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Function of myeloid NF-κB in inflammation-induced lung carcinoma proliferation

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献给我深爱的妻子和女儿

To my beloved wife and daughter

Abstract

Accumulating evidence indicates that inflammation has a strong association with development of cancer. Inflammation is suggested to cause DNA alterations and oncogenic mutations resulting in tumor initiation. Inflammation promotes tumor progression via inflammatory cytokines released by immune cells. Cigarette smoke (CS) is the most important risk factor for both lung cancer and chronic obstructive lung disease (COPD). Clinical data indicate that COPD-related inflammation is an important risk factor for the development of lung cancer. The aim of this study was to investigate the role of myeloid nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in the regulation of growth signalling of CS-induced pulmonary tumor growth. Furthermore, the impact of CS in the Wnt/β-catenin signalling pathway of tumor cells was studied and it was investigated whether the antimicrobial peptide LL-37 has tumor promoting functions.

In vitro, transwell inserts were used in a co-culture model to determine the impact of macrophages on lung cancer cells proliferation. In the co-culture model, macrophages increased the growth of the lung cancer cell line A549 shown by cell number count, FACS (carboxyfluorescein succinimidyl ester, CFSE labeling) and ELISA (5-bromodeoxyuridine, BrdU labeling) analysis. Furthermore, addition of cigarette smoke extract (CSE) to the co-culture enhanced macrophage induced growth of A549 cells. Real time PCR and ELISA assay showed that CSE increased the secretion of inflammatory factors from macrophages and A549 cells. Inhibition of the NF-κB pathway by a NF-κB inhibitor or by RNAi against RelA inhibited tumor cell proliferation.

A metastatic lung cancer mouse model which is established by intravenous injection of Lewis lung carcinoma cells was used to investigate lung cancer proliferation. After mainstream cigarette smoke (MCS) exposure, the number of tumor nodules was higher compared to air exposed control animals. However, in CS exposed $rela^{\Delta-/-}$ mice (deleted RelA/p65 in the myeloid lineage), the tumor growth was significantly lower. $rela^{\Delta-/-}$ mice survived longer as compared to wild type (WT) mice. CS exposure resulted in enhanced expression of the proliferation markers Ki-67, PCNA, c-myc, and cyclinD1 in lung tumors of wild type animals shown by immunoblot analysis and immunohistochemistry. This was not observed in $rela^{\Delta-/-}$ mice. In contrast to $rela^{\Delta-/-}$ mice, CS exposure of wild type mice resulted in the secretion of inflammatory factor such as IL-6, KC, and TNFα as well as in the activation

of STAT3 and c-jun signalling in tumor cells. Additionally, CS exposure significantly increased the number of infiltrated macrophages in tumsaor. However, in *rela*$^{\Delta-/-}$ mice, RelA/p65 ablation did not significantly affect the recruitment of macrophage.

To identify whether Wnt/β-catenin signalling pathway is implicated in tumor-promoting effect of CS, regulation of unphosphorylated β-cateninin were examined. Immunoblot and immunohistochemical analysis showed that the CS-induced activation of the Wnt/β-catenin signaling pathway in tumor cells is dependent on RelA/p65 of myeloid cells. In vitro data showed that TNF-α directly activates Wnt/β-catenin signaling in tumor cells by inducing glycogen synthase kinase 3β (GSK3β) phosphorylation and the activation of the Akt pathway.

Antimicrobial peptides, such as cathelicidins, are key effectors of the innate immune system and regulate host defense and inflammation. Previously we showed that the human cathelicidin LL-37/hCAP-18 is a growth factor for lung cancer cells. Whether cathelicidins play a role in CS-promoted lung carcinoma proliferation is poorly understood. Here, it is demonstrated that the murine cathelicidin cathelicidin-related antimicrobial peptide (CRAMP) mediates lung tumor growth. CS exposure increased the proliferation of lung tumors in wild type mice, but not in CRAMP deficient mice. Furthermore, CS exposure induced the recruitment of myeloid cells into tumor tissue in a CRAMP dependent manner. Mice specifically lacking RelA/p65 in myeloid cells showed impaired CS-induced CRAMP-positive myeloid cell recruitment into lung. Additionally, in vitro studies with human tissue showed that the CS induced LL-37/hCAP-18 expression in macrophages is mediated by cancer cells. The regulation of LL-37/hCAP-18 expression in macrophages induced by inflammation involved the vitamin D receptor and Cyp27B1 activation.

In summary, it is shown in this study that: (i) myeloid RelA/p65 is necessary to link smoke-induced inflammation with lung cancer growth and the activation of Wnt/β-catenin signaling in tumor cells; (ii) cathelicidin promotes cigarette smoke-induced lung carcinoma proliferation, which is associated with the recruitment of immune cells and activation of myeloid NF-κB.

Zusammenfassung

Eine Vielzahl an Studien legt nahe, dass es einen starken Zusammenhang zwischen der Entstehung von Krebs und Entzündung gibt. Es wird angenommen, dass Entzündungen DNA-Veränderungen und onkogene Mutationen verursachen, was zur Initiation von Tumoren beiträgt. Entzündungen begünstigen Tumorprogression mittels inflammatorischer Zytokine, die von Immunzellen abgegeben werden. Zigarettenrauch ist sowohl für Lungenkrebs als auch für die COPD (chronisch obstruktive Lungenerkrankung) der größte Risikofaktor. Klinisch Daten besagen, dass die mit COPD verbundene Entzündung ein bedeutender Risikofaktor für das Entstehen von Lungenkrebs ist. Es war das Ziel dieser Studie, die Rolle myeloiden NF-κBs bei der Regulation von Wachstumsfaktoren, die Zigarettenrauch induziertes Wachstum von Lungentumoren vermitteln, zu untersuchen. Es wurde darüberhinaus untersucht, ob Rauch den Wnt/β-catenin abhängigen Signalweg in Tumorzellen aktiviert und ob das antimikrobiellen Peptide LL-17 bei der Proliferation von Tumoren eine Rolle spielt

Transwell-Inserts wurden in einem in vitro Ko-Kulturmodell genutzt, um den Einfluss von Makrophagen auf die Proliferation von Lungenkrebszellen zu untersuchen. Makrophagen steigerten das Wachstum von der Lungenkrebszellline A549 in diesem Ko-Kulturmodell, was mittels Zellzahl, FACS (carboxyfluorescein succinimidyl ester, CFSE labeling) und ELISA (5-bromodeoxyuridine, BrdU labeling) gezeigt wurde. Zigarettenrauchextrakt verstärkte darüber hinaus das durch Makrophagen induzierte Wachstum von A549 Zellen in diesem Modell. Quantitative RT-PCR und ELISA Analysen zeigten, dass Zigarettenrauchextrakt die Abgabe von inflammatorischen Faktoren von Makrophagen und A549 Zellen steigert. Inhibition des NF-κB Signalwegs mit Inhibitoren für NF-κB oder RNAi gegen RelA verminderten die Proliferation von Tumorzellen.

Ein murines Metastase-Lungenkrebsmodell, das auf der intravenösen Gabe von Lewis Lung Carcinoma Zellen basiert, wurde genutzt, um Lungenkrebsproliferation zu untersuchen. Die Zahl der Tumorknoten war bei berauchten Tieren größer als bei Raumluft exponierten Tieren. Bei Rauch exponierten myeloid $rela^{\Delta-/-}$ defizienten Tieren (myeloid RelA/p65 war bei diesen Tieren ausgeschaltet) war das Tumorwachstum jedoch signifikant geringer. $rela^{\Delta-/-}$ defiziente Tiere zeigten größere Überlebensraten im Vergleich zu Wildtyp Mäusen. Rauchexposition führte zu einer gesteigerten Expression der Proliferationsmarker Ki-67, PCNA, c-myc und Cyclin D1 in Lungentumoren von Wildtyp Tieren, was mittels Westernblotanalyse und immunhistochemischer Färbung gezeigt wurde. Dies wurde in $rela^{\Delta-/-}$

defizienten Tieren nicht beobachtet. Im Gegensatz zu *rela*$^{\Delta-/-}$ defizienten Tieren führte Rauchexposition bei Wildtyp Mäusen zu der Abgabe von inflammatorischen Markern wie z.B. IL-6, KC und TNFα und zur Aktivierung der STAT3 und c-jun Signalkaskaden in Tumorzellen. Rauchexposition führte in den Tumoren auch zu einer gesteigerten Zahl an infiltrierten Makrophagen. In relaΔ-/- defizienten Tieren hatte die Ablation von RelA/p65 jedoch keinen Einfluss auf den Einstrom von Makrophagen in Tumorgewebe.

Um zu untersuchen, ob der Wnt/β-catenin Signalweg an den Tumor induzierenden Eigenschaften von Rauch beteiligt ist, wurde die Regulation von phosphoryliertem β-catenin untersucht. Westernblotanalysen und immunohistochemische Färbungen zeigten, dass die durch Rauch induzierte Aktivierung des Wnt/β-catenin Signalwegs in Tumorzellen von myeloidem RelA/p65 abhängt. In vitro Studien zeigten, dass TNFα den Wnt/β-catenin Signalweg in Tumorzellen direkt aktiviert. Dies geschieht mittels der Phosphorylierung der Glycogen-Synthase-Kinase 3β (GSKβ) und der Aktivierung des Akt Signalwegs.

Antimikrobielle Peptide wie Cathelizidine sind Schlüsselkomponenten des angeborenen Immunsystems und regulieren Wirtsabwehr und Entzündung. Unsere Gruppe hat bereits gezeigt, dass das Cathelizidin LL-37/hCAP-18 ein Wachstumsfaktor für Lungenkrebszellen ist. Ob Cathelizidine eine Rolle bei der durch Rauch verursachten Proliferation von Krebszellen eine Rolle spielt, ist nicht bekannt. In dieser Studie wird gezeigt, dass das murine Cathelizidin CRAMP Wachstum von Lungentumoren vermittelt. Rauch verstärkte die Proliferation von Lungentumoren in Wildtyp Mäusen, aber nicht in CRAMP defizienten Tieren. Darüberhinaus zeigte sich der Rauch induzierte Einstrom von Makrophagen CRAMP abhängig. Myeloid defiziente RelA/p65 Mäuse wiesen nach Rauchinkubation einen verminderten Einstrom von CRAMP positiven myeloiden Zellen in die Lunge auf. In vitro Studien mit humanem Gewebe zeigte darüber hinaus, dass die durch Rauch induzierte Expression von LL-17/hCAP-18 in Makrophagen durch Krebszellen vermittelt wird. An der Regulation der Expression von LL-37/hCAP-18 in Makrophagen bei Rauch induzierter Entzündung war der Vitamin D Rezeptor und Aktivierung von Cyp27B1 beteiligt.

Insgesamt wurde in dieser Arbeit gezeigt, dass (i) myeloides RelA/p65 die Rauch induzierte Entzündung mit der Proliferation von Lungentumoren und der Aktvierung des Wnt/β-catenin Signalwegs in Tumorzellen verbindet und dass (II) Cathelizidine die durch Rauch induzierte Proliferation von Carcinomen vermitteln, was mit der Rekrutierung von Immunzellen und der der Aktivierung myeloiden NF-κBs verbunden ist.
(The Zusammenfassung is kindly contributed by Dr. Christoph Beisswenger)

Contents

1 Introduction

1.1 Inflammation and cancer

'Most things in life are a double-edged sword' is a common wisdom, and today, it is widely believed that inflammation is a double-edged sword (Hagemann et al., 2007). Inflammation is a response to either internal or external environmental stimuli, and is a crucial function of host innate immunity, such as wound healing and infection (Coussens and Werb, 2002). Inflammation can be divided into acute inflammation and chronic inflammation. Acute inflammation is an initial stage of inflammation. It is a rapid and self-limiting process mediated by chemical mediators and immune cells. Continuous, long lasting inflammation is regarded as chronic inflammation (Balkwill et al., 2005). Accumulating evidence indicates that inflammation has a strong association with development of cancer (Grivennikov and Karin, 2010a). For example, cigarette smoke (CS), asbestos, and silica induce pulmonary inflammation and contribute to the initiation and promotion of lung cancer (Yoshimura, 2006; Takahashi et al., 2010). Some pulmonary inflammatory diseases such as chronic obstructive pulmonary disease (COPD) are associated with lung tumor development. Chronic inflammation induced by repetitive injury, infection with *Helicobacter pylori* or Hepatitis C virus (HCV), or by ulcerative colitis (UC) is associated with gastric cancer, liver cancer and colon cancer, respectively (Balkwill et al., 2005). Others inflammatory diseases, such as obesity-associated inflammation, may promote liver cancer (Park et al., 2010). Inflammatory bowel disease increases the risk of colitis-associated cancer (CAC) (Grivennikov and Karin, 2010a). Furthermore, acute and subacute inflammation caused by exogenous administration of CS, tumour necrosis factor α (TNFα), interleukin 1 (IL-1), and lipopolysaccharide (LPS) have been found to promote tumor growth and metastasis (Balkwill and Mantovani, 2001; Luo et al., 2004; Takahashi et al., 2010). Although inflammations has been implicated in tumor initiation, the major effect of inflammation on tumor development in experimental animal studies is tumor promotion (Karin and Greten, 2005; Takahashi et al., 2010).

1.1.1 Immune cells in tumorigenesis

The tumor microenvironment plays a critical role in tumor initiation and promotion and contains innate immune cells, lymphocytes and connective tissue, such as fibroblasts, endothelial cells, pericytes, and mesenchymal cells (Grivennikov et al., 2010). Innate immune cells (also known as myeloid cells) including macrophages, neutrophils, eosinophils, mast

cells, myeloid-derived suppressor cells, dendritic cells, and natural killer cells, express and release cytokines and chemokines and influence tunorigenesis (Yoshimura et al., 2006; Coussens and Werb, 2002; Grivennikov et al., 2010).

Macrophages play a pivotal role in inflammation. Macrophages can be divided into M1 or M2 phenotype (Sica et al., 2008). M1 macrophages exhibit a pro-inflammatory phenotype, and have an ability of killing microorganisms and reveal antitumor activity. They produce copious amounts of pro-inflammatory cytokines (e.g. TNFα, IL-1, IL-6, IL-12 or IL-23), express major histocompatibility complex (MCH) molecules, and inducible nitric oxide synthase and are activated by microbial products and interferon γ (IFNγ) (Hagemann et al., 2009; Sica et al., 2008). In contrast, M2 macrophages which are induced by IL-4, IL-10, IL-13, glucocorticoid hormones, and vitamin D_3 have an immunosuppressive phenotype and release cytokines that tune inflammatory response, promote angiogenesis, tissue remodelling, and cell proliferation (Hagemann et al., 2009; Mantovani et al., 2002; Murdoch et al., 2008). However, several studies have shown that ‘‘M1 cytokines’’ are also capable of promoting tumor proliferation, whereas some ‘‘M2 cytokines’’, such as IL-10, may inhibits tumor growth (Lin and Karin, 2007; Grivennikov et al., 2010).

Tumor-associated macrophages (TAMs) are an important component of inflammatory infiltrates in tumors and are derived from circulating monocytic precursors by chemoattractants, such as vascular endothelial growth factor, CXCL12 (SDF1) and monocyte chemotactic protein (MCP), secreted by both tumor and stromal cells (Mantovani et al., 2002; Pollard, 2004). Most solid tumors are abundantly populated with TAMs and these cells are mostly regarded as tumor promoters (Bingle et al., 2002). Clinical studies have confirmed that high populations of TAMs generally correlate with poor prognosis in lung, breast, prostate, ovarian, and cervical cancers (Koukourakis et al., 1998; Bingle et al., 2002; Hagemann et al., 2009). TAMs generally have a M2 phenotype and function, which promotes tumor cell proliferation, tissue remodelling, and angiogenesis (Mantovani et al., 2008). For example, TAMs downregulate MHC class II and IL-12 expression and increase expression of the anti-inflammatory cytokine IL-10, scavenger receptor A and arginase-1(Arg-1), display a poor antimicrobial and tumoricidal activity (Grivennikov et al., 2010). Moreover, TAMs also express factors that promote angiogenesis, such as vascular endothelial growth factor (VEGF) and cyclo-oxygenase-2 (COX-2)–derived prostaglandin E2 (Hagemann et al., 2009; Sica et al., 2008). However, accumulating evidence indicates that TAMs also have M1 characteristics which expressing ‘M1 cytokines’ such as TNFα, IL-1, and IL-6, and M1 macrophages enzyme (iNOS or NOS2,) (Grivennikov et al., 2010; Kusmartsev and Gabrilovich, 2005; Tsai

et al., 2007). Recent evidence has suggested that TAMs are plastic and their phenotype is variable with the stage of tumor development, and generally considered to be M1 phenotype as tumor initiation and M2 phenotype while tumor promotion (Murdoch et al., 2008).

