Aus dem Experimental and Clinical Research Center,

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin, and

Max-Delbrück-Center for Molecular Medicine

DISSERTATION

Identification of the key inflammatory cytokines on the regulation of the metastasis gene MACC1 in colorectal cancer

zur Erlangung des akademischen Grades

Doctor medicinae (Dr. med.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

von

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Datum der Promotion**: 1. März 2019**

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Contents

List of abbreviations

APC	Adenomatous polyposis coli
AP-1	Activating protein-1
AP2α	Adaptor protein 2α
AD	Alzheimer's disease
CRC	Colorectal cancer
CAC	Colitis associated cancer
CD	Crohn's disease
C/EBP	CCAAT-enhancer-binding proteins
CMV	Cytomegalovirus
DMSO	Dimethyl Sulfoxide
DD	Death domains
EH	Epsin 15 Homology motif
ERK	Extracellular signal-regulated kinase
EMT	Epithelial-mesenchymal transition
FAP	Familial adenomatous polyposis
FADD	Fas-associated protein with death domain
GAS	Gamma activated sequence
HGF	Hepatocyte growth factor
HNPCC	Hereditary nonpolyposis colorectal cancer
IBD	Inflammatory bowel diseases
IFN-γ	Interferon-gamma
IFNGR	Interferon-gamma receptor
IL-1, IL-4, IL-6	Interleukin-1, 4, 6
LT	Lymphotoxin
IKK	IκB kinase
JAK	Janus-activated kinase
JNK	c-Jun N-terminal kinases
K-Ras	V-Ki-ras2 Kirsten rat sarcoma viral oncogene

	homologue
LPS	Lipopolysaccharide
MACC1	Metastasis-associated in colon cancer 1
MAPK	Mitogen-activated protein kinase
MMP-9	Matrix metalloproteinase-9
NF-κB	Transcription factor of nuclear factor κB
PI3K	Phosphoinositide 3-kinase
REC	Rough endoplasmic reticulum
ROS	Reactive oxygen species
RIP	Receptor interacting protein
RA	Rheumatoid arthritis
SH3	Src-Homology 3
STAT1	Signal transducer and activator of transcription 1
Sp1	Specific protein 1
TNF-α	Tumor necrosis factor alpha
TACE	TNF-α converting enzyme
TRADD	TNFR-associated DD protein
TRAF2	TNFR-associated factor 2
UC	Ulcerative colitis
VEGFA	Vascular epithelial growth factor A

1a. Abstract

Background Colorectal cancer (CRC) is the third most common malignancy and one of the main causes of cancer-related mortality. One of the crucial oncogenes involved in molecular pathogenesis of colorectal cancer is *MACC1*, which has been identified as a metastasis-associated gene. Moreover, *MACC1* has been reported to be a prognostic biomarker for oncogenesis, metastasis formation and metastasis-free survival, inducing cell proliferation and migration *in vitro* and metastasis in CRC mouse models *in vivo*. Inflammation plays a pivotal role in tumorigenesis and tumor progression, and influences the metastatic process. Since certain cytokines, such as TNF-α and IFN-γ, are key risk factors in determining the contribution of the inflammatory process to CRC, knowledge of the connection between inflammation and the effects of TNF-α induction on *MACC1* remains unclear.

Methods The mRNA and protein expression of *MACC1*, with or without TNF-α stimulation, was evaluated using RT-qPCR and Western blotting, respectively. The pre-established transcription factor *c-Jun* mutation was used to investigate the effects of *MACC1* expression and cellular function following TNF-α treatment. In the cell model used, the TNF-α receptor responsible for *MACC1* induction was determined by neutralizing antibodies. To investigate signal transduction from the receptor to the effector molecules, knockdown experiments were performed for key molecules of the NF-κB signaling pathway.

Results The expression of *MACC1* and its transcription factor c-Jun were significantly increased in a dose-dependent manner following TNF-α treatment. Importantly, knockdown of *c-Jun* using siRNA caused a marked reduction in both *MACC1* and *c-Jun* expression following TNF-α stimulation. TNF-α promoted MACC1-induced cell migration and this phenotype was abolished following knock down of *MACC1*. Moreover, both *MACC1* and *c-Jun* expression were downregulated by blocking TNFR1, but not TNFR2. Furthermore, knock down of the NF-κB subunit, p65, reduced basal *MACC1* and *c-Jun* mRNA expression levels.

Conclusion The present study highlights the fact that TNF-α regulates the induction of

MACC1 via its transcription factor, *c-Jun*, to execute its function in CRC cells. The subunit of NF-κB, p65, which is involved in the activity of *c-Jun*, further governs *MACC1* induction. This finding may unravel a novel signaling pathway upstream of *MACC1* and provide a potential therapeutic target for the treatment of CRC patients.

1b. Zusammenfassung

Einleitung Das kolorektale Karzinom ist die dritthäufigste Krebserkrankung und eine der Hauptursachen für die krebsbedingte Sterblichkeit. *MACC1* wurde als metastasierungsassoziiertes Gen unter anderem im kolorektalen Karzinom identifiziert. Es ist eines der entscheidenden Onkogene für die molekulare Pathogenese dieser Entität. *MACC1* ist ein prognostischer Biomarker für die Krebsentwicklung, die Metastasenbildung und das metastasenfreie Überleben. *MACC1* induziert die Zellproliferation und –migration *in vitro* sowie die Metastasierung *in vivo*. In der Tumorentstehung und –progression spielen Entzündungsprozesse eine entscheidende Rolle. Daneben beeinflussen sie die Entstehung von Metastasen. Zytokine wie TNF-α und IFN-γ sind Schlüsselfaktoren von Entzündungsprozessen im Allgemeinen und während der Entwicklung des kolorektalen Karzinoms im Speziellen. Der Zusammenhang von entzündlichen Prozessen und der *MACC1* Expression ist nicht bekannt.

Methoden Die *MACC1* mRNA- und Proteinexpression vor und nach Zytokinstimulation wurde mit Hilfe von RT-qPCR (mRNA) bzw. Western-Blot (Protein) bestimmt. Mit Hilfe von Expressionsanalysen, Promotor-Reporter und knockdown Experimenten wurde der Einfluss von Transkriptionsfaktoren auf die *MACC1* Expression nach Zytokininduktion untersucht. Im verwendeten Zellmodell wurde durch neutralisierende Antikörper der für die MACC1-Induktion verantwortliche Rezeptor bestimmt. Um die Signalübertragung vom Rezeptor zu den Effektormolekülen zu untersuchen, wurden knockdown Experimente für Schlüsselmoleküle des NF-κB Signalweges durchgeführt.

Ergebnisse Die Expression von *MACC1* und des für die *MACC1* Expression wichtigen Transkriptionsfaktors *c-Jun* war nach TNF-α Behandlung dosisabhängig signifikant erhöht. IFN-γ hatte nur einen geringen Effekt auf die *MACC1* und *c-Jun* Expression. Der Knockdown von *c-Jun* mit Hilfe von siRNA führte zu einer deutlichen Verringerung sowohl der *MACC1* als auch der *c-Jun* Expression nach TNF-α Stimulation. Die Stimulation mit TNF-α erhöhte die MACC1-induzierte Zellmigration. Dieser Phänotyp wurde den *MACC1* knock down aufgehoben. Die Blockade des TNFR1 führte darüber

hinaus zum Fehlen der *MACC1* und *c-Jun* Induktion durch TNF-α. Dieser Effekt wurde für TNFR2 nicht beobachtet. Der knock down von der NF-κB Untereinheit p65 führte zu einer Reduktion des basalen *MACC1* und *c-Jun* mRNA Expressionsniveaus.

Schlussfolgerungen In der vorliegenden Arbeit konnte gezeigt werden, dass TNF-α *MACC1* über die Aktivierung von *c-Jun* induziert. Die NF-κB Untereinheit p65, die ebenfalls an der Aktivierung von *c-Jun* beteiligt ist, reguliert auch die *MACC1* Induktion durch TNF-α. Diese Ergebnisse beschreiben die Regulation von *MACC1* durch externe Stimuli und zeigen neue Ansätze für die Krebstherapie vor allem der hoch-Risiko Patienten mit erhöhter *MACC1* Expression.

2 Introduction

2.1 Colorectal cancer: Epidemiological features

Colorectal cancer (CRC) is a major cause of morbidity and mortality worldwide (Haggar and Boushey, 2009). It contributes to more than 8% of all cancer incidences that affect both men and women, making it the third most common cancer globally (Jemal et al., 2010). The highest incidence rates are seen in Western European Countries including, Australia, Canada, New Zealand, Germany and North America, whereas the lowest rates are found in Asia, Africa and South America (Torre et al., 2015). Countries with the highest incidence rates also suffer increased risk. Similarly, five-year survival rates in the countries with the lowest risk, such as Japan, Thailand and parts of Eastern Europe are rapidly declining (Haggar and Boushey, 2009; Marley and Nan, 2016). CRC is the most common cancer in Germany among the cancers that affect both genders, and is the second most common cause of cancer-related deaths (Majek et al., 2012). In Germany, although the age-standardized incidence numbers of new cases and deaths of CRC fell by 13.8% and 14.3% in men and women, respectively, from 2003-2012 as a result of colonoscopy screening, it remained the main cause of cancer-related mortality (Brenner et al., 2016). The increased incidence of CRC is attributed to a plethora of risk factors encompassing diet (high-fat, heavy consumption of red meat), “Western lifestyle” (obesity, heavy consumption of meat, high-calorie, high-fat and fiber-deficient diet and physical inactivity) (Sung et al., 2005; Stigliano et al., 2014), excessive alcohol and tobacco intake, environmental exposure and inflammatory bowel diseases (IBD) (ulcerative colitis (UC) and Crohn’s disease (CD)) (Pelucchi et al., 2011; Bishehsari et al., 2014). Regardless of the controllable risk factors, there exist non-modifiable risk factors including age and heredity (familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC)) (Wilmink, 1997). UC is responsible for 1% of all CRC cases due to chronic inflammation affecting the mucosa of the colon and rectum, and CD has also been shown to slightly increase the risk (Haggar and Boushey, 2009). The link between inflammation and CRC will be highlighted in the following sections.

2.2 CRC formation, progression and metastasis

Multiple factors are involved in the formation of CRC, including environmental influence and genetic susceptibility (Kinzler and Vogelstein, 1996). The majority of CRC tumorigenesis has been associated with environmental factors, whereas, the influence of genetic predisposition is a crucial portion of CRC carcinogenesis, which is mainly linked to the accumulation of somatic mutations in normal glandular epithelium (Rustgi, 2007; Grady and Markowitz, 2015). CRC pathogenesis is a complex long-term process; starting from the occurrence of aberrant crypts on normal epithelial cells, development of adenoma stages and carcinomas in situ, promotion to advanced neoplasms, transformation of tumors to invasive carcinomas, and finally metastases (De Robertis et al., 2011). There are several molecular mechanisms, such as mutations in the tumor suppressor gene, adenomatous polyposis coli (*APC*), which causes FAP by the formation of precursor lesions in colonic epithelium, as well as mutations in the oncogene, *K-Ras*, which arises during the adenomatous stage. In addition, epigenetic alterations including defects in DNA methylation or selective histone modifications are also involved in CRC formation and progression (Kondo and Issa, 2004; Hisamuddin and Yang, 2006; Yamagishi et al., 2016). Moreover, *APC* is a key player in the Wnt signaling pathway, regulating β-catenin stabilization, which is known to be a trigger of CRC carcinogenesis (Willert et al., 1999). The canonical adenoma-carcinoma sequence depicted by Fearon and Vogelstein revealed CRC tumorigenesis at the genetic level (Fig.1.1). Genes associated with cell proliferation, adhesion, apoptosis, tumor progression and DNA repair are involved in signal transduction that regulates the development of colorectal tumors (Fearon and Vogelstein, 1990).

Furthermore, of all CRC cases, approximately one-fifth presents chronic intestinal inflammation that precedes tumor development as a result of dysregulation of the immune system (Terzic et al., 2010). IBD is an inflammation-related CRC condition resulting from chronic infections, which initiate the appearance of cryptal dysplasia in normal mucosa and the development of neoplasms in the colon that profoundly increase CRC tumorigenesis (Bardhan and Liu, 2013). Finally, one of the main features of malignant neoplasms is metastasis, which is a series biological movements of tumor

cells from the primary neoplasm to distant sites, including surrounding tissue invasion, tumor cell survival, survival in the circulation, extravasation and growth at the disseminated site (Steeg, 2006). This property provides further evaluation criteria for CRC progression and carcinogenesis.

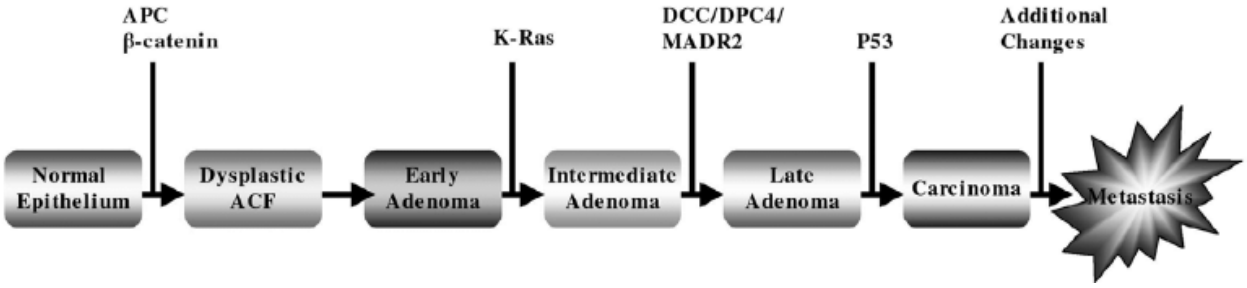

Fig.1.1. A genetic model of CRC tumorigenesis, progression and metastasis
Mutations in the tumor suppressor genes, *APC* and *K-Ras*, are involved in the early events of CRC tumorigenesis. Mutations in *APC* are responsible for early dysplasia, and lesions exhibiting *K-Ras* occur in small adenoma and produce a larger and more dysplastic tumor by clonal expansion. Mutations in the deleted in CRC (*DCC*) gene, which are caused by allelic loss in chromosome 18q, play a role in the progression from adenoma to carcinoma. Mutations in *p53* gene appears to be linked to late stage CRC (Fearon and Vogelstein, 1990). Adapted from Morin and Weeraratna (Morin and Weeraratna, 2003).

2.3 *MACC1*: a newly-identified metastatic gene in CRC

2.3.1 Discovery of *MACC1* and its features

A previously-undescribed gene, metastasis-associated in colon cancer 1 (*MACC1*), was identified by our group through a genome-wide differential expression analysis of colon mucosa, primary tumors and metastases of subjects with colon cancer (Stein et al., 2009). This novel gene was found to be highly expressed in malignant tissues as compared with normal tissues, and indicated little similarity to known genes (Stein et al., 2009). The *MACC1* gene is located on human chromosome 7 (7p21.1), and 7p21 is one of the most common chromosomal regions to gain mutations associated with the intestinal cancers (Morohara et al., 2006). Interestingly, among the nearest neighbors of *MACC1*, the genes, twist family bHLH transcription factor 1 (*TWIST1*), twist basic helix-loop-helix transcription factor 1 neighbor (*TWISTNB*), and integrin subunit beta 8

(*ITGB8*), have been reported to be involved in the tumorigenesis and metastasis of CRC (Yang et al., 2006). Moreover, the genes, MET proto-oncogene, receptor tyrosine kinase (*Met*) and hepatocyte growth factor (*HGF*), are also located on chromosome 7 at positions 7q31.2 and 7q21.1, respectively.

The unspliced primary transcript of MACC1 contains seven exons and six introns, with the longest known spliced mRNA having a length of 3,188 nucleotides (GeneBank: AK131400.1). The N-terminal region contains 130-150 amino acids, and several conserved motifs that contribute to putative protein interactions consist of a clathrin box, two epsin 15 homology motif (EH)-interacting sites and an adaptor protein 2α (AP2 α) binding site (Fig.1.2) (Stein et al., 2010). A ZU5 domain that follows the N-terminal region is concerned with mediating protein-protein interactions (Ipsaro et al., 2009). The C-terminus possesses a class I src-homology 3 (SH3) binding motif, for proline-rich consensus sequences, and a variant SH3 domain, which are essential for the translocation of *MACC1* into the nucleus for *Met* transcriptional activity (Stein et al., 2010). In addition, the C-terminal domain of *MACC1* contains two death domains (DD), which may be related to cell apoptosis, innate immunity, inflammation or migration (Reed et al., 2004).

On the basis of the structural features and genomic sequence, various biological functions of the *MACC1* gene have been discovered; however, the underlying molecular and cellular mechanisms need to be explored.

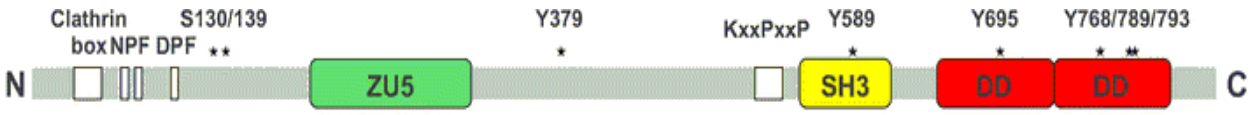

Fig.1.2. Structural domains of the *MACC1* protein

Linear sequence motifs (white boxes) and predicted structural domains of *MACC1* are shown: putative sites for posttranslational modifications (*asterisks*); ZU5 (green); SH3 (yellow), variant Src homology 3 domain (PF07653); DD (red), death domain (PF00531); NPF, Epsin homology 15 interacting motif; DPF, adaptor protein 2α interacting motif; KxxPxxP, class I SH3 interaction motif (Stein et al., 2010).

2.3.2 Functions of *MACC1* in CRC

A crucial role of *MACC1* has been illustrated in CRC, being a prognostic indicator of metastasis formation and metastasis-free survival (Stein et al., 2009). Both *MACC1* mRNA and protein are highly expressed in CRC tissues with metachronous metastases (those that occur after a period of three months postoperatively) compared with tumors without metastases. This suggests that *MACC1* represents an early prognostic marker for CRC metastasis, independent of age, sex, tumor infiltration, nodal status, and lymph vessel invasion (Arlt and Stein, 2009; Stein et al., 2012). The five-year survival falls to 15% for patients with high *MACC1* mRNA expression in the primary tumor as compared with 80% for patients with low *MACC1* expression, revealing that *MACC1* is also a prognostic marker for metastasis-free survival in CRC. Interestingly, high expression of the receptor tyrosine kinase, *Met*, in metachronous metastases are consistent with high *MACC1* expression, and are also linked to a shorter metastasis-free survival; however, this does not strengthen the predictive roles that *MACC1* plays alone (Stein et al., 2009; Stein, 2013). The metastatic phenotype caused by elevated *MACC1* expression can be found in more than 20 types of solid tumor, including gastric cancer (Wang et al., 2013), pancreatic cancer (Wang et al., 2012), hepatocellular carcinoma (Qiu et al., 2011), lung cancer (Guo et al., 2017), ovarian carcinoma (Zhang et al., 2011), osteosarcoma (Zhang et al., 2014), and nasopharyngeal carcinoma (Meng et al., 2013).

