three independent experiments. **(C&D)** are the representatives of KRAS siRNA knockdown western blot analysis. **(E&F)** protein bands of three independent experiments were densitometrically quantified. The relative bands indicated as percent change compared with scrambled siRNA in DLD1 and Caco2 cell lines. $5\times10^4$ cells were plated into 24 well plates and grown for 24 hours and then transfected with KRAS siRNA or scrambled (20nM) for 48h and 72h. Real Time PCR was performed for KRAS with gene specific primers and the expression was normalized to β-actin expression measured in the same sample as an internal control. For western blot cell lysates were size fractionated by SDS-PAGE, blotted and analyzed with antibodies specific for KRAS. ß-actin specific antibody to check for equal loading of total protein. Data presented are the means ± SEM of 3 independent experiments with double confirmation. * corresponds to $p< 0.05$.

### 4.14.2 Changes in chemokines mRNA expression due to KRAS knockdown in DLD1 and Caco2.

To further explore the consequences of decreased KRAS expression in the cell lines due to KRAS siRNA knockdown, we studied the chemokines gene expression at mRNA level.

A significant decrease in CXCL1 mRNA level was detected after 72h (0.31±0.07 fold; $p<0.05$ vs. scrambled control) transfection in the DLD1 cell line. Similarly CXCL10 also showed a decrease (0.30±0.08fold; $p<0.05$ vs. scrambled control) gene expression after 72h transfection due to KRAS inhibition. In contrast, CXCL8 (0.66±0.23fold; $p<0.05$ vs. scrambled control) mRNA expression was up-regulated in DLD1 after KRAS knockdown in comparison with its scrambled control **(Figure 26A, B&C; $p<0.05$).**

On the contrary to DLD1 (KRAS), in Caco2 (Wt) cell line, the KRAS knockdown resulted in up-regulation of CXCL1 (9.79±3.6fold; $p<0.05$ vs. Scrambled Control), CXCL8 mRNA (1.71±0.05fold; $p<0.05$ vs. Scrambled Control) and CXCL10 (18.40±12.80fold; $p<0.05$ vs. Scrambled Control) mRNA expression after 72h transfection (**Figure 26D, E &F; $p<0.05$)**.

To summarize, the results indicate a change in cytokine-gene expression of both cell lines after KRAS inhibition. Moreover, CXCL1 and CXCL10 showed an opposite expression in the two cell lines, whereas gene expression of CXCL8 was induced in both cell lines by KRAS knockdown.

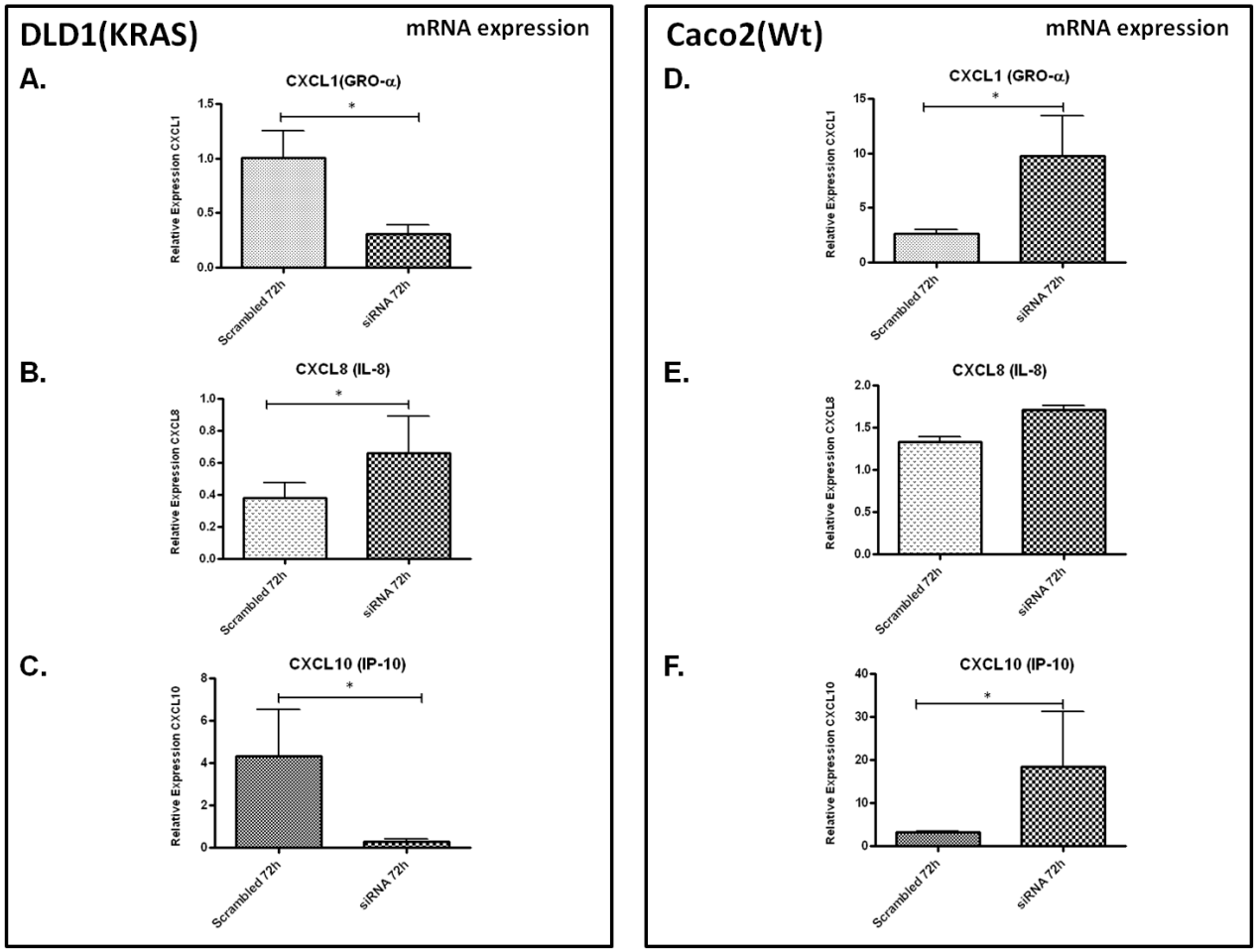



**Figure 26**: **Transient Transfection of KRAS siRNA in DLD1 (KRAS) and Caco-2(Wt) Cell line.** $5\times10^4$ cells were plated into 24 well plates and grown for 24 hours and then transfected with Kras siRNA or scrambled (20nM) for 72h. Real Time PCR was performed for CXCL1, CXCL8 and CXCL10 with gene specific primers and the expression was normalized to β-actin expression measured in the same sample as an internal control. Data presented are the means ± SEM of 4 independent experiments with double confirmation. * corresponds to $p< 0.05$.

### 4.14.3 Changes in protein expression of MAPK1and IκBα due to KRAS knockdown in DLD1 (KRAS) and Caco2.

To further evaluate the reason of change in chemokine gene expression by *KRAS*-knock down in both DLD1 and Caco2 cell lines, transcription factors MAPK1 and IκBα were analysed at protein level. MAPK1 specific protein band was detectable at 44kDa in DLD1 and Caco-2 cells. The MAPK1 desitometric analysis of three experiments showed no significant change after 48h and 72h by *KRAS* reduction. The data suggested that inhibition of *KRAS* has no significant effect in either cell lines for MAPK1 (Fig.27B&E).

To analyse the activation of the NF-κB pathway, cell lysates were analyzed for changes in the level of total IκBα (subunits of NF-κB), as phosphorylation of the p65 subunit and the degradation of IκBα are known to be associated with activation of the NF-κB classical pathway (Hayden and Ghosh 2008). However, inhibition of KRAS expression by siRNA affects significantly the levels of IκBα in Caco2. Our results revealed that after 48h of transfection, it reduced IκBα protein level to 46% and ~ 70% after 48h and 72h respectively. Furthermore, a non-significant decrease in DLD1 was also detected after KRAS gene knockdown **(Fig. 27C&F).**

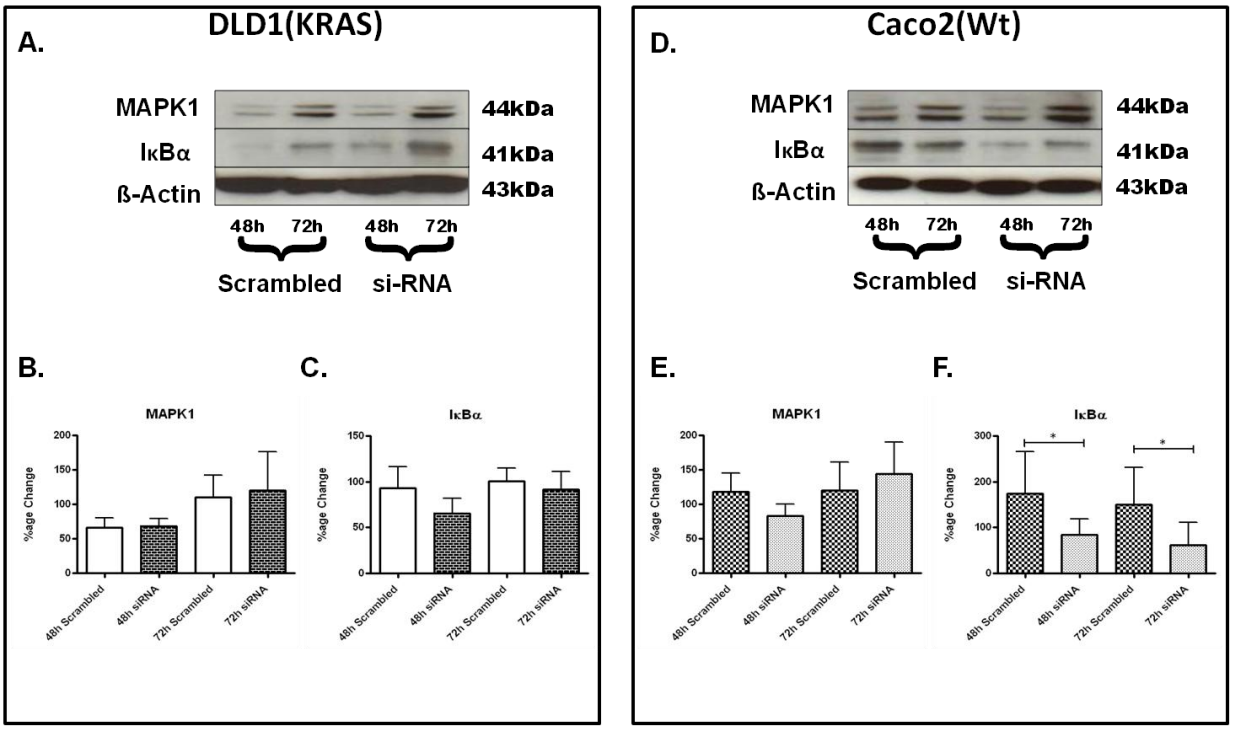



**Figure 27**: Effect of MAPK1 and IκBα protein expression in DLD1 (A) and Caco-2 cells (D) after KRAS knockdown. Proteins (20μg) from whole-cell lysates were size-fractionated by SDS-PAGE under transferred on to membranes, and incubated with antibodies as indicated. A and D, representative Western blots of MAPK-1 (44kDa) and IκBα (41kDa) proteins in DLD1 cells (A) and Caco-2 cells (D). B, C, E & F desitometric analysis of MAPK-1 and IκBα proteins in DLD1 cells and Caco-2 cells. Proteins were densitometrically quantified and expressed as percent increase or decrease compared with scrambled controls. Equal loading of total proteins were ensured by ß-Actin (43kDa). Data presented are the means ± SEM of 3 independent experiments with double confirmation. * corresponds to $p< 0.05$.

# 5. Discussion

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. Caco2 cells were previously described to possess characteristics of normal small intestinal epithelium (Jumarie and Malo 1995), HT-29 and DLD1 cells are known to possess the characteristics of surface villus cells (Panja et al. 1998).

The main aim of the study was to understand the influence of CRC-mutations (KRAS and BRAF) in view of the regulation and induction of inflammatory cytokines and chemokines and to find the signaling mechanism controlling these changes in colorectal carcinoma cell lines. (CRC). Pro-inflammatory cytokines are probably released by sub-mucosal immune cells of the tumor microenvironment or/and tumor cells themselves, which can further enhance the expression of inflammatory chemokines and respective transcription factors important for cell proliferation, angiogenesis and migration. The maintenance of a pro-oncogenic microenvironment regulated by the tumor-promoting cytokine – chemokine axis, might vary according to the underlying oncogenic driver mutation (i.e. KRAS). Therefore, it was further part of study to investigate how these cytokines affect the chemokine regulation in the KRAS mutated and non-mutated cell lines in the presence or absence of the KRAS gene. In addition, a comparative mutational analysis was conducted to validate the previously published mutations (KRAS and BRAF) in CRC cell lines.

## 5.1 Identification of Mutations in the Colorectal Cell lines.

KRAS and BRAF mutations occurs in CRC therefore, in the present study KRAS Codon (12, 13, 61 and 146) and BRAF (V600E) were systematically examined for all the mutations in the

RAS/RAF pathway through RFLP and real time PCR in six colorectal carcinoma cell lines. Our initial goal was to analyse the mutations in the different cell lines. The PCR/RFLP method is simple and has a higher reproducibility than the other techniques. However, the technique is highly specific, noninvasive, and cost-effective, and should provide a more sensitive and specific results for mass screening of CRC.