There is a growing body of evidence that TAMs promote the growth and survival of tumors, angiogenesis, invasion and metastases (Balkwill and Mantovani, 2001). In macrophage-deficient mice, tumor progression and metastatic ability were impaired and tumor growth was completed inhibited. This was associated with an increased survival rate in these mice (Lin et al., 2001; Aharinejad et al., 2002). TAMs produce high levels of potent angiogenic and lymphangiogenic growth factors, cytokines and proteases which support tumor progression (Sica et al., 2008). For example, the release of platelet derived growth factor (PDGF) from TAMs contributes to stroma formation and angiogenesis resulting in lung cancer promotion (Kataki et al., 2002). Moreover, TAMs release TNFα, IL-6, IL-1β, TGFβ, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and epidermal- growth-factor receptor (EGFR)-family ligands, all of which are implicated in tumor proliferation and angiogenesis (Pollard, 2004; Murdoch et al., 2008). TAMs also produce matrix-metalloproteases (e.g. MMP7, MMP9), which digest the extracellular matrix and break basement membrane, leading to tumor invasion (Pollard, 2004; Murdoch et al., 2008). In addition, TAMs can also inhibit antigen presentation of dendritic cells and induce T regulatory (Treg) cells through production of IL-10 and transforming growth factor (TGFβ) leading to immunosuppression that favours tumor promotion (Yoshimura, 2006). TAMs also suppress tumor-infiltrating T cell antitumor function by expression of hypoxia-inducible factor-1α thereby promoting breast tumor progression (Doedens et al., 2010).

There are also other immune cells that affect tumorigenesis. Many T cell subsets, such as $CD8^+$ T cells, IFNγ-producing Th1 cells, Th2 cells, Th17 cells, and Treg cells, are found to be involved in tumor promotion, progression, or metastasis in solid tumors (Grivennikov et al., 2010). It has been displayed that recruitment of Treg cells promotes lung carcinogenesis, which through suppressing T cell-mediated antitumor activity and regulation of immunosuppressive cytokine profile (Zaynagetdinov et al., 2011; Tao et al., 2012). In breast cancer, an increase in T cells with high $CD4^+/CD8^+$ and Th2/Th1 ratios is predictive of poor prognosis (Grivennikov et al., 2010). In melanoma and bladder cancers, IL-17 which is released by Th17 cells can promote tumor growth through an IL-6-Stat3 signalling pathway (Wang et al., 2009). Immature dendritic cells (iDC) in tumors promote tumor angiogenesis by secretion of pro-angiogenic cytokines, including TNFα, CXCL8 (IL-8), and VEGF (Murdoch

et al., 2008). In patients with bronchioloalveolar carcinoma, increased neutrophil numbers are correlated with poor prognosis. Tumor associated neutrophils promote tumor neo-vascularization and tumor development by production of cytokines, chemokines, proteases and ROS, including VEGF, MMP9, CXCL8, and CXCL1 (Murdoch et al., 2008; Fridlender et al., 2009).

1.1.2 Soluble mediators of inflammation in tumorigenesis

The inflammatory cytokine, chemokine, and enzymes expression profile of the tumor microenvironment is rich including TNFα, IL-6, IL-8, and MMPs and may be implicate in inflammation-associated tumor growth (Balkwill and Mantovani, 2001; Coussens and Werb, 2002). TNFα has a critical role in inflammatory reactions which are induced by a wide range of pathogenic stimuli. TNFα induces other inflammatory mediators and proteases that orchestrate inflammatory responses (Balkwill, 2002; Aggarwal et al., 2006). TNFα was initially described as a tumor killer. A high dose of TNFα selectively destroys tumor blood vessels and can stimulate anti-cancer immunity (Balkwill, 2002). However, a number of reports indicate that TNFα is also likely to be an important tumor-promoting cytokine contributing to the development of tumors (Aggarwal et al., 2006). TNFα production can be detected in various human cancers, such as lung carcinoma, ovarian carcinoma, renal carcinoma, CAC carcinoma, and breast carcinoma. Its presence is generally associated with a poor prognosis (Balkwill et al., 2005; Aggarwal et al., 2006). In the tumor microenvironment, TNFα is produced by inflammatory cells or tumor cells, supporting tumor proliferation and survival by activation of AP-1 and NF-κB signalling pathways (Grivennikov and Karin, 2011). TNFα is involved in tumor initiation by inducing the production of reactive oxygen and NO which leads to DNA damage and mutations (Balkwill, 2002). Exposure to 0.6 nM TNFα markedly enhanced transformation of BALB/3T3 cells initiated with 3-methylcholanthrene (Komori et al., 1993). *In vivo*, macrophages phagocytize asbestos and release TNFα that are susceptible to malignant transformation in asbestos-induced human malignant mesothelioma (Yang et al., 2006a). TNFα enhances tumor progression, including promotion of proliferation, angiogenesis, and invasion. For instance, TNFα can induces the expression of amphiregulin, EGFR, and TGFα in tumor cells, which mediate the proliferation of cells (Aggarwal et al., 2006). TNFα also induces angiogenic factors, such as VEGF, as well as the production of proteases that are important for invasion of tumor cells and macrophages, such matrix metalloproteases 9 (MMP9) (Balkwill and Mantovani, 2001).

The tumor-promoting effect of TNFα has been demonstrated in mice deficient in TNFα that showed markedly reduced lung tumor multiplicity and mortality after inoculation with Lewis lung carcinoma (LLC) cells (Kim et al., 2009). In another tumor transplantation model of lung cancer, TNFR1 deficiency led to depressed hepatocyte growth factor (HGF) expression and neovascularization resulting in apoptosis of tumor cells (Tomita et al., 2004). TNFα from host macrophages promotes LPS induced colon adenocarcinoma cell line growth in a mouse lung metastasis model by activation of NF-κB in tumor cells (Luo et al., 2004). TNFα deficient mice are resistant to carcinogen (7,12-dimethylbenz(a)anthracene) induced skin cancer (Moore et al., 1999). Deficiency of TNFR1 and TNFR1 in mice has also been found to reduce carcinogenesis of skin (Arnott et al., 2004). In a Mdr2-knockout model of Hepatocellular carcinoma (HCC), TNFα released by inflammatory cells promotes tumor development through activating NFκB in hepatocytes (Pikarsky et al., 2004). TNFα expression is upregulated during colitis accociated cancer (CAC) and lacking of TNFR1 or administration of etanercept, a specific antagonist of TNFα, results in decreased tumorigenicity and tumor growth (Popivanova et al., 2008).

IL-6 is a key inflammatory cytokine that is important for immune responses, cell survival, apoptosis, and proliferation through activation of Janus kinases (JAKs) and the downstream effectors signal transducers and activators of transcription factors 3 (STAT3), Shp2-Ras, and phosphatidylinositol 3-kinase (PI3K)-Akt (Grivennikov et al., 2009). The role of IL-6 in malignant cell proliferation and survival has been well documented. Elevated IL-6 expression levels in serum and tumor are associated with the pathogenesis of several cancers, including lung, breast, prostate, liver, ovarian, and colon cancers, and in general, this is correlated with an unfavourable outcome (Heikkila et al., 2008, Balkwill et al., 2005). In lung carcinoma, constitutively activation of STAT3 induced by IL-6 has been observed, which is important for development, proliferation, and survival of tumor cells (Hodge et al., 2005). In CAC, lamina propria myeloid cell derived IL-6 promotes proliferation of tumor cells and protects normal and premalignant intestinal epithelial cells from apoptosis (Grivennikov et al., 2009). Ablation of IL-6 in mice results in a decreased tumor number and a smaller tumor size in an azoxymethane dextran sulphate sodium induced CAC mouse model (Grivennikov et al., 2009).

Other important soluble mediators of inflammation also induce tumorigenesis and promotion of tumor, including IL-1β, IL-8, and MMPs (Aggarwal et al., 2006, Balkwill et al., 2005). IL-1β increases lung tumor growth by promoting the expression of several angiogenic factors in tumors and stromal cells (Balkwill et al., 2005). IL-8 has been reported to promote

growth and metastasis of several cancers including lung cancer, melanoma, and ovarian cancer (Aggarwal et al., 2006, Takahashi et al., 2010). Activation of K-ras in lung adenocarcinoma cells results in upregulation of IL-8. Autocrine production of IL-8 stimulates tumor inflammation, angiogenesis, and growth (Takahashi et al., 2010, Karin, 2005). Alveolar endothelial cell or TAM-induced MMP9 significantly promotes lung metastasis in tumor bearing mice dependent upon VEGFR-1/Flt-1 tyrosine kinase (VEGFR-1TK) (Hiratsuka et al., 2002). Reduced lung metastasis was also found in mice deficient in either MMP9 or VEGFR-1TK (Hiratsuka et al., 2002). MMP12 overexpression in lung epithelial cells induces emphysema to lung adenocarcinoma transition via upregulation of IL-6 and activation of STAT3 downstream of IL-6 (Qu et al., 2009).

1.1.3 Inflammation and tumor initiation

Carcinogenesis contains three stages: initiation, promotion, and progression, all are associated with inflammation (Fig. 1). Approximately 25% of cancer cases worldwide is associated with chronic infection and inflammation (Kundu and Surh, 2008). Growing evidence suggests that chronic inflammation induced by persistent stimulus might enhance tumor initiation, such as smoking, silica, asbestos, bacteria, and virus (Coussens and Werb, 2002). A strong association of chronic inflammation and tumor initiation has been shown in a model of colonic irritant dextran sodium sulphate (DSS) induced colitis. It was suggested that chronic inflammation can directly induce DNA damage and mutation and cause colitis-associated cancer (Meria et al., 2008). Various inflammatory cells infiltrate the site of infection or inflammation and induce DNA damage and genomic instability in proliferating cells through generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Karin and Greten, 2005). Some cytokines, such as TNFα, produced by inflammatory cells can induce ROS accumulation in epithelial cells (Grivennikov et al., 2010). It has been suggested that TNFα is a transforming agent and NO might be involved in the molecular mechanism of carcinogenesis (Balkwill and Mantovani, 2001). In *Helicobacter pylori* induced gastric and TNFα induced pulmonary carcinogenesis, a major biochemical hallmark is oxidative and mutagenic DNA damage (8-Oxo-7,8-dihydro-2'-deoxyguanosine) has been found (Coussens and Werb, 2002). Moreover, chronic inflammation induces oxidative damage and causes p53 mutation, resulting in p53 functional inactivation suggesting that chronic inflammation involves in tumor initiation (Balkwill and Mantovani, 2001).

Another mechanism through which inflammation might contribute to tumor initiation involves the up-regulation of activation-induced cytidine deaminase (AID). AID is an enzyme

induced by inflammatory cytokines in an NF-κB-dependent manner or by TGFβ, which induces genomic instability and increases mutation of critical tumor genes, such as Tp53, c-Myc, and Bcl-6. (Okazaki et al., 2007; Colotta et al., 2009). Furthermore, DNA damage induces cell death resulting in tumor initiation. In carcinogen diethylnitrosamine (DEN) induced hepatocellular carcinoma model, DEN causes hepatocyte DNA damage and contributes to necrotic cell death leading to inflammation and thereby promotes tumorigenesis (Maeda et al., 2005).

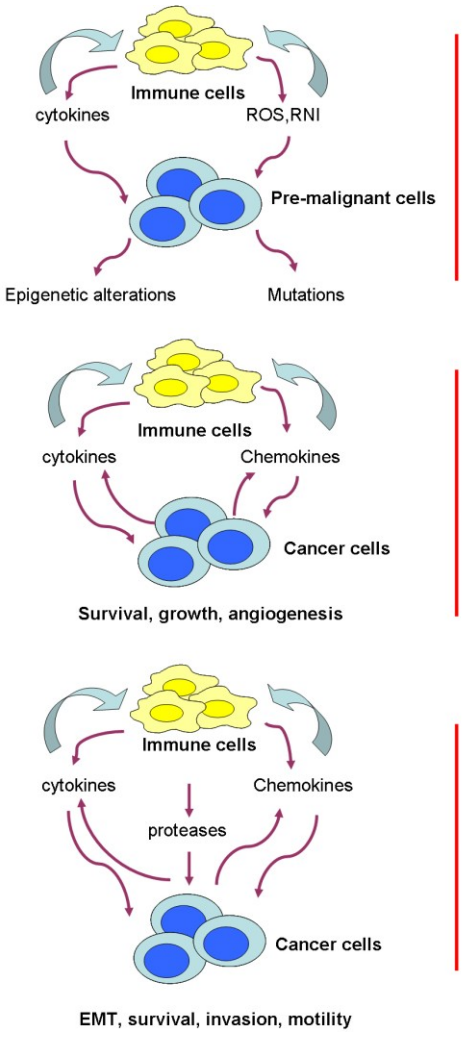


Figure 1: Role of inflammation in tumour carcinogenesis.
Inflammation acts at all phases of carcinogenesis. Reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) produced by immune /inflammatory cells contribute to tumor initiation by inducing DNA damage, mutations, genomic instability, and epigenetic modifications. During the second phase of carcinogenesis, cytokines and chemokines produced by immune and inflammatory cells increase tumur cells survival, growth and angiogenesis. As parts of positive feed-forward loops, cytokines and chemkines attract additional immune /inflammatory cells to the tumur microenvironment supporting tumur promotion. Finally, in the progression phase, immune cells further produce cytokines and chemkines to increase cancer cell survival, motility, invasion and epithelial mesenchymal transition (EMT) (adapted from Grivennikov and Karin, 2010).

1.1.4 Inflammation and tumor promotion

A single initiated cell undergoes proliferation to form a fully developed primary tumor, a process that is generally termed tumor promotion. Increased malignant cell proliferation and reduced malignant cell death result in tumor promotion, both are strongly modulated by various inflammatory mediators produced by immune cells from the tumor microenvironment

(Grivennikov and Karin, 2010a; Kundu and Surh, 2008). The tumor promoting effect of inflammation is well understood and inflammation is suggested to be implicated in tumor initiation. In animal models, the major enhancing effects of inflammation on tumors are exerted at the level of tumor promotion (Takahashi et al., 2010, Grivennikov et al., 2010). Inflammatory/immune cells are a major source of tumor promoting cytokines. The role of the cytokines in tumor promotion is more firmly established by several lines of evidence. In lung cancer models of carcinogen-treated and K-ras mutation mice, chronic and subacute inflammation induced by CS promote tumor proliferation through release of inflammatory cytokines by myeloid cells (Takahashi et al., 2010). Lipopolysaccharide (LPS) induced acute inflammatory responses in macrophages favour lung metastasis growth of colon adenocarcinoma cells (Luo et al., 2004). In a model of CAC, inactivation of NF-κB in myeloid cells decreased the production of inflammatory cytokines including IL-6, IL-11, TNFα, IL-1β, and IL-23, resulting in reduced tumor growth (Greten et al., 2004; Grivennikov et al., 2009; Grivennikov et al., 2010). In Mdr2-knockout mice, that develop cholestatic hepatitis followed by hepatocellular carcinoma, adjacent endothelial and inflammatory cells released TNFα contributes to tumor promotion in HCC (Pikarsky et al., 2004). The mechanisms by which inflammation induces tumor promotion involve acceleration of cell cycle progression and cell proliferation, evasion from apoptotic cell death, and stimulation of tumor neovascularization (Kundu and Surh, 2008).