MACC1 executes its functions by regulating the tyrosine kinase, *Met*, the receptor for HGF (Nguyen et al., 2009; Stein et al., 2009). *Met* is a transcriptional target of the *MACC1* gene, triggered by the activation of HGF-Met signaling pathway following binding to its ligand HGF*,* which leads to cell motility, invasion and metastasis (Takeuchi et al., 2003). Cellular and molecular evidence has been demonstrated that *MACC1* promotes cell proliferation, migration, invasion, colony formation, wound healing and HGF-induced scattering of CRC cells *in vitro* (Stein et al., 2009). Importantly, *MACC1* confers characteristic features by acting as a master regulator of the HGF-Met signaling pathway to form an HGF/Met/MACC1 feedback loop for CRC tumorigenesis and metastasis (Fig.1.3) (Arlt and Stein, 2009). Moreover, the implications of aberrant activation of HGF-Met signaling, resulting in tumor progression and distant dissemination,

has also been studied in other cancers (Birchmeier et al., 2003; Wang et al., 2013). This pathway plays a vital role in the carcinogenic pathway, since HGF induces cell scattering and tumor invasiveness through tumor-stromal cell interactions (Choi et al., 2009). *Met* is an essential oncogene for the metastatic potential in CRC, transmitting intracellular signals such as the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)-Akt pathways, which enhance migration, invasion and survival, and suppress apoptosis (Kammula et al., 2007; Sattler and Salgia, 2007). In addition, the capabilities of MACC1-induced tumor growth and metastasis in xenograft mouse models had been validated by gain-of-function and loss-of-function experiments (Stein et al., 2009).

Taken together, *MACC1* is a promising biomarker for CRC metastasis involving the transformation from a benign to a malignant phenotype (Shirahata et al., 2010), which serves as a reliable prognostic prediction for determining the risk of CRC recurrence, indicating *MACC1* as a promising therapeutic target for the treatment of CRC (Boardman, 2009).

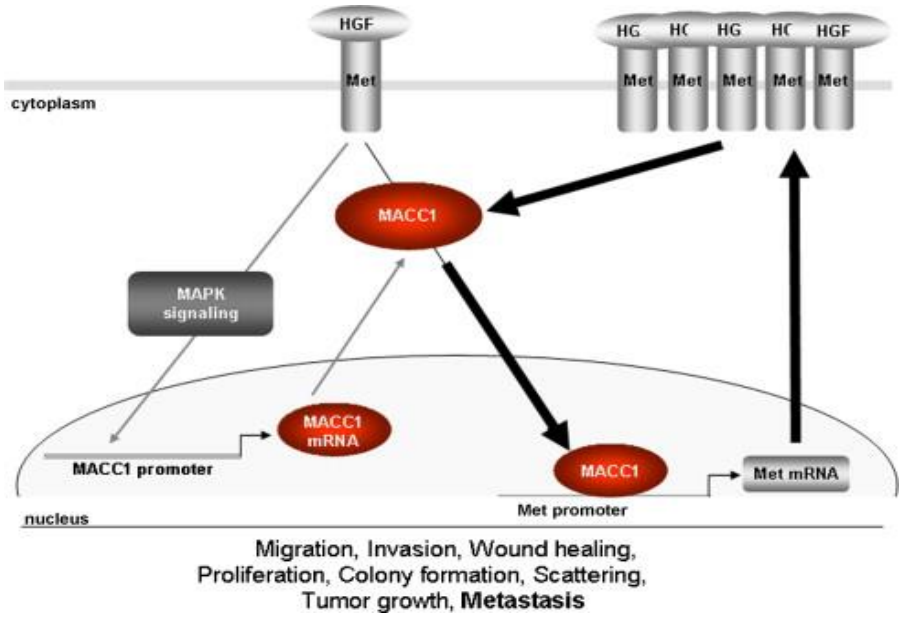

Fig.1.3. Schematic model of the MACC1/HGF/Met signaling feedback loop
HGF treatment induces translocation of *MACC1* to the nucleus, where it binds to the *Met* promoter. This strong transcriptional activation regulated by *MACC1* drives a positive feedback loop that induces the metastatic process. Adapted from Arlt and Stein (Arlt and Stein, 2009).

2.4 Inflammation and CRC

2.4.1 Inflammation and cytokines function

Inflammation is a defense mechanism carried out by the immune system, which is triggered by harmful stimuli such as pathogens, injuries and certain chemicals or radiation. The inflammation response results in redness, swelling, heat, pain and loss of function as the signs of clinical manifestation (Ferrero-Miliani et al., 2007). This protective response is an essential part of the healing process which is concerned with blood vessels and immune cells releasing different molecular mediators, including histamine and many cytokines, causing vasodilation, chemotaxis and increased vascular permeability (Kuriakose and Kanneganti, 2017). Inflammation can be classified as acute or chronic. Acute inflammation represents the rapid immune response to harmful irritations, whereas chronic inflammation refers to the long-term destruction and healing processes of tissue. If the agents can not be eliminated or certain factors interfere with the healing process, chronic inflammatory disease and even cancers eventually result (Feghali and Wright, 1997).

Cytokines are a family of small secreted proteins that affect cell signaling, induce cell interactions and communication during immune responses and stimulate cell chemotaxis to the site of inflammation, infection or trauma. It has been discovered that the cytokine family consists of interleukins, interferons (IFNs), lymphokines, chemokines and tumor necrosis factors. Cytokines are mainly produced by immune cells such as macrophages, T or B lymphocytes and mast cells. These immunomodulating molecules act through specific cell-surface receptors that participate in autocrine, paracrine and endocrine signaling, and modulate or alter the innate or adaptive immune system (Dinarello, 2007; Zhang et al., 2009). One of the primary cytokine-mediated functions is the pro-inflammatory effect dominating the fields of inflammation and cancer. The pro-inflammatory cytokines are predominantly produced by activated macrophages (Dinarello, 2007), such as IL-1, IL-6 and TNF-α as the key modulators of acute or chronic inflammation. These give rise to some inflammatory diseases including IBD, rheumatoid arthritis (RA) and certain neurological disorders (Turner et al., 2014). Here, two critical instances of pro-inflammatory cytokines will be illustrated further.

2.4.2 Classic pro-inflammatory cytokines signaling pathways

A plethora of investigations have established that pro-inflammatory cytokines can be divided into two groups; those associated with acute inflammation, such as IL-1, TNF-α and IL-6, and those involved in chronic inflammation, such as IL-6 (mediating the humoral response), IL-1, TNF-α and interferons (mediating the cellular response) (Feghali and Wright, 1997). Evidently, certain cytokines, such as IL-1 and TNF-α, are dedicated to both acute and chronic inflammation using several intracellular signaling pathways to mediate the inflammatory process and even tumor progression. Most importantly, a substantial body of evidence suggests that inflammation results from pro-inflammatory cytokines produced by activated innate immune cells, which participate in inflammation and further contribute to the promotion and/or exacerbation of tumor growth and progression (Lin and Karin, 2007). The molecular signaling pathways of the main inflammation-induced cytokines linked to tumor initiation and progression will be discussed.

IFNs are a subset of cytokines that were originally characterized by their anti-viral activity. IFNs can be classified into two categories; type I interferons, including IFN-α and IFN-β, and type Ⅱ interferon, termed immune interferon or IFN-γ (De Andrea et al., 2002). Type I interferons bind to common receptors, while IFN-γ binds to distinct receptors consisting of IFN-γR1 and IFN-γR2 subunits, inducing activation of the signal transduction pathway that activates a wide range of genes that are related not only via anti-viral properties but also via immunomodulatory specialties (Platanias, 2005). IFN-γ is known to be an important component of the pathogenesis of chronic inflammation (Heremans and Billiau, 1989), with a solid series of studies suggesting that it has pro-tumorigenic effects under certain conditions (Zaidi and Merlino, 2011). Conventionally, IFN-γ is mainly secreted by activated macrophages and has been identified to induce inflammation, apoptosis, and anti-viral and anti-tumor mechanisms (Mojic et al., 2017), regulating the differentiation and function of a variety of immune cells, intimately associated with all aspects of Th1-mediated immune responses (Agnello et al., 2003). CD belongs to the group of IBDs reported to be Th1-mediated as a result of increased production of IFN-γ (Strober and Fuss, 2011). The canonical signal transduction relevant to IFN-γ is the Janus

kinase/signal transducers and activators of transcription (JAK/STAT) pathway that affects gene regulation (Fig.1.4) (Zaidi and Merlino, 2011).

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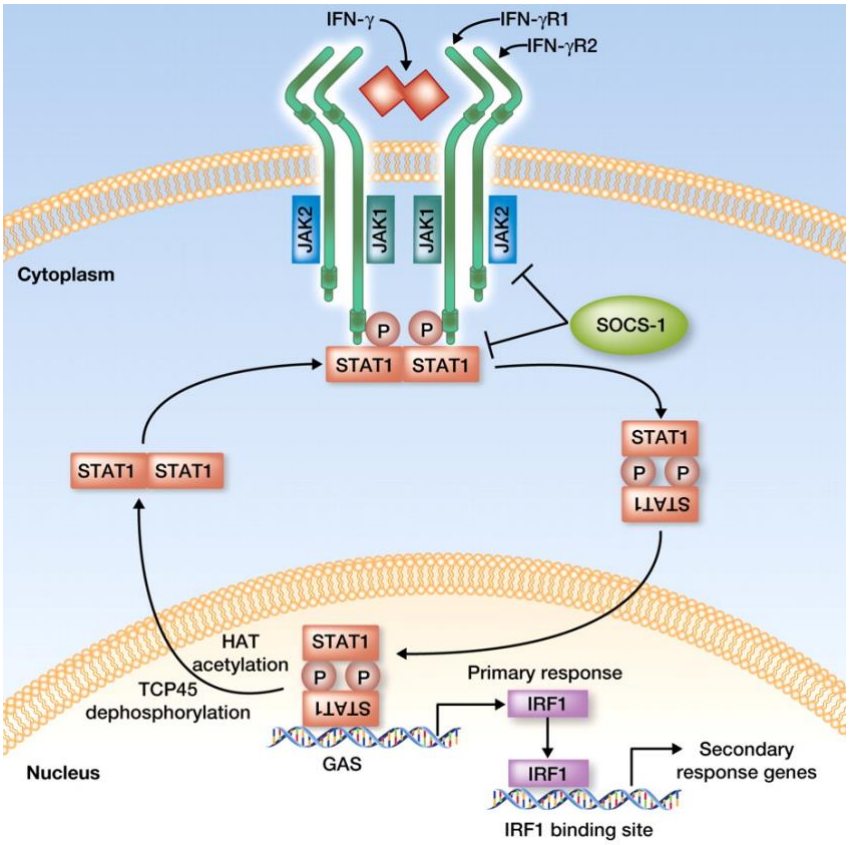


Fig.1.4. The classic JAK-STAT signaling pathway regulated by IFN-γ
The binding of IFN-γ to the extracellular domain of the receptor subunit IFN-γR1 causes recruitment of *JAK1*, followed by recruitment of *JAK2* by the IFN-γR2 subunit, which is responsible for intracellular signal transmission. The activated JAK kinase regulates the phosphorylation of *STAT1*, promoting its translocation into the nucleus, and leading to the downstream cascades of target genes. Adapted from Zaidi and Merlino (Zaidi and Merlino, 2011).

IFN-γ executes its function through binding to IFNR, activating the non-receptor tyrosine kinases *JAK1/2*, and leading to the phosphorylation of *STAT* proteins, primarily *STAT1*. This phosphorylation results in the translocation of *STAT1* to the nucleus, where it binds gamma activated sequence (GAS) elements to initiate transcription (Darnell, 1997). In addition, it has also been suggested that IFN-γ may regulate the activation of the PI3K-signalling pathway by serine phosphorylation of *STAT1* (Deb et al., 2003). Inquiringly, the function of IFN-γ is a 'double-edged sword' that comprises

anti-tumorigenic and pro-tumorigenic features that rely on the molecular, cellular and microenvironmental conditions.

2.4.3 Inflammation-associated CRC

As mentioned previously, inflammation is a protective immune action against multitudinous irritations; however, when inflammation persists, it leads to a chronic, dysregulated and subversive immune response, promoting the development of malignant neoplasms by changing the local tumor environment (Shalapour and Karin, 2015). By way of explanation, the tumor microenvironment, which is primarily orchestrated by inflammatory conditions, is an indispensable part of the malignant process, contributing to cell proliferation, survival, angiogenesis and metastasis (Coussens and Werb, 2002). Recent reports have shown that preexisting inflammatory conditions induce tumor occurrence in certain types of cancer, such as colon tumors. For other types of cancer, such as breast tumors, the oncogenic alterations trigger an inflammatory microenvironment and further foster tumor progression (Mantovani et al., 2008; Grivennikov, 2013). A connection between inflammation and cancer was first hypothesized and proposed by Virchow in the 19th century, suggesting that tumors arise at the site of chronic inflammation (Kraus and Arber, 2009). Links between inflammation and cancer have become widely accepted due to a plethora of convincing studies; with outstanding studies unraveling the molecular mechanisms of this linkage in CRC.

Colitis-associated cancer (CAC) is a type of CRC that has been identified as a classic model of inflammation-driven cancer (Feagins et al., 2009). Colitis results from inflammatory conditions and manifests itself as clinically-detectable IBD. With a longer duration of colitis, the risk of colon cancer increases greatly (Itzkowitz and Yio, 2004). IBD is a chronic relapsing disease of the colon and small intestine that arises as a result of environmental and genetic factors, generating inflammation and a disordered immune response (Baumgart and Carding, 2007). IBD primarily consists of Crohn's disease (CD) and ulcerative colitis (UC); CD affects almost the entire digestive tract, and inflammation occurs in all layers of the bowel associated with Th17 cytokines (Rhodes and Campbell,

2002; Yen et al., 2006), whereas, UC mainly affects the colon and rectum, and inflammation occurs in the mucosal region associated with Th2 cytokines (Fuss et al, 1996). Several reports have indicated that chronic inflammation is a crucial predisposed factor for increased risk of CRC pathogenesis in IBD (Francescone et al., 2015). The genetic mechanism and pathways underlying the relationship between inflammation and CRC are illustrated generally herein.

Cytokines are commonly induced in response to inflammation and are key mediators of inflammation linked to cancer (Fig.1.6). Overexpression of cytokines such as TNF-α, IFN-γ, IL-1 and IL-6 cause elevated reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) inside target epithelial cells, which induce oxidative stress to DNA, causing damage and mutations closely associated with CRC pathogenesis (Saraggi et al., 2017). Several transcription factors including nuclear factor kappa light-chain-enhancer of activated B cells (*NF-κB*) and *STAT3* are triggered by pro-inflammatory cytokines and become the major downstream signals activated in CRC. TNF-α and IL-1 have the ability to induce NF-κB signaling, which lead to cell proliferation, cell survival, angiogenesis, tumor growth and metastasis, serving as a bridge connecting inflammation and CRC (Greten et al, 2004). IL-6, as a potent activator of *STAT3,* extensively promotes cancer cell growth and CRC progression (Becker et al, 2005). In addition, mutations cause activation of the Wnt/β-catenin signaling pathway, which happens frequently in sporadic CRC and can be enhanced by several cytokines and inflammatory signaling through the NF-κB and AKT pathways (Kaler et al., 2009). Despite of the fact that many research studies have established a connection between inflammation and CRC, the mechanisms underlying inflammation in CRC carcinogenesis remain to be explored.

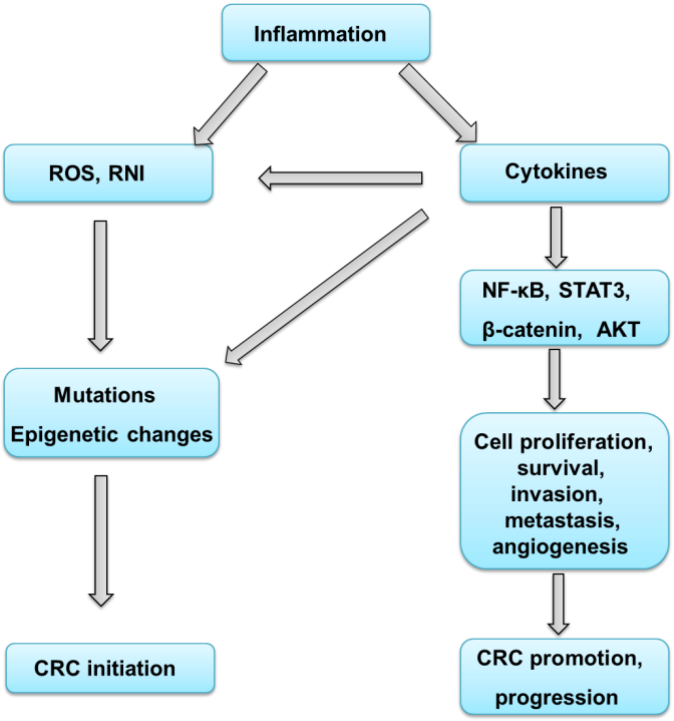


Fig.1.5. The role of inflammation in CRC
Cytokine-induced inflammation breaks down the immune response through the upregulation of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), leading to mutations and epigenetic alterations, and initiating tumor growth. Several key oncogenic pathways, such as *NF-κB*, *STAT3*, *β-catenin* and *AKT*, are activated by inflammation and are cooperatively involved in the pathophysiological processes of CRC, which induce cell proliferation, survival, invasion, angiogenesis and metastasis, promoting CRC development.

2.5 Tumor necrosis factor alpha

2.5.1 Origin and properties of TNF-α

TNF was first reported in 1975 as an endotoxin-induced serum factor, likely released by macrophages that resulted in tumor necrosis (Carswell et al., 1975). Subsequently, It was extensively described that excessive production of TNF causes malaria and endotoxin poisoning (Clark, 1982). In 1984, the cDNA encoding TNF was cloned and found to be distinct from lymphotoxin (LT, later named TNF-β), and as a result of sequence and functional homology, it was renamed TNF-α (Pennica, et al., 1984). The human *TNF-α* gene maps to chromosome 6p21.3, spans approximately 3 kb in length and contains 4 exons (Nedwin, et al., 1985). TNF-α is the typical member of type II

transmembrane proteins; following synthesis of a transmembrane precursor protein (mTNF-α) with a molecular weight of 26 kDa (Tracey et al., 2008), it is transported by the rough endoplasmic reticulum (REC) and Golgi complex to the cell surface (Stow et al., 2009). The TNF-α precursor is cleaved by the metalloprotease, TNF-α converting enzyme (TACE, also called ADAM17), releasing the 17 kDa soluble TNF-α (sTNF-α) containing the protein ligand (Black et al., 1997), which binds to the TNF receptors and activates a series of biological signaling.

TNF-α is not only one of the pleiotropic cytokines associated with the immune response, but is also a cell signaling molecule involved in several distinct signaling pathways through which it executes its biological functions. TNF-α is able to induce fever, cachexia, inflammation and apoptosis, being implicated in a diverse array of human diseases including Alzheimer's disease (AD), RA, IBD and cancer (Turner et al., 2014). Moreover, the pivotal effects of TNF-α are characterized as pro-inflammatory and are involved in the carcinogenesis of different tumors associated with inflammation.