The presence of KRAS mutation in histologically normal colonic mucosa has been described (Carvajal-Carmona et al. 2007). The KRAS mutation has been reported in approximately 40% of CRCs. 90% of the KRAS mutated CRCs have a mutation in codon G12D and G13D. Next to these mutations, the less frequent mutations at codon A61T and A146T were also analysed for the six CRC cell lines. The DLD1 cell line showed a G13D KRAS mutation. However, the other five cell lines did not show a mutation in the KRAS gene **(Table 5)**. Previous studies demonstrated that expression of mutated KRAS alleles could increase tumor metastasis in human cell line model systems (Nakano et al. 1984;Pulciani et al. 1982). Targeting activated KRAS could inhibit tumor growth in colon cancer cell lines (Ross et al. 2001).

In fact, KRAS is the central mediator of epidermal growth factor receptor (EGFR) signaling. Hence, the KRAS mutational status modifies the response of a tumor to anti-EGFR therapy (Feng et al. 2007). Two major key pathways RAS/RAF/MAPK and PI3K/AKT are known in CRC-cell lines which are immediately downstream of the EGFR, targeting the nucleus to drive cell proliferation, survival, angiogenesis, metastasis and invasion. The inhibition of either pathway could further strengthen the effect of EGFR inhibitors. Among them, targeting the RAS/RAF pathway is the most common (Servomaa et al. 2000). The mutations in this pathway lead to constitutive activation of the RAS oncoprotein in a guanosine triphosphate–bound state. The localization of activated RAS on the inner surface of the plasma membrane stimulates a series of subsequent signaling events leading to cellular proliferation. It is also mentioned that

targeting single Ras has been difficult, with more failures than successes, especially in gastro-intestinal malignancies including colorectal cancer (Bos 1989).

In our studies, the BRAF mutation V600E was confirmed in two cell lines HT-29 and Colo-205 through ARMS primer-PCR, whereas the other four cell lines did not show the BRAF mutation. It is known that the BRAF V600E mutation is several folds more oncogenic compared to wild type BRAF (Davies et al. 2002b). In the active form, GTP-bound Ras recruits Raf protein to the cell membrane and binds it directly, activating Raf kinase. The Raf then phosphorylates and activates downstream mediators of cell growth and proliferation, including mitogen-activated protein kinase (MEK-1, 2), ERK1 and ERK2 (Nagasaka et al. 2004;Wellbrock et al. 2004). It was previously shown that in CRC, BRAF mutations have been described in approximately 10% of tumors, and a recent study suggests that BRAF mutation is found in those tumors, not harboring KRAS mutations (Nagasaka et al. 2004). Thus, signal activation may occur through mutation in either BRAF or KRAS. In theory, direct inhibition of Raf could inhibit growth of tumors driven by mutations of either Raf or Ras (together accounting for more than 40% of CRC) (Nagasaka et al. 2004).

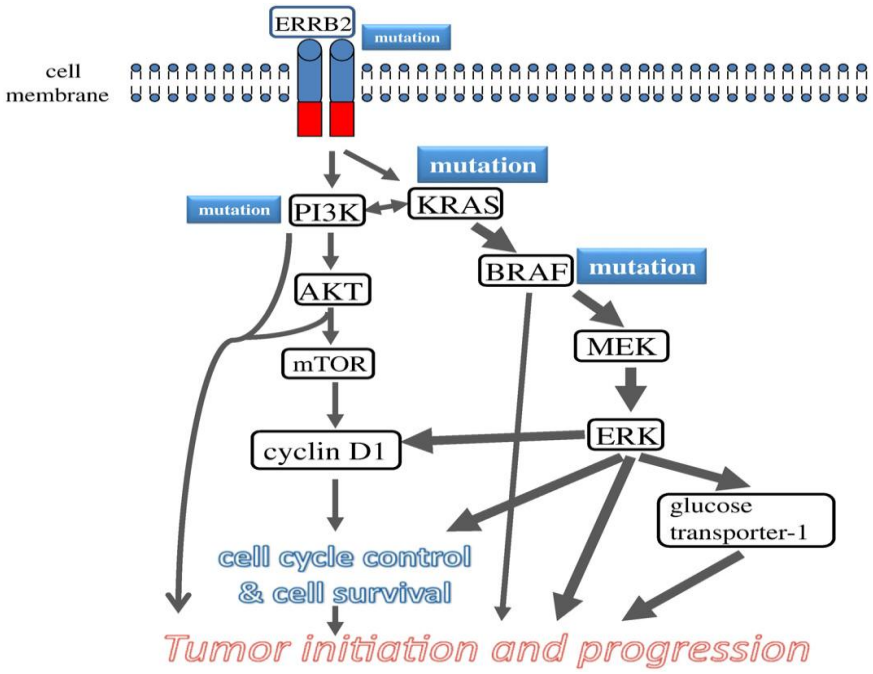



**Figure 28**: **Schematic illustration of pathway alterations involved in the development of CRC.** The cardinal molecular genetic changes include somatic mutations in *KRAS*, *BRAF*, and occasionally *ERRB2* (encoding Her2/Neu) and *PIK3CA*. The mutated gene products constitutively activate the signaling pathways that regulate cellular proliferation and survival and promote tumor initiation and progression. Adapted from (Kurman and Shih 2011).

## 5.2 Basal expression of cytokines and their receptors in mutated and wild type cell lines.

The CX-1 cell line was excluded for further experimentation due to its highly metastatic nature (Meterissian et al. 1993) and different growth properties including non-adherent nature. Experiments were continued with two wild type cell lines i.e Caco2 and Colo-320, as well as the mutated CRC cell lines DLD1 (KRAS), HT-29 (BRAF) and Colo-205 (BRAF).

The tumor microenvironment consists of tumor, immune, stromal, and inflammatory cells which produce cytokines, growth factors, and adhesion molecules that promote tumor progression and metastasis. A panel of basal cytokines (TNFα, IL-1ß, IFNγ and IL-6) and

cytokine receptors, which are more commonly involved in the tumor control and progression in colorectal carcinoma cell lines, was evaluated.

Significant differences were observed for basal TNF-α and IL-1β expression between mutated (KRAS and BRAF) and non-mutated CRC cell lines. A pro- as well as anti-cancer role of TNF has been described (Dalum et al. 1999). This anticancer effect is multi-factorial as TNF can cause vascular necrosis, a direct apoptotic effect on the cells and also free radical induced cell death. Gene expression of TNFα was found to be the lowest in KRAS and BRAF mutated cell lines compared to non-mutated. Based on these data, it could be suggested that a reduction in TNF-α level could be related to cell necrosis and apoptosis in mutated cell lines compared to wild type cell lines of CRC. In other words, reduction in TNF expression is probably necessary for mutated cells to survive.

Similar results were obtained for IL-1β basal expression with an exception of BRAF mutated cell lines which showed an induction of IL-1β compared to KRAS mutated or non-mutated cell lines. Of particular interest in this setting is interleukin-1 (IL-1), a pleiotropic cytokine with numerous roles in both physiological and pathological states. It is known to be up regulated in many tumor types and has been implicated as a factor in tumor progression via the expression of metastatic and angiogenic genes and growth factors (Lewis et al. 2006a).

A number of studies have reported that high IL-1 concentrations within the tumor microenvironment are associated with a more virulent tumor phenotype. In colon tumors, IL-1 has been shown to be up regulated and patients with IL-1 producing tumors have generally bad prognoses (Lewis et al. 2006b). Similarly, in our data, the most pronounced expression of IL-1β was detected in BRAF mutated cell lines and it is reported that patients with BRAF mutation have a bad prognosis. It shows that our data are in accordance with the clinical setting.

Strikingly, no gene expression of IFNγ and IL-6 was not detected in any of studied cell lines at basal level. However, IFNγ and IL-6 cytokines receptors were detected, as were TNFα and Il-1β receptors at basal level. The presence of the receptors points to a functional cytokine sensitivity. A role of IL-6 in tumor progression has already been described (Waldner et al. 2012). In fact, IFNs are proteins involved in many functions including, apoptosis, control of cell cycle and mediators of other cytokines (Gough et al. 2008;Slattery et al. 2011). IFN-γ is known for anti-proliferative and furthermore anti-tumor activity in CRC (Schroder et al. 2004). The lack of detection of these main pro-inflammatory cytokines in any of studied cell lines could explain that tumor cells are able to promote their microenvironment according to their needs. A regulation of apoptosis-related genes by IFNγ has been reported and IFNγ has also been hypothesized to regulate cell sensitivity to apoptosis (Tekautz et al. 2006). Additionally, IFNγ might induce the anti-tumorigenic role of TNF and thus to overcome the resistance of metastatic colon tumor cells to the TNF-induced classic apoptosis pathway (Liu et al. 2011a). Reduced expression of TNFα in mutated cell lines and the complete abolishment of IFN-γ expression in our data could be associated with increased tumor progression as has been suggested above.

## 5.3 Basal and cytokine induced expression of chemokines in mutated and wild type cell lines.

Chemokines are known to attract leukocytes during stress conditions (Mackay 2001) and promote tumor development (Mukaida and Baba 2012). Carcinogenesis of colon cancer has been correlated with mutation of the RAS/RAF family of genes in the MAP kinase pathway, suggesting the importance of mutation of these genes in the early stage of malignant transformation in colon cancer (Rajagopalan et al. 2002b). Mutations of KRAS/BRAF have been identified in many colorectal cancer cell lines, but the correlation with cytokine-mediated expression of chemokines by intestinal epithelial cell lines has not been studied.

In the current study, the regulation of pro- (CXCL1, CXCL8) and anti-angiogenic (CXCL10) CXC-chemokines was studied by RT-PCR. The expression of CXCL1 and CXCL8 was found to be highest in KRAS and BRAF mutated cell lines as compared to wild type (Caco2 and Colo-320) cell lines at mRNA level. However, CXCL10 basal expression was highest in wild type as compared to KRAS and BRAF mutated cell lines. Previously, CXCL1 protein secretion was found to be enhanced in highly metastatic cell lines as compared to a cell line with low metastatic potential with the wild type Caco2 cells having shown the lowest CXCL1 levels (Li et al. 2004). This is in accordance with our results. Recent studies have also shown a basal difference of chemokine expression in intestinal epithelial cells of normal and IBD patients. It was suggested that the differences in the basal expression of CXCL1, CXCL8 and CXCL10 in the cell lines mimic in vivo conditions of normal intestinal epithelium (Banks et al. 2003). Moreover, in the tumor microenvironment, the balance between pro-and anti-angiogenic chemokines may determine the degree of angiogenesis and the ensuing tumor progression. Accordingly, I observed a reduced expression of the anti-angiogenic chemokine CXCL10 in wild type compared to mutated cell lines. The opposite was true in case of pro-angiogenic chemokines (CXCL1 and CXCL8). Indeed, the difference in basal chemokine expression could be due to the activation of transcription factors responsible for the expression of these chemokines.

In addition to the basal chemokine expression, the obtained results showed that cytokine stimulation differentially influences the induction of target chemokines in the mutated (KRAS and BRAF) and non-mutated cell lines, suggesting the activation of differential or/and multiple pathways. TNFα and IFNγ were the main inducers for CXCL1, CXCL8 and CXCL10 gene expression in mutated cell lines compared to wild type (Caco2) cell line. However, an exception was observed for CXCL8 which showed a higher induction in wild type than in mutated cell lines after IL1β administration.

Another major finding was the observation that specific knockdown of mutant and wild type *KRAS* through siRNA disrupts chemokine gene expression. Specific siRNA inhibition of the KRAS gene in a KRAS-mutated cell line (DLD1) showed reduced gene expression of CXCL1 and CXCL10 whereas the opposite was found in case of a wild type cell line Caco2. These results clearly show that mutation of the RAS gene in colon cancer influences gene regulation of chemokines, which is in accordance to previous reports. A previous report stated a reduced expression of chemokines after mutant KRAS inhibition in a human cancer cell line (Cunningham et al. 2004). Furthermore, a stable knockdown of oncogenic KRAS led to reduced proliferation rates and anchorage independent growth in lung adenocarcinoma cell lines (Sunaga et al. 2011). It suggests that mutant KRAS may affect the chemokine levels by shifting to additional pathways. Increased chemokine levels mean increased recruitment of inflammatory cells which are known to play a role in tumor growth and metastasis (Hingorani et al. 2003;Milne et al. 2009;Schubbert et al. 2007).

As for increased levels of chemokines and cytokines in tumor tissues a role in tissue remodelling and angiogenesis has been documented (Coussens and Werb 2002;Karin 2006;Sica et al. 2008), our study could give insight to this process as KRAS-mutation may lead to an increased tumor growth or metastasis by enhancing the recruitment of inflammatory cells. Further studies are required to explore the molecular mechanism controlling this process. In fact, previous studies on human cancers have shown a complex chemokine network that regulates the extent and phenotype of the infiltrating leukocytes, as well as the an effect on tumor growth, survival, migration, and angiogenesis (Balkwill 2004a). The pattern of chemokine-receptor and ligand expression in a tissue is generally correlated with the numbers and types of infiltrating cells that are present in the tumor microenvironment (Balkwill 2004b;Karin and Greten 2005;Rossi and Zlotnik 2000;Sica et al. 2008).