Inflammatory mediators released by tumor infiltrating immune cells activate the oncogenic transcription factors NF-κB and STAT3 in cancer cells and induce the expression of a variety of target genes important for cell proliferation and survival. This is a major tumor-promoting mechanism (Grivennikov et al., 2010; Yoshimura, 2006). The NF-κB or STAT3 regulated target genes include proliferative genes (PCNA, Cyclins, c-Myc), anti-apoptotic genes (c-IAP, Bcl-xL, Bcl-2, c-FLIP), stress-response genes (SOD2, ferritin heavy chain, hsp70), chemokines, and pro-angiogenic molecules (VEGF, bFGF, CXCL12) (Luo et al., 2004; Grivennikov et al., 2010). On the contrary, NF-κB or STAT3 activation in immune cells controls the production of pro-inflammatory cytokines, including TNFα, IL-1, IL-6, and IL-23, which induce NF-κB and STAT3 activation in malignant cells (Karin, et al., 2005; Grivennikov and Karin, 2010b). Recent reports showed that IL-6 produced by lamina propria myeloid cells enhanced proliferation of tumor initiating cells and protects premalignant intestinal epithelial cells from apoptosis through transcription factor STAT3 (Grivennikov, et al., 2009). In addition, NF-κB and STAT3 also directly regulate the expression of some

important proangiogenic genes, such as IL-8, CXCL1, CXCL8, VEGF, and hypoxia-inducible factor1 alpha (HIF1α) in inflammatory/immune cells (Rius et al., 2008; Kujawski et al., 2008).

1.1.5 Inflammation and tumor progression

It has been suggested that promotion of tumor progression is another important effect of inflammation. TAMs are an important partner for tumor cell progression. They produce colony stimulating factor 1(CSF1), IL-6, IL-1, and TNFα resulting in tumor progression. Co-culture with macrophages by transwell inserts leads to increased invasive capacity of the tumor cells in a TNFα, NF-κB, and matrix metalloprotease dependent manner (Hagemann et al., 2005). Studies with CSF1 deficient of breast cancer transgenic mice showed that the incidence or growth of the primary tumors is normal, however, the tumors are unable to develop invasive, metastatic pulmonary carcinomas, and this was associated with an impaired infiltration of macrophages into the primary tumor (Lin et al., 2001).

Immune cells produced cytokines, chemokines, and proteases are generally considered to be responsible for tumor metastasis. For example, TGFβ, TNFα, IL-6, and IL-1 regulate the process of epithelial to mesenchymal transition (EMT) which is crucial for tumor progression. EMT is mediated via the production of various EMT regulators, such as Snail, Twist, and Kiss in STAT3 and NF-κB-mediated manner (Grivennikov et al., 2010; Yu et al., 2009). TNFα can increase vascular permeability, augment expression of adhesion molecules on endothelial cells, and facilitates tumor cells migration (Grivennikov et al., 2006; Balkwill and Mantovani, 2001). MMPs, such as MMP9 and MMP2, are produced by inflammatory /immune cells and by stromal cells and degrade the extracellular matrix and facilitate invasion and extravasation of tumor cells (Grivennikov et al., 2010). Chemokines direct the movement of tumor cells towards blood vessels and inflammatory cytokines, such as TNFα, IL-6, and IL-1β, possibly increase this effect through up-regulation of chemokine-receptor expression in malignant cells (Mantovani et al., 2008).

1.2 NF-κB and the role in cancer

NF-κB was identified in 1986 as a nuclear factor bund to the kappa immunoglobulin gene enhancer in B cells (Aggarwal, 2004). However, NF-κB has since been shown to be ubiquitously expressed in the cytoplasm of every cell type. NF-κB is a key coordinator of inflammatory and immune response and has recently been found to play a pivotal role in carcinogenesis of a number of cancers (Karin, 2006). It is noteworthy that the pro-inflammatory cytokines and chemokines mentioned above have been linked to carcinogenic

processes in humans and mice, and are regulated by the NF-κB pathway. These pro-inflammatory factors are mostly produced by inflammatory/immune cells, such as myeloid cells, and ultimately activate NF-κB transcription factor. In myeloid cells, NF-κB generally contributes to the expression of inflammatory mediators, cytokines, and growth factors. However, in tumor cells, NF-κB influences tumor initiation, promotion, and progression. Therefore, NF-κB has different roles in different cell types during tumor development.

1.2.1 NF-κB pathway

NF-κB is assembled by the dimerization of five homologous subunits: RelA (p65), c-Rel, RelB, NF-κB1 (p50/p105), and NF-κB2 (p52/p100) (Luo et al., 2005; Ghosh and Karin, 2002). These members share a highly conserved Rel homology domain (RHD) which is responsible for DNA binding, dimerization, and interaction with the NF-κB inhibitor κBα (IκBα) (Luo et al., 2005; Ghosh and Karin, 2002). A wide range of stimuli can activate NF-κB and more than 100 genes are induced on its activation. There are two distinct and evolutionarily conserved NF-κB signalling pathways described (Fig. 2). The classical pathway is normally triggered by a variety of inflammatory stimuli including microbial stressors, viral infections, and cytokines, such as LPS, TNFα, and IL-1 (Hoffmann and Baltimore, 2006). In resting stage of cell, most NF-κB are retained in the cytoplasm by IκBα. On activation, cell stimulation results in activation of the three subunit IκB kinase (IKK) complex consisting of two catalytic subunits (IKKα and IKKβ) and a regulator component (IKKγ/NEMO). The activated IKK complex then phosphorylates IκBα followed by ubiquitination and eventual proteasomal degradation. The liberated NF-κB dimers, mostly p50:RelA dimers, translocate to the nucleus where they modulate the transcriptional activation of several hundred target genes encoding cytokines, growth factors, cell adhesion molecules, and antiapoptotic proteins (Karin, 2006; Aggarwal, 2004; Yoshimura, 2006). The other pathway, the alternative pathway, is activated by B-cell-activating factor of TNF family, CD40, lymphotoxin-β, LPS, and latent membrane protein (LMP)-1 of Epstein-Barr virus (EBV) via selective activation of IKKα homodimers by the upstream kinase NIK (Perkins and Gilmore, 2006). Activation of IKKα homodimers results in phosphorylation of p100/NF-κB2 protein, inducing its proteolytic processing to p52, then p52:RelB dimers enter the nucleus (Perkins and Gilmore, 2006; Hoffmann and Baltimore, 2006). The classical pathway is responsible for innate immunity and inflammation and the alternative pathway is mostly involved in the generation of secondary lymphoid organs and B-cell maturation and survival

(Karin, 2006; Karin and Lin, 2002). Although both pathways of NF-κB have been implicated in tumor development, the classical pathway is likely more important for carcinogenesis (Karin and Greten, 2005; Naugler and Karin, 2008).

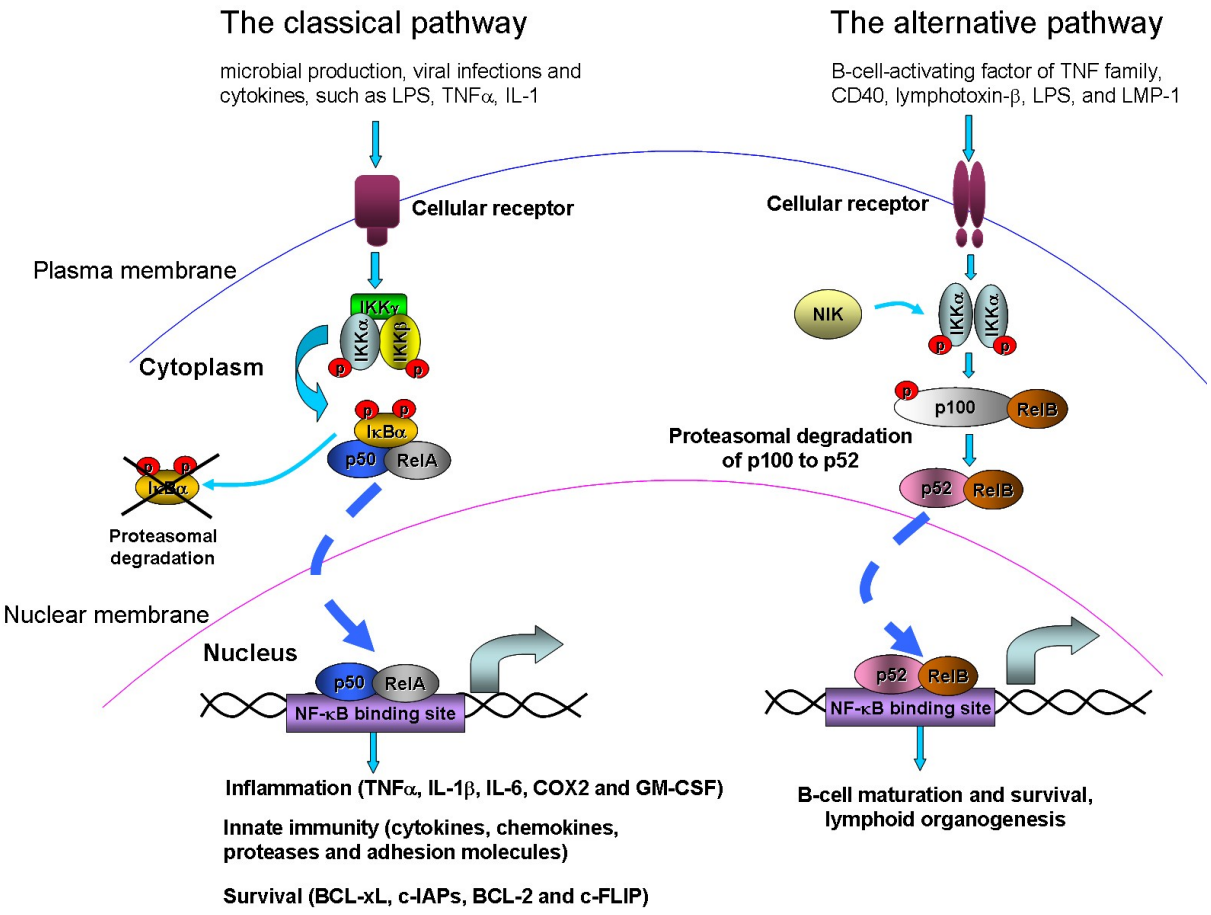


Figure 2: Two NF-κB signalling pathways.
The classical pathway is normally triggered by a variety of inflammatory stimuli including microbial products, viral infections, and cytokines. The activated IKK complex phosphorylates IκBα followed by ubiquitination and eventual proteasomal degradation. The liberated NF-κB dimers (p50:RelA dimmers) translocate to the nucleus where they modulate the transcriptional activation of several hundred target genes encoding cytokines, growth factors, cell adhesion molecules and antiapoptotic proteins. The other pathway, the alternative pathway, is activated by B-cell-activating factor of TNF family, CD40, lymphotoxin-β, LPS, and latent membrane protein (LMP)-1 of Epstein-Barr virus (EBV) via selective activation of IKKα homodimers by the upstream kinase NIK. Activation of IKKα homodimers results in phosphorylation of p100/ NF-κB2 protein inducing its proteolytic processing to p52, then p52:RelB dimers enter the nucleus. The alternative pathway is involved in the generation of secondary lymphoid organs and B-cell maturation and survival. Bcl-2, B-cell lymphoma 2; BCL-xL, B-cell lymphoma xL; cFLIP, cellular caspase-8 (FLICE)-like inhibitory protein; cIAPs, inhibitor of apoptosis protein; COX-2, cyclooxygenase-2; GM-CSF, granulocyte-macrophage colony stimulating factor; IκBα, inhibitor of κBα; IKK, IκB kinase; IL-1β, interleukin 1 β; IL-6, interleukin 6; LPS, lipopolysaccharide; TNFα, tumour necrosis factor α (adapted from Karin, 2006; Aggarwal, 2004; Yoshimura, 2006).

1.2.2 NF-κB pathway and tumor development

Constitutive activation of NF-κB has been observed in most tumor cells, both solid and hematologic tumors, including lung cancer, breast cancer, colon cancer pancreatic cancer, and various types of leukaemia (Naugler and Karin, 2008). Inhibition of NF-κB in these tumor cells results in abrogation of proliferation and in increased apoptosis indicating the crucial role of NF-κB in cell proliferation and survival (Aggarwal, 2004). Furthermore, sustained NF-κB activation in tumor associated inflammatory cells including myeloid cells has been reported (Karin et al., 2006, Grivennikov and Karin, 2010a). Based on many functions of the classical NF-κB pathway target genes, it has been proposed that the classical NF-κB pathway has a crucial role in inflammation caused tumor promotion and progression (Karin and Greten, 2005) (Fig. 3). It has been accepted that many pro-inflammatory cytokines produced by myeloid cells, such as TNFα, IL-6, and IL-1, are growth factors of tumors and regulated by NF-κB. On the contrary, these pro-inflammatory cytokines stimulate NF-κB activation and induce transcription of NF-κB target genes including cyclinD1, c-Myc, COX-2, Bcl-X_L, Bcl-2, and XIAP in malignant cells, which regulate cell proliferation, survival, angiogenesis, invasion, and metastasis (Grivennikov and Karin, 2010a; Naugler and Karin, 2008; Aggarwal et al. 2009) (Fig. 4).

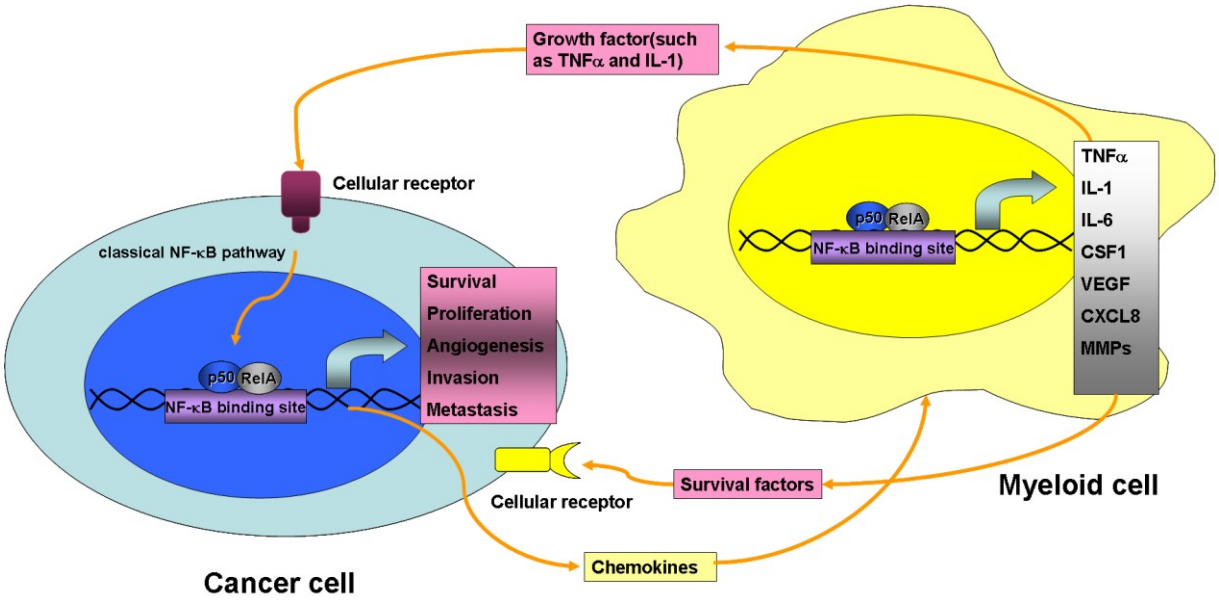


Figure 3: NF-κB activation has a crucial role in inflammation to tumour promotion and progression. Many pro-inflammatory cytokines and chemokines, such as TNFα, IL-6, and IL-1, are growth and survival factors of tumors and are produced by myeloid cells and other inflammatory cells in a NF-κB dependent manner. These pro-inflammatory cytokines and chemokines stimulate NF-κB activation and induce transcription of NF-κB target genes in cancer cells, which regulate cell proliferation, survival, angiogenesis, invasion, and metastasis. CSF1, colony stimulating factor 1; CXCL8, CXC-chemokine ligand 8, also known as IL-8; IL-1, interleukin 1; IL-6, interleukin 6; MMPs, matrix metalloproteinases; TNFα, tumour necrosis factor α; VEGF, vascular endothelial growth factor (adapted from Karin and Greten, 2005).