2.5.2 The general TNF-α signaling pathway

The molecular effects of TNF-α begin with binding to two membrane receptors: TNFR1 (also known as p55/60, CD120a or TNFRSF1A) and TNFR2 (also known as p75/80, CD120b or TNFRSF1B) (Mukai et al., 2009). TNFR1 is extensively expressed on the plasma membrane of a variety of cell types, whereas, TNFR2 exists predominantly in immune and endothelial cells (Bradley, 2008). Unlike TNFR2 that only binds to mTNF-α, TNFR1 is activated by both mTNF-α and sTNF-α (Fig.1.7). The binding of TNF-α to TNFR1 initiates signaling by assembling with an intracellular death domain (DD) and releasing the DD of silencer of DD protein (SODD) (Chen and Goeddel, 2002). The dissociation of SODD enables the TNFR-associated DD protein (TRADD) to bind to DD. The recruitment of the adaptor molecule, TRADD, is essential for signaling transduction. Following TRADD binding, two additional molecules, TNFR-associated factor 2 (TRAF2) and receptor interacting protein (RIP), are recruited to form a complex that triggers the downstream signals cascades leading to pro-inflammatory gene expression (Hsu et al.,

1996). Further, TNFR2 lacks the DD, thus the biological events of TNF-α appear to be mediated primarily through TNFR1.

TNF-α activates the NF-κB signaling pathway by recruiting the multiprotein IκB kinase (IKK) complex through TRAF2. Subsequently, the IKK complex is activated by RIP, which mediates phosphorylation-dependent ubiquitination and degradation of the inhibitory protein of IKK, IκBα, leading to the translocation of the transcription factor of NF-κB to the nucleus, inducing cell survival, inflammation and oncogenesis (Israel, 2000). In addition, TNF-α regulates the mitogen-activated protein kinase (MAPK) signaling pathway through binding to TNFR1. Among the three major MAPK cascades, TNF-α induces strongest activation of the c-Jun N-terminal kinases (JNK) group related to stress stimuli, and evokes a moderate response of the p38-MAPK and extracellular signal-regulated kinase (ERK). An activated JNK translocates to the nucleus and controls the induction of transcription factors such as *c-Jun*, which is involved in apoptosis, cell differentiation and proliferation (Urschel and Cicha, 2015). TNF-α can also induce death signaling by the binding of TRADD to Fas-associated protein with death domain (FADD), following the recruitment of caspase-8, which contributes to cell apoptosis (Gaur and Aggarwal, 2003). Moreover, activation of TNFR2 by TNF-α initiates the PI3K/AKT pathway, which is associated with angiogenesis in endothelial cells (Zhang et al., 2003). Interestingly, TNF-α mediation of signaling through binding of TNFR1 is not always independent, and the often-conflicting effects indicate the existence of extensive cross-talk, which needs to be further explored.

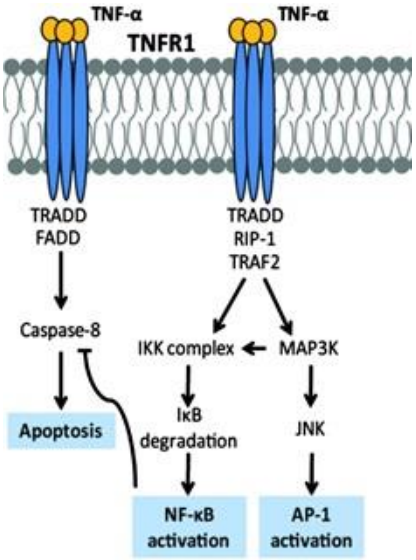


Fig.1.6. TNF-α -mediated signaling pathway

TNF-α mediates its functions by binding to two different receptors, TNFR1 or TNFR2. Following binding, TNFR1 recruits adaptor molecules to form a complex of TRADD/TRAF2/RIP. Subsequently, this complex activates the major signaling pathways, NF-κB, MAPK and JNK. Adapted from Moelants et al. (Moelants et al., 2013).

2.5.3 The role of TNF-α in CRC

Abundant evidence indicates that TNF-α is an important cytokine mediating several immune responses, in particular its pro-inflammatory effects associated with inflammation and diseases caused by inflammatory disorders. TNF-α acts as a key regulator in establishing a complicated connection between inflammation and cancer. The function of TNF-α as a master switch involved in CRC tumorigenesis can be generalized in two manners: an extrinsic pathway caused by inflammatory conditions such as IBD that increase the risk of CRC (Mantovani et al., 2008), for instance blocking TNF-α in mice reduces colitis-associated colorectal carcinogenesis (Popivanova et al., 2008); and an intrinsic pathway driven by genetic alterations. TNF-α is produced by tumor cells and mediates different signaling transductions that promote cell proliferation, survival, and angiogenesis in CRC. Moreover, TNF-α induces the NF-κB-Snail pathway that contributes to migration and invasion in CRC (Wu and Zhou, 2008).

2.6 Aim of the dissertation

1. Regulation of *MACC1* by pro-inflammatory cytokines
2. Impact of pro-inflammatory cytokines on *MACC1* function
3. Deciphering pro-inflammatory signaling for *MACC1* regulation

3 Materials and Methods

3.1 Materials

3.1.1 Devices and Equipment

Table 1. Devices and Equipment

Product	Company
Cell culture incubator	Heraeus instruments (Hanau, Germany)
Countess™ automated cell counter	Invitrogen (Karlsruhe, Germany)
Fluorescence microscope Axio Vert.A1	Zeiss (Jena, Germany)
Cooling Centrifuge 5804 R	Eppendorf (Hamburg, Germany)
NanoDrop 1000	Thermo Fisher Scientific (Wilmington, USA)
Professional TRIO Thermocycler	Biometra (Jena, Germany)
Light Cycler® 480II	Roche Diagnostic (Mannheim, Germany)
Infinite F200 PRO	Tecan (Berlin, Germany)
The Belly Dancer	Stovall Life Science (Greensboro, USA)
XCell Sure Lock™	Invitrogen (Karlsruhe, Germany)
Trans-blot® Turbo™ Transfer System	BioRad Laboratories Inc., Singapore
FUJI MEDICAL X-RAY Film	Minato (Tokio, Japan)
ChemiDoc™ MP Imaging System	Bio-Rad (München, Germany)
Spectrafluor plus	Tecan (Berlin, Germany)
Standard Power Pack P25	Biometra (Jena, Germany)
Tabletop centrifuge	Eppendorf (Hamburg, Germany)
Votex Genie 2™	Zurich, Switzerland
Centrifuge 5810R	Eppendorf (Hamburg, Germany)
Sorval LYNX 6000 centrifuge	Thermo Scientific (Wilmington, USA)
Incubator Shaker Series Excella E24	New Brunswick Scientific (New Jersey, USA)

3.1.2 Reagents and chemicals

Table 2. Reagents and chemicals

Reagents/Chemicals	Company
Fetal calf Serum	PAA Laboratories (Cölbe, Germany)
DMEM medium	PAA Laboratories (Cölbe, Germany)
Trypsin-EDTA	PAA Laboratories (Cölbe, Germany)
Trypan-Blue	Invitrogen (Karlsruhe, Germany)
Recombinant Human TNF-α	Peprotech (Rocky Hill, USA)
DMSO	Carl Roth (Karlsruhe, Germany)
MycoAlert™ Mycoplasma Detection Kit	Lonza (Basel, Switzerland)
Recombinant Human IFN-γ	R&D System (Wiesbaden-Nordenstadt, Germany)
GeneMatrix Universal RNA purification kit	EURx® Molecular Biology Products (Berlin, Germany)
$MgCl_2$ (25 mM)	Applied Biosystems (Foster City, USA)
10 x PCR-buffer II	Invitrogen (Karlsruhe, Germany)
dNTPs	Applied Biosystems (Foster City, USA)
RNase Inhibitor	Applied Biosystems (Foster City, USA)
Random Hexamers	Applied Biosystems (Foster City, USA)
MuLV Reverse Transcriptase	Applied Biosystems (Foster City, USA)
Ethanol	Carl Roth (Karlsruhe, Germany)
GoTaq® qPCR Master Mix	Promega (Madison, USA)
PBS	PAA Laboratories (Cölbe, Germany)
BSA Standard	Pierce (Rockford, USA)

Pierce™ BCA Protein Assay Kit	Thermo Scientific (Wilmington, USA)
NuPAGE® LDS Sample Buffer	Invitrogen (Karlsruhe, Germany)
DTT	Sigma-Aldrich (Taufkirchen, Germany)
NuPAGE® 10% Bis-Tris Gel	Invitrogen (Karlsruhe, Germany)
Spectra™ Multicolor Broad Range Protein Ladder	Fermentas (Sankt Leon-Rot, Germany)
Ponceau S solution	Sigma-Aldrich (Taufkirchen, Germany)
Tween® 20	Carl Roth (Karlsruhe, Germany)
WesternBright™ ECL	Invitrogen (Karlsruhe, Germany)
PureYield™ Plasmid Midiprep System	Promega (Madison, USA)
Isopropanol	Carl Roth (Karlsruhe, Germany)
Acetic acid	DIFCO (Heidelberg, Germany)
Opti-MEM medium	PAA Laboratories (Cölbe, Germany)
TransIT 2020	Invitrogen (Karlsruhe, Germany)
Dual-Luciferase® Reporter Assay System	Promega Corporations (Madison, USA)
Silencer® Select Pre-designed siRNA	Ambion® (Carlsbad, USA)
Lipofectamine® RNAiMAX Reagent	Invitrogen (Karlsruhe, Germany)

3.1.3 Plasmids / Vectors

Table 3. Plasmids / Vectors

Plasmids / Vectors	Character
pGL4.17 empty vector	Empty (promoter less) vector
pGL4.74 Renilla luciferase vector	Internal control
pGL4.17-MACC1p-Luc	MACC1 promoter driving firefly luciferase expression

3.1.4 Buffers

Table 4. Buffers

Buffer Name	Ingredients
1 x Phosphate Buffered Saline (PBS)	155 mM NaCl, 0.2 g, 1 mM KH_2PO_4, 3 mM Na_2HPO_4
1 x RIPA Buffer	50 mM TRIS pH 7.5 150 mM NaCl 1% Nonidet P-40 0.5% sodium deoxycholate, Proteaseinhibitor
20 x MOPS Buffer	50 mM MOPS, 50 mM TRIS, 0.1% SDS, 1mM EDTA
1 x Transfer Buffer	25 mM Tris-HCl pH 7.5, 200 mM Glycine, 0.1% SDS, 20% Methanol
1 x TBST	50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1%, Tween®20
Blocking Buffer	1 x TBST + 5% skimmed milk
TAE Buffer	40 mM Tris-NaOH 1 mM EDTA-Na_2-Salz 40 mM Acetic acid pH 8
LB Medium	10 g/L Trypton, 5 g/L NaCl, 5 g/L Yeast extract
Mild Stripping Buffer	15 g/L Glycine, 1 g/L SDS, 10 ml Tween 20, pH2.2

3.1.5 Antibodies

Table 5. Antibodies

Primary antibody	Dilution	Manufacturer
Anti-human MACC1 (Rabbit, polyclonal IgG)	1:1500 in 5% BSA in TBST	Sigma Aldrich (München,Germany)
Anti-human c-Jun (Rabbit, polyclonal IgG)	1:1000 in 5% BSA in TBST	Cell Signaling technology (Danvers, USA)
Anti-β-actin (Mouse, monoclonal IgG)	1:20000 in 5% BSA in TBST	Pierce Thermo Scientific (Wilmington, USA)
Anti-Human TNFRI (Mouse, monoclonal IgG)	1:500 in 5% BSA in TBST	R&D Systems, Inc. (Minneapolis, USA)
Anti-Human TNFRII (Mouse, monoclonal IgG)	1:500 in 5% BSA in TBST	R&D Systems, Inc. (Minneapolis, USA)
Secondary antibody	**Dilution**	**Manufacturer**
Anti-Rabbit-HRP	1:10000 in TBST	Promega Corporations (Madison, USA)
Anti-mouse-IgG-HRP	1:30000 in TBST	Pierce Thermo Scientific (Wilmington, USA)

3.1.6 Software

Table 6. Software

Software	Manufacturer
Magellan 7	Tecan (Berlin, Germany)
GraphPad Prism v5.0	GraphPad Software (La Jolla, USA)
Microsoft Office 2010	Microsoft Corporation (Washington, USA)

3.2 Methods

3.2.1 Cell culture

The human colorectal cancer cell line, HCT116, was obtained from the American Type Culture Collection (ATCC). Cells were cultured at 37 °C, 100% atmospheric humidity and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were passed every 3-4 days using trypsin-EDTA and fresh medium. Cells were regularly verified as mycoplasma-negative using a detection kit.

3.2.2 Cell counting

For determination of the cell number, cells were removed from the bottom of the flask or dish using trypsin-EDTA and incubated at 37 °C. The reaction was stopped with medium containing FCS. From this cell suspension 20 µl were added to 20 µl trypan blue. Trypan blue is an anionic diazo dye that can penetrate the membrane of dead cells, however, the living cells do not absorb the dye. Ten µl of the resulting mixture was added to a counting slide. Cell number was determined using an automated cell counter.

3.2.3 TNF-α treatment

Recombinant human TNF-α was obtained from R&D System and stored at -20 °C following reconstitution at 0.1 mg/ml in sterile, deionized water. To maintain the stability of the cytokine, small aliquots were created to avoid repeated freeze-thaw cycles. Briefly, 1×10^6 cells/well were seeded onto 6-well plates and allowed to adhere for 24 h. Subsequently, cells were treated with increasing concentrations (1, 10, 100 ng/ml) of TNF-α, and harvested after 24 and 48 h. Each experiment was performed in triplicate.

3.2.4 siRNA transfections

Predesigned siRNA targeting *c-Jun*, p65 and IKKγ, as well as scrambled siRNA serving as a negative control, were obtained from Ambion. 3×10^5 HCT116 cells were seeded onto 6-well plates and cultured for 24 h. Three µl siRNA (10 µM) in 150 µl Opti-MEM medium, and 7 µl Lipofectamine RNAiMAX transfection reagent in 150 µl Opti-MEM

medium were incubated separately for 5 min. Subsequently, the 150 µl Lipofectamine RNAiMAX reagent was added dropwise to 150 µl siRNA, and incubated for 20 min at room temperature. 250 µl transfection complex was added dropwise to the cells on 6-well plates in 2 ml media and incubated for 24-48 h.

3.2.5 Cell migration assay

For the evaluation of cell migration, the Boyden chamber assay was used according to the manufacturer's protocol. Inserts with a pore size of 8 µm were used in 24-well plates. Cells were serum-starved overnight, and the following day, 3×10^5 cells in 300 µl medium containing 1% FCS, with or without TNF-α (1, 10, 100 ng/ml), were seeded onto each transwell chamber. The transwell chambers were presoaked in 200 µl FCS-free medium for 30 min prior to cell seeding. 600 µl medium containing 10% FCS, with or without TNF-α (1, 10, 100 ng/ml), was added to each lower chamber. Following 24 h culture, the medium in the upper chamber was removed. The cells that had migrated to the lower chamber were incubated for 5 min with 500 µl trypsin/EDTA. The cell suspension was added to the medium from the lower chamber, mixed, and centrifuged at 5000 rpm for 5 min at room temperature. 100 µl medium was mixed with the cell pellet, the solution transferred to a white 96-well plate and 25 µl Cell Titer-Glo reagent was added. After incubation for 10 min in the dark, luminescence intensity was analyzed with an integration time of 1 sec using a 96 well plate reader. Each migration assay was performed three times in triplicate.

3.2.6 Transfection and the dual luciferase reporter gene assay

For transient promoter construct transfections, 7.5×10^4 cells were seeded onto 24-well plates. Briefly, 0.5 µg empty pGL4.17 plasmid or pGL4.17 plasmid containing a *MACC1* promoter fragment or predesigned mutant *MACC1* promoter constructs and 0.05 µg Renilla plasmid were incubated in 50 µl Opti-MEM for 5 min. The DNA mixtures were incubated with separately prepared 50 µl Opti-MEM containing 1.5 µl TransIT 2020 for 20 min at room temperature. The lipid DNA complex was added dropwise to 70%

confluent cells. Afterwards, cells were cultivated for 24 h.

For the reporter assay, the activities of the firefly and Renilla luciferases were measured using the Dual-Luciferase® Reporter Assay System. Cells transiently expressing the luciferase constructs were lysed in 100 µl passive lysis buffer (PBL) with gentle shaking for 15 min at room temperature. 25 µl obtained lysate was placed in a white 96-well plate. Following addition of 25 µl luciferase substrate (LARII), the firefly luminescence was quantitated using a luminometer. Upon addition of 25 µl Stop&Glo® reagent, the firefly luminescence was quenched and the Renilla luciferase was simultaneously initiated and quantitated. Renilla luciferase intensity was used as for normalization and determination of transfection efficiency.

3.2.7 Total RNA isolation, cDNA synthesis and quantitative real-time PCR

The total RNA was isolated using the GeneMatrix Universal RNA Purification Kit (EURX), according to the manufacturer's instructions. Briefly, cells were harvested and lysed in 400 µl RL buffer. Then RNA was bound to the column matrix by centrifugation. After washing of column material with 350 µl ethanol (70%), 400 µl Wash DN1, 650 µl Wash RBW, sequentially, RNA was eluted with 50 µl nuclease-free H_2O. RNA concentration was quantified photometrically using NanoDrop microvolume spectrophotometer. The samples were stored at -80 °C freezer until further use.

Fifty ng total RNA was reverse-transcribed to complementary DNA (cDNA). Reverse transcription was performed with random hexamers in 5 mM $MgCl_2$, 1x PCR buffer, 4mM dNTPs pool, 1 U/µl RNAse inhibitor and 2.5 U/µl MuLV reverse transcriptase. The reaction was carried out at 42 °C for 45 min, 99 °C for 5 min and 5 °C for 5 min. cDNA was stored at -20 °C or used directly for quantitative PCR.

Quantitative PCR was performed using SYBR Green dye chemistry in a LightCycler 480II. In parallel, the quantitation of housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as normalization. The data were evaluated by the LightCycler® 480 Software release 1.5.0 SP3. For each sample, the mean value of the duplicates was taken and normalized to the associated mean value of GAPDH.

Table 7. Primers used for RT- qPCR.

Primer	Sequence
MACC1 F	5'- TTCTTTTGATTCCTCCGGTGA -3'
MACC1 R	5'- ACTCTGATGGGCATGTG TG -3'
c-Jun F	5'- CAGGTGGCACAGCTTAAACA -3'
c-Jun R	5′- GTTTGCAACTGCTGCGTTAG -3′
Sp1 F	5'- GCTCTGAACATCCAGCAAAA -3'
Sp1 R	5'- CAGAGTTTGGAACAGCCTGA -3'
NEMO F	5'- AGAGTCTCCTCTGGGGAAGC -3'
NEMO R	5'- GCTTGGAAATGCAGAAGCTC -3'
p65 F	5'- ACAACCCCTTCCAAGTTCCT -3'
p65 R	5'- ATCTTGAGCTCGGCAGTGTT -3'
GAPDH F	5'- GAAGATGGTGATGGGATTTC -3'
GAPDH R	5'- GAAGGTGAAGGTCGGAGT -3'
G6PDH F	5'- ATCGACCACTACCTGGGCAA -3'
G6PDH R	5'- TTCTGCATCACGTCCCGGA -3'

3.2.8 Protein extraction, quantitation and Western blotting

For total protein extraction, cells were harvested using trypsin and washed twice in 1 ml of PBS and centrifuged at 113 g for 5 min. Cells were lysed in 150 µl RIPA buffer for 15 min on ice. Following centrifugation at 16260 g for 20 min at 4 °C, the supernatant was transferred to a clean 1.5 ml tube and stored at -80 °C or applied directly to Western blotting analysis.