On the other hand, these chemokines may promote tumor progression and invasion by stimulating the process of neo-angiogenesis and the activation of matrix proteases (Murphy, 2001; Strieter et al., 2006). Previous studies have shown a role of cytokine-induced up-regulation of CXCL1 and CXCL8 for tumor growth and angiogenesis (Dhawan and Richmond 2002;Haghnegahdar et al. 2000;Wang et al. 1998). In our findings TNFα strongly induced CXCL1 mRNA and protein expression in DLD1 KRAS mutant cell line in comparison with BRAF and wild type. Further analysis of cytokine receptors in this work illustrated the possible effect of mutation in the cell lines. We found that TNFα Rec1 and IFNγ Rec1 up-regulation in the BRAF mutated cell lines could cause deviation from the RAS-RAF pathway in inducing the cell lines.

Higher expression of IFNγ Rec1 in BRAF mutated cell lines was demonstrated in a study that IFNγ sensitizes human colon carcinoma cells to TRAIL-mediated apoptosis due to caspase-8 expression (Langaas et al. 2001). Here we can conclude that IFNγ Rec1 could play a role in activating cytokine pathway to induce the gene expression of cell lines.

Furthermore, migration and invasion play important roles in the tumor-host interaction that contribute to the aggressiveness and metastatic potential of tumor cells (Liotta and Kohn 2001).

Up-regulation of CXCL8 has been recently linked to β-catenin activation. The study was based on microarray analysis of differentially expressed genes regarding 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.

Nevertheless, its underlying mechanisms and true impact on tumor progression are yet to be determined. The results of this study may be helpful to build a solid rationale for novel therapeutic interventions targeted to specific inflammatory molecules, and to identify novel prognostic CRC markers.

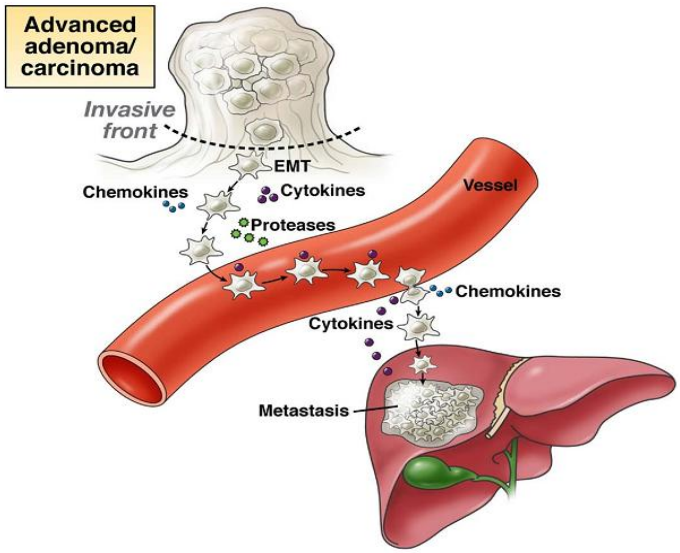



**Figure 29**: **Inflammation in angiogenesis and metastasis cytokines, chemokines and proteases (MMP) produced by inflammatory cells influence the processes of angiogenesis and metastasis.** They alter the migratory properties and behaviour of cancer cells, degrade the extra cellular matrix (ECM), and increase the tumor's blood supply and access to vasculature. Immune cells also create chemokine gradients that guide the migration of metastatic cells through the tissue to distant organs and facilitate other aspects of metastasis. **Adapted from (Terzic et al. 2010).**

## 5.4 The Pathway studies (STAT3, NF-κB and MAPK1) in mutated and wild type cell lines cell lines

In order to find out the mechanisms involved in the induction of chemokines in mutated (KRAS) and wild type cell lines at basal level and after cytokines treatment, the basal expression of candidate transcription factors (MAPK1, NF-κB and STAT3) was analyzed. A marked difference in basal expression of the transcription factors was observed between the KRAS mutated and wild type cell line. In comparison to wild type, a constitutive expression of transcription factors in a KRAS-mutated cell line could suggest that mutation could independently influence the activation of these pathways which can be further enhanced by treatment of cytokines.

A link between inflammation and colon cancer has already been established. Inflammatory cytokines can serve as tumor suppressors or tumor progressors as well as survival factors, and

can promote tumor growth by promoting angiogenesis and suppressing immune-mediated tumor elimination (Karin and Greten 2005). They act through their transcriptions factors such as NF-κB, MAPK1 and STAT-3 (Duyao et al. 1992;Terzic et al. 2010).

A large fraction of CRC tumors and cell lines exhibit constitutive activation of transcription factors that are essential components of multiple inflammatory pathways, namely nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) (Popivanova et al. 2008;Terzic et al. 2010;Vallabhapurapu and Karin 2009) and MAPK (Terzic et al. 2010;Kreeger et al. 2009).

MAPK is an important transcription factor in the inflammatory signaling pathway (Cohen et al. 2005). The primary function of Raf is to phosphorylate and activate MEK (also MAP-kinase (MAPK) (Cobb and Goldsmith 1995). MEK, in turn, catalyzes the phosphorylation of ERK1 and ERK2 (MAPKs), the only known substrates for MEK phosphorylation. Phospho-ERK (p-ERK) then dimerizes and translocates to the nucleus where it leads to transcription-factor activation and cell proliferation, differentiation, survival, invasion, and metastasis (Sebolt-Leopold and Herrera 2004). MAPK is constitutively activated with high frequency in colon cancers (Hoshino et al. 1999). Thus, the ERK/MAPK step of the transcription activation pathway may represent an important therapeutic target, given its position as the final common pathway for cell proliferation and other activities relevant to cancer pathogenesis. In preclinical models it has been demonstrated that MEK inhibition via a small molecule inhibitor results in cell death (Wang et al. 2004). This effect is most pronounced in p-53 wild-type cells, where it is postulated that tumor cells may be more dependent on MEK/MAPK signaling for cell survival in the presence of intact p53(Shaw et al. 1991;Terzic et al. 2010).

NF-κB, one of the major transcriptional regulators of many genes, is known to be highly activated in colon cancer (Yeruva et al. 2008a) by cytokines like TNF through its receptors

(TNFR) (Waldner and Neurath 2008). It has been demonstrated that several TNFR members translocate into the nucleus to interact with different NF-κB Rel subunits and activate the transcription of NF-κB target genes (Locksley et al. 2001). NF-κB acts through either the classical or the canonical pathway (Yeruva et al. 2008a).

In chronic inflammation, cytokines and chemokines produced by inflammatory cells, propagate a localized inflammatory response and also enhance the survival of premalignant cells by activating NF-κB (Lin and Karin 2007). Activation of the IKK/ NF-κB pathway is one key survival mechanism in a variety of cancer types (Karin and Greten 2005;Schulze-Bergkamen and Krammer 2004;Yeruva et al. 2008b). Numerous studies have indicated that NF-κB can block apoptosis by regulating the anti-apoptosis proteins such as inhibitor of apoptotic proteins (IAPs) (Kucharczak et al. 2003). Another mechanism whereby NF-κB may inhibit apoptosis is through its ability to inhibit prolonged c-Jun N-terminal kinase (JNK) activation and accumulation of reactive oxygen species (ROS) (Luo et al. 2005).

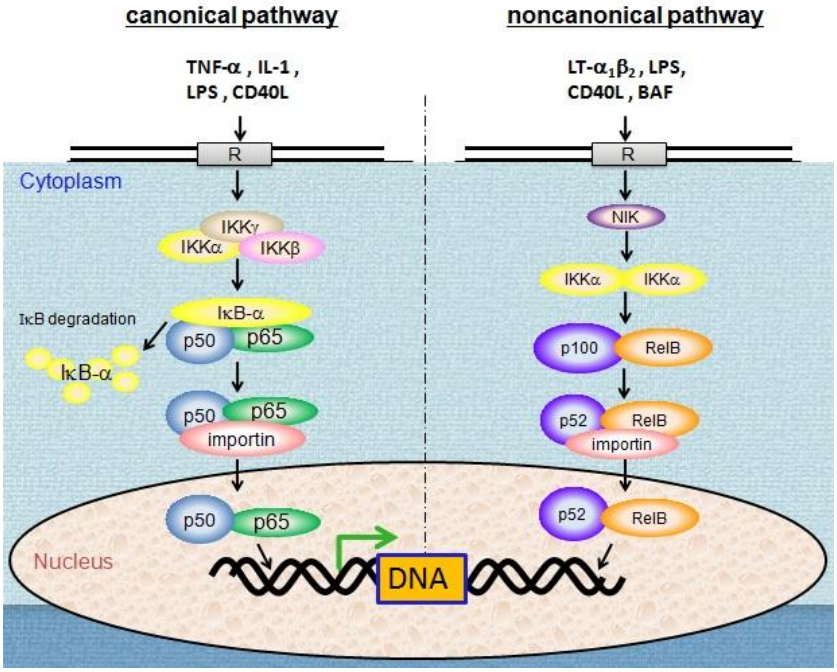



**Figure 30**: **Canonical and non canonical NF-κB activating pathways. Source: Inhibition of tumor growth by NF-kappa B inhibitors. Adapted from (Umezawa 2006)**

NF-κB activation in intestinal epithelial cells is also a major regulator of chemokine gene expression (Yeruva et al. 2008b). A number of studies have revealed that LPS induced cytokines like IL-1β and TNFα change the expression of several chemokines through the transcription factor NF-κB in IECs (Elewaut et al. 1999;Kim et al. 2004;Kim et al. 2002;Vallee et al. 2004;Yeruva et al. 2008b). NF-κB is a complex transcription factor which consists of several subunits. The degradation (reduction) of IκBα-level and the activation of p65 subunit is known as classical NF-κB pathway (Karin and Greten 2005). Several inhibitors are present which inhibit NF-κB activation (Kim et al. 2002). Some of them are non specific and some are specific inhibitors. A previous study reported that inhibition of NF-κB (p65) reduces chemokine gene expression which could imply a pharmacological importance in treating IBD (Yeruva et al. 2008b) . These results are in accordance with our study, where inhibition of KRAS by the siRNA approach induced NF-κB (reduced IκBα-level) followed by an increase in chemokine gene expression in the wild type cell line.

STAT-3 is a down-stream target in the IL-6 pathway. A striking finding of the current study was the detection of its phosphorylation by IFNγ treatment in KRAS mutated DLD1. The role of STAT-3 as pro- or anti-tumor agent has been controversially documented. STAT3 was shown to be repeatedly activated in many types of human solid and blood cancers, including colon cancer (Buettner et al. 2002;Corvinus et al. 2005;Kusaba et al. 2005;Ma et al. 2004;Turkson and Jove 2000), however its phosphorylated form (p-STAT-3) has not been evaluated so far in this setting.

In mouse models of colitis-associated cancer (CAC) and liver cancer, the genetic and pharmacological manipulation of NF-κB and STAT3 activation has revealed the complex

interaction of the signaling cascades in various cell types and that tumor cells have intrinsic and extrinsic mechanisms to regulate apoptosis and proliferation (Eaden et al. 2001).

Previous studies have shown that blocking the signaling to STAT3 inhibits cancer cell growth, indicating that STAT3 is crucial to the survival and growth of tumor cells (Buettner et al. 2002;Ling and Arlinghaus 2005;Turkson and Jove 2000) and is an attractive therapeutic target for cancer. In fact, a main effort to target constitutive STAT3 signaling was focused on a multitude of cancer cells. Whilst it has been shown that is activated in colon cancer–initiating cells, no approach has been initiated to explore the STAT3 as a possible therapeutic target in colon cancer–initiating cells.

However, STAT3 has also been shown to have tumor suppressor role depending on the mutational background of the tumor (de la Iglesia et al. 2008). Accordingly, we could show a lower expression in WT compared to KRAS colon cancer cell lines.

In our studies, we further showed that elevated p-STAT3 is detected in the DLD1 (KRAS) colon cancer cell line and can be further induced by IFNγ. We could however show, that at basal level, colon cancer cell lines do not express IFNγ nor IL-6 (another upstream inducer of the STAT-3 pathway). Thus, the STAT-3 pathway might be switched down in wild type CRC-cell lines, whilst in KRAS mutated cells, it takes on pro-tumorigenic roles.

These results suggest that activated STAT3 is indeed a novel therapeutic target, especially in KRAS mutated, colon cancer–initiating cells. Previous studies showed that, co-operation between IFNγ induced STAT1 and that the constitutive or inducible NF-κB is necessary for the transcriptional activity of IFNγ inducible chemokines (Hiroi and Ohmori 2003).

This work illustrates, that tight regulation of cytoplasmic with nuclear transcriptional processes is necessary, and that external stimuli affect by i.e. cytokines can affect intra-cellular processes, as well as the underlying gene mutation.