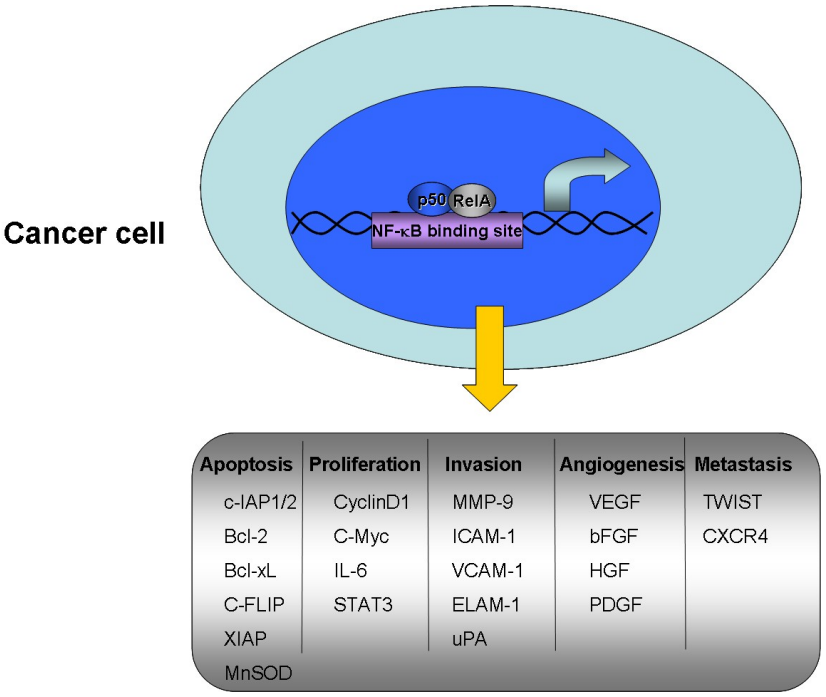


Figure 4: Target genes of NF-κB signalling pathway that mediate tumourigenesis.
Bcl-2, B-cell lymphoma 5; BCL-xL, B-cell lymphoma xL; bFGF, basic fibroblast growth factor; cFLIP, cellular caspase-8 (FLICE)-like inhibitory protein; cIAP1/2, inhibitor of apoptosis protein 1/2; CXCR4, CXC chemokine receptor-4; ELAM-1, endothelial cell leukocyte adhesion molecule-1; HGF, hepatocyte growth factor; ICAM-1, intercellular adhesion molecule-1; MMP-9, matrix metalloproteinase-9; MnSOD, manganese superoxide dismutase; PDGF, platelet-derived growth factors; STAT3, signal transducer and activator of transcription 3; uPA, urokinase-type plasminogen activator; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; XIAP, X-linked inhibitor of apoptosis protein (adapted from Grivennikov et al., 2010; Naugler and Karin, 2008; Aggarwal et al. 2009).

Numerous lines of evidence demonstrate a role of NF-κB in inflammation promoted tumor development. Karin's group found that IKKβ ablation (leading to decreased NF-κB activity) in myeloid cells reduces the CS induced inflammatory response in the lung and abrogates lung tumor development (Takahashi et al., 2010). In a metastatic colon cancer mouse model, LPS- induced expression of TNFα in host cell (most likely macrophages) resulting in NF-κB mediated growth of metastatic cancer in the lung, whereas abolished NF-κB in colon cancer cells resulted in tumor regression by TNF-related-apoptosis-inducing-ligand (TRAIL), also known as Apo2 ligand, is a type II transmembrane protein of the TNF family (Luo et al, 2004). In a mouse model of CAC, deletion of IKKβ in enterocytes and myeloid cells resulted in reduced tumor multiplicity and tumor size by different mechanisms. IKKβ deficiency in enterocytes resulted in a 80% decrease in tumor multiplicity without affecting tumor size and

increased apoptosis of enterocytes, suggesting NF-κB in enterocytes contributes to early stages tumor initiation and/or promotion (Greten et al., 2004). When IKKβ was deleted in myeloid cells, tumor size was considerably smaller compared to controls and expression of pro-inflammatory cytokines, such as TNFα, IL-6, and IL-1, was also markedly reduced (Greten et al., 2004). However, deletion IKKβ in myeloid cells had no effect on azoxymethane (AOM)-induced tumor apoptosis. Thus in myeloid cells, NF-κB activation promotes tumor growth. This effect is mainly due to enhanced tumor cell proliferation via the production of TNFα, IL-6, and other cytokines (Greten et al., 2004; Grivennikov and Karin, 2010a). In a mouse model of diethylnitrosamine (DEN) induced hepatocellular carcinoma (HCC), deletion of the gene encoding IKKβ in hepatocytes resulted in a remarkable increase in the number of tumors as well as in tumor size, growth rate and aggressiveness. However, simultaneous deletion of IKKβ in both hepatocytes and Kupffer cells (liver macrophage) resulted in the reduction of number and size of HCC tumors. In this case, NF-κB inactivation in hepatocytes increased DEN-induced cell apoptosis or necrosis and resulted in hepatocytes compensatory proliferation. Compensatory proliferation is mainly due to production of pro-inflammatory cytokines (including TNFα, IL-6) by NF-κB activation in Kupffer cells that respond to necrotic hepatocyte, and ablation of IKKβ prevent induction of these cytokines after DEN administration resulting in a remarkable decrease in tumor load (Maeda et al., 2005; Naugler et al., 2007; Karin and Greten, 2005). Additionally, Umar and colleagues found NF-κB mediated hyperproliferative effects of progastrin on proximal colonic crypts by enhanced Wnt/β-catenin signaling (Umar et al., 2009).

NF-κB also regulates tumor metastasis and angiogenesis by modulating the transcription of relevant gene products (Aggarwal, 2004). For example, some critical proteases involved in tumor invasion and metastasis, such as MMP9, MMP2, and urokinase type of plasminogen activator (uPA), are regulated by NF-κB (Aggarwal, 2004, Naugler and Karin, 2008). Metastasis requires various adhesion molecules including ICAM-1, VCAM-1, and ELAM-1, which are regulated by NF-κB (Naugler and Karin, 2008). As described above, EMT is crucial for metastasis. In a breast cancer model, NF-κB activation was implicated in EMT and its target genes included E-cadherin, Bcl2, MMP2/9, VCAM-1, and ELAM-1 were found responsible for this action (Chua et al., 2007, Wang et al., 2007, Naugler and Karin, 2008). TAMs promote tumor progression by increasing the formation of new blood vessels, which is dependent on NF-κB signalling (Karin and Greten, 2005). In TAMs, the NF-κB regulated inflammatory cytokines TNFα, IL-6, and IL-1 can up-regulate COX2 expression by NF-κB

activation. This leads to hypoxia-inducible factor 1 α (HIF1α) expression resulting in VEGF production (Jung et al., 2003).

1.3 Wnt/β-catenin pathway and cancer

The Wnt family of proteins constitutes 19 secreted cysteine-rich glycoproteins that not only play a critical role in the embryonic development and maintenance of homeostasis in mature tissue but also in cellular proliferation, differentiation, motility, and survival and /or apoptosis. Wnt ligands bound to their receptor activate multiple signalling pathways including the canonical Wnt/β-catenin pathway, the noncanonical planar cell polarity pathway, the Wnt/Ca^{2+} pathway, and the protein kinase A pathway (Takahashi-Yanaga and Kahn, 2010; Neumann et al., 2010). Of these four, the canonical Wnt/β-catenin pathway is by far the best understood (Fig. 5). The activity of the Wnt/β-catenin pathway is dependent on cytoplasmic β-catenin protein. In the absence of Wnt signalling, cytoplasmic β-catenin is phosphorylated on several serine and threonine residues by the destruction complex containing glycogen synthase kinase-3β (GSK3β), casein kinase1 (CK1), axin and adenomatous polyposis coli (APC). Phosphorylation leads to ubiquitin-proteasome-mediated degradation of β-catenin. Binding of Wnt ligands to Frizzled and to low-density lipoprotein receptor-related protein (LRP) receptors leads to activation of the phosphoprotein Dishevelled (Dsh or Dvl) and the inhibition of the destruction complex including Axin, GSK3β, which reduces the phosphorylation and degradation of β-catenin. Nonphosphorylated β-catenin translocates into the nucleus where it binds to T cell factor/lymphocyte enhancer factor (TCF/LEF) family transcription factors and modulates expression of a broad range of target genes, such as cyclinD1 and c-Myc (Moon et al., 2004; Reya and Clevers, 2005; Fodde and Brabletz, 2007).

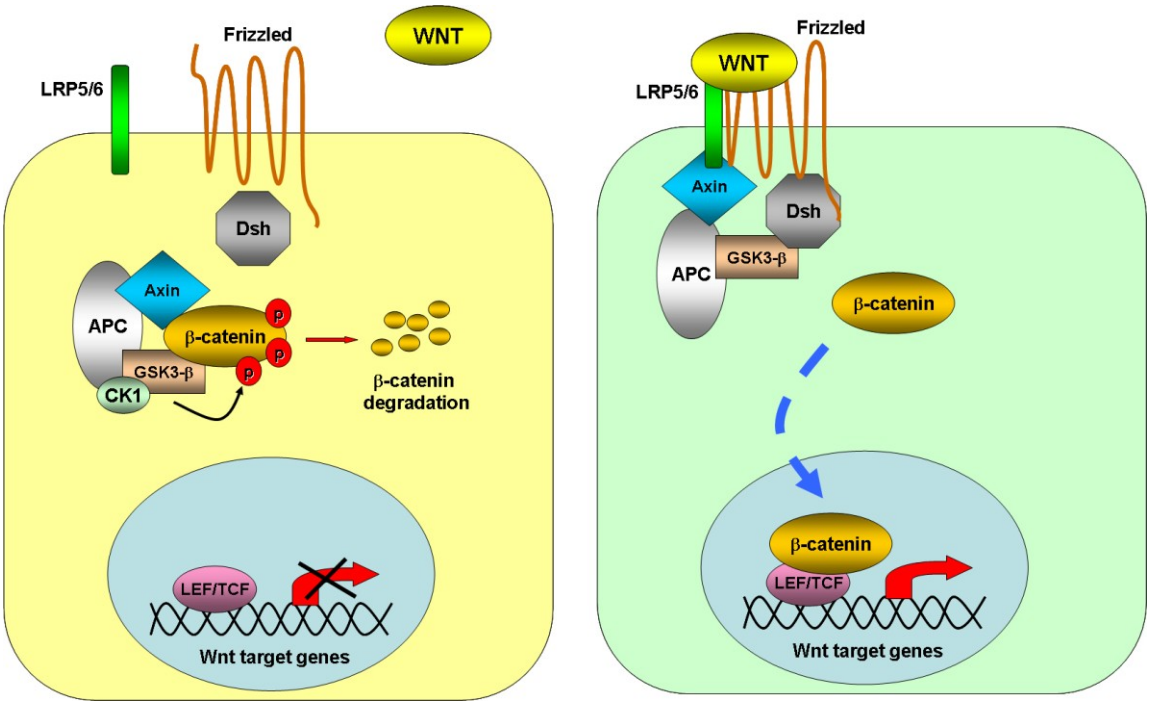


Figure 5: the canonical Wnt/β-catenin pathway.
In the absence of Wnt signalling, cytoplasmic β-catenin is phosphorylated on several serine and threonine residues by the destruction complex containing glycogen synthase kinase-3β (GSK3β), casein kinase1 (CK1), Axin and adenomatous polyposis coli (APC) leading to ubiquitin-proteasome-mediated degradation. Binding of Wnt ligands to Frizzled and low-density lipoprotein receptor-related protein (LRP) receptors leads to activation of the phosphoprotein Dishevelled (Dsh or Dvl), and the inhibition of destruction complex including Axin, GSK3β, which reduces the phosphorylation and degradation of β-catenin. Nonphosphorylated β-catenin translocates into the nucleus where it binds to T cell factor/lymphocyte enhancer factor (TCF/LEF) family transcription factors and modulate expression of a broad range of Wnt target genes (adapted from Moon et al., 2004; Reya and Clevers, 2005; Fodde and Brabletz, 2007).

The Wnt/β-catenin signalling pathway is essential to lung morphogenesis. However, recent research showed that its activation is involved in the initiation and promotion phase of lung tumorigenesis (Moon et al., 2004; Konigshoff and Eickelberg, 2010). Although mutations in key Wnt signalling gene, such as β-catenin or APC, are rare in lung cancer, activation of Wnt/β-catenin signalling pathway has been found in various non-small cell lung cancer (NSCLC) cell lines and lung cancer (Licchesi et al., 2008; Retera et al., 1998). Overexpression of cyclinD1 and COX2 in lung adenocarcinomas suggests that the Wnt/β-catenin signalling pathway may be constitutively active (Licchesi et al., 2008). In NSCLC, overexpression of the intracellular signal transducer Dsh increased β-catenin expression and promoted TCF-dependent transcriptional activity in Wnt/β-catenin signalling pathway resulting in increased tumor cell growth (Uematsu et al., 2003; Konigshoff and Eickelberg,

2010). Hommura and colleagues examined specimens of 217 surgically resected primary NSCLC and found that increased β-catenin expression is associated with enhanced cell proliferation and a better prognosis (Hommura et al., 2002). Activation of Wnt/β-catenin signalling is also found in colon cancer, gastric cancer, skin cancer, and breast cancer, and Wnt activation has been demonstrated to be a major cause for the development of these cancers (Oguma et al., 2008; Fodde and Brabletz, 2007; Clevers, 2006). Although the constitutive activation of the Wnt/β-catenin pathway by mutations in APC or the β-catenin gene contributes to many tumors tumorigenesis, accumulating evidences suggests that further promotion of the Wnt/β-catenin pathway activity has an important function in tumor growth and progression (Fodde and Brabletz, 2007; Oguma et al., 2008). For example, increased nuclear entry of β-catenin in colon and gastric cancer cells promotes tumor cell growth (Castellone et al., 2005; Oguma et al., 2008). Moreover, in the invasive front of colon cancer, increased nuclear β-catenin accumulation is also observed (Brabletz et al., 1998).

Wnt/β-catenin pathway activation has been implicated in inflammation associated cancer. It has been reported that macrophages increase Wnt/β-catenin signalling activity in gastrointestinal tumor cells through production of some inflammatory cytokines such as TNFα and IL-1β, which contributes to tumors growth (Oguma et al., 2008; Kaler et al., 2009). For example, in gastric tumor cells, macrophage-derived TNFα promotes Wnt/β-catenin signalling through inhibition of GSK3β, whereas in colon cancer cells, macrophage-derived IL-1β acts same function (Oguma et al., 2008; Kaler et al., 2009). Castellone et al. also provided evidence that inflammation directly influences the mutationally activated Wnt/β-catenin signalling, which is responsible for growth of colorectal cancer (Castellone et al., 2005). They found that inflammatory prostaglandin E2 stimulates colon cancer cell growth through enhanced accumulation of β-catenin in the nucleus. This resulted in enhanced activation of Wnt/β-catenin signalling, already activated to some degree in these cells (owing to the mutant APC gene) (Castellone et al., 2005). Although the mechanistic details of above reports remain to be addressed, a clear link among inflammation, Wnt/β-catenin signalling and growth of tumor cells is established.

1.4 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are key effectors of the innate immune system with direct antimicrobial function. AMPs further act as inflammatory mediators and modulate processes such as wound repair, cell proliferation, and angiogenesis (Zasloff, 2002; Wah et al., 2006).

According to size, tertiary structure or predominant amino acids sequence, there are two main families of AMPs in human and other mammals: the defensins and the cathelicidins. They are expressed in immune cells and at epithelial surfaces (Chromek et al., 2006; Herr et al., 2007).

1.4.1 Defensins

Defensins are a family of cysteine-rich, cationic peptides, which includes two major subgroups: α-defensins and β-defensins, classified by the pattern of disulfide bonding and each subfamily consist of several members (Coffelt and Scandurro, 2008). α-defensins 1-4 are located in neutrophil primary (azurophilic) granules and are also called human neutrophil peptides (HNP1-4). Human α-defensin 5 (HD5) and 6 (HD6) are primarily secreted from Paneth cells of the small intestine (Herr et al., 2007). Generally, β-defensins are expressed by epithelial cells, macrophages and lymphocytes, and consists of a variety of members, such as human β-defensin1 (hBD-1), human β-defensin 2 (hBD-2), human β-defensin3 (hBD-3), and human β-defensin4 (hBD-4) (Beisswenger et al., 2005). Moreover up to 28 new human and 43 new mouse β-defensin genes in 5 syntenic chromosomal regions have been identified by computer-based screening of the human and murine genomes (Beisswenger et al., 2005).