The protein concentration of the supernatant was determined by a BCA (bicinchoninic acid) protein assay, according to the manufacturer's instructions. The lysates were diluted 1:5 in 1×PBS, and 2 mg/ml BSA solution was used to prepare a standard curve. From each sample or standard 25 µl were used. Following addition of 200 µl BCA working mixture to each well, the samples were incubated for 30 min at room

temperature. The absorbance was measured at 560 nm using the Tecan infinite 200 PRO. For Western blotting, 20 µg protein was mixed with 1×NUPAGE sample buffer and supplemented with 10% DTT, and heated for 10 min at 95 °C. Proteins were separated on 10% NuPAGE® Bis-Tris gels in 500 ml 1×MOPS buffer at 150 V for 1 h, using 10 µl protein marker as a size reference. Subsequently, proteins were transferred to a nitrocellulose membrane in semi-dry turbo-blot electrotransfer apparatus at 20 V for 7 min. The membrane was the stained with Ponceau S solution to visualize protein bands, washed in 1×TBST for 5 min and blocked in freshly prepared milk/1×TBST for 1 h at room temperature. Following blocking, the membrane was washed three times in 1×TBST for 10 min and incubated with a relevant primary antibody (Table 5) at 4 °C with gentle shaking overnight. The following day, the membrane was washed three times in 1×TBST for 10 min and incubated with a species-appropriate secondary antibody (Table 5). After washing the membrane a further six times in 1×TBST for 10 min, activated WesternBright ECL substrate was used to detect the proteins using a chemiluminescence imager.

3.2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 5. Comparison of the control with multiple groups was carried out using a one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test. Comparison between two groups was carried out using an unpaired t-test. Statistical significance was set at $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

4 Results

4.1 Effects of cytokines on *MACC1* expression in CRC cells

4.1.1 TNF-α regulates *MACC1* mRNA and protein expression levels

To evaluate the effect of inflammation on *MACC1* in epithelial CRC cells, we chose the potent pro-inflammatory cytokines, TNF-α and IFN-γ, to stimulate an inflammatory response. HCT116WT (Fig.4.1 A, B), HCT116/GFP (Fig.4.1 C, D) and HCT116/MACC1-GFP (Fig.4.1 E, F) cell lines were stimulated with increasing concentrations of TNF-α for 24 and 48 h, which express lower or higher *MACC1*, respectively. *MACC1* mRNA and protein expression levels were determined by RT-qPCR and Western blotting, respectively. As shown in Fig.4.1, HCT116WT cells, expressing lower levels of *MACC1*, were treated with TNF-α for 24 and 48 h (Fig.4.1 A). *MACC1* mRNA expression levels were significantly increased upon treatment with 1 ng/ml (p<0.05), 10 ng/ml (p<0.01), and 100 ng/ml (p<0.01) TNF-α, as compared with the untreated group. However, following 48 h treatment (Fig.4.1 B), the increase in mRNA expression levels of *MACC1* were not significant. Consistent with the increase in mRNA expression levels, *MACC1* protein expression was also upregulated following 24 h TNF-α treatment in a dose-dependent manner. However, this increase was not significant at 48 h. HCT116/GFP cells, which express lower *MACC1*, showed significantly elevated *MACC1* mRNA levels following 24 h (Fig.4.1 C) and 48 h (Fig.4.1 D) treatment (p<0.001) with TNF-α, although the fold changes at 48 h were less than at 24 h. *MACC1* protein expression levels were upregulated in accordance with *MACC1* mRNA expression levels in HCT116/GFP cells. For the ectopically-high MACC1-expressing cells, HCT116/MACC1-GFP, the expression levels of *MACC1* mRNA were upregulated predominantly in a dose-dependent manner following 24 h (Fig.4.1 E) TNF-α treatment (p<0.001), however, at 48 h (Fig.4.1 F), the fold changes in *MACC1* mRNA expression levels were not significant. Ten ng/ml TNF-α treatment was able to regulate *MACC1* protein expression, but the other concentrations triggered no induction. Taken together, the data suggests that TNF-α upregulates *MACC1* expression at both the mRNA and protein levels in a dose-dependent manner in HCT116 cells, more

remarkably following 24 h treatment than 48 h; with the optimum concentration condition being 10 ng/ml.

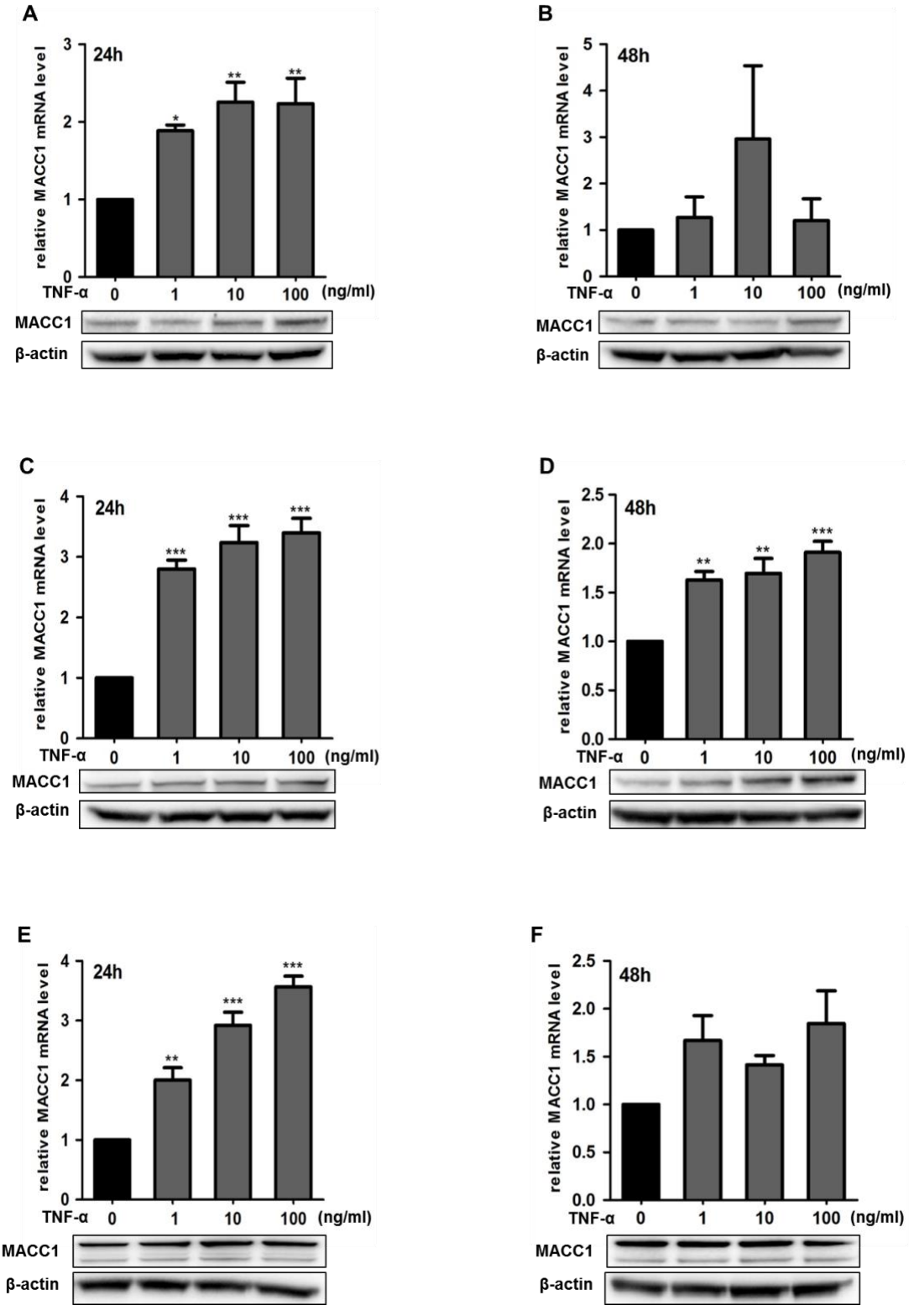


Fig.4.1. Effects of TNF-α stimulation on *MACC1* expression
HCT116WT (A, B), HCT116/GFP (C, D), and HCT116/MACC1-GFP (E, F) cells were treated with increasing concentrations of TNF-α (1, 10, 100 ng/ml) for 24 and 48 h. Cells without TNF-α treatment served as a control. *MACC1* mRNA expression levels were determined by RT-qPCR and normalized to GAPDH. Evaluation of *MACC1* protein expression levels was performed by Western blotting, and β-actin served as a loading control. Results are representative of three independent experiments. The asterisks show the level of statistical significance: **p*≤0.05; ***p*≤0.01; ****p*≤0.001.

4.1.2 IFN-γ has marginal effects on *MACC1* expression

HCT116WT (A, B), HCT116/GFP (C, D) and HCT116/MACC1-GFP (E, F) cell lines were stimulated with increasing concentrations of IFN-γ for 24 and 48 h. *MACC1* mRNA and protein expression levels were determined by RT-qPCR and Western blotting, respectively. As shown in Fig.4.2, HCT116WT cells were treated with IFN-γ for 24 h (Fig.4.2 A) and 48 h (Fig.4.2 B); however, the change in the *MACC1* mRNA levels showed no significance compared with the control group. Conversely, *MACC1* protein expression showed an upregulated trend following treatment with 1 ng/ml and 10 ng/ml IFN-γ, especially at 24 h. The same phenomenon was also seen in HCT116/GFP cells, where *MACC1* mRNA expression levels were elevated at 24 h following treatment with 10 ng/ml IFN-γ ($p<0.05$) (Fig.4.2 C), but not at 48 h (Fig.4.2 D). In HCT116/MACC1-GFP cells, the *MACC1* mRNA levels were increased following both 24 h (Fig.4.2 E) and 48 h (Fig.4.2 F) treatment with IFN-γ, nevertheless, no increase in *MACC1* protein expression was seen. The data demonstrates that stimulation with a lower concentration of IFN-γ was able to upregulate *MACC1* mRNA expression to a certain extent in HCT116 cells following stimulation for 24 h. Furthermore, since TNF-α showed a stronger and more sustained effect on *MACC1* expression, this cytokine was analyzed in more detail.

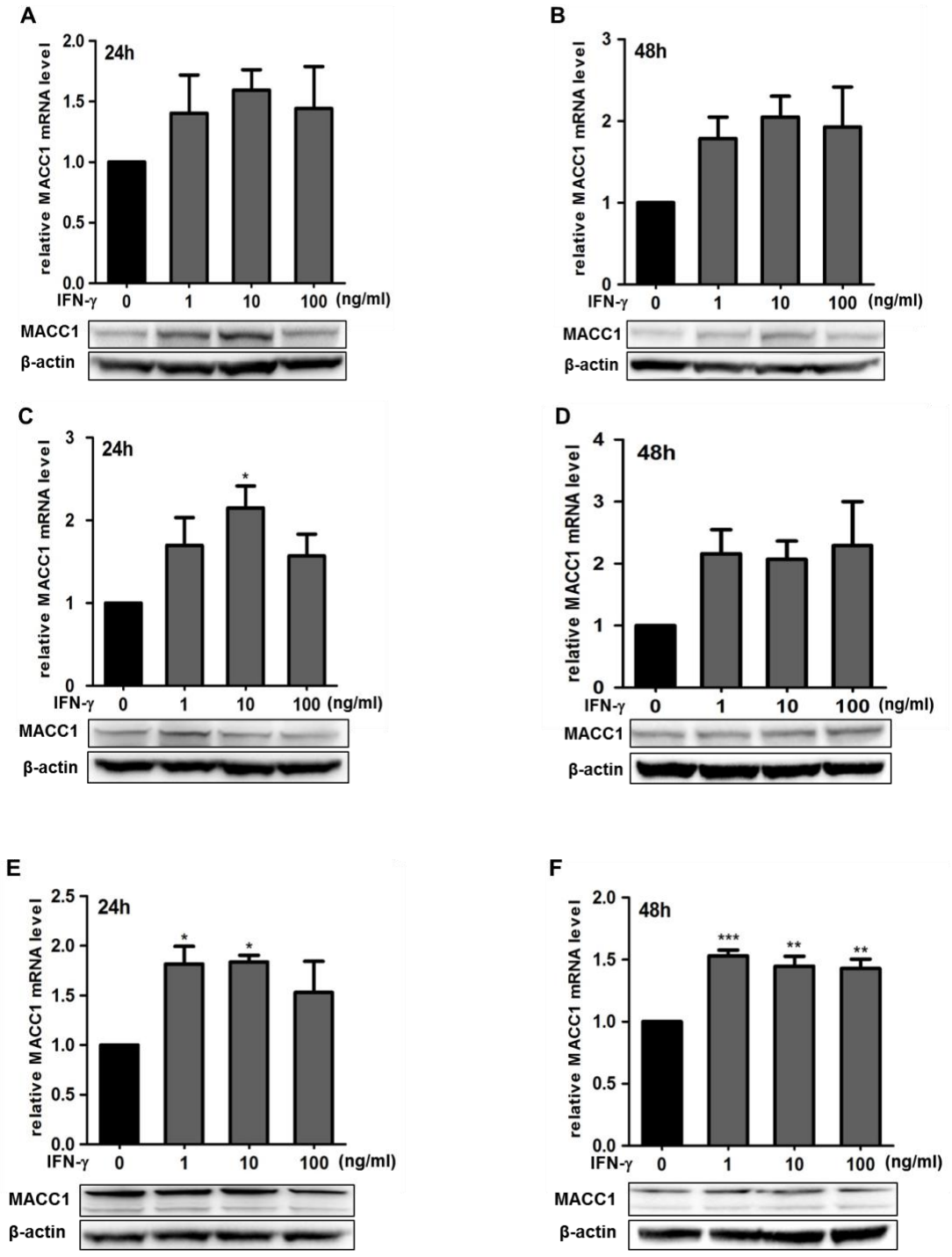


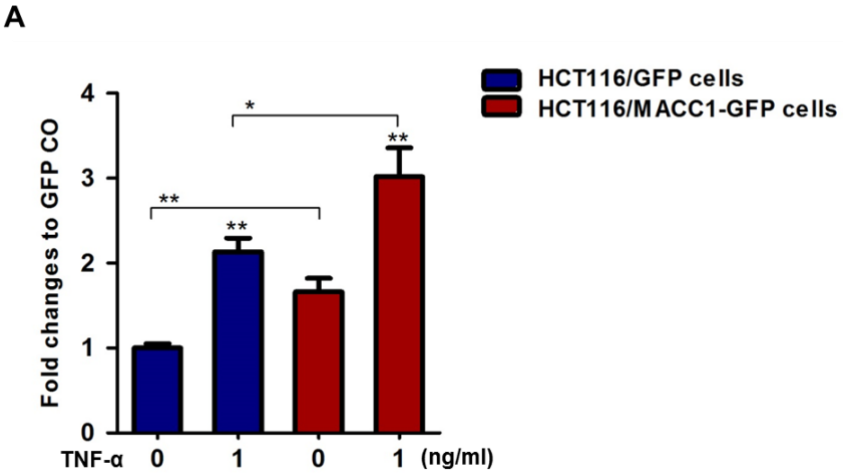
Fig.4.2. Effects of IFN-γ stimulation on *MACC1* expression

HCT116WT (A, B), HCT116/GFP (C, D), and HCT116/MACC1-GFP (E, F) cells were treated with increasing concentrations of IFN-γ (1, 10, 100 ng/ml) for 24 and 48 h. Cells without IFN-γ treatment served as a control. *MACC1* mRNA expression was determined by RT-qPCR and normalized to GAPDH. Evaluation of *MACC1* protein expression levels was performed by Western blotting, and β-actin served as a loading control. Results are representative of three independent experiments. The asterisks show the level of statistical significance: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

4.2 Effects of cytokines on MACC1-induced migration in CRC cells

4.2.1 TNF-α induces cell migration

To explore the role of the pro-inflammatory cytokine, TNF-α, on cell motility, its involvement in cell migration was determined. HCT116/GFP and HCT116/MACC1-GFP cells were treated with 1 ng/ml, 10 ng/ml and 100 ng/ml TNF-α for 24 h. As shown, TNF-α induced cell migration by more than two-fold in HCT116/GFP cells and three-fold in HCT116/MACC1-GFP cells at a concentration of 1 ng/ml (Fig.4.2 A), as compared with unstimulated cells. Upon treatment with 10 ng/ml (Fig.4.3 B) TNF-α, cell migration was strongly induced by four-fold in HCT116/GFP cells and more than ten-fold in HCT116/MACC1-GFP cells, as compared with the control. At 100 ng/ml (Fig.4.3 C) TNF-α, cell migration was induced by less than two-fold in both cell-lines, as compared with the control. However, the fold change in HCT116/MACC1-GFP cells was lower than that in HCT116/GFP cells. Without TNF-α treatment, HCT116/MACC1-GFP cells, which express higher *MACC1* levels, migrated more as compared with HCT116/GFP cells, which express lower *MACC1* levels. Upon treatment with TNF-α, cell migration was remarkably induced in HCT116/MACC1-GFP cells compared with HCT116/GFP cells, in particular at 10 ng/ml. However, at 100 ng/ml TNF-α treatment in HCT116/MACC1-GFP cells resulted in inhibition of cell migration. The data clearly indicates that TNF-α was able to induce cell migration *in vitro* in a dose-dependent manner, with the optimum concentration being 10 ng/ml.



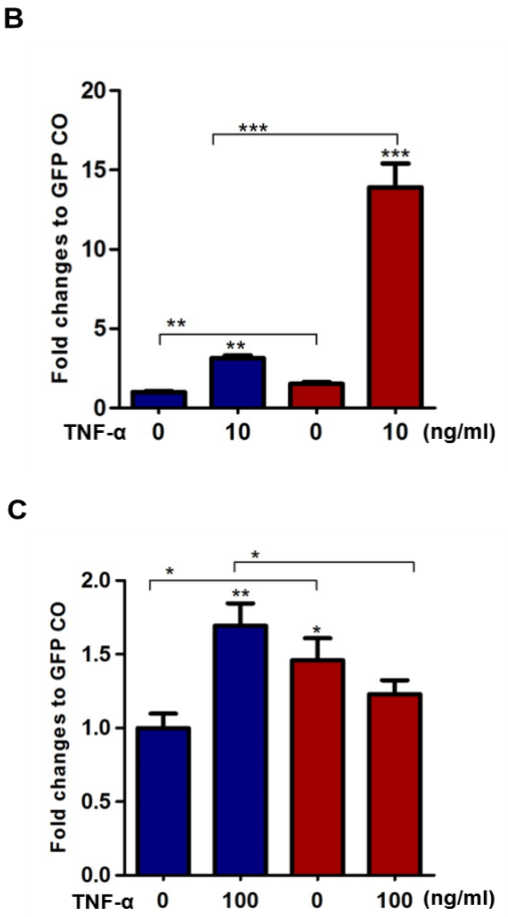


Fig.4.3. Impact of TNF-α treatment on cell migration
HCT116/GFP, and HCT116/MACC1-GFP cells were stimulated with increasing concentrations of TNF-α at 1 ng/ml (A), 10 ng/ml (B), and 100 ng/ml (C) for 24 h, and cell migration was subsequently measured. Cells without TNF-α treatment served as a control. Results are representative of three independent experiments. The asterisks show the level of statistical significance: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

4.2.2 TNF-α regulates MACC1-induced migration

MACC1 has been identified as a gene involved in CRC metastasis. In order to affirm the role of TNF-α in MACC1-induced cell motility, its effect on cell migration was elevated by knock down of *MACC1*. As shown above, 10 ng/ml TNF-α was the most efficient concentration for the induction of cell migration in HCT116 cells. Thus, this concentration of TNF-α was used in the further experiments. Treatment of HCT116/GFP cells with siRNA against *MACC1* dramatically inhibited cell migration, with or without 10 ng/ml TNF-α stimulation for 24 h, as compared with the scrambled control group ($p \leq 0.001$)

(Fig.4.4). Cells treated with 10 ng/ml TNF-α showed extensive migratory capability. In summary, knock down of *MACC1* abates cell migration, despite stimulation with TNF-α; therefore, it can be suggested that increased *MACC1* expression leads to a higher migratory potential in CRC cells.