# 6. References

Reference List


Aggarwal BB, Pocsik E (1992) Cytokines: from clone to clinic. Arch Biochem Biophys 292:335-359

Andresen L, Jorgensen VL, Perner A, Hansen A, Eugen-Olsen J, Rask-Madsen J (2005) Activation of nuclear factor kappaB in colonic mucosa from patients with collagenous and ulcerative colitis. Gut 54:503-509

Andus T, Daig R, Vogl D, Aschenbrenner E, Lock G, Hollerbach S, Kollinger M, Scholmerich J, Gross V (1997) Imbalance of the interleukin 1 system in colonic mucosa--association with intestinal inflammation and interleukin 1 receptor antagonist [corrected] genotype 2. Gut 41:651-657

Anisowicz A, Bardwell L, Sager R (1987a) Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. Proc Natl Acad Sci U S A 84:7188-7192

Anisowicz A, Bardwell L, Sager R (1987b) Constitutive overexpression of a growth-regulated gene in transformed Chinese hamster and human cells. Proc Natl Acad Sci U S A 84:7188-7192

Armaghany T, Wilson JD, Chu Q, Mills G (2012a) Genetic alterations in colorectal cancer. Gastrointest Cancer Res 5:19-27

Armaghany T, Wilson JD, Chu Q, Mills G (2012b) Genetic alterations in colorectal cancer. Gastrointest Cancer Res 5:19-27

Askling J, Dickman PW, Karlen P, Brostrom O, Lapidus A, Lofberg R, Ekbom A (2001) Family history as a risk factor for colorectal cancer in inflammatory bowel disease. Gastroenterology 120:1356-1362

Atreya I, Atreya R, Neurath MF (2008) NF-kappaB in inflammatory bowel disease. J Intern Med 263:591-596

Atreya I, Neurath MF (2008) Immune cells in colorectal cancer: prognostic relevance and therapeutic strategies. Expert Rev Anticancer Ther 8:561-572

Atzeni F, Sarzi-Puttini P, Gorla R, Marchesoni A, Caporali R (2011) Switching rheumatoid arthritis treatments: an update. Autoimmun Rev 10:397-403

Baggiolini M, Imboden P, Detmers P (1992) Neutrophil activation and the effects of interleukin-8/neutrophil-activating peptide 1 (IL-8/NAP-1). Cytokines 4:1-17

Baldwin AS, Jr. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol 14:649-683

Balkwill F (2004a) Cancer and the chemokine network. Nat Rev Cancer 4:540-550

Balkwill F (2004b) Cancer and the chemokine network. Nat Rev Cancer 4:540-550

Balkwill FR (2012) The chemokine system and cancer. J Pathol 226:148-157

Banks C, Bateman A, Payne R, Johnson P, Sheron N (2003) Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in both ulcerative colitis and Crohn's disease. J Pathol 199:28-35

Becker S, Quay J, Koren HS, Haskill JS (1994) Constitutive and stimulated MCP-1, GRO alpha, beta, and gamma expression in human airway epithelium and bronchoalveolar macrophages. Am J Physiol 266:L278-L286

Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, Strieter RM (2000) CXC chemokines in angiogenesis. J Leukoc Biol 68:1-8

Boimel PJ, Smirnova T, Zhou ZN, Wyckoff J, Park H, Coniglio SJ, Qian BZ, Stanley ER, Cox D, Pollard JW, Muller WJ, Condeelis J, Segall JE (2012) fContribution of CXCL12 secretion to invasion of breast cancer cells. Breast Cancer Res 14:R23

Bollrath J, Greten FR (2009) IKK/NF-kappaB and STAT3 pathways: central signalling hubs in inflammation-mediated tumour promotion and metastasis. EMBO Rep 10:1314-1319

Bos JL (1989) ras oncogenes in human cancer: a review. Cancer Res 49:4682-4689

Breese E, Braegger CP, Corrigan CJ, Walker-Smith JA, MacDonald TT (1993) Interleukin-2- and interferon-gamma-secreting T cells in normal and diseased human intestinal mucosa. Immunology 78:127-131

Buettner R, Mora LB, Jove R (2002) Activated STAT signaling in human tumors provides novel molecular targets for therapeutic intervention. Clin Cancer Res 8:945-954

Calkins BM, Mendeloff AI (1986) Epidemiology of inflammatory bowel disease. Epidemiol Rev 8:60-91

Cappell MS (2008) Pathophysiology, clinical presentation, and management of colon cancer. Gastroenterol Clin North Am 37:1-24, v

Carvajal-Carmona LG, Howarth KM, Lockett M, Polanco-Echeverry GM, Volikos E, Gorman M, Barclay E, Martin L, Jones AM, Saunders B, Guenther T, Donaldson A, Paterson J, Frayling I, Novelli MR, Phillips R, Thomas HJ, Silver A, Atkin W, Tomlinson IP (2007) Molecular classification and genetic pathways in hyperplastic polyposis syndrome. J Pathol 212:378-385

Casini-Raggi V, Kam L, Chong YJ, Fiocchi C, Pizarro TT, Cominelli F (1995) Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. J Immunol 154:2434-2440

Chaly YV, Selvan RS, Fegeding KV, Kolesnikova TS, Voitenok NN (2000) Expression of IL-8 gene in human monocytes and lymphocytes: differential regulation by TNF and IL-1. Cytokine 12:636-643

Clevers H (2004) At the crossroads of inflammation and cancer. Cell 118:671-674

Cobb MH, Goldsmith EJ (1995) How MAP kinases are regulated. J Biol Chem 270:14843-14846

Cohen SJ, Cohen RB, Meropol NJ (2005) Targeting signal transduction pathways in colorectal cancer--more than skin deep. J Clin Oncol 23:5374-5385

Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A (2009) Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis 30:1073-1081

Corvinus FM, Orth C, Moriggl R, Tsareva SA, Wagner S, Pfitzner EB, Baus D, Kaufmann R, Huber LA, Zatloukal K, Beug H, Ohlschlager P, Schutz A, Halbhuber KJ, Friedrich K (2005) Persistent STAT3 activation in colon cancer is associated with enhanced cell proliferation and tumor growth. Neoplasia 7:545-555

Coussens LM, Werb Z (2002) Inflammation and cancer. Nature 420:860-867

Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, Bets D, Mueser M, Harstrick A, Verslype C, Chau I, Van CE (2004) Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. N Engl J Med 351:337-345

Dalum I, Butler DM, Jensen MR, Hindersson P, Steinaa L, Waterston AM, Grell SN, Feldmann M, Elsner HI, Mouritsen S (1999) Therapeutic antibodies elicited by immunization against TNF-alpha. Nat Biotechnol 17:666-669

Darnell JE, Jr. (2007) Interferon research: impact on understanding transcriptional control. Curr Top Microbiol Immunol 316:155-163

Davidson NJ, Leach MW, Fort MM, Thompson-Snipes L, Kuhn R, Muller W, Berg DJ, Rennick DM (1996) T helper cell 1-type CD4+ T cells, but not B cells, mediate colitis in interleukin 10-deficient mice. J Exp Med 184:241-251

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA (2002a) Mutations of the BRAF gene in human cancer. Nature 417:949-954

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA (2002b) Mutations of the BRAF gene in human cancer. Nature 417:949-954

de la Iglesia N, Konopka G, Puram SV, Chan JA, Bachoo RM, You MJ, Levy DE, Depinho RA, Bonni A (2008) Identification of a PTEN-regulated STAT3 brain tumor suppressor pathway. Genes Dev 22:449-462

De FC, Luceri C, Caderni G, Pacini M, Messerini L, Biggeri A, Mini E, Tonelli F, Cianchi F, Dolara P (2002) Mutations of the APC gene in human sporadic colorectal cancers. Scand J Gastroenterol 37:1048-1053

Dhawan P, Richmond A (2002) Role of CXCL1 in tumorigenesis of melanoma. J Leukoc Biol 72:9-18

Dhomen N, Marais R (2007) New insight into BRAF mutations in cancer. Curr Opin Genet Dev 17:31-39

Dinarello CA (1994) The interleukin-1 family: 10 years of discovery. FASEB J 8:1314-1325

Dinarello CA, Thompson RC (1991) Blocking IL-1: interleukin 1 receptor antagonist in vivo and in vitro. Immunol Today 12:404-410

Downward J (2003) Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 3:11-22

Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, van de Putte LB, Lipsky PE (1998) Cyclooxygenase in biology and disease. FASEB J 12:1063-1073

Dunn GP, Koebel CM, Schreiber RD (2006) Interferons, immunity and cancer immunoediting. Nat Rev Immunol 6:836-848

Duyao MP, Kessler DJ, Spicer DB, Bartholomew C, Cleveland JL, Siekevitz M, Sonenshein GE (1992) Transactivation of the c-myc promoter by human T cell leukemia virus type 1 tax is mediated by NF kappa B. J Biol Chem 267:16288-16291

Dwinell MB, Lugering N, Eckmann L, Kagnoff MF (2001) Regulated production of interferon-inducible T-cell chemoattractants by human intestinal epithelial cells. Gastroenterology 120:49-59

Eaden JA, Abrams KR, Mayberry JF (2001) The risk of colorectal cancer in ulcerative colitis: a meta-analysis. Gut 48:526-535

Elewaut D, DiDonato JA, Kim JM, Truong F, Eckmann L, Kagnoff MF (1999) NF-kappa B is a central regulator of the intestinal epithelial cell innate immune response induced by infection with enteroinvasive bacteria. J Immunol 163:1457-1466

Elson CO, Sartor RB, Tennyson GS, Riddell RH (1995) Experimental models of inflammatory bowel disease. Gastroenterology 109:1344-1367

Erreni M, Bianchi P, Laghi L, Mirolo M, Fabbri M, Locati M, Mantovani A, Allavena P (2009) Expression of chemokines and chemokine receptors in human colon cancer. Methods Enzymol 460:105-121

Fais S, Capobianchi MR, Pallone F, Di MP, Boirivant M, Dianzani F, Torsoli A (1991) Spontaneous release of interferon gamma by intestinal lamina propria lymphocytes in Crohn's disease. Kinetics of in vitro response to interferon gamma inducers. Gut 32:403-407

Fearon ER, Vogelstein B (1990a) A genetic model for colorectal tumorigenesis. Cell 61:759-767

Fearon ER, Vogelstein B (1990b) A genetic model for colorectal tumorigenesis. Cell 61:759-767

Feig LA, Cooper GM (1988) Relationship among guanine nucleotide exchange, GTP hydrolysis, and transforming potential of mutated ras proteins. Mol Cell Biol 8:2472-2478

Feldman ED, Weinreich DM, Carroll NM, Burness ML, Feldman AL, Turner E, Xu H, Alexander HR, Jr. (2006) Interferon gamma-inducible protein 10 selectively inhibits proliferation and induces apoptosis in endothelial cells. Ann Surg Oncol 13:125-133

Feng XD, Huang SG, Shou JY, Liao BR, Yingling JM, Ye X, Lin X, Gelbert LM, Su EW, Onyia JE, Li SY (2007) Analysis of pathway activity in primary tumors and NCI60 cell lines using gene expression profiling data. Genomics Proteomics Bioinformatics 5:15-24

Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P (2007) Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol 18:581-592

Fernandez EJ, Lolis E (2002) Structure, function, and inhibition of chemokines. Annu Rev Pharmacol Toxicol 42:469-499

Finco TS, Baldwin AS, Jr. (1993) Kappa B site-dependent induction of gene expression by diverse inducers of nuclear factor kappa B requires Raf-1. J Biol Chem 268:17676-17679

Fujimoto J, Aoki I, Toyoki H, Khatun S, Tamaya T (2002) Clinical implications of expression of ETS-1 related to angiogenesis in uterine cervical cancers. Ann Oncol 13:1598-1604

Fusunyan RD, Quinn JJ, Ohno Y, Macdermott RP, Sanderson IR (1998) Butyrate enhances interleukin (IL)-8 secretion by intestinal epithelial cells in response to IL-1beta and lipopolysaccharide. Pediatr Res 43:84-90

Futreal PA, Wooster R, Stratton MR (2005) Somatic mutations in human cancer: insights from resequencing the protein kinase gene family. Cold Spring Harb Symp Quant Biol 70:43-49

Garces S, Demengeot J, Benito-Garcia E (2012) The immunogenicity of anti-TNF therapy in immune-mediated inflammatory diseases: a systematic review of the literature with a meta-analysis. Ann Rheum Dis

Garrity-Park MM, Loftus EV, Jr., Bryant SC, Sandborn WJ, Smyrk TC (2008) Tumor necrosis factor-alpha polymorphisms in ulcerative colitis-associated colorectal cancer. Am J Gastroenterol 103:407-415

Gasperini S, Marchi M, Calzetti F, Laudanna C, Vicentini L, Olsen H, Murphy M, Liao F, Farber J, Cassatella MA (1999) Gene expression and production of the monokine induced by IFN-gamma (MIG), IFN-inducible T cell alpha chemoattractant (I-TAC), and IFN-gamma-inducible protein-10 (IP-10) chemokines by human neutrophils. J Immunol 162:4928-4937

Gough DJ, Levy DE, Johnstone RW, Clarke CJ (2008) IFNgamma signaling-does it mean JAK-STAT? Cytokine Growth Factor Rev 19:383-394

Grand RJ, Owen D (1991) The biochemistry of ras p21. Biochem J 279 ( Pt 3):609-631

Grilli M, Memo M (1999) Nuclear factor-kappaB/Rel proteins: a point of convergence of signalling pathways relevant in neuronal function and dysfunction. Biochem Pharmacol 57:1-7

Haghnegahdar H, Du J, Wang D, Strieter RM, Burdick MD, Nanney LB, Cardwell N, Luan J, Shattuck-Brandt R, Richmond A (2000) The tumorigenic and angiogenic effects of MGSA/GRO proteins in melanoma. J Leukoc Biol 67:53-62

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57-70

Hayden MS, Ghosh S (2008) Shared principles in NF-kappaB signaling. Cell 132:344-362