The expression of defensins in the lung can be induced by pro-inflammatory stimuli or microorganisms. They directly kill microbes and disrupt their cell membranes, resulting in increased permeability and leakage of small molecules (Herr et al., 2007). Additionally, defensins also have other biological functions. For example, in bronchial epithelial cells, α-defensins-induced IL-8 increases inflammatory reaction and subsequent recruitment of neutrophils (Van et al., 2002). Moreover, α-defensins bind to protease inhibitors such as α1-antitrypsin, and inactivate their antiprotease activity, which increase inflammation (Panyutich et al., 1995). Furthermore, defensins also are chemoattractants for leukocytes and other immune cells (Herr et al., 2007). Several evidences display that the defensins involve in cancer process, and have different effects to tumor development. In prostate cancer and renal clear cell carcinoma samples, the expression of hBD-1 almost is suppressed, but followed by reintroduction of hBD-1 to prostate and renal cancer cells lines, proliferation is inhibited and apoptosis is induced (Sun et al., 2006; Bullard et al., 2008). However, a study found that murine β-defensin 29 (mBD-29), the homologue of human hBD-2, has a proangiogenic, protumorigenic founction (Conejo-Garcia et al., 2004). This study suggest mBD-29 and vascular endothelial growth factor-A (Vegf-A) cooperate to promote mouse ovarian tumor growth by accumulation of dendritic cell precursors (Conejo-Garcia et al., 2004).

1.4.2 Cathelicidin

Human cathelicidin antimicrobial protein LL-37/hCAP18 is the only known cathelicidin in man (Beisswenger et al., 2005). LL-37/hCAP18 is constitutively produced by professional host defense cells, such as macrophages or neutrophils, by epithelial cells of the skin, gastrointestinal tract and urinary tract, by the epididymis and by respiratory epithelial cells (Shaykhiev et al., 2005; Chromek et al., 2006). hCAP18 consists of 3 domains: the N-terminal signal peptide, the highly conserved cathelin-like domain and the C-terminal peptide termed LL-37 peptide (Coffelt and Scandurro, 2008). LL-37 peptide is maintained in its pro-peptide form until secretion after cleavage by protease 3 (Coffelt et al., 2008; Beisswenger et al., 2005). In mice, the gene encoding for cathelicidin is called *Cnlp*. Cnlp is very similar to the human gene. The mouse cathelicidin is termed cathelicidin related antimicrobial peptide (CRAMP) showing several close similarities to human LL-37 including an α-helical structure, antimicrobial function, and analogous tissue distribution (Kurosaka et al., 2005; Chromek et al., 2006; Koczulla et al., 2003).

A variety of stimuli found in infection and inflammation, such as pro-inflammatory cytokines, growth factors, nutrients, and bacterial products, as well as injury can up-regulated expression of LL-37/hCAP18 (Bucki et al., 2010). It has been shown that the expression of LL-37/hCAP18 is increased in the airways of patients with inflammatory and infectious lung diseases (Shaykhiev et al., 2005). In human myeloid cells, 1,25-dihydroxyvitamin D_3 strongly stimulates the expression of LL-37/hCAP18 gene via the vitamin D receptor (Gombart et al., 2005; Liu et al., 2006). However, induction of CRAMP in murine cells has not been observed as there is no vitamin D response element (VDRE) in the CRAMP gene promoter (Gombart et al., 2005). Liu et al., reported that Toll-like receptor activation of human macrophages results in up-regulated expression of the vitamin D receptor and 25-hydroxyvitamin D-1-a-hydroxylase (CYP27B1). This led to the induction of LL-37/hCAP18 and killing of intracellular mycobacterium tuberculosis (Liu et al., 2006). The NF-κB signalling pathway also plays an important role in the regulation of cathelicidin. Inhibition of NF-κB by siRNA or over-expression of IκBα reduced both basal and induced levels of CRAMP mRNA in murine mast cells (Li et al., 2009). In macrophages, activation of NF-κB is also important for LL-37-mediated cytokine release (Mookherjee et al., 2009). HIF1α is also involved in up-regulation of LL-37/hCAP18 in myeloid cells and keratinocytes (Peyssonnaux et al., 2008).

Beyond its activity against microorganisms, LL-37/hCAP18 is involved in various processes in different immune reactions, including immune modulation, inflammatory reaction, cell proliferation, angiogenis, and antiapoptosis (Bucki et al., 2010). In various

inflammatory lung diseases, LL-37 is a regulator of immune responses by stimulation of IL-8 production in human airway smooth muscle cells (Zuyderduyn et al., 2006). In human bronchial epithelial cells, LL-37 enhances cellular activation by LPS resulting in increased release of the inflammatory cytokines IL-6 and IL-8 (Shaykhiev et al., 2010). LL-37 can bind to formyl peptide receptor-like 1 (FPRL1), a promiscuous receptor expressed on a variety of cells including neutrophils, monocytes, and lymphocytes, and attracts neutrophils, monocytes and $CD4^+$ T cells, and activates mast cells (Herr et al., 2007). Moreover, LL-37 induces wound healing, cell proliferation, and migration of airway epithelial cells, suggesting that this peptide is likely involved in the regulation of tissue homeostasis in the airways (Shaykhiev et al., 2005). Deficiency of CRAMP in mice resulted indecreased vascularization during wound repair indicating important action of cathelicidins during angiogenesis (Koczulla et al., 2003).

A growing body of evidence indicates that LL-37/hCAP18 is related to tumorigenesis in prostate, breast, ovarian, and lung cancers (Bucki et al., 2010, Hensel et al., 2010). For example, LL-37 is strongly expressed in human lung cancer and treatment with synthetic biologically active LL-37 peptide or transgenic expression LL-37 shows a significant increase in the proliferation of lung tumor cells. This research suggests that LL-37acts as growth factor for human lung cancer (Von Haussen et al., 2008). The pro-inflammatory peptide LL-37 also contribute to ovarian proliferation, invasion, and progression through direct stimulation of tumor cells, initiation of angiogenesis and recruitment of immune cells (Coffelt et al., 2008; Coffelt et al., 2009). Additionally, LL-37 also strongly expressed in breast cancer and it is considered to be a growth factor for epithelial cells (Heilborn et al., 2005). A recent report showed that CRAMP knock-down in a murine prostate cancer model results in decreased proliferation and invasion of tumors, which involved Erk1/2 and Akt singnalling (Hensel et al., 2010). Accordingly, these findings support the hypothesis that LL-37 is a growth factor of tumor cell.

1.5 Cigarette smoke and lung cancer

Lung cancer is the most common cause of cancer mortality in the world, causing more than one million deaths worldwide (Walser et al., 2008). In the European Union (EU), lung cancer accounts for more than 25% or 12% of total cancer deaths in men or women, respectively (Malvezzi et al., 2011). Although lung cancer death rates have a decliny trend in men since the late 1980s in the EU, lung cancer mortality in women has increased up to 2007, and the upward trend is likely to continue until 2011(Levi et al., 2004; Malvezzi et al., 2011). Lung cancer is divided into two broad categories: Small cell lung carcinoma (SCLC) and non-small

cell lung cancer (NSCLC) (Webb et al., 2010, Sun et al., 2007). SCLC is the less common form of lung cancer, making up approximately 15% of lung cancer cases, which has neuroendocrine characteristics and are a highly malignant tumor type (Webb et al., 2010). Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases and are further classified into three major types: adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (Herbst et al., 2008). Among lung cancers, pulmonary adenocarcinoma is the predominant histological type and represents approximately 31% of lung cancers (Beadsmoore et al., 2003).

Cigarette smoke (CS) is the most important risk factor for lung cancer, which is associated with almost 90% of case in men and 70 to 80% of case in women (Jemal et al., 2003; Walser et al., 2008). Clinical studies have shown that smokers have as much as 30-fold increased risk of developing cancer relative to nonsmokers (Walser et al., 2008), and even after smoking cessation, the relative risk never returns to that of a non-smoker during the first 5 years (Hecht et al., 1999). Multifocal premalignant lesions have been found in bronchial tree of smokers and as in lung cancer patients, bronchial epithelium of smokers displays multistage foci of genetic changes which contribute to lung cancer risk (Wistuba et al., 1997, Yashima et al., 1997). Furthermore, by high-density gene expression arrays analysis, gene changes have been found even in histological normal airway epithelial cells of smokers (Spira et al., 2004, Spira et al., 2007). Tumor-promoting effect of CS exposure has been identified in a mice model of Lewis lung cancer and chemically or genetically initiated lung cancers (Zhu et al., 2003, Takahashi et al., 2010). CS has been also found to activate prometastatic oncogene synuclein-γ (SNCG) and may contribute to lung cancer progression (Liu et al., 2007). CS is a complex mixture and contains over 4000 identified chemical agents including over 60 carcinogens: polycyclic aromatic hydrocarbons (PAHs), N-nitrosamines and aromatic amines are the strongest carcinogens (Dasgupta et al., 2009; Takahashi et al., 2010; Hecht et al., 2002). For example, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) are known to form DNA adducts that mutate vital growth regulatory genes like p53 and Ras, initiate lung cancer (Dasgupta et al., 2009, Hecht et al., 2002). In addition, nicotin which is a major component of CS has been shown to promote angiogenesis and to accelerate tumor growth and atherosclerosis (Zhu et al., 2003, Villablanca et al., 1998, Dasgupta et al., 2006). Moreover, Dasgupta and colleagues found that nicotine induces cell proliferation, invasion and EMT mediated by the nicotinic acetylcholine receptors (nAChRs) and that it inhibits apoptosis induced by chemotherapeutic drugs by up-regulation XIAP and survivin proteins expression in lung cancer cells (Dasgupta et al., 2006, Dasgupta et al., 2009).

It is understood that CS exposure increases the pulmonary inflammatory response. Long time or short time CS exposure of mice increases recruitment of inflammatory cells (macrophages, neutrophils, lymphocytes and dendritic cells), activates the NF-κB pathway and induces production of inflammatory mediators (TNFα, IL-6, IL-8) (D'hulst et al., 2005; Vlahos et al., 2006; Takahashi et al., 2010). Some evidences indicated that chronic and subacute inflammation induced by CS is also clearly linked to cancer development (Yoshimura, et al., 2006; Takahashi et al., 2010). Chronic obstructive pulmonary disease (COPD) which is characterized by abnormal inflammatory features in the lung and systemically is mostly caused by cigarette smoke and is associated with the greatest risk for lung cancer (Walser et al., 2008, Yang et al., 2008; Punturieri et al., 2009). From clinical investigation it is known that the presence of COPD in smokers is associates with a 1.3 to 4.9 fold increased risk of lung cancer compared to smokers without COPD (Moghaddam et al., 2008). Moghaddam and coworkers used products of nontypeable *Haemophilus influenzae* which colonizes the airway of patients with COPD to demonstrate that COPD-like airway inflammation promotes lung carcinogenesis in a background of a G12D-activated K-ras allele in airway secretory cells (Moghaddam et al., 2008). Subacute exposure to second hand smoke promotes tumor angiogenesis and growth in a murine model of Lewis lung cancer which is associated with increased plasma levels of angiogenic cytokines including VEGF and monocyte chemoattractant protein-1 (MCP-1) as well as circulating endothelial progenitor cells (Zhu et al., 2003). Takahashi et al. found that CS induced chronic and subacute inflammatory responses increase the proliferation of lung tumor cells and alveolar epithelial cells in the lungs of mice (Takahashi et al. 2010).

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

β-Mercaptoethanol	Roth, Karlsruhe, Germany
Polyacrylamide gels (37.5:1)	Roth, Karlsruhe, Germany
APS	Sigma, Steinheim, Germany
Bromophenol blue	Fluka, Buchs (CH) , Germany
BSA	Calbiochem, Darmstadt, Germany
Glycerin	Roth, Karlsruhe, Germany
Glycin	Roth, Karlsruhe, Germany
H_2SO_4	Roth, Karlsruhe, Germany
HCl	Roth, Karlsruhe, Germany
Methanol	Center of chemical storage, University of Saarland
NaCl	Roth, Karlsruhe, Germany
NaH_2PO_4. H_2O	Merck, Darmstadt, Germany
Na_2HPO_4	Fluka, Buchs (CH), Germany
NaOH	AppliChem, Darmstadt, Germany
Paraformaldehyde	Roth, Karlsruhe, Germany
Protein Standard (SeeBlue® Plus2)	Invitrogen, Carlsbad, CA, USA
Skim milk	Roth, Karlsruhe, Germany
SDS	Roth, Karlsruhe, Germany
TEMED	Sigma, Steinheim, Germany
Tris base	Sigma, St. Louis, MO,USA
Tween® 20	AppliChem, Darmstadt, Germany
Citric acid monohydrate	Roth, Karlsruhe, Germany
Ethanol	Roth, Karlsruhe, Germany
Recombinant human TNFα	Invitrogen, San Diego, CA, USA
Recombinant human IL-1β	eBioscience, San Diego, CA, USA
Recombinant human IFNγ	eBioscience, San Diego, CA, USA
Recombinant human biglycan	R&D systems, Minneapolis, MN, USA
Recombinant human EGF	Sigma, Steinheim, Germany
Bay 11-7085	Sigma, Steinheim, Germany

6-Amino-4-(4-phenoxyphenylethylamino) Quinazoline	Merck, Darmstadt, Germany
Pyrrolidine dithiocarbamate	Sigma, Steinheim, Germany
Pam2CSK4	Invivogen, San Diego, CA, USA
$1,25(OH)_2D_3$	Calbiochem, Darmstadt, Germany

2.1.2 Buffer

- 10×TBS buffer:

Tris base	24.2g
NaCl	80g
Water	1000ml
Adjust pH to 7.6	

- TBS-T buffer: TBS containing 0.1% Tween-20

- SDS-polyacrylamide gels:

Running Gel Solution

Concentration (%)	Total volume (ml)	Distilled water (ml)	30%Polyacrylamide (ml)	1.5M Tris-HCl, pH 8.8 (ml)	10% SDS (μl)	10%APS (μl)	TEMED (μl)
10	10	4	3.3	2.5	100	100	5
12	10	3.3	4	2.5	100	100	5

Stacking Gel Solution

Concentration (%)	Total volume (ml)	Distilled water (ml)	30%Polyacrylamide (ml)	1M Tris-HCl, pH 6.8 (ml)	10% SDS (μl)	10%APS (μl)	TEMED (μl)
5	5	3.4	0.83	0.63	50	50	5

- 2× Loading buffer:

100mM Tris-HCl, pH 6.8
4% SDS
0.2% bromophenol blue
20% glycerine
5% β-mercaptoethanol

- 5×SDS-PAGE running buffer:

Tris base	15.1g
Glycin	94.0g
SDS	5g
Water	1000ml

- SDS-PAGE transfer buffer:

Tris base	5.8g
Glycin	2.9g
SDS	0.37g
Methanol	200ml
Water	1000ml

- 5% Skim milk

Skim milk	5g
TBS	100ml

- 10x Citrate buffer

Citric acid monohydrate, pH 6.0	21g
Water	1000ml

- 4 % Paraformaldehyde fixative (0.1 M. Sodium Phosphate Buffer, pH 7.2):

0.2M sodium phosphate buffer, pH 7.2:	Sodium phosphate monobasic ($NaH_2PO_4.H_20$, FW 137.99)	3.17g
	Sodium phosphate dibasic (Na_2HPO_4, FW 141.96)	10.93g
	Water	500ml
8% Paraformaldehyde solution:	Paraformaldehyde	40 g
	Water	500ml

Heat the paraformaldehyde solution to 60 - 65°C while stirring. Reduce heat and add 2 to 3 ml of 1.0 M NaOH, dropwise. After the solution has cleared, filter and add an equal volume of 0.2 M phosphate buffer. This fix is best used fresh and should be used within 72 h.

- 2% BSA

BSA	2g
TBS-T	100ml

2.2 Methods

2.2.1 Cell culture

The human lung adenocarcinoma cell line A549 cells, mouse Lewis lung carcinoma (LLC) cells, human monocytic cell line U937 cells, and mouse macrophage cell line RAW264.7 cells were cultured in Dulbeccos's modified Eagles medium (DMEM) (Invitrogen, Grand Island, NY, USA). The medium was supplemented with 10% fetal calf serum (FCS) (Invitrogen, Grand Island, NY, USA), 100 U/mL penicillin and 100U/mL streptomycin (PAA Laboratories GmbH, Pasching, Austria). Cell culture was performed at 37°C in humidified air with 5% CO_2.