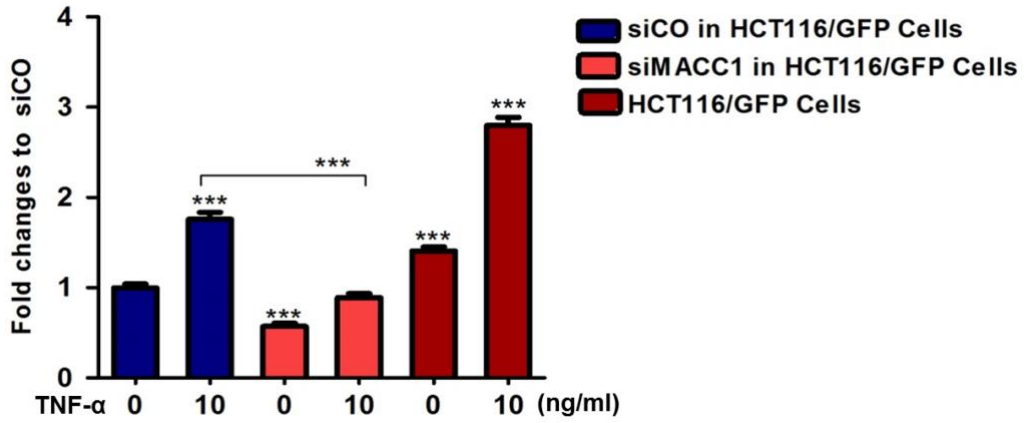


Fig.4.4. Migratory ability of MACC1-siRNA treated cells
Cells were transfected with scrambled control or *MACC1* siRNA for 24 h. Cells were stimulated with TNF-α for 24 h and cell migration was subsequently measured. Results are representative of four independent experiments. The asterisks show the level of statistical significance: **p*≤0.05; ***p*≤0.01; ****p*≤0.001.

4.2.3 IFN-γ induces cell migration slightly

To examine the role of the pro-inflammatory cytokine, IFN-γ, on cell migration, the cells were treated with increasing concentrations of IFN-γ. As can be seen from the data, IFN-γ induced cell migration by four-fold in HCT116/MACC1-GFP cells at a concentration of 1 ng/ml (Fig.4.5 A) as compared with the unstimulated control. However, this treatment did not result in significant migratory changes in HCT116/GFP cells. At 10 ng/ml (Fig.4.5 B) IFN-γ, both HCT116/GFP and HCT116/MACC1-GFP cells showed enhanced cell migration compared with the control; however, the migratory capability of MACC1-overexpressing cells was greater than that of the lower-expressing cells. Furthermore, cells showed no migratory potential at the higher concentration, 100 ng/ml (Fig.4.5 C) IFN-γ. The data reveal that IFN-γ can induce cell migration at a lower concentration, with 10 ng/ml being the most efficient concentration; however, the effect of TNF-α on cell migratory ability is far more significant.

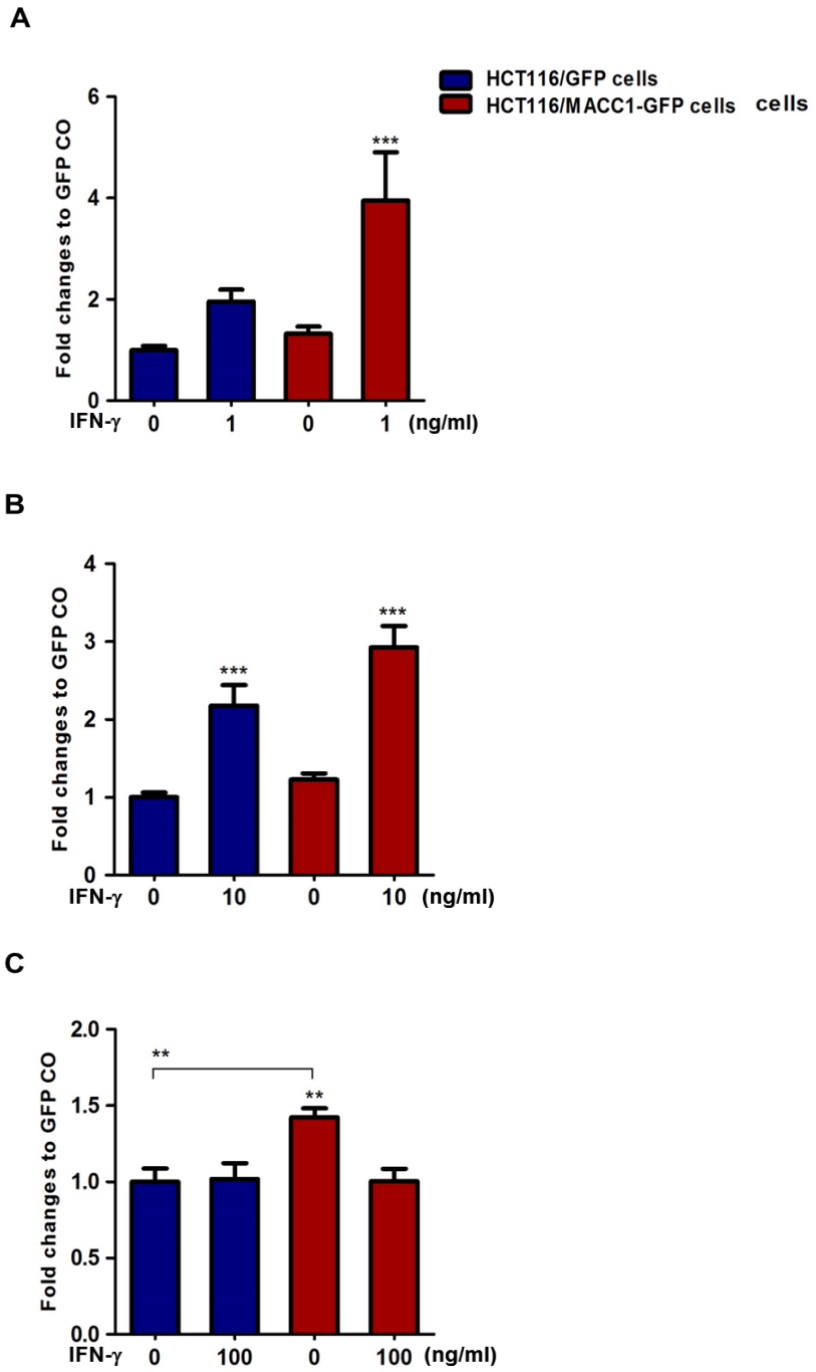


Fig.4.5. Impact of IFN-γ treatment on cell migration

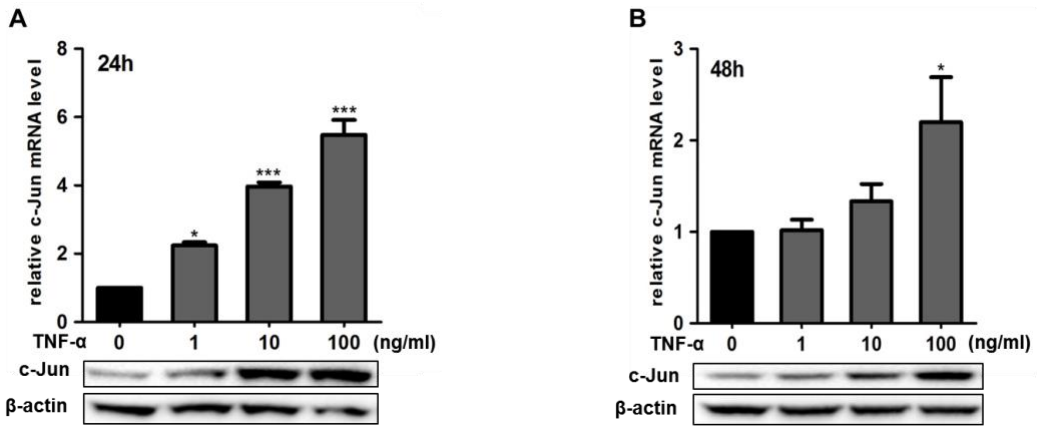
HCT116/GFP, and HCT116/MACC1-GFP cells were stimulated with increasing concentrations of IFN-γ at 1 ng/ml (A), 10 ng/ml (B), and 100 ng/ml (C) for 24 h, and cell migration was subsequently measured. Cells without IFN-γ treatment served as a control. Results are representative of three independent experiments. The asterisks show the level of statistical significance: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

4.3 TNF-α induces activity of the transcription factor *c-Jun*

4.3.1 TNF-α stimulates high expression of *c-Jun* at both the mRNA and protein levels

The gene *c-Jun* is an essential component of the transcription factor *AP-1*. TNF-α stimulated *AP-1* activity has been described in human breast cancer cells (Qiao et al., 2016). Here, the role of TNF-α on *c-Jun* was investigated in CRC cells. The HCT116 cell line was treated with increasing concentrations of TNF-α for 24 and 48 h. TNF-α potently stimulates *c-Jun* expression in a concentration-dependent manner at both the mRNA and protein levels at 24 h in HCT116WT (Fig.4.6 A) and HCT116/GFP (Fig.4.6 C) cells, which both express lower *MACC1* levels. The same phenomenon was also seen in HCT116/MACC1-GFP (Fig.4.6 E) cells, which express higher *MACC1* levels.

As shown, treatment with 100 ng/ml TNF-α for 48 h induced an increase in the mRNA levels of *c-Jun* in HCT116WT (Fig.4.6 B), HCT116/GFP (Fig.4.6 D) and HCT116/MACC1-GFP (Fig.4.6 F) cells; however, the lower concentrations of TNF-α had no significant effect on *c-Jun* mRNA expression. Additionally, *c-Jun* protein expression levels were strongly induced with TNF-α stimulation for 24 h. These data clearly confirm that TNF-α induced an increase in the expression of both *c-Jun* mRNA and protein in a concentration-dependent manner, in particular at 24 h.



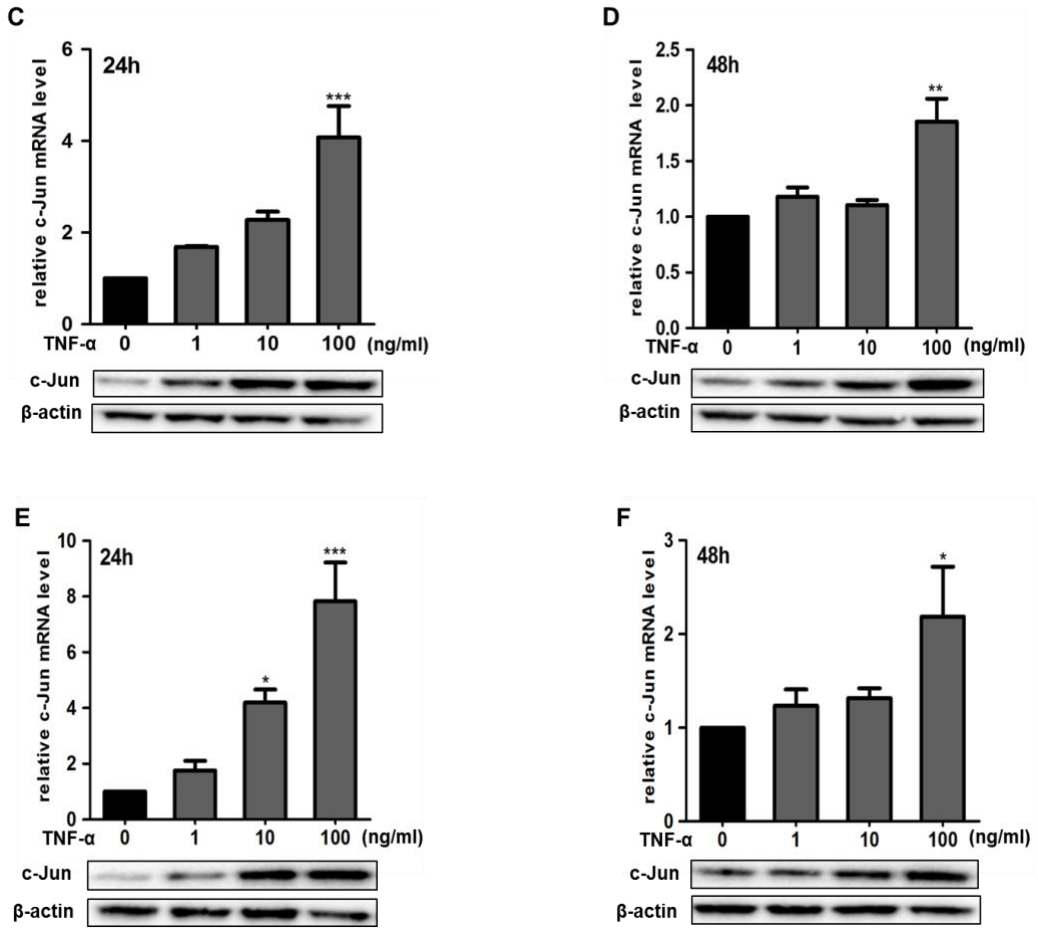


Fig.4.6. Implication of TNF-α stimulation on the transcription factor *c-Jun*
HCT116WT (A, B), HCT116/GFP (C, D) and HCT116/MACC1-GFP (E, F) cells were treated with increasing concentrations of TNF-α for 24 and 48 h. Cells without TNF-α treatment served as a control. The mRNA and protein expression levels of *c-Jun* were measured by RT-qPCR and Western blotting, respectively. Results are representative of three independent experiments. The asterisks show the level of statistical significance: **p*≤0.05; ***p*≤0.01; ****p*≤0.001.

4.3.2 TNF-α does not induce the transcription factor *Sp1*

The gene *Sp1* has been identified as a transcription factor of *MACC1*, which can bind to the core promoter of *MACC1* and regulate its transcription and expression (Juneja et al., 2013). The effects of TNF-α stimulation on *Sp1* is not yet clear, thus, to ascertain its effect on *Sp1,* HCT116 cells expressing inherently low *MACC1* levels were treated with increasing concentrations of TNF-α for 24 h (Fig.4.7 A) and 48 h (Fig.4.7 B), and *Sp1* mRNA levels were subsequently determined by RT-qPCR. The mRNA levels of *Sp1* were unchanged following TNF-α stimulation; therefore, it appears that the induction of

the transcription factor, *Sp1,* has no correlation with TNF-α treatment. Based on these results, we conclude that TNF-α stimulated the transcription factor, *c-Jun*, in order to increase *MACC1* expression.

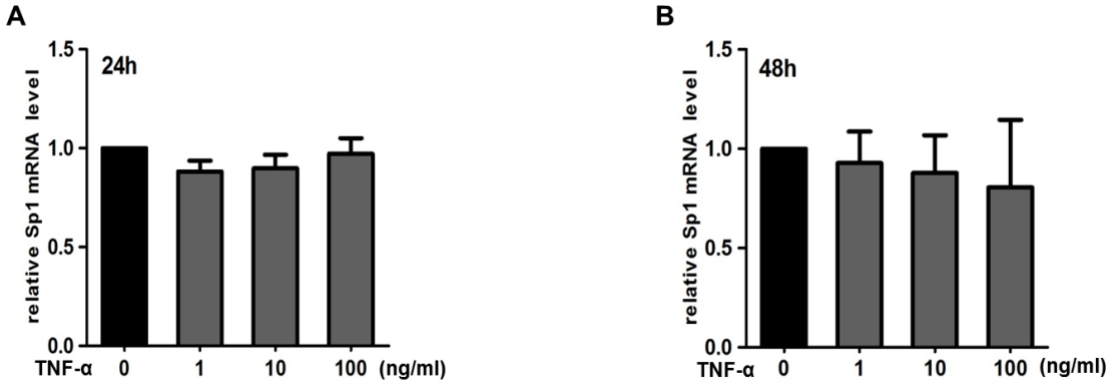


Fig.4.7. Effects of TNF-α treatment on the transcription factor *Sp1*
HCT116 cells were treated with increasing concentrations of TNF-α for 24 h (A) and 48 h (B). Cells without TNF-α treatment served as a control. The mRNA expression level of *Sp1* was determined by RT-qPCR and normalized to GAPDH. Results are representative of three independent experiments.
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4.3.3 IFN-γ does not induce the transcription factor *c-Jun*

As previously described, TNF-α strongly induces *c-Jun*. The role of the other pro-inflammatory cytokine, IFN-γ, on the induction of *c-Jun* was also explored. HCT116WT, HCT116/GFP and HCT116/MACC1-GFP cells were treated with increasing concentrations of IFN-γ, and harvested after 24 h or 48 h for analysis of *c-Jun* mRNA expression levels. No induction of *c-Jun* mRNA was seen in HCT116WT (Fig.4.8 A, B) following IFN-γ stimulation for 24 h or 48 h. Similarly, there was no significant change in *c-Jun* mRNA expression levels in either HCT116/GFP (Fig.4.8 C, D) or HCT116/MACC1-GFP (Fig.4.8 E, F) cells, suggesting that the induction of *c-Jun* was not regulated by IFN-γ treatment.