Hingorani SR, Petricoin EF, Maitra A, Rajapakse V, King C, Jacobetz MA, Ross S, Conrads TP, Veenstra TD, Hitt BA, Kawaguchi Y, Johann D, Liotta LA, Crawford HC, Putt ME, Jacks T, Wright CV, Hruban RH, Lowy AM, Tuveson DA (2003) Preinvasive and invasive ductal pancreatic cancer and its early detection in the mouse. Cancer Cell 4:437-450

Hiroi M, Ohmori Y (2003) Constitutive nuclear factor kappaB activity is required to elicit interferon-gamma-induced expression of chemokine CXC ligand 9 (CXCL9) and CXCL10 in human tumour cell lines. Biochem J 376:393-402

Hoch RC, Schraufstatter IU, Cochrane CG (1996) In vivo, in vitro, and molecular aspects of interleukin-8 and the interleukin-8 receptors. J Lab Clin Med 128:134-145

Holmes WE, Lee J, Kuang WJ, Rice GC, Wood WI (2009) Structure and functional expression of a human interleukin-8 receptor. Science. 1991. 253: 1278-1280. J Immunol 183:2895-2897

Hoshino R, Chatani Y, Yamori T, Tsuruo T, Oka H, Yoshida O, Shimada Y, Ari-i S, Wada H, Fujimoto J, Kohno M (1999) Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. Oncogene 18:813-822

Iida N, Grotendorst GR (1990) Cloning and sequencing of a new gro transcript from activated human monocytes: expression in leukocytes and wound tissue. Mol Cell Biol 10:5596-5599

Ilyas M, Straub J, Tomlinson IP, Bodmer WF (1999) Genetic pathways in colorectal and other cancers. Eur J Cancer 35:335-351

Inoue K, Slaton JW, Eve BY, Kim SJ, Perrotte P, Balbay MD, Yano S, Bar-Eli M, Radinsky R, Pettaway CA, Dinney CP (2000) Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. Clin Cancer Res 6:2104-2119

Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M (1993) Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. Nature 363:558-561

Jass JR (2006) Colorectal cancer: a multipathway disease. Crit Rev Oncog 12:273-287

Jobin C, Haskill S, Mayer L, Panja A, Sartor RB (1997) Evidence for altered regulation of I kappa B alpha degradation in human colonic epithelial cells. J Immunol 158:226-234

Jumarie C, Malo C (1995) Ornithine decarboxylase activity is associated with proliferation but not with T3-induced differentiation of Caco-2 cells. J Cell Physiol 165:639-646

Karin M (2006) Nuclear factor-kappaB in cancer development and progression. Nature 441:431-436

Karin M, Greten FR (2005) NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 5:749-759

Keeley EC, Mehrad B, Strieter RM (2008) Chemokines as mediators of neovascularization. Arterioscler Thromb Vasc Biol 28:1928-1936

Kiefer F, Siekmann AF (2011) The role of chemokines and their receptors in angiogenesis. Cell Mol Life Sci 68:2811-2830

Kim JA, Kim DK, Jin T, Kang OH, Choi YA, Choi SC, Kim TH, Nah YH, Choi SJ, Kim YH, Bae KH, Lee YM (2004) Acanthoic acid inhibits IL-8 production via MAPKs and NF-kappaB in a TNF-alpha-stimulated human intestinal epithelial cell line. Clin Chim Acta 342:193-202

Kim JM, Cho SJ, Oh YK, Jung HY, Kim YJ, Kim N (2002) Nuclear factor-kappa B activation pathway in intestinal epithelial cells is a major regulator of chemokine gene expression and neutrophil migration induced by Bacteroides fragilis enterotoxin. Clin Exp Immunol 130:59-66

Kim JM, Oh YK, Kim YJ, Oh HB, Cho YJ (2001) Polarized secretion of CXC chemokines by human intestinal epithelial cells in response to Bacteroides fragilis enterotoxin: NF-kappa B plays a major role in the regulation of IL-8 expression. Clin Exp Immunol 123:421-427

Kreeger PK, Mandhana R, Alford SK, Haigis KM, Lauffenburger DA (2009) RAS mutations affect tumor necrosis factor-induced apoptosis in colon carcinoma cells via ERK-modulatory negative and positive feedback circuits along with non-ERK pathway effects. Cancer Res 69:8191-8199

Kucharczak J, Simmons MJ, Fan Y, Gelinas C (2003) To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. Oncogene 22:8961-8982

Kurman RJ, Shih I (2011) Molecular pathogenesis and extraovarian origin of epithelial ovarian cancer--shifting the paradigm. Hum Pathol 42:918-931

Kusaba T, Nakayama T, Yamazumi K, Yakata Y, Yoshizaki A, Nagayasu T, Sekine I (2005) Expression of p-STAT3 in human colorectal adenocarcinoma and adenoma; correlation with clinicopathological factors. J Clin Pathol 58:833-838

Lakatos PL, Lakatos L (2008) Risk for colorectal cancer in ulcerative colitis: changes, causes and management strategies. World J Gastroenterol 14:3937-3947

Langaas V, Shahzidi S, Johnsen JI, Smedsrod B, Sveinbjornsson B (2001) Interferon-gamma modulates TRAIL-mediated apoptosis in human colon carcinoma cells. Anticancer Res 21:3733-3738

Laroux FS, Pavlick KP, Wolf RE, Grisham MB (2001) Dysregulation of intestinal mucosal immunity: implications in inflammatory bowel disease. News Physiol Sci 16:272-277

Lewis AM, Varghese S, Xu H, Alexander HR (2006a) Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. J Transl Med 4:48

Lewis AM, Varghese S, Xu H, Alexander HR (2006b) Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. J Transl Med 4:48

Li A, Varney ML, Singh RK (2004) Constitutive expression of growth regulated oncogene (gro) in human colon carcinoma cells with different metastatic potential and its role in regulating their metastatic phenotype. Clin Exp Metastasis 21:571-579

Lin L, Liu A, Peng Z, Lin HJ, Li PK, Li C, Lin J (2011) STAT3 is necessary for proliferation and survival in colon cancer-initiating cells. Cancer Res 71:7226-7237

Lin Q, Lai R, Chirieac LR, Li C, Thomazy VA, Grammatikakis I, Rassidakis GZ, Zhang W, Fujio Y, Kunisada K, Hamilton SR, Amin HM (2005) Constitutive activation of JAK3/STAT3 in colon carcinoma tumors and cell lines: inhibition of JAK3/STAT3 signaling induces apoptosis and cell cycle arrest of colon carcinoma cells. Am J Pathol 167:969-980

Lin WW, Karin M (2007) A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest 117:1175-1183

Ling X, Arlinghaus RB (2005) Knockdown of STAT3 expression by RNA interference inhibits the induction of breast tumors in immunocompetent mice. Cancer Res 65:2532-2536

Liotta LA, Kohn EC (2001) The microenvironment of the tumour-host interface. Nature 411:375-379

Liu F, Hu X, Zimmerman M, Waller JL, Wu P, Hayes-Jordan A, Lev D, Liu K (2011a) TNFalpha cooperates with IFN-gamma to repress Bcl-xL expression to sensitize metastatic colon carcinoma cells to TRAIL-mediated apoptosis. PLoS One 6:e16241

Liu M, Guo S, Hibbert JM, Jain V, Singh N, Wilson NO, Stiles JK (2011b) CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. Cytokine Growth Factor Rev 22:121-130

Locksley RM, Killeen N, Lenardo MJ (2001) The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 104:487-501

Loetscher H, Gentz R, Zulauf M, Lustig A, Tabuchi H, Schlaeger EJ, Brockhaus M, Gallati H, Manneberg M, Lesslauer W (1991) Recombinant 55-kDa tumor necrosis factor (TNF) receptor. Stoichiometry of binding to TNF alpha and TNF beta and inhibition of TNF activity. J Biol Chem 266:18324-18329

Lugering A, Schmidt M, Lugering N, Pauels HG, Domschke W, Kucharzik T (2001) Infliximab induces apoptosis in monocytes from patients with chronic active Crohn's disease by using a caspase-dependent pathway. Gastroenterology 121:1145-1157

Luo JL, Kamata H, Karin M (2005) IKK/NF-kappaB signaling: balancing life and death--a new approach to cancer therapy. J Clin Invest 115:2625-2632

Ma XT, Wang S, Ye YJ, Du RY, Cui ZR, Somsouk M (2004) Constitutive activation of Stat3 signaling pathway in human colorectal carcinoma. World J Gastroenterol 10:1569-1573

Macdermott RP (1999) Chemokines in the inflammatory bowel diseases. J Clin Immunol 19:266-272

Mackay CR (2001) Chemokines: immunology's high impact factors. Nat Immunol 2:95-101

Mahida YR, Patel S, Gionchetti P, Vaux D, Jewell DP (1989) Macrophage subpopulations in lamina propria of normal and inflamed colon and terminal ileum. Gut 30:826-834

Malik IA, Moriconi F, Sheikh N, Naz N, Khan S, Dudas J, Mansuroglu T, Hess CF, Rave-Frank M, Christiansen H, Ramadori G (2010) Single-dose gamma-irradiation induces up-regulation of chemokine gene expression and recruitment of granulocytes into the portal area but not into other regions of rat hepatic tissue. Am J Pathol 176:1801-1815

Malik IA, Naz N, Sheikh N, Khan S, Moriconi F, Blaschke M, Ramadori G (2011) Comparison of changes in gene expression of transferrin receptor-1 and other iron-regulatory proteins in rat liver and brain during acute-phase response. Cell Tissue Res 344:299-312

Malumbres M, Barbacid M (2003) RAS oncogenes: the first 30 years. Nat Rev Cancer 3:459-465

Mantovani A, Savino B, Locati M, Zammataro L, Allavena P, Bonecchi R (2010) The chemokine system in cancer biology and therapy. Cytokine Growth Factor Rev 21:27-39

Marra F (2002) Chemokines in liver inflammation and fibrosis. Front Biosci 7:d1899-d1914

McDonald SA, Palmen MJ, Van Rees EP, MacDonald TT (1997) Characterization of the mucosal cell-mediated immune response in IL-2 knockout mice before and after the onset of colitis. Immunology 91:73-80

Meterissian S, Steele GD, Jr., Thomas P (1993) Human and murine Kupffer cell function may be altered by both intrahepatic and intrasplenic tumor deposits. Clin Exp Metastasis 11:175-182

Milne AN, Carneiro F, O'Morain C, Offerhaus GJ (2009) Nature meets nurture: molecular genetics of gastric cancer. Hum Genet 126:615-628

Moore BB, Arenberg DA, Addison CL, Keane MP, Strieter RM (1998) Tumor angiogenesis is regulated by CXC chemokines. J Lab Clin Med 132:97-103

Moser B, Clark-Lewis I, Zwahlen R, Baggiolini M (1990) Neutrophil-activating properties of the melanoma growth-stimulatory activity. J Exp Med 171:1797-1802

Mukaida N, Baba T (2012) Chemokines in tumor development and progression. Exp Cell Res 318:95-102

Muller WA (2003) Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol 24:327-334

Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev 52:145-176

Nagasaka T, Sasamoto H, Notohara K, Cullings HM, Takeda M, Kimura K, Kambara T, MacPhee DG, Young J, Leggett BA, Jass JR, Tanaka N, Matsubara N (2004) Colorectal cancer with mutation in BRAF, KRAS, and wild-type with respect to both oncogenes showing different patterns of DNA methylation. J Clin Oncol 22:4584-4594

Nakano H, Yamamoto F, Neville C, Evans D, Mizuno T, Perucho M (1984) Isolation of transforming sequences of two human lung carcinomas: structural and functional analysis of the activated c-K-ras oncogenes. Proc Natl Acad Sci U S A 81:71-75

Norris JL, Baldwin AS, Jr. (1999) Oncogenic Ras enhances NF-kappaB transcriptional activity through Raf-dependent and Raf-independent mitogen-activated protein kinase signaling pathways. J Biol Chem 274:13841-13846

Oka H, Chatani Y, Hoshino R, Ogawa O, Kakehi Y, Terachi T, Okada Y, Kawaichi M, Kohno M, Yoshida O (1995) Constitutive activation of mitogen-activated protein (MAP) kinases in human renal cell carcinoma. Cancer Res 55:4182-4187

Owen JD, Strieter R, Burdick M, Haghnegahdar H, Nanney L, Shattuck-Brandt R, Richmond A (1997) Enhanced tumor-forming capacity for immortalized melanocytes expressing melanoma growth stimulatory activity/growth-regulated cytokine beta and gamma proteins. Int J Cancer 73:94-103

Panja A, Goldberg S, Eckmann L, Krishen P, Mayer L (1998) The regulation and functional consequence of proinflammatory cytokine binding on human intestinal epithelial cells. J Immunol 161:3675-3684

Podolsky DK (1991) Inflammatory bowel disease (1). N Engl J Med 325:928-937

Podolsky DK (2002) The current future understanding of inflammatory bowel disease. Best Pract Res Clin Gastroenterol 16:933-943

Popivanova BK, Kitamura K, Wu Y, Kondo T, Kagaya T, Kaneko S, Oshima M, Fujii C, Mukaida N (2008) Blocking TNF-alpha in mice reduces colorectal carcinogenesis associated with chronic colitis. J Clin Invest 118:560-570

Pulciani S, Santos E, Lauver AV, Long LK, Aaronson SA, Barbacid M (1982) Oncogenes in solid human tumours. Nature 300:539-542

Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest 101:746-754

Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE (2002a) Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 418:934

Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B, Velculescu VE (2002b) Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 418:934

Ramadori G, Armbrust T (2001) Cytokines in the liver. Eur J Gastroenterol Hepatol 13:777-784

Ramadori G, Christ B (1999) Cytokines and the hepatic acute-phase response. Semin Liver Dis 19:141-155

Ramadori G, Moriconi F, Malik I, Dudas J (2008) Physiology and pathophysiology of liver inflammation, damage and repair. J Physiol Pharmacol 59 Suppl 1:107-117

Richmond A, Thomas HG (1988) Melanoma growth stimulatory activity: isolation from human melanoma tumors and characterization of tissue distribution. J Cell Biochem 36:185-198

Roessner A, Kuester D, Malfertheiner P, Schneider-Stock R (2008) Oxidative stress in ulcerative colitis-associated carcinogenesis. Pathol Res Pract 204:511-524

Rogler G, Andus T (1998a) Cytokines in inflammatory bowel disease. World J Surg 22:382-389

Rogler G, Andus T (1998b) Cytokines in inflammatory bowel disease. World J Surg 22:382-389

Ross PJ, George M, Cunningham D, DiStefano F, Andreyev HJ, Workman P, Clarke PA (2001) Inhibition of Kirsten-ras expression in human colorectal cancer using rationally selected Kirsten-ras antisense oligonucleotides. Mol Cancer Ther 1:29-41

Rossi D, Zlotnik A (2000) The biology of chemokines and their receptors. Annu Rev Immunol 18:217-242

Ruben SM, Narayanan R, Klement JF, Chen CH, Rosen CA (1992) Functional characterization of the NF-kappa B p65 transcriptional activator and an alternatively spliced derivative. Mol Cell Biol 12:444-454

Rustgi AK (2007a) The genetics of hereditary colon cancer. Genes Dev 21:2525-2538

Rustgi AK (2007b) The genetics of hereditary colon cancer. Genes Dev 21:2525-2538

Sakamoto K, Maeda S, Hikiba Y, Nakagawa H, Hayakawa Y, Shibata W, Yanai A, Ogura K, Omata M (2009) Constitutive NF-kappaB activation in colorectal carcinoma plays a key role in angiogenesis, promoting tumor growth. Clin Cancer Res 15:2248-2258

Sartor RB (1994) Cytokines in intestinal inflammation: pathophysiological and clinical considerations. Gastroenterology 106:533-539

Schenkel AR, Mamdouh Z, Chen X, Liebman RM, Muller WA (2002) CD99 plays a major role in the migration of monocytes through endothelial junctions. Nat Immunol 3:143-150

Schimanski CC, Linnemann U, Berger MR (1999) Sensitive detection of K-ras mutations augments diagnosis of colorectal cancer metastases in the liver. Cancer Res 59:5169-5175

Schindler CW (2002) Series introduction. JAK-STAT signaling in human disease. J Clin Invest 109:1133-1137

Schroder K, Hertzog PJ, Ravasi T, Hume DA (2004) Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 75:163-189

Schubbert S, Shannon K, Bollag G (2007) Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer 7:295-308

Schulze-Bergkamen H, Krammer PH (2004) Apoptosis in cancer--implications for therapy. Semin Oncol 31:90-119

Schumacher C, Clark-Lewis I, Baggiolini M, Moser B (1992) High- and low-affinity binding of GRO alpha and neutrophil-activating peptide 2 to interleukin 8 receptors on human neutrophils. Proc Natl Acad Sci U S A 89:10542-10546

Sebolt-Leopold JS, Herrera R (2004) Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat Rev Cancer 4:937-947

Servomaa K, Kiuru A, Kosma VM, Hirvikoski P, Rytomaa T (2000) p53 and K-ras gene mutations in carcinoma of the rectum among Finnish women. Mol Pathol 53:24-30

Shaw P, Tardy S, Benito E, Obrador A, Costa J (1991) Occurrence of Ki-ras and p53 mutations in primary colorectal tumors. Oncogene 6:2121-2128

Sheng H, Shao J, Williams CS, Pereira MA, Taketo MM, Oshima M, Reynolds AB, Washington MK, Dubois RN, Beauchamp RD (1998) Nuclear translocation of beta-catenin in hereditary and carcinogen-induced intestinal adenomas. Carcinogenesis 19:543-549

Sica A, Allavena P, Mantovani A (2008) Cancer related inflammation: the macrophage connection. Cancer Lett 267:204-215

Singer G, Oldt R, III, Cohen Y, Wang BG, Sidransky D, Kurman RJ, Shih I (2003) Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma. J Natl Cancer Inst 95:484-486

Slattery ML, Lundgreen A, Bondurant KL, Wolff RK (2011) Interferon-signaling pathway: associations with colon and rectal cancer risk and subsequent survival. Carcinogenesis 32:1660-1667

Smith DR, Polverini PJ, Kunkel SL, Orringer MB, Whyte RI, Burdick MD, Wilke CA, Strieter RM (1994) Inhibition of interleukin 8 attenuates angiogenesis in bronchogenic carcinoma. J Exp Med 179:1409-1415

Stevens C, Walz G, Singaram C, Lipman ML, Zanker B, Muggia A, Antonioli D, Peppercorn MA, Strom TB (1992) Tumor necrosis factor-alpha, interleukin-1 beta, and interleukin-6 expression in inflammatory bowel disease. Dig Dis Sci 37:818-826

Struyf S, Gouwy M, Dillen C, Proost P, Opdenakker G, Van DJ (2005) Chemokines synergize in the recruitment of circulating neutrophils into inflamed tissue. Eur J Immunol 35:1583-1591

Sunaga N, Shames DS, Girard L, Peyton M, Larsen JE, Imai H, Soh J, Sato M, Yanagitani N, Kaira K, Xie Y, Gazdar AF, Mori M, Minna JD (2011) Knockdown of oncogenic KRAS in non-small cell lung cancers suppresses tumor growth and sensitizes tumor cells to targeted therapy. Mol Cancer Ther 10:336-346

Tekautz TM, Zhu K, Grenet J, Kaushal D, Kidd VJ, Lahti JM (2006) Evaluation of IFN-gamma effects on apoptosis and gene expression in neuroblastoma--preclinical studies. Biochim Biophys Acta 1763:1000-1010

Ten HT, van MC, Peppelenbosch MP, van Deventer SJ (2002) Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. Gut 50:206-211

Terzic J, Grivennikov S, Karin E, Karin M (2010) Inflammation and colon cancer. Gastroenterology 138:2101-2114

Tsai HH, Frost E, To V, Robinson S, Ffrench-Constant C, Geertman R, Ransohoff RM, Miller RH (2002) The chemokine receptor CXCR2 controls positioning of oligodendrocyte precursors in developing spinal cord by arresting their migration. Cell 110:373-383

Tsareva SA, Moriggl R, Corvinus FM, Wiederanders B, Schutz A, Kovacic B, Friedrich K (2007) Signal transducer and activator of transcription 3 activation promotes invasive growth of colon carcinomas through matrix metalloproteinase induction. Neoplasia 9:279-291

Turkson J, Jove R (2000) STAT proteins: novel molecular targets for cancer drug discovery. Oncogene 19:6613-6626

Uguccioni M, Gionchetti P, Robbiani DF, Rizzello F, Peruzzo S, Campieri M, Baggiolini M (1999) Increased expression of IP-10, IL-8, MCP-1, and MCP-3 in ulcerative colitis. Am J Pathol 155:331-336

Umezawa K (2006) Inhibition of tumor growth by NF-kappaB inhibitors. Cancer Sci 97:990-995

Vallabhapurapu S, Karin M (2009) Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol 27:693-733

Vallee S, Laforest S, Fouchier F, Montero MP, Penel C, Champion S (2004) Cytokine-induced upregulation of NF-kappaB, IL-8, and ICAM-1 is dependent on colonic cell polarity: implication for PKCdelta. Exp Cell Res 297:165-185

van Dullemen HM, van Deventer SJ, Hommes DW, Bijl HA, Jansen J, Tytgat GN, Woody J (1995) Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). Gastroenterology 109:129-135

Vogelstein B, Kinzler KW (2004) Cancer genes and the pathways they control. Nat Med 10:789-799

Waldner MJ, Foersch S, Neurath MF (2012) Interleukin-6--a key regulator of colorectal cancer development. Int J Biol Sci 8:1248-1253

Waldner MJ, Neurath MF (2008) Cytokines in colitis associated cancer: potential drug targets? Inflamm Allergy Drug Targets 7:187-194

Wang D, Wang H, Brown J, Daikoku T, Ning W, Shi Q, Richmond A, Strieter R, Dey SK, Dubois RN (2006) CXCL1 induced by prostaglandin E2 promotes angiogenesis in colorectal cancer. J Exp Med 203:941-951

Wang JM, Deng X, Gong W, Su S (1998) Chemokines and their role in tumor growth and metastasis. J Immunol Methods 220:1-17

Wang Z, Li Y, Liu ET, Yu Q (2004) Susceptibility to cell death induced by blockade of MAPK pathway in human colorectal cancer cells carrying Ras mutations is dependent on p53 status. Biochem Biophys Res Commun 322:609-613

Wasmuth HE, Tacke F, Trautwein C (2010) Chemokines in liver inflammation and fibrosis. Semin Liver Dis 30:215-225

Weitz J, Koch M, Debus J, Hohler T, Galle PR, Buchler MW (2005) Colorectal cancer. Lancet 365:153-165

Wellbrock C, Karasarides M, Marais R (2004) The RAF proteins take centre stage. Nat Rev Mol Cell Biol 5:875-885

Xiong H, Zhang ZG, Tian XQ, Sun DF, Liang QC, Zhang YJ, Lu R, Chen YX, Fang JY (2008) Inhibition of JAK1, 2/STAT3 signaling induces apoptosis, cell cycle arrest, and reduces tumor cell invasion in colorectal cancer cells. Neoplasia 10:287-297

Yang SK, Eckmann L, Panja A, Kagnoff MF (1997) Differential and regulated expression of C-X-C, C-C, and C-chemokines by human colon epithelial cells. Gastroenterology 113:1214-1223

Ye S, Dhillon S, Ke X, Collins AR, Day IN (2001) An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res 29:E88

Yeruva S, Ramadori G, Raddatz D (2008a) NF-kappaB-dependent synergistic regulation of CXCL10 gene expression by IL-1beta and IFN-gamma in human intestinal epithelial cell lines. Int J Colorectal Dis 23:305-317

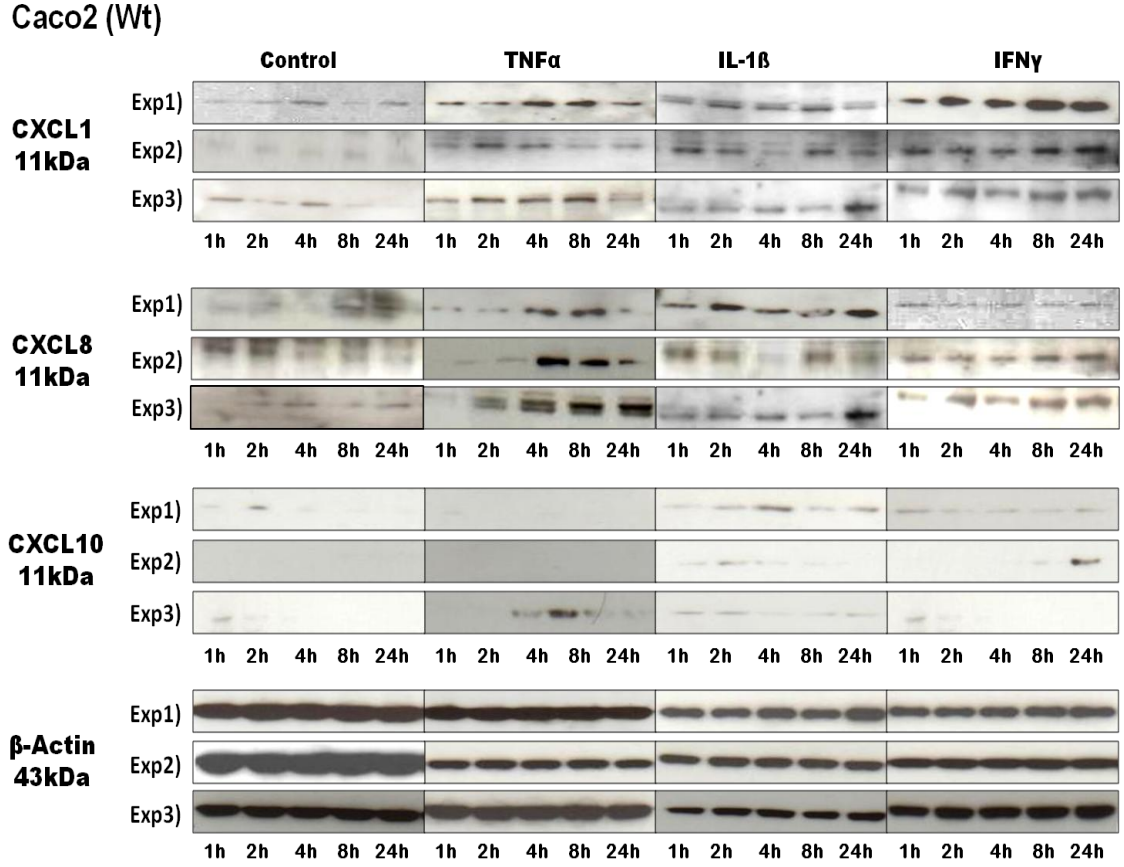
Yeruva S, Ramadori G, Raddatz D (2008b) NF-kappaB-dependent synergistic regulation of CXCL10 gene expression by IL-1beta and IFN-gamma in human intestinal epithelial cell lines. Int J Colorectal Dis 23:305-317