2.2.2 Generation of human peripheral blood monocyte-derived macrophages

Buffy coats of healthy donors were obtained from the blood bank of the University hospital of Marburg. Buffy coats (about 7.5 ml) were diluted 1:2 with PBS (PAA Laboratories GmbH, Pasching, Austrlia). The diluted blood was gently overlaid on top of a 20 ml Ficoll-Paque (GE Healthcare, Uppsala, Sweden) in a 50 ml conical tube and centrifuged at 500 g for 30 min. at room temperature (RT) and stopped without braking. The layer containing white blood cells was recovered and transfered to a new 50 ml conical tube. 40 ml PBS were added and the cells were centrifuged at 400 g for 10 minutes at RT. The supernatant was discarded and 10 ml RBC lysis buffer were added (155 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM Na_2EDTA; sterile filtered) to the pellet. The pellet was completely resuspended and incubated for 5 minutes at RT and occasionally mixed gently. 30 ml PBS were added and the cells were centrifuged at 200 g for 5 minutes at RT. The supernatant was discarded and the pellet was washed with 40 ml PBS followed by centrifugation at 200 g for 5 minutes at RT. The supernatant was discarded and the cells were resuspended in 30 ml DMEM + 1% heat–inactivated human serum. The sample was divided in 1×10^6 ml to 75 cm^2 tissue culture flasks. Cells were allowed to adhere to culture flasks for 1 h at 37°C, 5% CO2 and non-adherent cells were removed by vigorous washing 2-3 times with warm PBS. Adherent cells were cultured in 20 ml DMEM (10% FCS) supplemented with 50 ng/ml macrophage colony stimulating factor (M-CSF) (eBioscience, San Diego, CA, USA) for 7 days to allow differentiation to macrophages.

2.2.3 Monocytic differentiation

U937 cells were treated with 10nM phorbol-12myristat-13acetat (PMA) (Sigma Aldrich, Steinheim, Germany) for 48 hour resulting in adherence of the cells to culture flasks. After 48 hours, cells were washed and cultured for additional 24 hours in PMA-free DMEM (10% FCS) in order to reduce possible effects of residual PMA. Cells were detached by incubation with 0.05% (v/v) trypsin-EDTA (Invitrogen, Grand Island, NY, USA) for 5 mins. Cells were enumerated by using a hemocytometer (Innovatis AG, Reutlingen, Germany) and seeded in multi-well cell culture plate in indicated number for further experiment.

2.2.4 Cell transduction

For establishing cell lines with stable knock down of RelA/p65, the pSuppressorNeo (Imgenex, San Diego, CA, USA) RNA interference (RNAi) plasmids RelA were constructed according to the protocols previously described (Takada et al., 2003). siRNA sequences for *RelA* gene were:CCCCTTCCAAGTTCCTATA and TATAGGAACTTGGAAGGGG. siRNA sequences of mock RNAi plasmids: GCGCGCTTTGTAGGATTCG and CGAATCCTACA AAGCGC GC.

Log growth U937 cells were washed 1 time with PBS and resuspended at 2×10^7 cells/ml in 1 ml of Gene Pulser electroporation buffer reagent (Bio-Rad, Hercules, CA, USA), mixed with 20 μg of RelA RNAi plasmids or mock RNAi plasmids. Electroporations were performed using a Gene-Pulser (Bio-Rad, Hercules, CA, USA) at 280V and 960 μF in 0.4 cm cuvette (Bio-Rad, Hercules, CA, USA). The samples were transferred to culture flasks containing complete DMEM medium with10% FCS in 25 cm^2 and incubated at 37°C in 5% CO_2. 48 hours later, the growth medium was changed and G418 (PAA Laboratories GmbH, Pasching, Austria) was added at a concentration of 800 μg/ml. The culture medium was changed every 4 days using fresh growth medium (containing 800μg/ml G418). After 4 weeks, positive polyclonal populations (pools) were identified based on western blot analysis for p65 expression. Individual positive clones were eventually isolated by limiting dilution analysis in 96-well plates.

2.2.5 Preparation of cigarette smoke extracts (CSE)

CSE was prepared by bubbling smoke from 2 3R4F reference cigarette into 20 ml of serum-free DMEM at a rate of 1 cigarette/min. This medium (defined as 100%) was adjusted to pH 7.4, and was sterile filtered with a 0.2 μm filter. CSE stock solutions were frozen in aliquots at -80°C.

2.2.6 Co-culturing lung cancer cells and macrophages

Cells were rendered quiescent by serum starvation for 24 h. For co-culture studies with A549 cells and macrophages (human primary macrophage or PMA-U937 cells), macrophages were seeded into the bottom of mutil-well cell culture plates and A549 cells were placed in transwell inserts (0.4 um, 6 wells from Dickinson and Company, Franklin Lakes, NJ, USA or 12 or 24wells from Corning Incorporated, Corning, NY, USA) with a membrane permeable for liquids but not for cells. Cells were incubated overnight. The transwells were inserted into the well of mutil-well culture plate and cultured for indicated time. For CSE stimulation, co-cultures, macrophages, and A549 cells were stimulated with indicated concentration of CSE. For inhibition of the NF-κB pathway, the NF-κB inhibitors Bay 11-7085, QNZ and PDTC were added at various concentrations as indicated 1-2 hours before co-culture and/or CSE stimulation. For TNFα neutralization, neutralizing antibody (Clone # 6401, R&D Systems, Minneapolis, MN, USA) was added at 10 μg/ml.

2.2.7 Cell proliferation assays

2.2.7.1 Cell number count

Cells were rendered quiescent by serum starvation for 24 h. Human primary macrophages (4×10^4 cells) and A549 cells (2×10^4 cells) were seeded in 12-well trans-well or cell culture plates and incubated overnight. Cells were co-cultured and treated with 0, 0.1, 0.25, and 0.5% CSE for 5 days. A549 cells were harvested after 5 days by trypsinization and counted with a hemocytometer (Innovatis AG, Reutlingen, Germany).

2.2.7.2 CFSE cell proliferation assay

Carboxyfluorescein diacetate, succinimidyl ester (CFSE) is a fluorescent cell staining dye. It diffuses freely inside the cells and intracellular esterases cleave the acetate groups converting it to a fluorescent, membrane impermeable dye. The label is inherited by daughter cells after cell division or cell fusion, but is not transferred to adjacent cells. CFSE is retained by the cell in the cytoplasm and does not adversely affect cellular function. During each round of cell division, relative fluorescence intensity of the dye is decreased by half. CellTrace™ CFSE Cell Proliferation Kit (Invitrogen, Eugene, OR, USA) was used in this experiment. A549 cells were digested by incubation with 0.05% (v/v) trypsin-EDTA for 5 mins. Cells were washed 3 times with PBS and resuspend in prewarmed PBS/0.1%BSA at a final concentration of 1×10^6 cells/ml. CFSE solution stock was added (CFSE dissolved in DMSO) to the cell suspension for a final working concentration of 5 μM. Cell were incubated at 37°C for 15 min. To stop

staining, 5 volumes of ice cold medium containing 10% FCS was added and the cells were incubated 5 min on ice. The cells were centrifugation and washed 3 times with fresh media. CFSE stained A549 cells were seeded in 12-well cell culture plate (2×10^4 cells) and incubated overnight. Co-cultured of CFSE stained A549 cells and primary human macrophages (4×10^4 cells) were stimulated with or without CSE for 5 days in DMEM with 5% FBS. A549 cells were harvested at day 5 by trypsinization. Cells were washed 3 times in PBS and CFSE fluorescence was monitored by FACS (BD FACS Calibur, BD Biosciences, San Jose, CA, USA) and data analysis was performed using the BD CellQuest Pro software.

2.2.7.3 BrdU ELISA cell proliferation assay

A549 cell proliferation was determined by using the commercially available Cell Proliferation ELISA, BrdU (colorimetric) Kit (Roche, Mannheim, Germany). Co-cultured A549 cells (1×10^4 cells) and primary human macrophages (2×10^4 cells) were stimulated with or without CSE for 5 days in 24 well plates. At day 5, supernatants of A549 cells were aspirated and 400 µl/well growth media containing 10µM BrdU was added. Cells were incubated for additional 2 h at 37°C. Labelling medium was removed by tapping off and the cells were fixed by adding 500 µl FixDenat. FixDenat solution was thoroughly removed by tapping. 400µl/well anti-BrdU-POD working solution was added and cells were incubated for 90 min at RT. The antibody conjugate was removed by flicking off and wells were washed 3 times with PBS. After that, 400 µl/well of a substrate solution were added and the solution was incubated at RT for 5-30 min. 400µl 3N H_2SO_4 was added to each well and the plates were incubated for 1 min on the shaker. The absorbance was measured using ELISA reader at 450nm.

2.2.8 Animal experiments

CRAMP$^{-/-}$ mice were kindly provided by Prof. Richard L. Gallo (Nizet et al., 2001). *rela*$^{F/F}$ LysMCre (*rela*$^{\Delta-/-}$) mice with myeloid specific truncated RelA/p65 (deletion of exons 7-10 of the *rela* gene) have been described previously (Hess et al.,2010; Algul et al., 2007; Clausen et al., 1999). Briefly, to generate a constitutive knockout of RelA/p65 in myeloid cells, *rela*$^{F/F}$ mice were crossed with LysMCre animals to generate a *rela*$^{F/F}$ LysMCre mouse line. *rela*$^{wt/wt}$ LysMCre (WT) mice were used as control mice. All strains used in tumor studies had a C57BL6 background. Mice were maintained under a pathogen-free condition, and all animal experiments were approved by the Landesamt für Soziales, Gesundheit und Verbraucherschutz of the State of Saarland following the national guidelines for animal treatment.

2.2.9 Generation of lung cancer metastasis model in mice

A lung cancer metastasis model in mice was generated by intravenous injection of LLC cells. LLC cells were derived from mouse Lewis lung carcinoma. Briefly, subconfluent LLC cells were harvested and passed through a 40µm cell strainer (BD Biosciences, Bedford, MA, USA), washed three times with PBS, resuspended in serum free DMEM and injected at a concentration of 2×10^5 or 5×10^5 cells per mouse into the tail vein.

2.2.10 Cigarette smoke exposure

Mice were placed in plexiglas smoke box and exposed to mainstream cigarette smoke (MCS) generated by burning 3R4F reference cigarettes (College of Agriculture, Reference Cigarette Program, University of Kentucky, Lexington, Kentucky, USA) using a smoking machine (Ugo Basile 7025 rodent ventilator, Comerio VA, Italy). Cigarette smoke was generated in 270 ml/minute and diluted with fresh air (1800 ml/minute), and directed into a plexiglas smoke box. The average total suspended particulate (TSP) matter in the chamber approximately was 400-425 mg/m^3. Mice were exposed to CS for 20 minutes 5 times per day for 7 days, followed by 7 days rest. Control groups were incubated with filtered air. For examining CS induced subacute inflammation and tumor cell proliferation in lungs, 7 days after intravenous injection of LLC cells, mice were exposed to CS for 7 consecutive days, 24h after the last CS exposure, mice were sacrificed and lungs were removed. For the lung tumor promotion study, mice were killed after 7 days rest interval and lungs were removed, weighed and histologically examined. Some mice were kept until 8 weeks and survival data were obtained. Lung tumor nodules were carefully microdissected from lungs using an 18G needle under a microscope. The entire tumor-bearing lung was used for ELISA and RNA analysis. The smoke exposure protocol is graphically displayed in Figure 11B.

2.2.11 Bronchoalveolar lavage

Bronchoalveolar lavage fluid (BALF) was determined as described previously (Hess et al., 2010). In brief, lungs from mice were instilled with 3×1ml sterile PBS through the trachea. 3ml of BALF were collected from each mouse. Cytospin preparations of BALF cells were prepared with 200µl volume at 500 rpm for 5 min on cytospin centrifuge (Tharmac, Waldsolms, Germany). BALF cells were stained with DiffQuik (Medion Diagnostics AG, Düdingen, Switzerland) and percentages of leukocyte types were determined by light microscopy. The total number of leukocyte in the BALF was determined by using a hemocytometer (Innovatis AG, Reutlingen, Germany). Alveolar macrophages were isolated

for western blot as previously described (Vlahos et al., 2006). Briefly, the BALF was seed in DMEM and maintained in cell culture dish. Cells were incubated at 37°C in humidified air with 5% CO_2 for 1 hour. The dish then was washed 3 times with PBS, and the adherent cells, predominantly macrophages, were collected for protein extract.

2.2.12 Human tissue specimens

Formalin-fixed, paraffin-embedded anonymous human lung tumor tissues were obtained from 82 patients at the Hospital of University of Münster, Germany. According to the WHO classification, tumor specimens were classified as squamous cell carcinoma in 37 patients and adenocacinoma in 45 patients. Lung tissues obtained from 3 healthy donors were used as controls. Clinical TNM staging was performed according to the IUCC/AJCC recommendations (Sobin and Fleming, 1997). None of these patients received prior radiation or chemotherapy.

2.2.13 Preparation of cell nuclear protein extract

Quiescent human peripheral blood monocyte derived macrophages (2×10^6 cells) and A549 cells (1×10^6 cells) were co-incubated in 6-well plates as described in 3.2.6. Cell lysates and nuclear extracts were prepared 1 hour after co-culture and/or CSE stimulation using nuclear extract kit (Active motif, Carlsbad, CA, USA). The protocol was as followed: The medium was aspirated out of the 6 wells plates and the trans-wells and cells were washed 3 times with 2 ml ice-cold PBS containing phosphatase inhibitors. Cells were removed from trans-wells and plates by gently scraping with a cell scraper and transfered to 1.5 ml microcentrifuge tubes; the cells were centrifuged for 5 min at 3000 rpm at 4ºC, supernatants were discarded and 200 µl 1X hypotonic buffer were added. The suspensions were mixed by pipetting up and down, and incubated for 15 min on ice. 10 µl detergent were added and the suspensions were vortexed for 10 seconds at highest setting and centrifuged for 60 seconds at 13000 rpm at 4ºC. Supernatants were discarded and 20 µl of the lysis buffer (10 mM DTT, Lysis Buffer AM1, Protease Inhibitor Cocktail) were added. The suspensions were vortexed for 10 seconds at highest setting and cell lysates were incubated for 30 min on ice and centrifuged for 15 min at 13000 rpm (4ºC). Supernatants (nuclear protein) were collected and protein concentrations were measured with a BCA protein assay kit (Thermo scientific, Rockford, IL, USA). Proteins were frozen at -80°C for further detection.

2.2.14 Preparation of cell total protein extract

Lung tumor nodules were carefully microdissected using an 18G needle from lungs under a microscope. 10 mg tumor nodule were homogenized in the 500 ul cell lysis buffer (Cell Signalling Technology, Danvers, MA, USA) containing 5mM PMSF and protease inhibitors (Roche, Mannheim, Germany) using rotor-stator homogenizer (IKA works. Inc, Wilmington, NC, USA). CSE or TNFα treated A549 cells were washed 3 times in PBS, the pellets were lysed in the cell lysis buffer containing 5mM PMSF and protease inhibitors. For ELISA assay, the entire tumor-bearing lung was used. Lungs were homogenized in 2 ml cell lysis buffer containing 20 mM Hepes (pH 7.8), 100mM NaCl, 1% NP-40, 5mM PMSF, and protease inhibitors using rotor-stator homogenizer. After 60 min incubation on ice, homogenates were cleared by centrifugation for 15 min (13,000 rpm at 4°C). Protein concentrations were measured with BCA protein assay kit, and proteins were frozen at -80°C.