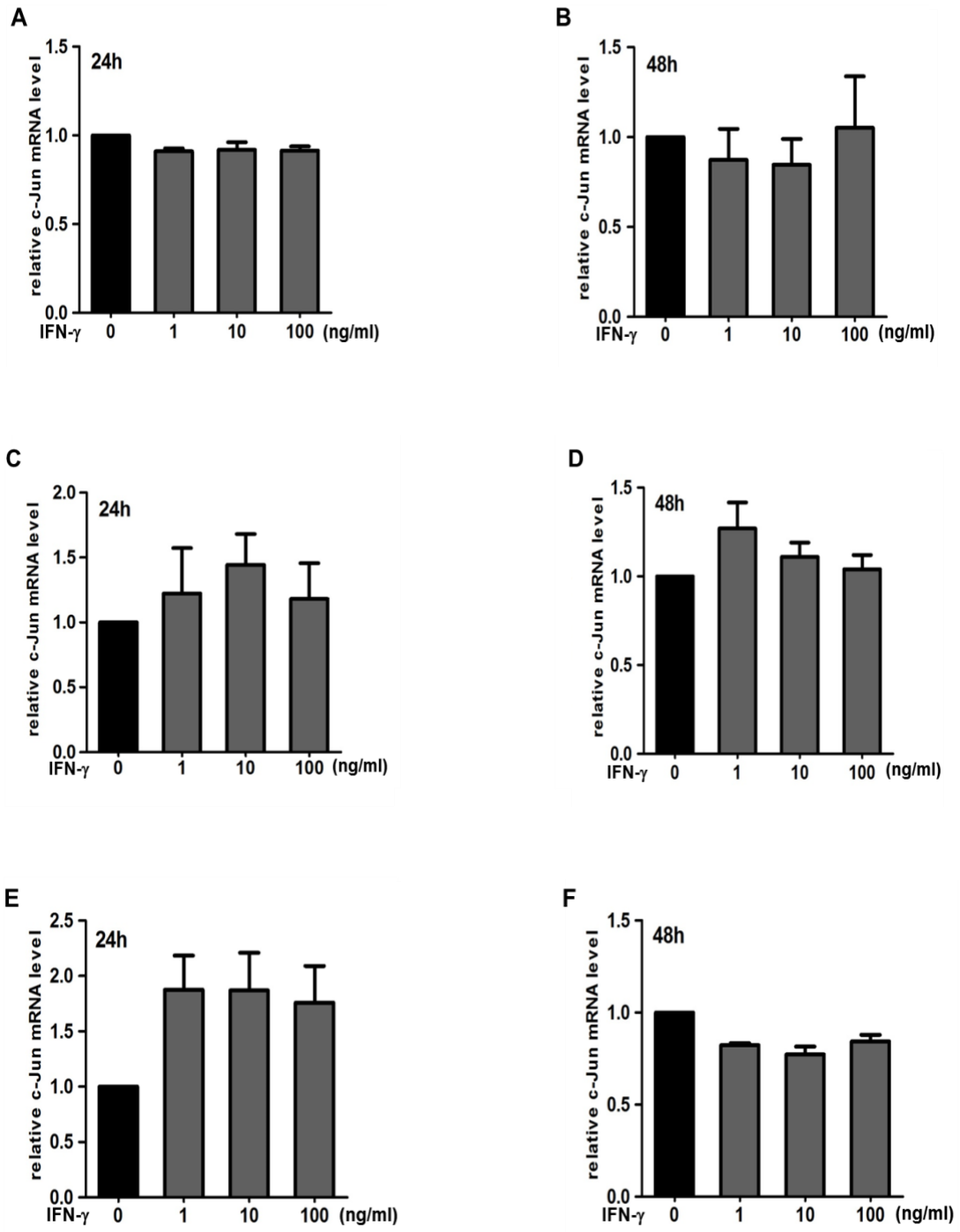


Fig.4.8. Implication of IFN-γ stimulation on the transcription factor *c-Jun*
HCT116WT (A, B), HCT116/GFP (C, D) and HCT116/MACC1-GFP (E, F) cells were treated with increasing concentrations of IFN-γ. Cells without IFN-γ treatment served as a control. Total RNA was isolated after 24 h or 48 h, reverse transcribed and quantitated by real-time PCR. The data is normalized to GAPDH. Results are representative of three independent experiments.

4.3.4 IFN-γ does not induce the transcription factor *Sp1*

The role of IFN-γ on the induction of *Sp1* was also explored. HCT116 cells were treated with increasing concentrations of IFN-γ for 24 h (Fig.4.9 A) and 48 h (Fig.4.9 B), and *Sp1* mRNA expression levels were subsequently measured by RT-qPCR. As expected, similar results were seen with IFN-γ stimulation as with TNF-α. No induction of *Sp1* mRNA expression was detected at any treatment concentration or time point, indicating that IFN-γ has no effect on *Sp1* expression. Furthermore, we verified that TNF-α induces the expression of *c-Jun*, which may further control *MACC1* activity.

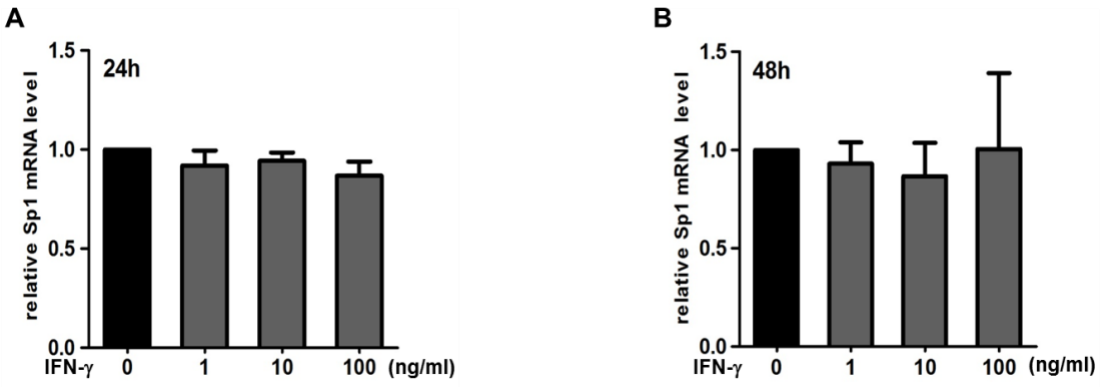


Fig.4.9. Effect of IFN-γ treatment on the transcription factor *Sp1*
HCT116 cells were treated with increasing concentrations of IFN-γ for 24 h (A) and 48 h (B). Cells without IFN-γ treatment served as a control. Total RNA was isolated after 24 h or 48 h, reverse transcribed and quantitated by real-time PCR. The data is normalized to GAPDH. Results are representative of three independent experiments

4.4 TNF-α regulates *MACC1* through an *AP-1* functional binding site

4.4.1 *c-Jun* is one of the transcription factors of *MACC1*

It has been identified that the *MACC1* promoter harbors the functional binding sites for *AP-1* and *Sp1*. These transcription factors have been shown to be involved in the regulation of *MACC1* activity (Juneja et al., 2013). Considering the necessity for these experiments, point mutations in the DNA binding sites of the *MACC1* promoter for *AP-1* and *Sp1* were introduced to confirm the importance of these two transcription factors on *MACC1* function. Furthermore, close attention was paid to the implications of

pro-inflammatory cytokines on the transcription factor of *MACC1*, *c-Jun*, which is the major constituent of *AP-1*. As described above, TNF-α showed a stronger and more sustained effect on *MACC1* and *c-Jun* expression as compared with IFN-γ. HCT116 cells were transiently transfected with *AP-1* and *Sp1* mutant promoter plasmids together with a Renilla control plasmid for 24 h. Following TNF-α treatment for a further 24 h, the functional relevance of the two mutated binding sites in the *MACC1* promoter was analyzed using a Dual Luciferase reporter gene assay. Both the mutated *AP-1* and *Sp1* sites (Fig.4.10) markedly reduced *MACC1* promoter activity, accounting for the crucial role of the two binding sites for its promoter function. The activity of the *MACC1* promoter was increased by TNF-α treatment but reduced by a mutation in the *AP-1* binding site. In summary, the *AP-1* binding site is an indispensable element for the transcriptional activation of the *MACC1* gene.

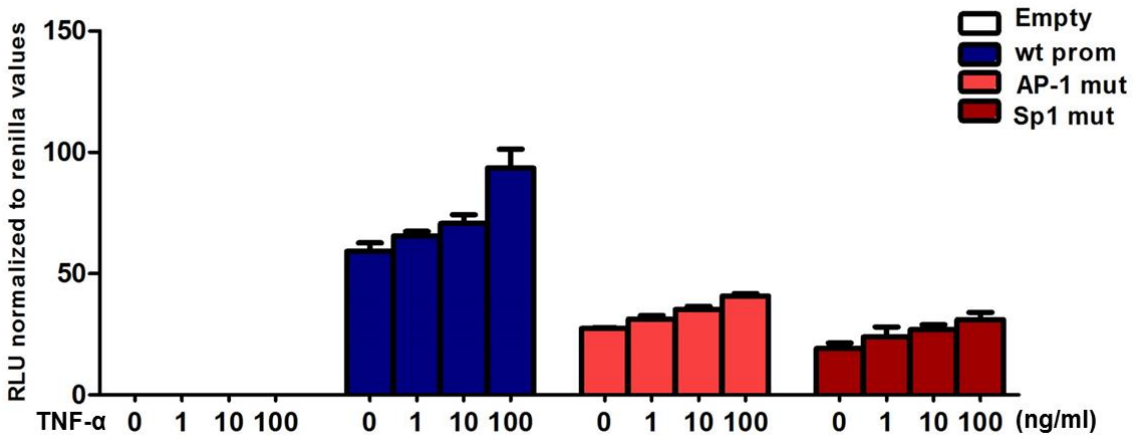


Fig.4.10. Identification of the transcription factors controlling *MACC1* expression
HCT116 cells were transiently transfected with the mutated *AP-1* and *Sp1* promoter plasmids together with the Renilla control plasmid for 24 h. Cells were subsequently treated with increasing concentrations of TNF-α, and the luciferase activity was measured and normalized to Renilla luciferase activity. The columns represent Empty: pGL4.17 vector without promoter; wt Prom: wild type MACC1 promoter; mut: mutated promoter at the *AP-1* and *Sp1*binding sites.

4.4.2 *c-Jun* knock down significantly reduce *MACC1* expression

Subsequently, the relevance of *c-Jun* in the control of *MACC1* by TNF-α stimulation was explored. A siRNA strategy was established to knock down *c-Jun* in HCT116 cells, and

its effect on the mRNA and protein expression levels of *MACC1* were evaluated. HCT116WT, HCT116/GFP and HCT116/MACC1-GFP cells were transfected with the target-specific predesigned *c-Jun* siRNA or scrambled control for 24 h. Cells were then treated with increasing concentrations of TNF-α for a further 24 h, and harvested for the analysis of *c-Jun* and *MACC1* expression at both the mRNA and protein levels by RT-qPCR and Western blotting, respectively. As shown, the mRNA expression level of *c-Jun* (Fig.4.11 A) reduced dramatically as compared with the scrambled control, indicating sufficient efficacy of the *c-Jun* siRNA knock down. Further, mRNA expression level of *c-Jun* in cells where *c-Jun* had been knocked down was unable to be induced by TNF-α; however, in the scrambled control group, the mRNA expression level of *c-Jun* was upregulated by TNF-α treatment in a dose-dependent manner. In accordance with the patterns of mRNA expression levels, the *c-Jun* protein expression levels showed the same tendency. Consistent with *c-Jun* expression, *MACC1* expression (Fig.4.11 B) at both the mRNA and protein levels was also significantly downregulated by *c-Jun* knockdown, and was no induced with TNF-α stimulation. In HCT116/GFP cells, which express lower *MACC1* levels similar to HCT116WT, the mRNA and protein expression levels of *c-Jun* (Fig.4.11 C) and *MACC1* (Fig.4.11 D) demonstrated the same phenomenon, as expected. In HCT116/MACC1-GFP cells, both the mRNA and protein expression levels of *c-Jun* (Fig.4.11 E) and *MACC1* (Fig.4.11 F) were markedly reduced by *c-Jun* knockdown, showing no effects with TNF-α treatment. In addition, the fact that reduction in *MACC1* mRNA and protein expression levels was not statistically significant with *c-Jun* knockdown, may be an artifact of the ectopic CMV promoter-driven *MACC1* expression. Taken together, these data directly or indirectly illustrate that *MACC1* expression was markedly downregulated by knocking down *c-Jun,* levels of which could not be induced by TNF-α treatment. This indicates that the transcription factor *c-Jun* is strongly associated with governing *MACC1* expression and is indispensable in the induction of *MACC1* via TNF-α stimulation.

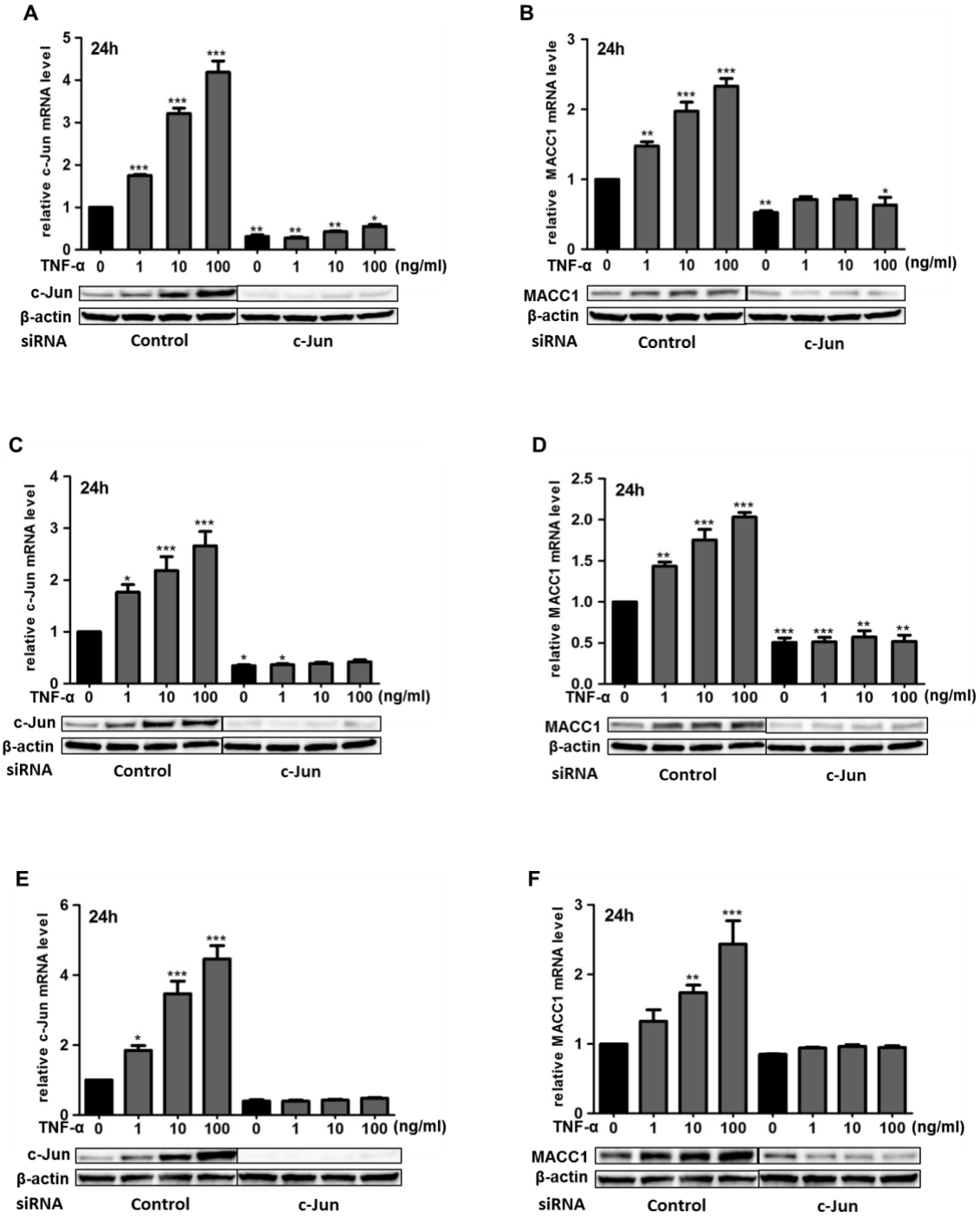


Fig.4.11. Effects of *c-Jun* knock down on *MACC1* expression

HCT116WT (A, B), HCT116/GFP (C, D) and HCT116/MACC1-GFP (E, F) cells were transfected with the target-specific predesigned *c-Jun* siRNA or scrambled control for 24 h. Cells were subsequently stimulated with increasing concentrations of TNF-α, for a further 24 h. Cells without TNF-α treatment served as a control. Cells were analyzed to assess the *c-Jun* and *MACC1* mRNA and protein expression levels using RT-qPCR and Western blotting, respectively. The data are normalized to GAPDH and β-actin, respectively. The asterisks show the level of statistical significance: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

4.4.3 TNFR1 is responsible for signal transduction leading to *MACC1* induction

It has been reported that TNF-α triggers several intracellular signaling pathways to execute its function through binding to two membrane receptors, TNFR1 or TNFR2 (Moelants et al., 2013). TNFR1 is extensively expressed in different cell types; however, TNFR2 is selectively found in immune and endothelial cells (Moelants et al., 2013). Now, we understand that TNF-α controls *MACC1* activity by regulating the expression of the transcription factor *c-Jun*. It is still unknown, however, which TNF-α binding receptor is involved in the signaling activation. To investigate this, HCT116WT cells were treated with 10 ng/ml TNF-α, which was established to be the most efficient concentration. One h prior to each stimulation, cells were pre-incubated with a specific blocking antibody for either TNFR1 or TNFR2. Following 24 h TNF-α treatment, cells were harvested and analyzed for *c-Jun* and or *MACC1* expression at both the mRNA and protein levels. The results show that TNF-α treatment upregulated both *c-Jun* and *MACC1* expression in the control group; however, the induction of *c-Jun* (Fig.4.12 A) disappeared at both the mRNA and protein levels upon pretreatment with a TNFR1-specific blocking antibody, which were not evaluated in response to TNF-α treatment. On the contrary, TNF-α treatment upregulated *c-Jun* expression upon pretreatment with a TNFR2-specific blocking antibody. In accordance with the *c-Jun* expression pattern, the mRNA and protein expression levels of *MACC1* (Fig.4.12 B) were also reduced upon pretreatment with a TNFR1-specific blocking antibody and showed no increase with TNF-α treatment; however, the upregulation of *MACC1* still occurred upon pretreatment with a TNFR2-specific blocking antibody. Based on this data, it can be concluded that blocking TNFR1, but not TNFR2, inhibited both *c-Jun* and *MACC1* induction at both the mRNA and protein levels, and TNF-α executed its function through TNFR1, but not TNFR2, to drive the activity of *c-Jun* and *MACC1* in HCT116 cells.