Yoneda J, Kuniyasu H, Crispens MA, Price JE, Bucana CD, Fidler IJ (1998) Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice. J Natl Cancer Inst 90:447-454

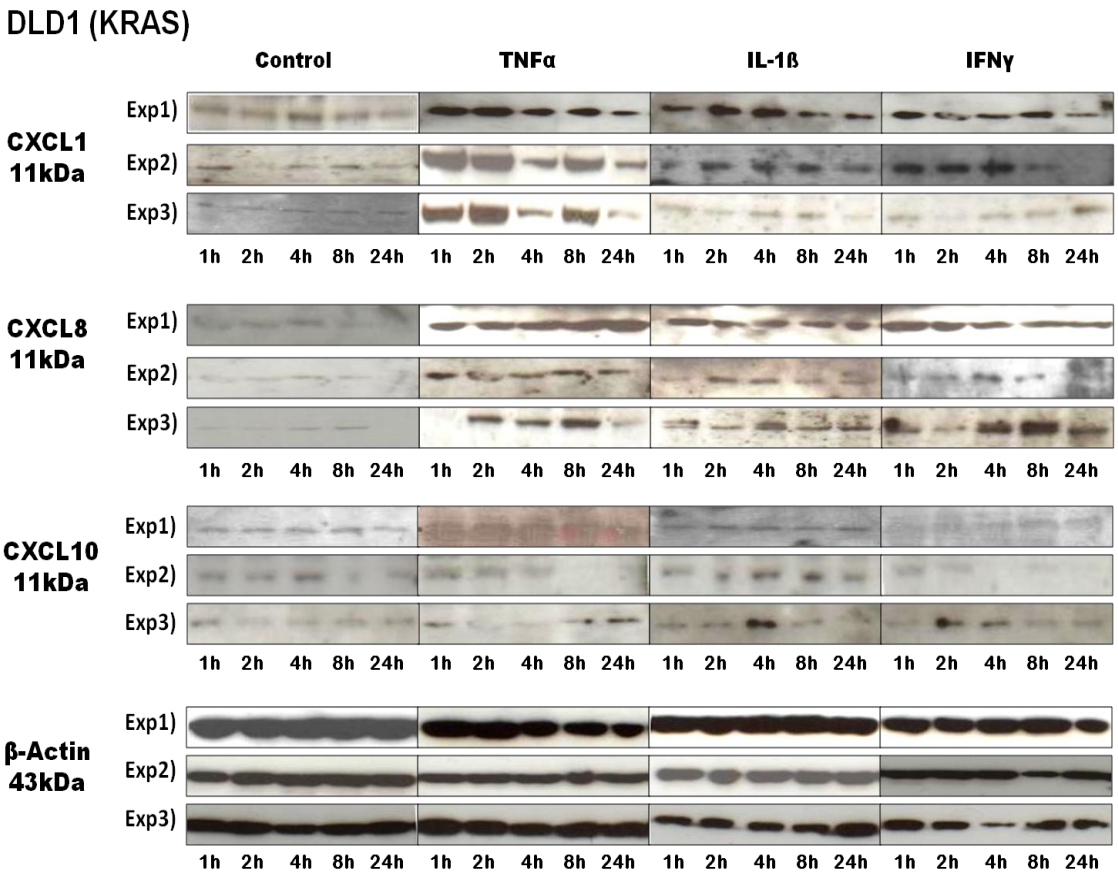
Yu H, Pardoll D, Jove R (2009) STATs in cancer inflammation and immunity: a leading role for STAT3. Nat Rev Cancer 9:798-809

Zhang W, Gordon M, Lenz HJ (2006) Novel approaches to treatment of advanced colorectal cancer with anti-EGFR monoclonal antibodies. Ann Med 38:545-551

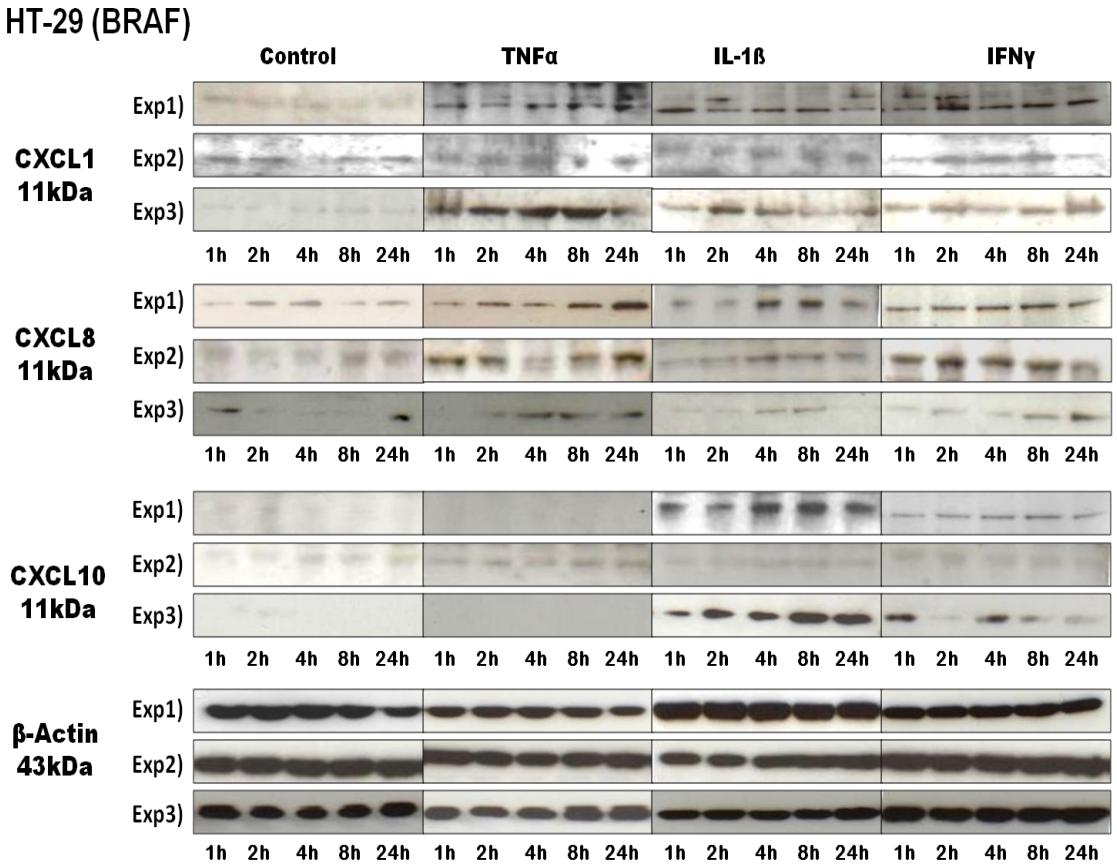
**Appendix 1:** Results of Chemokines in Caco-2 (Wt) treated with cytokines (TNFα, IL-1ß and IFNγ) in three Independent Experiments.



**Appendix 2:** Results of Chemokines DLD1 (Wt) treated with cytokines (TNFα, IL-1ß and IFNγ) in three Independent Experiments.

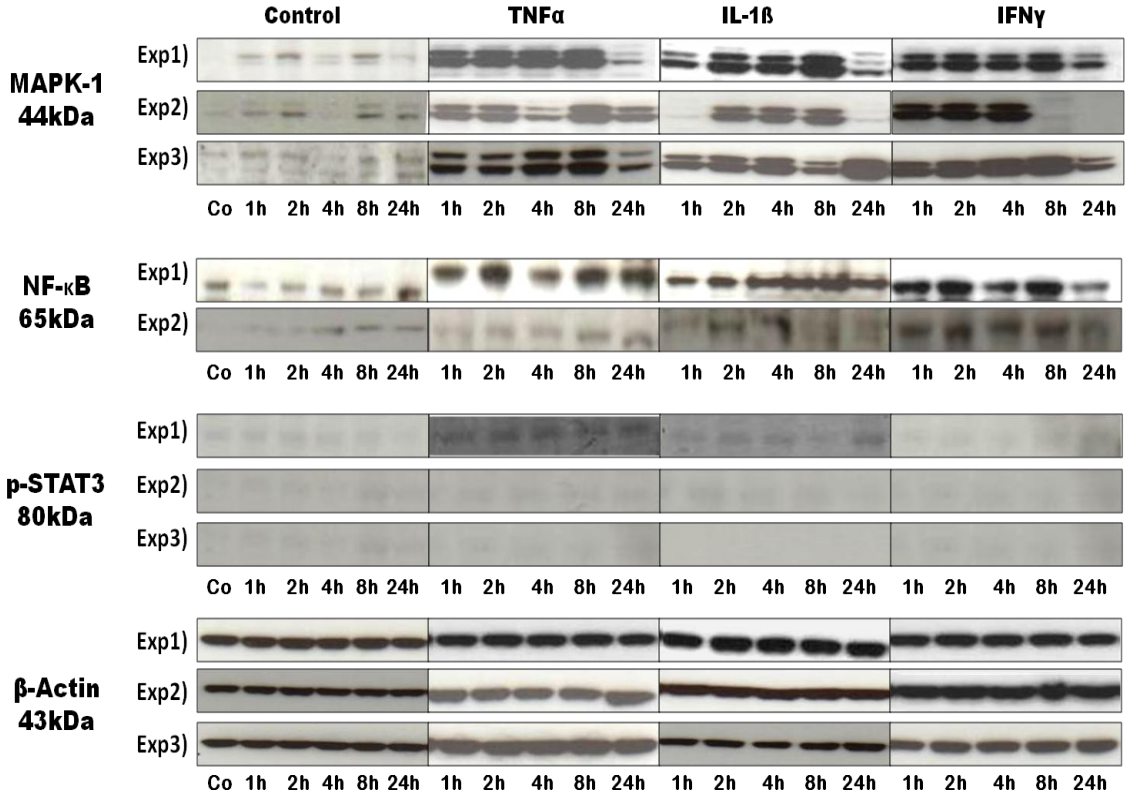


**Appendix 3:** Results of Chemokines HT-29 (BRAF) treated with cytokines (TNFα,IL-1ß and IFNγ) in three Independent Experiments.



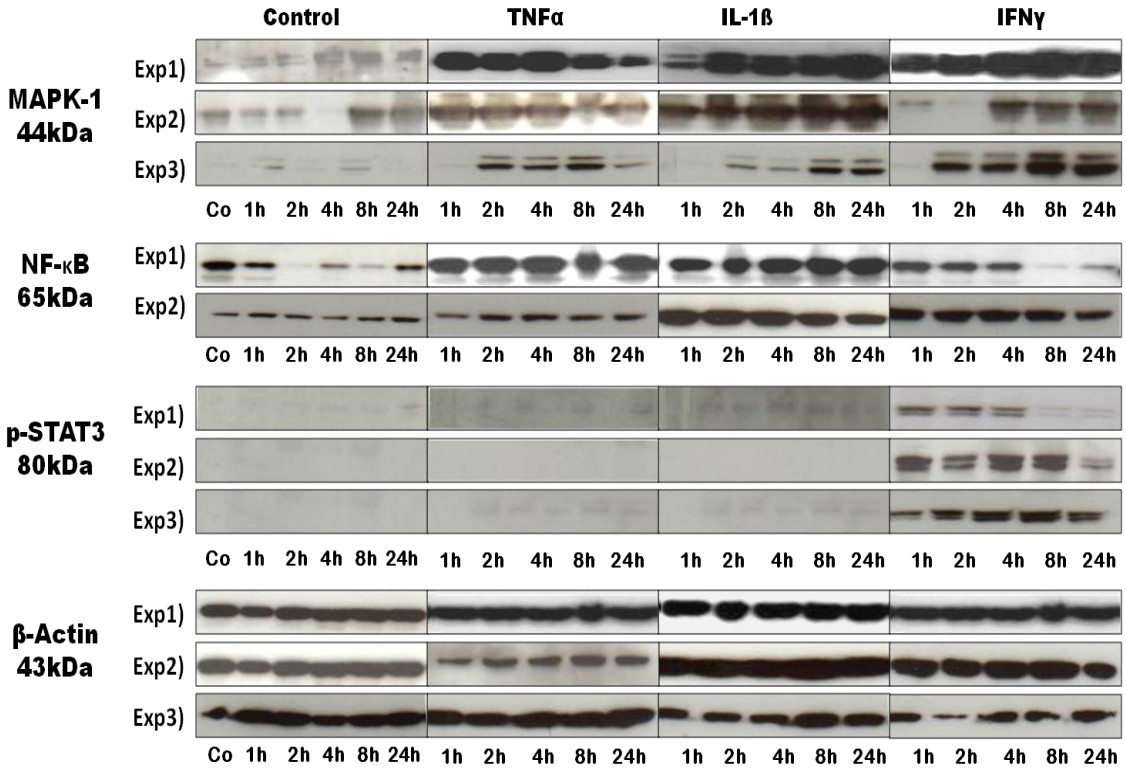
**Appendix 4:** Results of Transcription factors in Caco-2 (Wt) treated with cytokines (TNFα, IL-1ß and IFNγ) in three Independent Experiments.