2.2.15 Western blot

For western blot analysis, 30 μg total protein or 5 μg nuclear protein extracts were mixed with 2× loading buffer, and were then loaded on 10-12% SDS-polyacrylamide gels, subjected to electrophoresis at a constant power of 150 V. The separated samples were blotted onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) at a constant power of 100 V for 2.0 hours. After transfer, the membranes were blocked with 5% skim milk in TBS buffer for 1 hour, incubated with a specific antibody against the indicated protein in TBS-T /5% skim milk overnight at 4°C. Subsequently, membranes were washed 3 times in TBS-T (3×10min) and were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit, anti-goat or anti-mouse secondary antibody for 1 hour at room temperature. After three 10-minute washes of membranes with TBS-T, detection was performed by an enhanced chemiluminescence kit (Cell Signalling Technology, Danvers, MA, USA) according to the manufacturer's instruction on Kodak scientific imaging films (Eastman Kodak, Rochester, N.Y., USA). Primary antibodies used for western blot analysis included: goat anti-LaminB (1:500), rabbit anti-p65 (1:40000), mouse anti-cyclinD1 (1:5000), mosue anti-c-jun (1:1000) (all three from Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-STAT3 (1:10000), rabbit anti-STAT3(pTyr705) (1:1000), rabbit anti-c-myc (1:500), rabbit anti-Erk (1:1000) (pThr202/Tyr204), rabbit anti-total β-catenin (1:1000), rabbit anti-GSK3β (pSer9) (1:1000), and rabbit anti-Akt (pSer473) (1:2000) (all seven from Cell Signalling Technology, Danvers, MA, USA), mouse anti-unphosphorylated β-catenin (1:1000) (Millipore, Temecula, CA, USA), mouse anti-PCNA (1:10000) (Abcam, Cambridge, UK) and mouse anti-β-actin

(1:40000) (Sigma Aldrich, Steinheim, Germany). Secondary antibodies include HRP-conjugated goat anti-rabbit (1:2000) (Abcam, Cambridge, UK), rabbit anti-mouse secondary antibody (1:1000) (Dako, Glostrup, Denmark) or HRP-conjugated donkey anti-goat (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.2.16 Real time RT-PCR

The entire tumor-bearing lungs were homogenized in the 3 ml Trizol reagent (Invitrogen, Carlsbad, CA, USA) using rotor-stator homogenizer. Human primary macrophage, A549, and RAW264.7 cells were lysed in the 1 ml Trizol reagent. Total RNA was isolated according to manufacturer’ s recommendations. 1.5 μg of total RNA was reverse transcribed using the First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) applying oligo(dT)18. cDNA was diluted 1:9 and 5 μl was used as template in a 25-mL SYBR-Green-PCR mix. Real-time PCR was performed using the SensiMix SYBR & Fluorescein Kit (Bioline, Luckenwalde, Germany) on iCycler machine (Bio-Rad, Munich, Germany). The amplication condition was:

	Cycle	Temperature	Time
polymerase activation	1	95°C	15 min
denaturation anneal extension	45	95°C T*m* 72°C	30 sec 30 sec 45 sec
final extension	1	72°C	3 min
denaturation	1	95°C	1 min
start-temperature	1	55°C	1 min
melt temperature	80	55°C (+0.5°C /cycle)	10 sec
hold		10°C	∞

Specificity of RT-PCR was controlled by ‘‘no reverse transcription’’ controls and melting curve analysis. Quantitative PCR results were obtained using the ΔΔCT (cycle threshold) method. Data were normalised to β-actin or GAPDH levels in each sample.

The following PCR primers (Metabion, Martinsried, Germany) were used:

Name	Sense primer	Antisense primer	T*m*
mouse β-actin	5'-AGCCTCGCCTTTGCCGA-3'	5'-CTGGTGCCTGGGGCG-3'	60℃
mouse TNFα	5'-AGCCCCCAGTCTGTATCCTT-3'	5'-CTCCCT TTGCAGAACTCAGG-3'	60℃
mouse IL-6	5'-CCGGAGAGGAGACTTCACAG-3'	5'-TC CACGATTTCCCAGAGAAC-3'	60℃
mouse CRAMP	5'-AATTTTCTTGAACCGAAAGGGC-3'	5'-TGTTTTCTCAGATCCTTGGGAGC'	60℃
human GAPDH	5'-AGGTCGGAGTCAACGGATTTGGT-3'	5'-GTGCAGGAGGCATTGCTGATGAT-3'	60℃
human LL-37/hCAP18	5'- CCACCATGGGCCTGGTGATGCCT CTGGCCATC-3'	5'- TGTACACTAGGACTCTGTCCTGGGTACAAG-3'	59℃
human Cyp27B1	5'- ACCCGACACGGAGACCTTC-3'	5'-CACAGGTGCG ACAACTGGTA-3'	60℃
human VDR	5'- AAGGACAACCGACGCCACT-3'	5'- ACACAC CTGTAGCCGTACTA-3'	60℃
human TNFα	5'-CCCAGGCAGTCAGATCATCTTC-3'	5'-AGCTGCCCCTCAGCTTGA	60℃
human IL-6	5'-TCACCAGGCAAGTCTCCTCATTG	5'-ACTCCTTCTCCACAAGCGCCTT-3'	58℃
human IL-8	5'-TGCAGCTCTGTGTGAAGGTGCAGT	5'-TGAATTCTCAGCCCTCTTCAAAAACTTC-3'	58℃

2.2.17 Histologic and immunohistochemical analyses

Mice were euthanized and the lungs were removed and infused with 4% paraformaldehyde, then immediately immersed in 4% paraformaldehyde before embedding in paraffin. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Other sections were prepared for immunohistochemical analysis. Sections were incubated at 60°C overnight and were deparaffinized. Sections were heated in microwave oven at 100°C for 20 min in 0.01 M citrate buffer (pH 6.0) for antigen retrieval. Unspecific tissue peroxidases were blocked by 3% (v/v) H_2O_2 followed by incubation in blocking solution (2% BSA in TBS-T) for 60 min to prevent nonspecific binding. Slides were incubated with primary antibodies at 4°C overnight. Secondary antibody incubation and staining were performed using the EnVision®+ System–HRP (AEC) kit (Dako, Carpinteria, CA, USA) according to manufacturer's recommendations. The sections were counterstained with hematoxylin. The following antibodies were used: rabbit anti-CD68 (for mouse, 1:200, Abbiotec, San Diego, CA, USA), mouse anti-CD68 (1:100) (for human, 1:100, DAKO, Glostrup, Denmark), rabbit anti-TNFα (1:100), mouse anti-Ki-67 (1:100) (all two from Abcam, Cambridge, UK), and mouse anti-unphosphorylated β-catenin (1:50) (Millipore, Temecula, CA, USA), rabbit anti-CRAMP (1:10000) (Pineda-Antikörper-Service, Berlin, Germany). The number of CD68-positive stained cells in randomly selected lung tumors was counted and the mean values were calculated. The number of Ki-67-positive tumor cells and the total number tumor cells was measured in six microscopic fields of randomly selected tumor and then the mean value was calculated as the percentage of Ki-67-positive tumor cells.

3.2.18 Cytokines ELISA assay

Samples were prepared as described above. Before measuring cytokines, the total protein concentrations were measured with BCA protein assay kit and adjusted to the same total protein concentration. TNFα, IL-6, and KC were measured by commercially available sandwich-type ELISA (R&D Systems, Minneapolis, MN, USA).

3.2.19 Statistical analysis

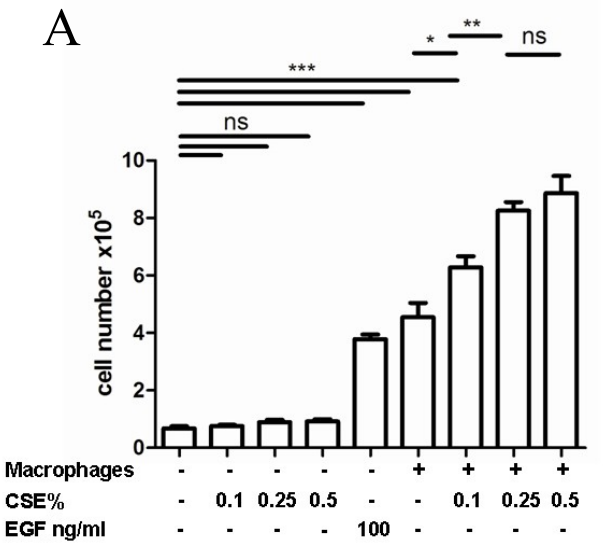
Values are displayed as mean plus or minus SEM. Comparisons between groups were analyzed by the t test (two-sided) or ANOVA for experiments with more than two subgroups or Kaplan-Meier survival analysis. Results were considered statistically significant for P values less than 0.05.

3 Results

3.1 Macrophages promote proliferation of lung cancer cells and cigarette smoke increases this effect

To determine the effect of macrophages on proliferation of lung cancer cells, transwell inserts were used in a co-culture model. After 5 days of co-culture, human macrophages derived from peripheral blood monocytes increased the growth of the lung cancer cell line A549 (Fig. 6A). Furthermore, addition of different concentrations of CSE (0.1 %, 0.25 %, 0.5 %) to the co-culture enhanced macrophage induced growth of A549 cells (Fig. 6A). Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent dye that is used to identify cell division by its intensity determined by flow cytometry. After each round of cell division, relative fluorescence intensity of the dye is decreased by half. Cell division of A549 cells was strongly promoted by co-culture with macrophages (Fig. 6B). In response to CSE treatment, the cell division rate of A549 cells co-cultured with macrophages was further increased (Fig. 6B).

To assess whether macrophage induced growth of A549 cells was due to altered cell proliferation, BrdU incorporation which incorporates into newly synthesized DNA strands of actively proliferating cells was detected using anti-BrdU antibodies ELISA assay kit. Consistent with the changes in the number of cells and mitotic activity, DNA synthesis of A549 cells was remarkably increased when cells were co-cultured with macrophages. Incubation with CSE enhanced this effect (Fig. 6C).



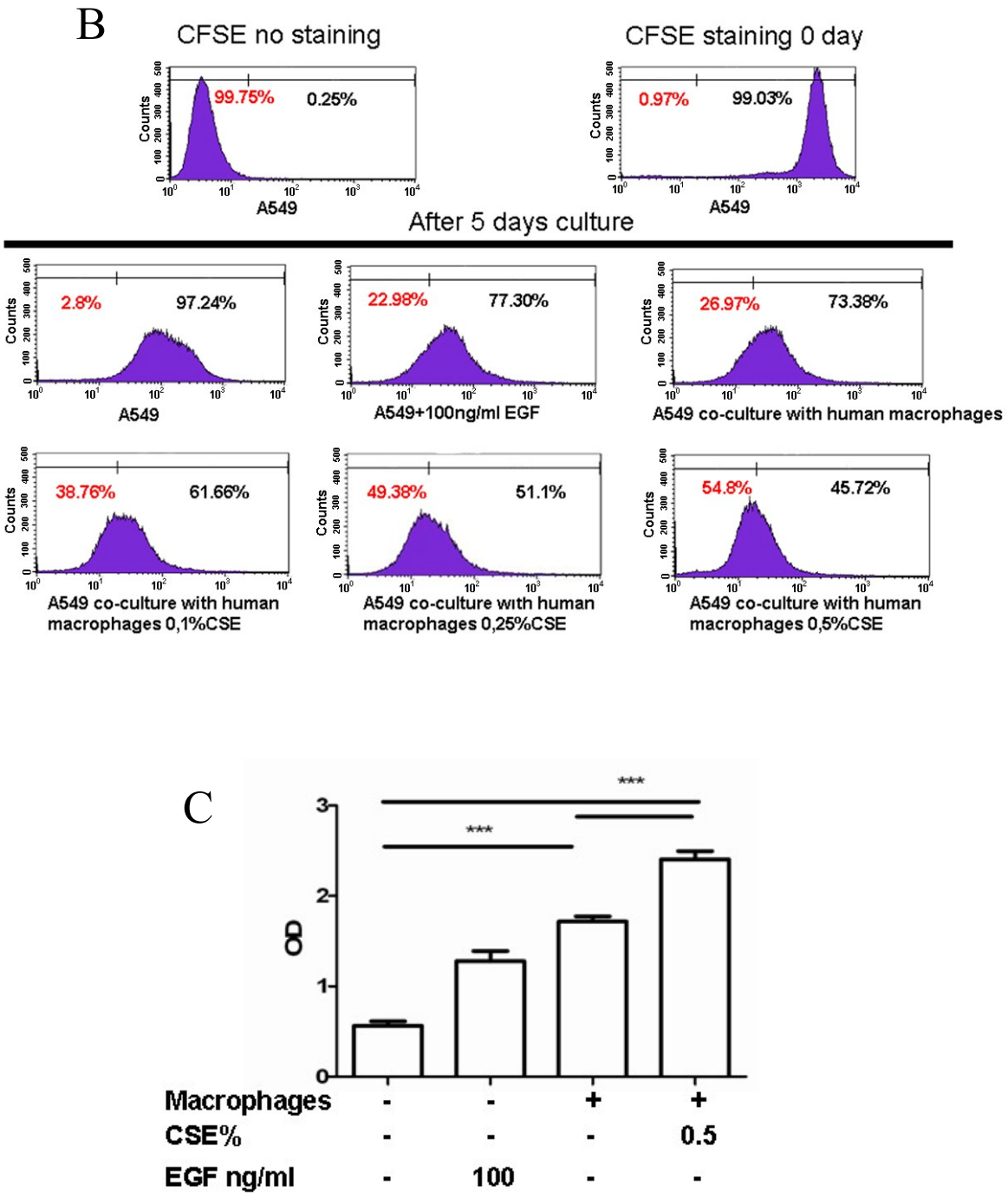


Figure 6: Macrophages promote proliferation of lung cancer cells and cigarettes smoke increase this effect

Cells were rendered quiescent by serum starvation for 24 h. Thereafter, for the co-culture of A549 cells with macrophages, human peripheral blood monocyte-derived macrophages (4×10^4 cells) were placed in transwell inserts and A549 cells (2×10^4 cells) were seeded on the bottom of 12-well plates. The cells were exposed to indicated concentration of CSE for 5 days. EGF was used as control. (A) The proliferation of lung cancer cells A549 was measured by cell number count. (B) Cell division of A549 cells was analyzed by FACS (carboxyfluorescein succinimidyl ester, CFSE labeling). (C) Cell proliferation was measured by ELISA (5-bromodeoxyuridine, BrdU labeling) analysis. Results are means ± SEM, significant difference,* p<0,05;** p<0,01; *** p<0,001; ns, not significant.

3.2 CSE induces NF-κB activity and NF-κB-dependent expression of pro-inflammatory factors in coculture of macrophages and A549 cells

It has been shown that CS exposure induces lung inflammation by activation of transcription factor NF-κB (Yang et al., 2006b). NF-κB activation plays a pivotal role in regulation of inflammatory processes which are associated with inflammation-promoting tumor growth (Karin et al., 2006; Pikarsky et al., 2004). In order to examine whether the NF-κB signalling pathway is activated after co-culture of macrophages with lung cancer cells and after CSE incubation, nuclear translocation of the NF-κB subunit p65/RelA was detected by western blot analysis of nuclear protein extracts of macrophages. As shown in figure 7A, the levels of p65/RelA protein were increased in nuclei of macrophages after 1 h of co-culture with A549 cells. Addition of CSE further increased translocation of p65/RelA protein into nuclei of macrophages. Interestingly, co-culture with macrophages and addition of CSE also increased NF-κB activation in A549 cells (Fig. 7B).