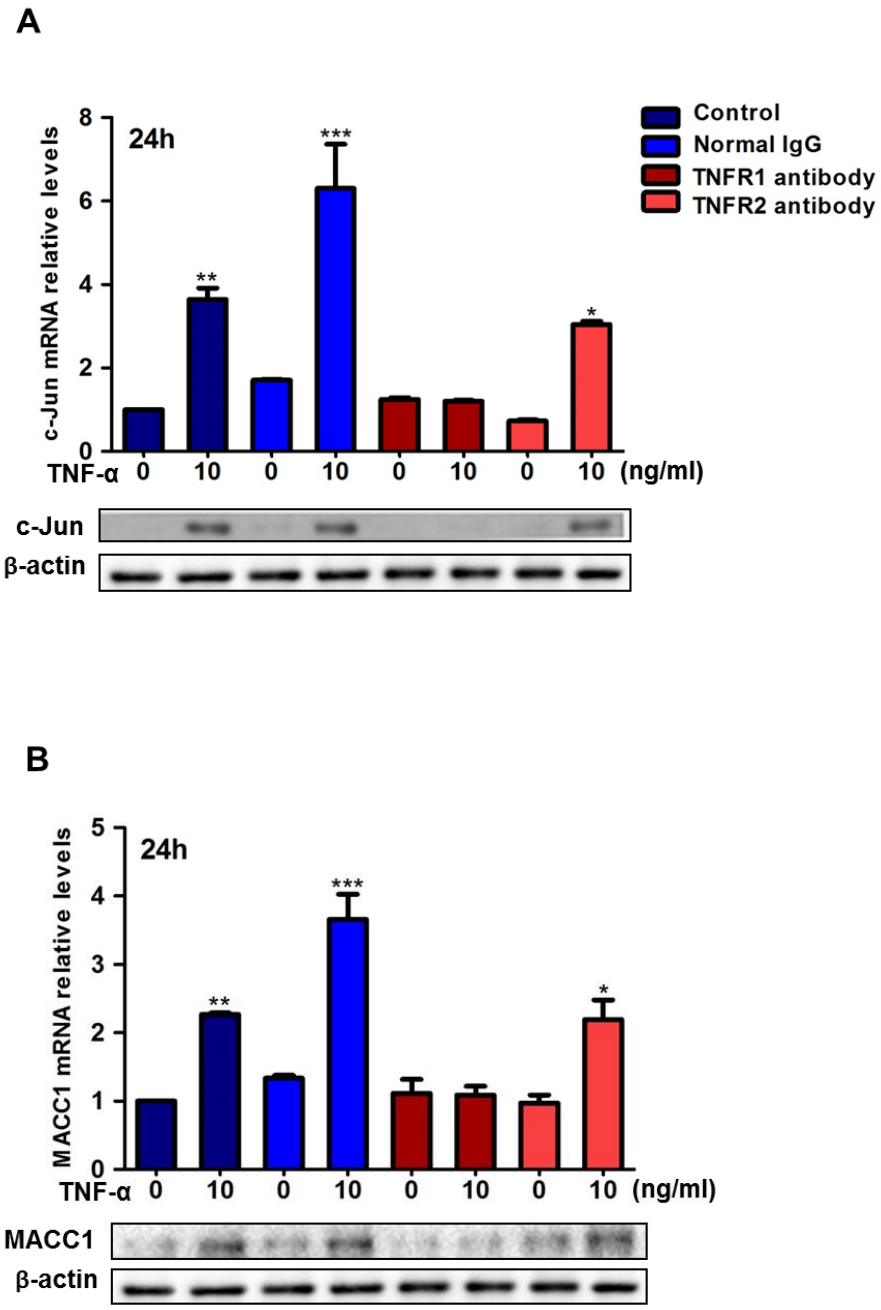


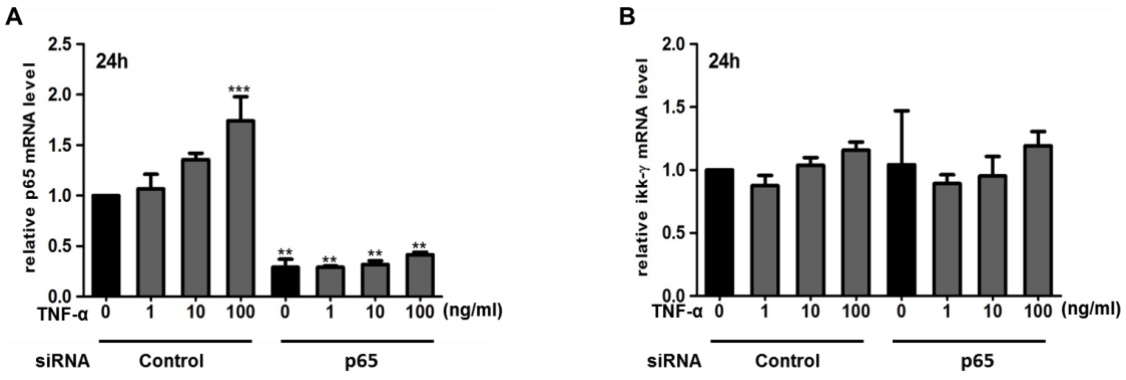
Fig.4.12. Effect of blocking TNFRs on the expression of *c-Jun* and *MACC1*

HCT1116WT cells were pre-incubated with specific blocking antibodies targeting TNFR1 or TNFR2 1 h prior to the addition of 10 ng/ml TNF-α. Following a 24 h stimulation with TNF-α, cells were harvested and analyzed to assess the *c-Jun* (A) and *MACC1* (B) mRNA and protein expression levels using RT-qPCR and Western blotting, respectively. The data are normalized to GAPDH and β-actin, respectively. Normal IgG-specific antibody served as a positive control. Ab represents antibody. The asterisks show the level of statistical significance: *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

4.5 NF-κB influence *c-Jun* gene expression via its subunit p65 to control the *MACC1* gene

4.5.1 p65 knock down reduces *c-Jun* and *MACC1* expression at mRNA level

NF-κB pathway is considered to be a prototypical pro-inflammatory signaling pathway, which includes at least two separate pathways for the activation of NF-κB (Lawrence, 2009). One of the principle pathways is the "canonical" pathway triggered by TNF-α, which requires the IKKγ subunit and results in the activation of p65 that regulates the inflammatory response (Lawrence, 2009). Despite the fact that *c-Jun* appears to be necessary for the induction of *MACC1* by TNF-α, the interaction between the classic NF-κB pathway and *c-Jun* is not satisfactory. To explore this, HCT116 cells were transfected with siRNA against p65 for 24 h and subsequently treated with increasing concentrations of TNF-α for a further 24 h. As can be seen, the mRNA expression levels of p65 (Fig.4.13 A) were increased in a concentration-dependent manner upon TNF-α αtreatment. The efficacy of p65 knock down was significant. Moreover, TNF-α did not influence IKKγ mRNA expression levels (Fig.4.13 B) following p65 knockdown. Next, the mRNA expression levels of *c-Jun* and *MACC1* were examined, and shown to be upregulated by TNF-α treatment. Most importantly, knock down of p65 abated basal mRNA expression levels of *c-Jun* (Fig.4.13 C) and *MACC1* (Fig.4.13 D), but still showed a marginal dose-dependent response to TNF-α treatment. The data clearly suggest that *c-Jun* and *MACC1* mRNA expression were downregulated by knock down of p65, indicating that the subunit of NF-κB, p65, was directly involved in the induction of c-Jun that governs the *MACC1* gene.



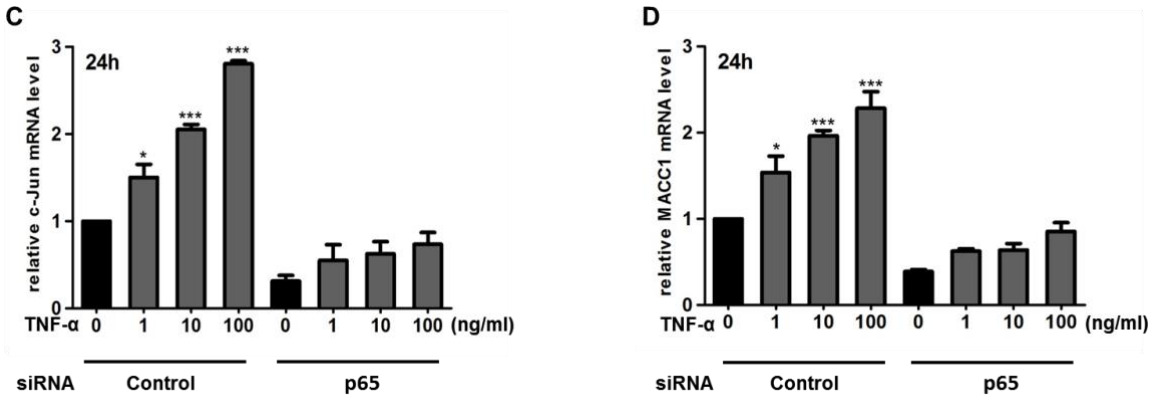


Fig.4.13. Implications of p65 knock down on *c-Jun* and *MACC1* expression at the mRNA level

HCT116 cells were transfected with p65 siRNA or scrambled control for 24 h. Cells were subsequently treated with increasing concentrations of TNF-α for a further 24 h. Total RNA was extracted, reverse transcribed and the mRNA levels of p65 (A), IKKγ (B), *c-Jun* (C) and *MACC1* (D) were quantitated using real-time PCR. The data is normalized to GAPDH. The asterisks show the level of statistical significance: **p*≤0.05; ***p*≤0.01; ****p*≤0.001.

4.5.2 IKKγ knock down has no effects on the mRNA expression levels of *c-Jun* and *MACC1*

IKKγ belongs to the IκB kinase (IKK) complex, which is a crucial component for the regulation of NF-κB activation (Hinz and Scheidereit, 2014). Canonical and alternative NF-κB signaling pathways have differential requirements for IKK subunits. IKK activation leads to phosphorylation of IκB and p65, which requires the IKKγ subunit. Based on this knowledge, IKKγ siRNA was designed. HCT116 cells were transfected with siRNA against IKKγ for 24 h. Cells were subsequently treated with increasing concentrations of TNF-α for a further 24 h. As shown in Fig.4.14 A, the efficacy of IKKγ knockdown was verified by reduced IKKγ mRNA expression. The basal mRNA expression levels of p65 (Fig.4.14 B) were reduced via the IKKγ knockdown strategy, *c-Jun* and *MACC1* mRNA expression levels were regulated by TNF-α. Interestingly, it was observed that both *c-Jun* (Fig.4.14 C) and *MACC1* (Fig.4.14 D) mRNA expression levels were still upregulated by TNF-α treatment in a dose-dependent manner following IKKγ knockdown. Taken together, these data indicate that IKKγ knock down has no influence on *c-Jun* and

MACC1 mRNA expression, which further illustrates that NF-κB is involved in the regulation of the *c-Jun* gene via its subunit p65, but not IKKγ, to control *MACC1* activity.

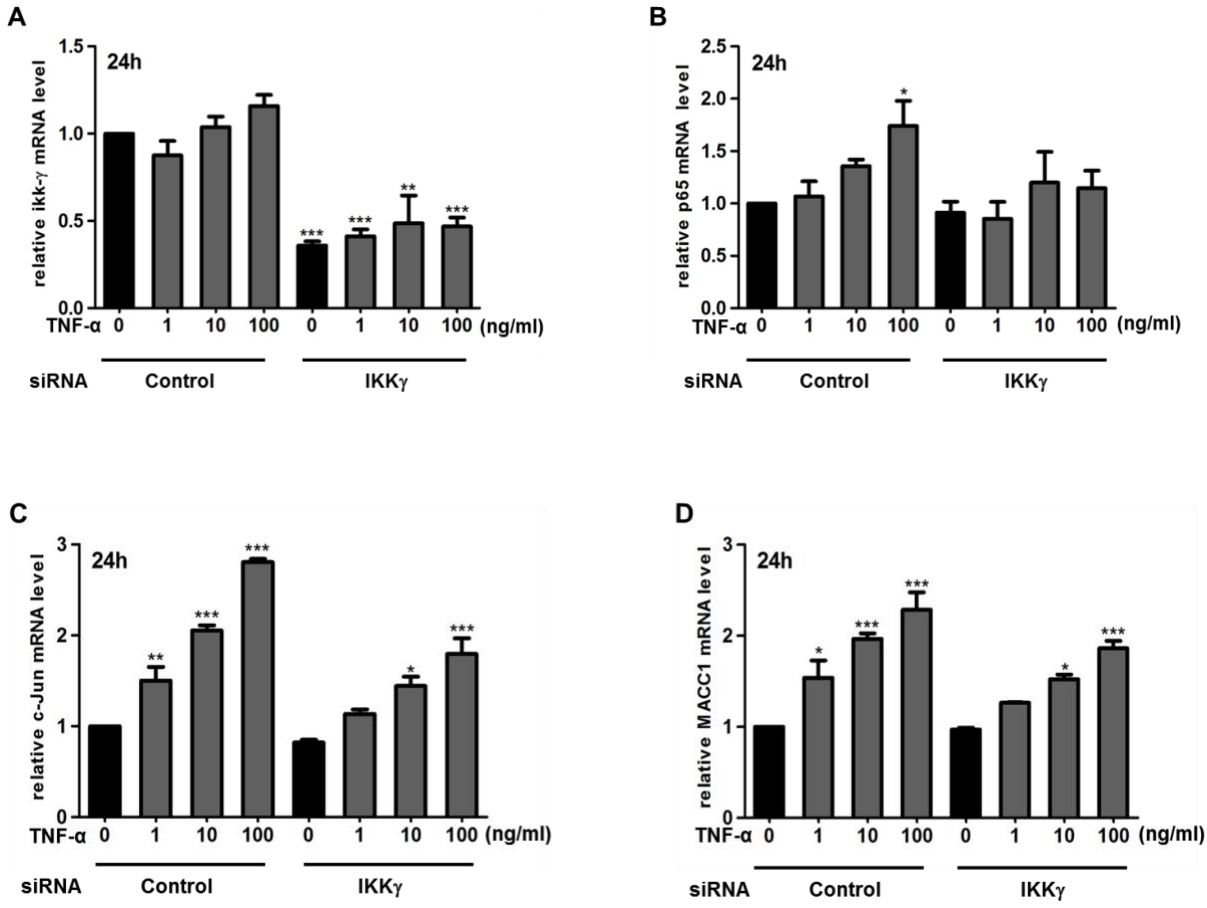


Fig.4.14. Implication of IKKγ knock down on *c-Jun* and *MACC1* expression at the mRNA level

HCT116 cells were transfected with IKKγ siRNA or scrambled control for 24 h. Cells were subsequently treated with increasing concentrations of TNF-α for a further 24 h. The mRNA levels of IKKγ (A), p65 (B), *c-Jun* (C) and *MACC1* (D) were quantitated using real-time PCR. The data is normalized to GAPDH. The asterisks show the level of statistical significance: **p*≤0.05; ***p*≤0.01; ****p*≤0.001.

5 Discussion

5.1 Regulation of the *MACC1* gene by pro-inflammatory cytokines

In numerous studies involving pro-inflammatory cytokines, inflammation was found to be associated with cancer–particularly CRC. However, the link between inflammation and cancer metastasis is not well understood. Expression of the *MACC1* gene, particularly in CRC, can yield tumor invasion and metastasis. Therefore, an examination of the *MACC1* gene can help to clarify the relationship between inflammation and metastasis in CRC. Other investigators have demonstrated that *MACC1* expression can be induced by IL-4 and lipopolysaccharide (LPS) in bone marrow-derived macrophages, suggesting that this gene might be involved in inflammation or inflammatory processes (El Chartouni and Rehli, 2010). Moreover, 78 gene regions containing *MACC1* were found to be likely associated with conditions of inflammatory dysregulation leading to Crohn's disease (Elding et al., 2013).

5.1.1 Regulation of *MACC1* by TNF-α

We hypothesized that, TNF-α–a strong pro-inflammatory cytokine – regulates MACC1 gene expression in CRC cells. Herein, we demonstrated that TNF-α regulates both transcriptional and translational products of *MACC1*. Specifically, *MACC1* mRNA and protein levels are increased by TNF-α stimulation. Recently, short-term stimulation with TNF-α was shown to trigger abnormal inflammatory processes and tumorigenesis (Wang et al., 2017). We found that, when TNF-α exposure is continued for a longer duration, levels of *MACC1* expression is lower than after a shorter treatment time. Thus, the inflammatory microenvironment sustained by TNF-α might be an important condition of CRC progression. Researchers have determined that inflammatory settings are crucial for tumors progression. In this context, inflammation regulates aspects of proliferation, angiogenesis, invasion, and metastasis; however, the underlying mechanisms of these processes are not well understood (El-Kenawi and Ruffell, 2017). We found that, TNF-α concentrations affect levels of *MACC1* mRNA and protein expression in a dose dependent manner. Kim et al. provided evidence that high levels of

TNF-α are associated with prevalence of colorectal adenomas (Kim et al., 2008). The concentrations of TNF-α also are found to positively correlated with growth of breast cancer tissues (Cai et al., 2017).

TNF-α also influences the function of the *MACC1* gene. We demonstrated that, TNF-α is a strong inducer of cell migration in CRC cells. TNF-α contributes to migration of CRC cells primarily through the epithelial-mesenchymal transition (EMT) (Bates and Mercurio, 2003). Shen et al. determined that microRNA-105 is involved in the EMT induced by TNF-α; inhibition of microRNA-105 in HCT116 cells diminishes TNF-α-enhanced cell migration (Shen et al., 2017). The *MACC1* gene induces cell mobility in several cancer types, and we found that low concentrations of TNF-α augment MACC1-induced cell migration. Conversely, high dose of TNF-α hinders cell migration in CRC cells overexpressing *MACC1*. Silencing of *MACC1* RNA abrogates the effects of TNF-α on cell migration and precludes cell responsiveness to TNF-α treatment. Hence, TNF-α might increase cell migration by acting through the *MACC1* gene, thereby augmenting the migratory potential of *MACC1* in CRC.

TNF-α not only promotes cell migration, but also promotes invasion and metastasis in CRC. Zhao and Zhang demonstrated that TNF-α stimulates migration and invasion of colon cancer cells by upregulating TROP-2 via ERK1/2 signaling (Zhao and Zhang, 2018). TNF-α–induced cell invasion and metastasis also has been described in other certain malignant tumors, such as gastric cancer (Chen et al., 2017), hepatocellular carcinoma (Xu et al., 2017), and breast cancer (Cai et al., 2017). Further investigation of TNF-α–mediated and MACC1-induced cell mobility is warranted. The molecular mechanisms and signal transduction pathways that yield cell growth, invasion, and tumor progression owing to TNF-α and *MACC1* remain unsatisfactory. Studies that address migration–related metastasis have become popular and might provide a new direction for exploring CRC development.

5.1.2 Regulation of *MACC1* by IFN-γ

IFN-γ, another pro-inflammatory cytokine, also upregulates *MACC1* mRNA and protein

expression at low concentrations, although the effects of IFN-γ stimulation are less intense than those of TNF-α. Exposure of cells to low levels of IFN-γ induces migration and enhances MACC1–expression–induced migration. However, exposure to high concentrations of IFN-γ attenuates the effect of MACC1–expression–induced cell mobility. Increasing levels of IFN-γ yield progressive inhibition of the stimulatory effects of *MACC1* on cell migration. These results suggest that progression of CRC is complex and involves multiple immune mediators.

IFN-γ is a pleiotropic effector molecular that has been long regarded as exhibiting excellent anti-tumor immunity: augmenting cytotoxic function of NK cells and $CD8^+$ T lymphocytes, inhibiting cell proliferation and tumor angiogenesis, and promoting apoptotic cell death (Parker et al., 2016). Investigators have demonstrated that IFN-γ inhibits cell proliferation in APC-mutated HT-29 colon cancer cells and terminates proliferation of HCT116 cells expressing endogenous *APC* expression. Moreover, IFN-γ deficiency promotes EGFR/Erk1/2 and Wnt/β-catenin signaling (Wang et al., 2015). However, other researchers have reported pro-tumorigenic effects of IFN-γ based, in part, on clinical observations. Specifically, IFN-γ has been found to initiate tumor growth and progression and enable escape of cancer cells from immunosurveillance (Mojic et al., 2017). If the level of IFN-γ that is appropriate for immunosurveillance of tumors is disrupted, the effects of this molecule can include enhancement of tumorigenesis and an immunoevasive phenotype; this can give rise to an immunosuppressive tumor environment in which immunosuppressive cells are recruited, immunosuppressive factors are secreted and the responses of cytotoxic T lymphocytes are suppressed (Lin et al., 2017). The chronic inflammation produced by IFN-γ contributes to all stages of tumorigenesis. In fact, IFN-γ is essential for spontaneous development of colorectal carcinomas via STAT1 signaling (Hanada et al., 2006). However, the threshold level of IFN-γ exposure needed to stimulate tumor development is not known.