Caco2 (Wt)



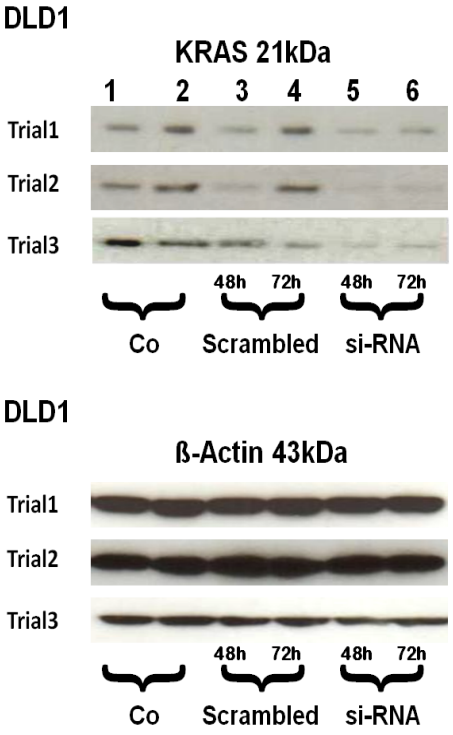
**Appendix 5:** Results of Transcription factors in DLD1 (Wt) treated with cytokines (TNFα, IL-1ß and IFNγ) in three Independent Experiments.

DLD1 (KRAS)

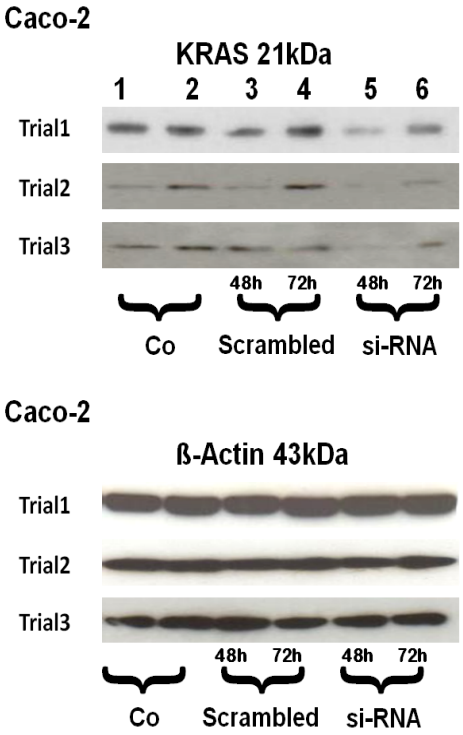


**Appendix 6:** KRAS siRNA knockdown Experiments in DLD1 (KRAS) and Caco2(Wt).

DLD1 (KRAS)

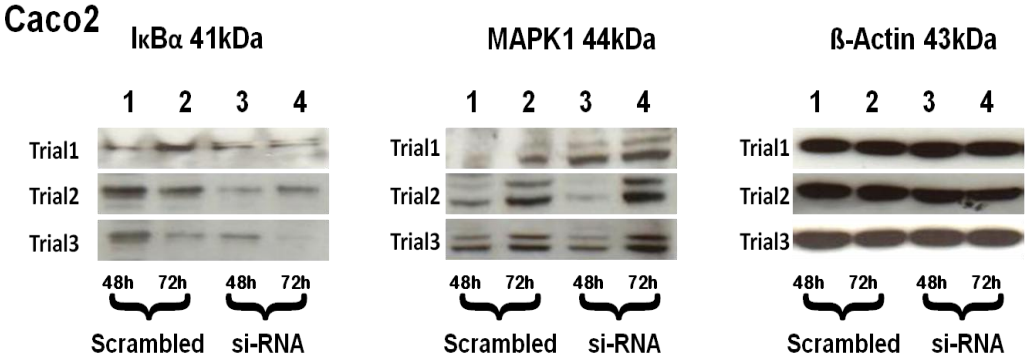



Caco2 (Wt)

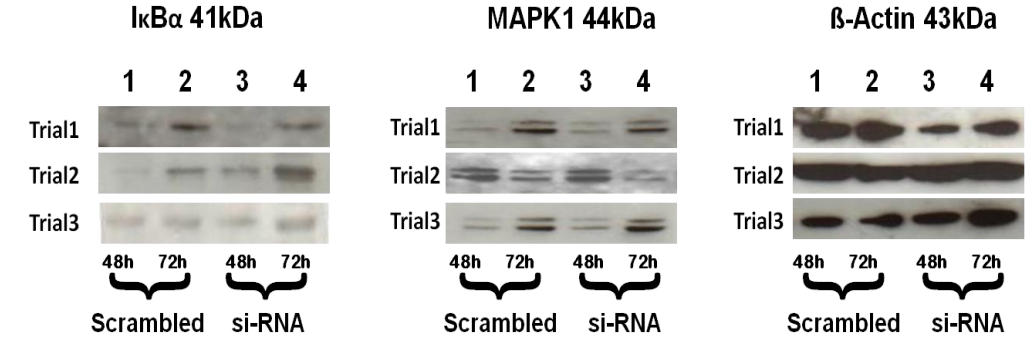


**Appendix 7:** Protein Expression of NFkB and MAPK1 in KRAS siRNA knockdown Experiment in DLD1 (KRAS) and Caco2(Wt).

## Caco2




## DLD1



# Acknowledgments

To begin with the name of Almighty who revealed through his beloved prophet Muhammad (PBUH) Read! In the Name of your Lord, Who has created (all that exists).
I gratefully acknowledge the continuous support, encouragement and insightful guidance of Prof. Dr. Giuliano Ramadori who gave me the chance to work in his laboratory and supported me financially. At the end of my studies I could not forget the assistance and fruitful suggestions from Prof. Dr. Tilman Sauerbruch that encouraged me and would help me in my career.

I also wish to express my gratitude to Prof. Tomas Pieler and Prof. Ahmed Mansouri for agreeing to be members of my PhD committee and for their time and insightful comments.

Dr. Ihtzaz Malik deserves special thanks for all the assistance, patience and inspiring comments he offered me during my thesis writing and for his invaluable advices and support ever since I enrolled in the PhD program. I would greatly appreciate the undemanding support of Dr. Silke Cameron who never let me down and whenever I needed her help she stood by me.

Moreover, additional thanks to Mrs. Elke Neumann who taught me a lot and I learned a lot from her during my stay in the lab. Many thanks to Ms.Daniela Gerke and Mrs.Corinna Dunaiski for all their technical and logistic support in their labs. Thanks are due to other members of my lab Ms. Jutta Otto, Mrs. Jutta Blumberg and Ms.Sonya Heyroth.

I also thank my lab fellows who stood by me through every thick and thin: Dr. Naila Naz, Dr. Martina Balschke Dr. Sadaf Sultan, Professor Hassan Dihazi, Shakeel Ahmed, Salamah Alwahsh, Min Xu, Dr.Federico Moriconi and Dr. Ahmed Amanzada at last but not the least Dr. Hazir rahman and Dr. Muhammad Qasim from Clinical chemistry department and Naveed Akhtar from Botany department. All of them helped me and gave useful suggestions during my PhD work. Regards and blessings to all those whose name have been unintentionally omitted who supported me in any respect during the completion of the project.

Finally, and most importantly, I would like to thank my little family including my wife (Mrs.Farzana Khan and my little angel Jawaria Khan) for their support, encouragement; quiet patience and unwavering love were undeniably the bedrock upon which the past years of my

life have been built. I thank my parents, for their faith in me and allowing me to be as ambitious as I wanted. It was under their watchful eye that I gained so much drive and an ability to tackle challenges head on.

**Sajjad Khan**
**Göttingen (March, 2013)**

# Curriculum Vitae


| | |
|---|---|
| Name: | Sajjad Khan (khan.goettingen@gmail.com) |
| Date and Place of birth: | 12th August 1974, Peshawar, Pakistan |
| Nationality: | Pakistani/ Canadian Citizenship |


## Educational Background

| | |
|---|---|
| 2009–till date | Ph.D (Biology) from Georg-August University, Goettingen, Germany. **(Supervised by Dr.Ihtzaz Malik and Prof. Dr. Giuliano Ramadori)** |
| 2003–2006 | M.Sc (Molecular Entomology & Phytopathology) Georg-August University,Goettingen, Germany. |
| 1996–1999 | M.Sc (Hons) (Agricultural Sciences) Khyber Pakhtunkhwa Agriculture University Peshawar Pakistan. |
| 1993–1996 | B.Sc (Hons) (Agricultural Sciences) Khyber Pakhtunkhwa Agriculture University Peshawar Pakistan. |

## Publications


1. Malik IA, Moriconi F, Sheikh N, Naz N, **Khan S**, Dudas J, Mansuroglu T, Hess CF, Rave-Frank M, Christiansen H, Ramadori G (2010) Single-dose gamma-irradiation induces up-regulation of chemokine gene expression and recruitment of granulocytes into the portal area but not into other regions of rat hepatic tissue. Am J Pathol 176:1801-1815

2. Malik IA, Naz N, Sheikh N, **Khan S**, Moriconi F, Blaschke M, Ramadori G (2011) Comparison of changes in gene expression of transferrin receptor-1 and other iron-regulatory proteins in rat liver and brain during acute-phase response. Cell Tissue Res 344:299-312

3. Malik IA, Triebel J, Posselt J, **Khan S**, Ramadori P, Raddatz D, Ramadori G (2012) Melanocortin receptors in rat liver cells: change of gene expression and intracellular localization during acute-phase response. Histochem Cell Biol 137:279-291

4. Moriconi F, Malik IA, Amanzada A, Blaschke M, Raddatz D, **Khan S**, Ramadori G (2012) The anti-TNF-alpha antibody infliximab indirectly regulates PECAM-1 gene expression in two models of in vitro blood cell activation. Lab Invest 92:166-177

5. Naz N, Malik IA, Sheikh N, Ahmad S, **Khan S**, Blaschke M, Schultze F, Ramadori G (2012) Ferroportin-1 is a 'nuclear'-negative acute-phase protein in rat liver: a comparison with other iron-transport proteins. Lab Invest 92:842-856

6. Wojcik M, Ramadori P, Blaschke M, Sultan S, **Khan S**, Malik IA, Naz N, Martius G, Ramadori G, Schultze FC (2012) Immunodetection of cyclooxygenase-2 (COX-2) is restricted to tissue macrophages in normal rat liver and to recruited mononuclear phagocytes in liver injury and cholangiocarcinoma. Histochem Cell Biol 137:217-233

7. Naz N, Moriconi F, Ahmad S, Amanzada A, **Khan S**, Mihm S, Ramadori G, Malik IA (2013) Ferritin L is The Sole Serum Ferritin Constituent and a Positive Hepatic Acute Phase Protein. Shock

8. Moriconi F, Ramadori P, Schultze FC, Blaschke M, Amanzada A, **Khan S**, Ramadori G (2013) Characterization of the erythropoietin/erythropoietin receptor axis in a rat model of liver damage and cholangiocarcinoma development. Histochem Cell Biol 139:473-485

9. Naz N, Moriconi F, Ahmad S, Amanzada A, **Khan S**, Mihm S, Ramadori G, Malik IA (2013) Ferritin L is The Sole Serum Ferritin Constituent and a Positive Hepatic Acute Phase Protein. Shock

10. Amanzada A, Malik IA, Blaschke M, **Khan S**, Rahman H, Ramadori G. Identification of CD68+ neutrophil granulocytes in in vitro model of acute inflammation and inflammatory bowel disease. International Journal of Clinical and Experimental Pathology (2013).

11. **Khan S**, Cameron S, Blaschke M, Naz N, Moriconi F, Amanzada A, Ramadori G, Malik IA (2013). Differential gene expression of chemokines in KRAS and BRAF mutated colorectal cell lines: Role of cytokines (Under process).

## Abstracts Published

**1.** Malik IA, Triebel J, Posselt J, Ramadori P, Raddatz D, **Khan S** and Ramadori G. "Melanocortin receptors in rat liver cells: change of gene expression and localization during acute-phase-response". German Association for the study of the liver 27th Annual Meeting 2011 (GASL 2011) Regensburg, Germany.

**2.** Malik IA, Moriconi F, Sheikh N, Naz N, **Khan S,** Dudas J, Mansuroglu T, Hess CF, Rave-Frank M, Christiansen H, Ramadori G (2010). Single-dose gamma irradiation induces up-regulation of chemokine gene expression and recruitment of granulocytes into the portal area but not into other regions of rat hepatic tissue.American Society of Clinical Oncology (ASCO, 2010) Chicago, USA.

**3.** **Khan S**, Malik IA, Blaschke M, Naz N, Cameron S, Moriconi F, Ramadori G (2011). Differential gene expression of chemokines in KRAS and BRAF mutated colorectal cell lines: Role of cytokines (Academic Drug Development in Oncology - Translating Basic Science Research into Innovative Treatments. November 2011 in Berlin).

**4.** **Khan S**, Malik IA, Blaschke M, Naz N, Cameron S, Moriconi F, Ramadori G (2011). Differential gene expression of chemokines in KRAS and BRAF mutated colorectal cell lines: Role of cytokines. (Falk Workshop "Inflammation and Cancer" January 2012 Hamburg) Accepted.

**Sajjad Khan**
**(Göttingen, April 2013**)