Conventionally, cytokines have been considered as mediators of inflammation and carcinogenesis by redundant mechanisms; several types of cytokines can bind to the same type of receptor and function in the same signaling pathway. TNF-α and IFN-γ induce tumorigenesis synergistically through NF-κB signaling (Wang et al., 2013).

TNF-α cooperates with IL-6 to promote the growth of CRC cells by activating STAT3 and the NF-κB signaling transducer (De Simone et al., 2015). Thus, cytokines can establish an inflammatory microenvironment that can lead to degenerative diseases or malignant tumors via complex signal transducer networks. Research is needed to better understand these pathways that link inflammation and cancer.

5.2 TNF-α regulates transcription factors that bind the *MACC1* gene

5.2.1 Influence of specific pro-inflammatory cytokines on transcription factors

TNF-α stimulates c-Jun N-terminal kinase (JNK) to induce activation of *c-Jun*. This classical signaling pathway that is known to be involved in inflammation and cancer. We analyzed *c-Jun* mRNA and protein levels in response to TNF-α treatment and found, that TNF-α induces transcription and translation of *c-Jun* in a dose dependent manner in CRC cells. Hence, TNF-α can facilitate a variety of pathophysiological activities directly or indirectly by regulating *c-Jun* induction.

Other investigators have shown that TNF-α is expressed abundantly in CRC, despite of inhibition of JNK (Stanilov et al., 2011). TNF-α triggers JNK activation, which results in its nucleus translocation. There, it phosphorylates *c-Jun*, thereby enhancing its transcriptional activity. TNF-α has been shown to induce the activation of *c-Jun*, which could indicate a role in tumor initiation and progression. Wang and Tai reported that the substrate of JNK, *c-Jun*, promotes cell proliferation and contributes to the progression of hepatocellular carcinoma (Wang and Tai, 2016). Sato et al. found that JNK/c-Jun signaling is activated in pancreatic cancer and yields accelerated tumor development in mice (Sato et al., 2017). The level of *c-Jun* is elevated in advanced nasopharyngeal carcinoma, and patients with high levels of *c-Jun* have a poor 5-year survival rates (Zhong et al., 2017).

In contrast, the mechanisms by which TNF-α regulates *c-Jun* protein have not been established, and fewer studies have addressed the function of *c-Jun* in advanced CRC, therefore, we undertook a study to examine the regulation of *c-Jun* on migratory, invasive, and metastatic cell phenotypes. We found that, the long-term TNF-α stimulation affects

c-Jun expression less strongly than does short-term time treatment. Therefore, *c-Jun* activity might require a persistent inflammatory condition. Additionally, we demonstrated that TNF-α does not significantly induce the transcription factor, *Sp1*, in CRC cells. TNF-α is able to increase *Sp1* expression in rheumatoid fibroblast-like synoviocytes (Ikuta et al., 2012).

We determined herein that IFN-γ shows no capacity to regulate transcription of *c-Jun* or *Sp1* in CRC cells. IFN-γ has been a subject of debate regarding its apparent anti-tumor and pro-tumor immune mechanisms. *STAT1* (i.e., signal transducer and activator of transcription 1), a component of the JAK/STAT signaling cascade, has been shown to affect the anti-tumor/pro-tumor status of IFN-γ in solid tumors.

Several compelling lines of evidences have indicated that *STAT1* functions as a tumor suppressor. Klover et al. reported that mammary epithelial cells that lack *STAT1* give rise to the development of tumors in mice (Klover et al., 2010). Ramana et al. demonstrated that IFN-γ induces the expression of *c-Jun* in Stat1-null fibroblasts, whereas *c-Jun* expression was repressed in wild-type cells (Ramana et al., 2001). We speculate that the potential of IFN-γ to induce *c-Jun* might be repressed by *STAT1* activity. In recent years, more studies have been undertaken on the mechanisms of IFN-γ tumorigenesis. Results of one study indicated that *STAT1* could act as a promoter of tumor cell proliferation and invasion and could confer resistance to cancer immunotherapy (Meissl et al., 2017).

5.2.2 The *c-Jun* gene and known transcription factors of the *MACC1* gene

Juneja et al. specified the *MACC1* promoter region as nucleotides -992 to -18; this region can drive transcription of the *MACC1* gene (Juneja et al., 2013). The minimal essential core promoter region of *MACC1* lies within nucleotides -426 to -18. It encompasses all information needed for *MACC1* transcription, including initiation of transcription and basal activation of the *MACC1* gene. This core promoter region contains functional binding sites for transcription factors, including *AP-1*, *Sp1*, and *C/EBPs*. These transcription factors have been shown to regulate *MACC1* expression at the levels of mRNA and protein.

AP-1 regulates the expression of various genes expression and controls multiple cellular processes: cell growth, proliferation, differentiation, and apoptosis. The *c-Jun* protein is a crucial active constituent of the *AP-1* complex. Moreover, *c-Jun* is involved in cell cycle progression, proliferation, and angiogenesis and acts as a proto-oncogene by mediating tumor initiation and progression. Authors have determined that *c-Jun* can accelerate the cell cycle and promote proliferation of breast tumor cells by repressing Wee1 expression (Xu et al., 2015). Lin et al. found that phosphorylated *c-Jun* activates the expression of the vascular epithelial growth factor A (VEGFA) to induce tumor angiogenesis during prostate cancer progression (Lin et al., 2017). Knirsh et al. noted that the role of *c-Jun* could be augmented by microRNA 10b to boost migration and invasion of human cancer cells (Knirsh et al., 2016). Moreover, in the context of *c-Jun* and *Sp1* knockdown, MACC1-induced migration in CRC cells is abated. Hence, these molecules may regulate MACC1-associated cell mobility. The levels of *c-Jun* and *Sp1* have been found to positively correlate with *MACC1* expression in CRC patients, thus contributing to distant metastasis of tumors.

5.3 Regulation of the *MACC1* gene by TNF-α is mediated by its transcription factor

5.3.1 TNF-α regulates the induction of *MACC1* via *c-Jun*

The results of previous investigations indicated that TNF-α could induce the expression and function of *MACC1* in CRC cells. As a *MACC1* transcription factor, *c-Jun* might contribute to this induction. In response to *c-Jun* siRNA exposure, *c-Jun* and *MACC1* mRNA and protein expression were found to decrease sharply. Furthermore, in the context of *c-Jun* knockdown, TNF-α stimulation was ineffective at upregulating *MACC1* expression. In the absence of *c-Jun* siRNA treatment, TNF-α up-regulated *c-Jun* and *MACC1* expression significantly and in a dose-dependent manner.

Our findings indicate that *c-Jun* is involved in the regulation of TNF-α on the *MACC1* gene in CRC cells. Therefore, *c-Jun* could act as a master mediator of CRC metastasis. Liu et al. determined that *c-Jun* is a strong stimulant of cell invasion and metastasis that functions by regulating the induction of β-1,3-N-Acetylglucosaminyltransferase 8

(β3GnT8) in CRC (Liu et al, 2017). Our findings support the hypothesis that inhibition of *c-Jun* activity may be beneficial as treatment for progressive CRC.

The role of *c-Jun* in tumorigenesis is gradually being elucidated. Many investigators have explored the underlying mechanisms in this process. Jung and Lee demonstrated that apomorphine was a potential anti-cancer agent that inhibits *c-Jun* activity, leading to suppression of TNF-α–induced cell invasion in breast carcinoma (Jung and Lee, 2017). Kim et al. found that Salvia miltiorrhiza extract blocks transcriptional activation of *c-Jun* yielding inhibition of matrix metalloproteinase-9 (MMP-9)–induced cell invasion in breast cancer; therefore, this extract could be regarded as having anti-cancer effects (Kim et al., 2017). Further, ascofuranone effectively suppresses LPS-regulated inflammatory processes, thereby reducing tumor occurrence by inhibiting nuclear translocation of *c-Jun* (Park et al., 2017).

In summary, TNF-α directs the induction of *MACC1* through *c-Jun*. Activated *c-Jun* binds to the *MACC1* promoter directly and produces transcriptional activation of *MACC1*, thereby promoting tumor invasion and metastasis. Moreover, *c-Jun* might function as a mediator of pro-inflammatory signaling in tumor development, rather than in the inflammatory response. This represents a novel mechanism of tumor metastasis and provides direction for a potential CRC treatment.

5.3.2 TNF-α drives the induction of *c-Jun* and *MACC1* via TNFR1

TNF-α mediates a variety of cell-signaling processes involved in the immune response and carcinogenesis, primarily via its interaction with TNFR1 and/or TNFR2. TNFR1 is a central regulator of signal transduction pathways. As a continuation of our previous study of the effects of TNF-α on c-Jun/MACC1 signaling, we exposed CRC cells to specific antibodies to block TNFR1 or TNFR2. Addition of anti-TNFR1 antibody resulted in a precipitous drop in *c-Jun* and *MACC1* expression and a loss of responsiveness of *c-Jun* and *MACC1* mRNA and protein expression to TNF-α stimulation. In contrast, exposure to anti-TNFR2 antibody did not preclude the stimulation of *c-Jun* and *MACC1* by TNF-α. These results show that TNF-α induces *c-Jun* and *MACC1* via action of TNFR1, but not

TNFR2. Thus, these findings define a signaling axis comprising TNFR, *c-Jun*, and *MACC1* signaling (Fig.5.1) that potentially mediates tumor progression and metastasis. TNFR1 is an upstream signaling molecule that influences cellular responses, such as pathogenesis of solid tumors. Hosono et al. confirmed that serum levels of TNFR1 are highly elevated in patients with colorectal adenoma, and the expression of TNFR1 correlates with that of JNK in CRC epithelial cells. Hence, TNFR1 may have utility as a biomarker of early stages of CRC carcinogenesis (Hosono et al., 2012). Oshima et al. indicated that TNF-α/TNFR1 signaling plays a crucial role in facilitating the development of gastric tumors via NADPH oxidase organizer 1 (Noxo1) and G protein alpha 14 (Gna14) (Oshima et al., 2014). TNFR1 is highly sensitive to interactions with TNF-α, which promote lymphatic metastasis in human tumors via VEGF-C/VEGFR3 signaling (Cao et al., 2015). TNFR2 has been found to contribute to cell growth and proliferation in CRC by activating the PI3K/AKT signaling pathway (Zhao et al., 2017). Therefore, TNF-α/TNFR1 signaling has potential as a target in cancer prevention and therapy. Further, investigations of the signaling processes modulated by TNFRs are warranted to better understand the relationship between inflammation and cancer.

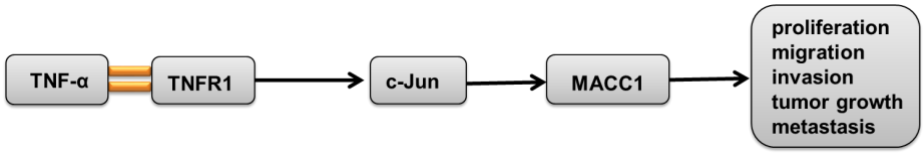


Fig.5.1. The TNFR1/c-Jun/MACC1 signaling axis
Binding of TNF-α to TNFR1 evokes the activity of *c-Jun* and leads to the induction of MACC1. This, in turn, induces cell mobility, tumor growth and metastasis.

5.4 Regulation of *c-Jun* and *MACC1* induction by NF-κB

NF-κB is a multifunctional transcription factor with essential roles in a variety of biological activities and cellular responses. NF-κB comprises a family of 5 molecules: NFKB1 (p105) and NFKB2 (p100) (precursor proteins of p50 and p52, respectively), RelA (p65), RelB and c-Rel. These members share a Rel homology domain, which is necessary for dimerization and for interactions with IκBs.

NF-κB subunits form various homo- and heterodimers. In resting cells, the subunits bind to IκB proteins and are maintained in an inactive state (Hayden and Ghosh, 2004). Degradation of IκB is a prerequisite for activation of the NF-κB pathway. This degradation is mediated by an activated IκB kinase (IKK) complex, which is composed of 2 serine-threonine kinases (IKKα and IKKβ) and a "master" regulatory protein, IKKγ (also termed NEMO). In the canonical pathway, NF-κB is activated by pro-inflammatory cytokines, such as TNF-α, and recruits IKK complexes. Activated IKK phosphorylates IκB and p65 in a process that is dependent on IKKγ. IκB phosphorylation gives rise to ubiquitination and proteasomal degradation, which results in translocation of NF-κB dimers into the nucleus. Once there, NF-κB dimers bind to target promoters and induce multiple pathologic responses (Hinz and Scheidereit, 2014).

TNF-α constitutively induces NF-κB to activate signal transductions processes. Consistent with previous studies, we determined that TNF-α activates *c-Jun* to regulate the induction of *MACC1* in CRC cells. Further analyses are needed to ascertain whether NF-κB associates with the induction of *c-Jun* and *MACC1*. We explored the effects of NF-κB signaling on *c-Jun* and *MACC1* by knocking down IKKγ and p65. Our results showed that TNF-α increases the levels of p65 mRNA expression in a dose-dependent manner. In the context of p65 knockdown, the basal levels of *c-Jun* and *MACC1* mRNA were lower. These levels could be increased slightly by TNF-α treatment. Treatment with p65 siRNA did not alter the level of IKKγ mRNA. Therefore, p65—a subunit of NF-κB —directly or indirectly induce transcription of *c-Jun* and controls the induction of *MACC1* in CRC cells. Li et al. demonstrated that truncation of Rel/p65 in myeloid cells sharply lowers the expression of *c-Jun* in lung tumor cells; hence, p65 protein might facilitate lung cancer growth via *c-Jun* activation (Li et al., 2014). In addition, TNF-α elicits marginal levels of IKKγ mRNA expression in CRC cells. In response to IKKγ knockdown, we noted a tendency toward lower levels of p65, *c-Jun*, and *MACC1* mRNA expression. In contrast, the levels of *c-Jun* and *MACC1* mRNA expression still could be significantly upregulated by TNF-α stimulation in a dose dependent manner. Therefore, IKKγ appears to have no influence on *c-Jun* and *MACC1* activities.

Collectively, TNF-α triggers cellular signaling by binding to TNFR1. Binding of TNFR1 induces the activity of *c-Jun* directly or indirectly through p65, a subunit of NF-κB. This process governs the expression of the *MACC1* gene in CRC cells (Fig.5.2). Few studies have addressed the mechanisms of NF-κB signaling in the induction of *c-Jun* and *MACC1* in solid tumors. Our findings indicate a notable signaling network involved in cancer development.

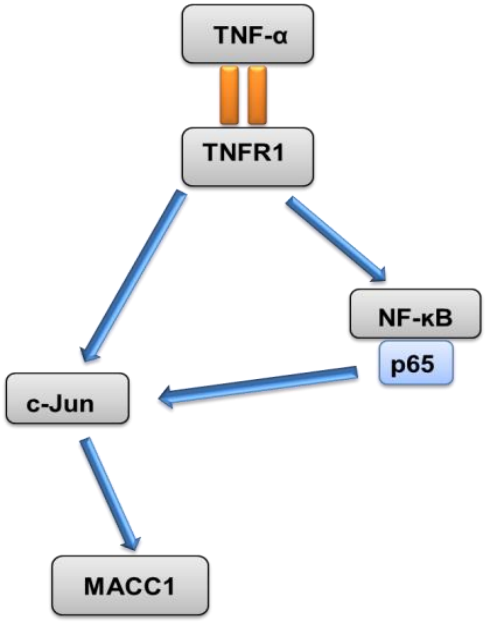


Fig.5.2. Signal transduction involved in the regulation of *c-Jun* and *MACC1*
TNF-α interacts with TNFR1 resulting in the induction of *MACC1* through *c-Jun* activation. In parallel, TNF-α activates p65, a subunit of NF-κB which also acts on *c-Jun*.

TNF-α drives NF-κB signaling, thereby contributing to inflammation, cell survival, proliferation, angiogenesis, tumor promotion, and metastasis. The transcription factor NF-κB is a fundamental link between inflammation and cancer and is involved in nearly every stage of cancer development, especially invasion and metastasis. Li et al. demonstrated that activated NF-κB promotes tumor metastasis by regulating epithelial mesenchymal-transition (EMT) in CRC (Li et al., 2017). Cho et al. determined that TNF-α, secreted by pro-inflammatory macrophages, enhances the metastatic potential of ovarian tumor cells via activation of the NF-κB signaling pathway (Cho et al., 2018). NF-κB can be activated by TNF-α application, which results in greater invasiveness and

metastatic capacity in oral squamous cell carcinoma (Tang et al., 2017). Given these multiple functions of NF-κB signaling, we advocate for further probes of the roles of intracellular transduction in cancer progression.

Taken together, these findings support the hypothesis that the oncogenic transcription factors *c-Jun* and NF-κB could be considered as a potential molecular target in CRC therapy. Control of inflammation offers an effective approach for repressing tumor metastasis. Knowledge of the interplay between inflammation and cancer provides a foundation for disease prognosis and clinical treatment.

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Eidesstattliche Versicherung

„Ich, Chenyu.Zhang, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: ***Identification of the key inflammatory cytokines on the regulation of the metastasis gene MACC1 in colorectal cancer*** selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.
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Datum Unterschrift

Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Acknowledgements

First of all, I would like to thank my supervisor Prof. Dr. Ulrike Stein, who gave me such opportunity to work in brilliant group with excellent colleagues, and led to the field of solid tumor researches that I am really interested in. Meanwhile, I would like to thank Prof. Dr. Britta Siegmund for her co-supervision.

Secondly, particular thanks go to Dr. Dennis Kobelt and Prof. Dr. Wolfgang Walther who offered me lots of help and patience on every step of my project, and guided me to solve the research problems when I felt confused. Thanks also go to Cynthia Voss who taught me new skills at the beginning of my work and assisted me to finish important parts of the experiments. I would also thank my outstanding colleagues: Dr. Ilm Katharina for helping with the RT-qPCR experiment; Dr. Jessia Pahle for teaching me western blotting; Benedikt Kortüm for solving many details occurred in my study; Dr. Mathias Dahlmann for helping with some statistical analyses. Besides, I want to sincerely thank my brilliant colleagues- Müge Erdem, Francesca Imbastari, Inna Zaimenko, Fabian Zincke and Harikrishnan Radhakrishnan who make my life abundant and colorful. I also would like to thank our diligent technicians: Janice Smith, Pia Herrmann and Margarita Mokrizkij for their organization of materials and assistance with my experiments. Special thanks go to the collaborators, Dr. Rainer Glauben, who will provide mouse models for further investigation of my project; Prof. Dr. Claus Scheidereit, who kindly provided siRNA for further exploring the molecular signaling of my study.

Last but not least, I would like to express my gratitude to my beloved parents and boyfriend, who always stand on my side, support every decision that I made, and encourage me when I feel depressed. All my achievements are inseparable from their support.