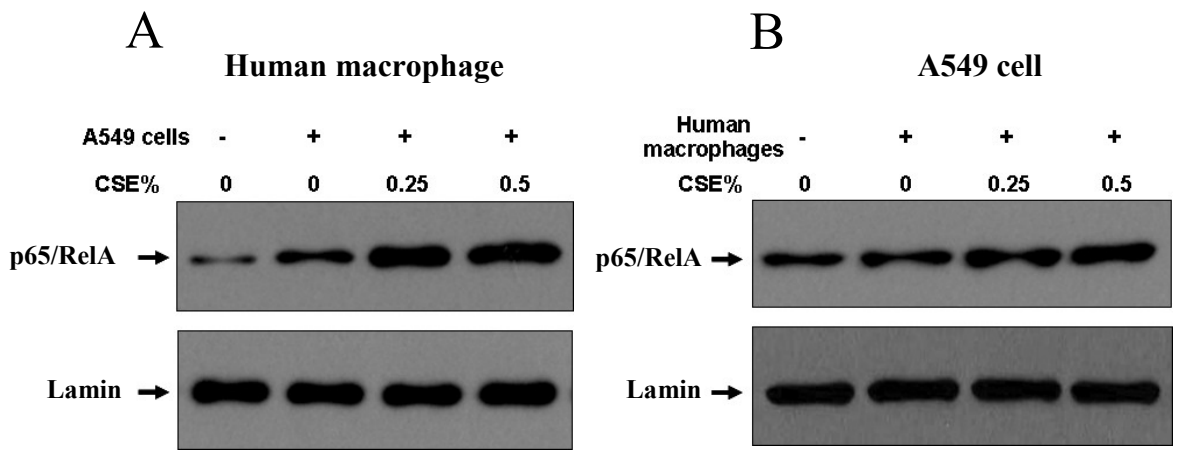
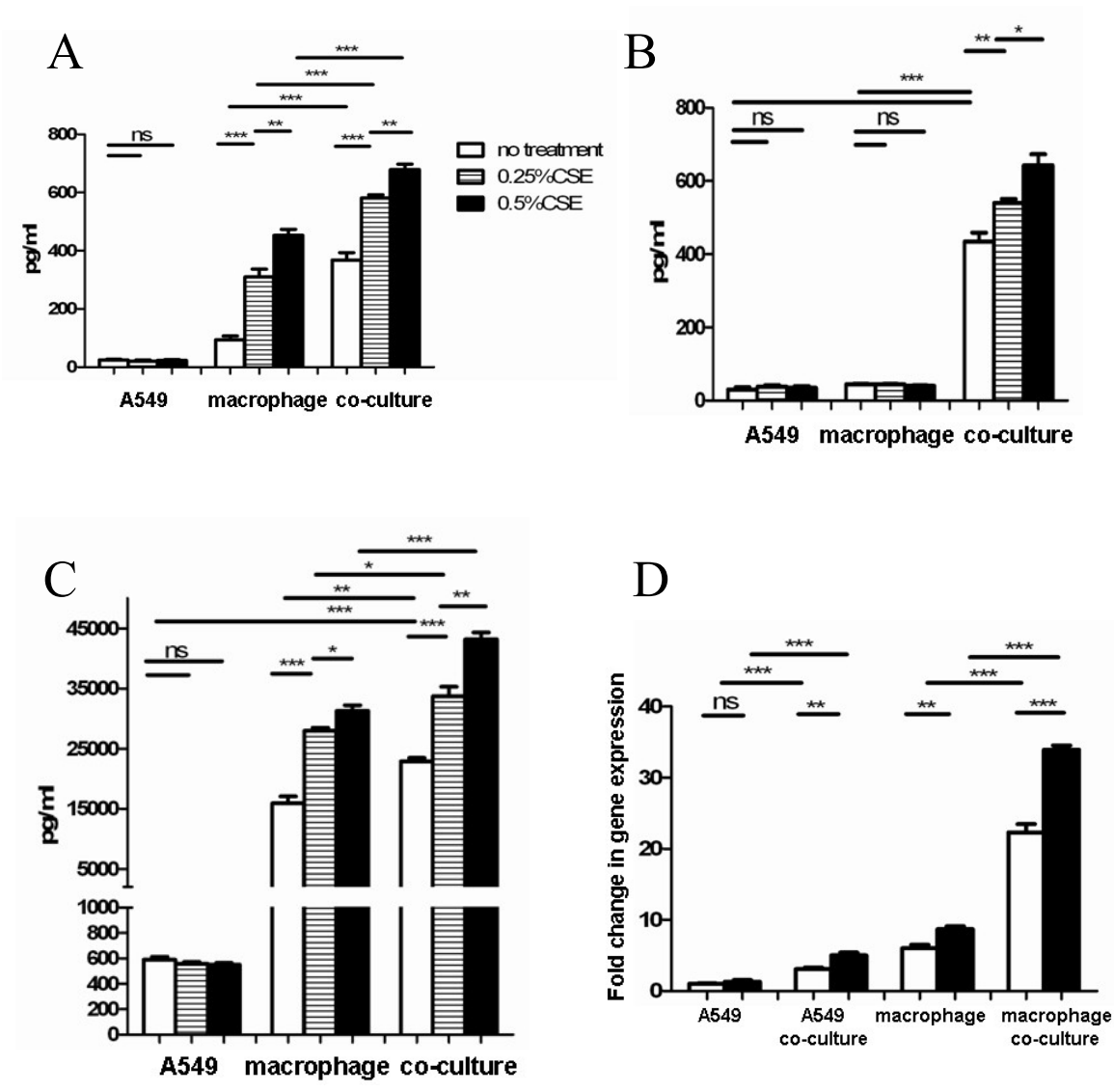


Figure 7: NF-κB-p65 activation in macrophages and A549 cells.
Quiescent human peripheral blood monocyte-derived macrophages (2×10^6 cells) and A549 cells (1×10^6 cells) were co-incubated as described in Fig 6. Nuclear extracts were prepared 1 hour after co-culture and/or CSE exposure and were analyzed by western blot. Lamin B served as loading control. (A) human macrophage. (B) A549 cells.

NF-κB is known to be central in the induction and regulation of inflammatory responses. Next, the effect of co-culture and CSE exposure on the NF-κB-dependent release of pro-inflammatory factors including TNFα, IL-6, and IL-8 was determined. When A549 cells were treated with CSE for 8 hours, there was no effect on the release of TNFα, IL-6, and IL-8,

compared to controls (Fig. 8A-C). In contrast, there was an increased release of TNFα and IL-8 when macrophages were treated with CSE whereas IL-6 levels were not affected (Fig. 8A-C). Moreover, when both macrophages and A549 cells were present, the production of TNFα, IL-6, and IL-8 was remarkably up-regulated and CSE further increased the release of these pro-inflammatory factors after 8 hours of treatment (Fig. 8A-C). To understand which type of cell is responsible for the release of pro-inflammatory factors when macrophages were co-cultured with A549 cells, mRNAs expression patterns were analysed in both cell types. Interestingly, as determined by real-time PCR, co-culture or co-culture combinated with CSE incubation strongly induced *Tnfα, Il6*, and *IL8* gene expression in macrophages as well as in A549 cells indicating that both cell types contributed to the enhanced release of pro-inflammatory factors (Fig. 8D-F).



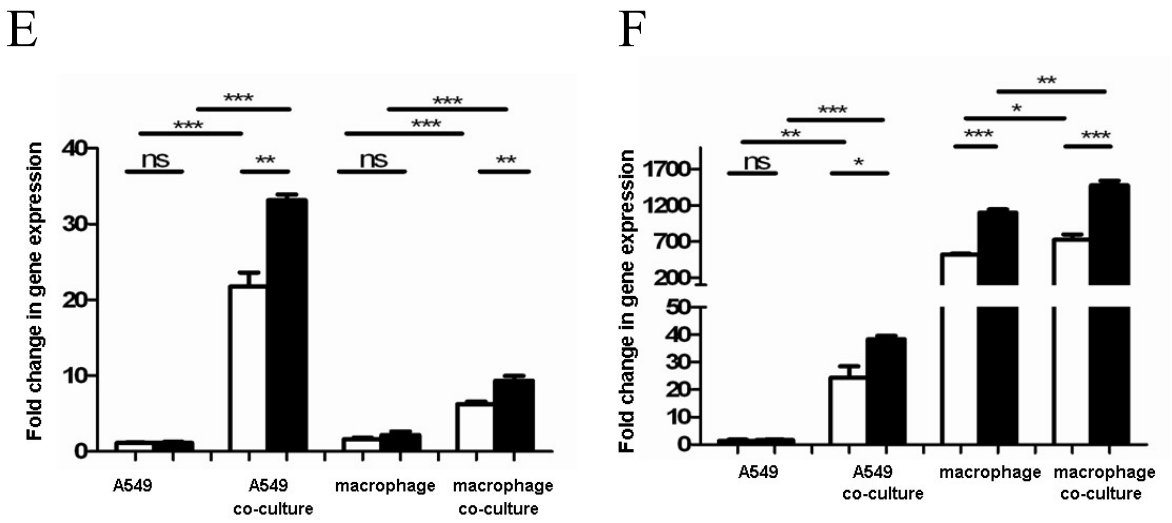


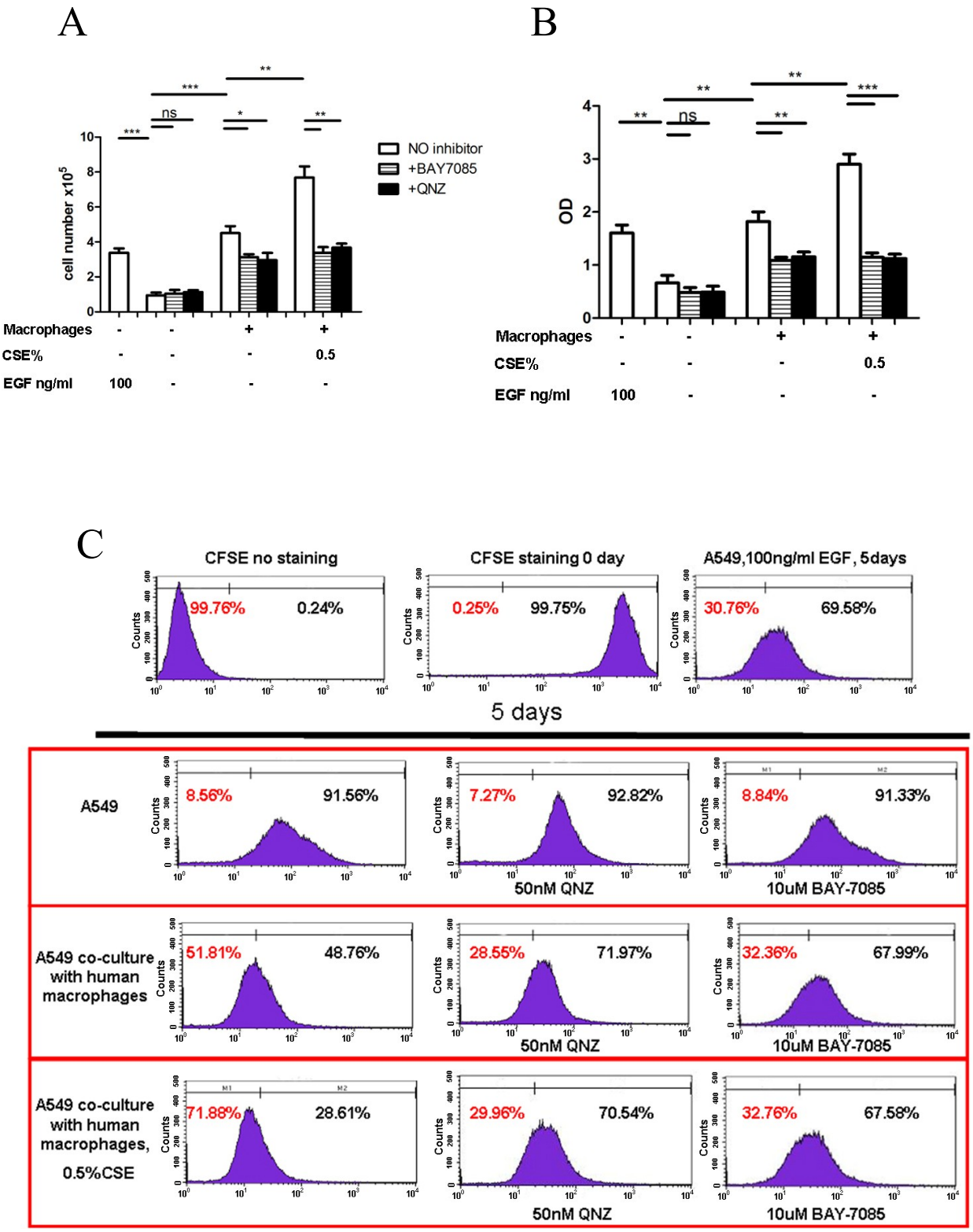
Figure 8: Macrophage and CSE induce expression of pro-inflammatory factors.
A549 cells and human peripheral blood monocyte-derived macrophages were co-cultured and were exposed to indicate concentration of CSE for 8 h in DMEM without FBS. (A) TNFα (B) IL-6 and (C) IL-8 levels in culture supernatants of A549 cells, of macrophages, and of macrophages co-cultivated with A549 cells were analyzed by ELISA. Induction of the inflammatory cytokines (D) *Tnfα*, (E) *Il6* and (F) *Il8* was measured by real-time PCR. Total RNAs of A549 cells and macrophages were isolated 8 hours after CSE exposure. Mean fold change ± SEM, significant difference,* $p<0,05$;** $p<0,01$; *** $p<0,001$; ns, not significant.

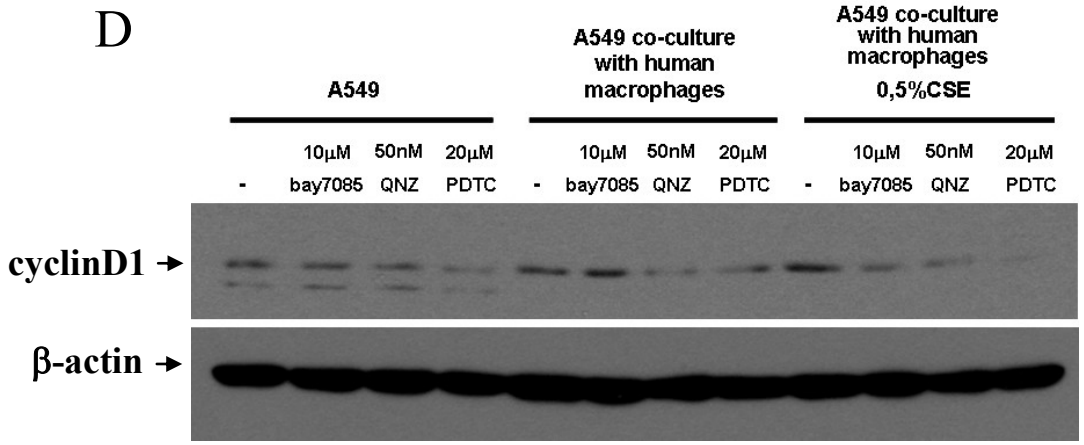
3.3 Inhibition of NF-κB activation reduces macrophage-promoted tumor cells proliferation

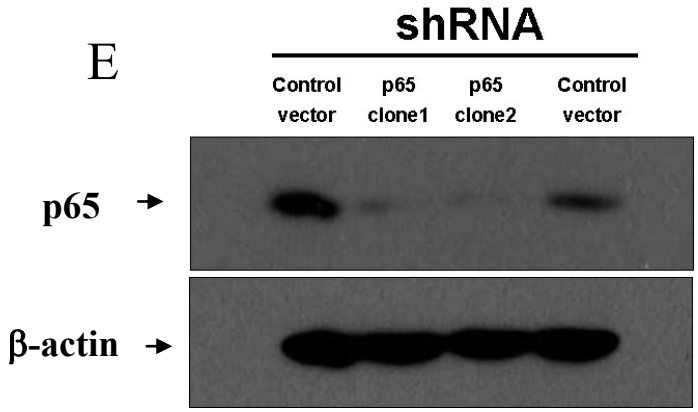
To examine the effect of NF-κB on proliferation of tumor cells, the NF-κB activation inhibitors Bay 11-7085, 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline (QNZ) or pyrrolidine dithiocarbamate (PDTC) were used to prevent NF‑κB transcriptional activity. After 5 days of co-culture, the NF-κB activation inhibitor Bay 11-7085 or QNZ reduced macrophage induced proliferation of A549 cells compared to control levels, even though 0.5% CSE exposure from results of cell count, BrdU and CFSE analyses (Fig. 9A-C). Next, these results were confirmed by western blotting of protein extract of A549 with or without NF-κB activation inhibitor. Cell cycle protein cyclinD1 was decreased after treatment with Bay 11-7085, QNZ or PDTC in A549 cells. Addition of 0.5% CSE failed to protect cyclinD1 protein reduction (Fig. 9D).

Because addition of NF-κB activation inhibitor in co-culture, NF-κB activation were inhibited both macrophages and A549 cells. To further define the role of NF-κB in macrophages, stable U937 cell lines containing short hairpin RNA (shRNA) specific to p65/RelA were generated (Fig. 9E). p65/RelA silenced U937 cells (U937 p65ko) or mock

U937 (U937 control) cells were co-cultures with A549 cells. After 5 days of co-incubation, silencing of p65 in U937 inhibited co-culture induced proliferation of A549 cells compared with mock U937 cells even when 0.5% CSE was added (Fig. 9F-H).




D
A549
A549 co-culture with human macrophages
A549 co-culture with human macrophages 0,5%CSE
10μM 50nM 20μM
- bay7085 QNZ PDTC
cyclinD1
β-actin


E
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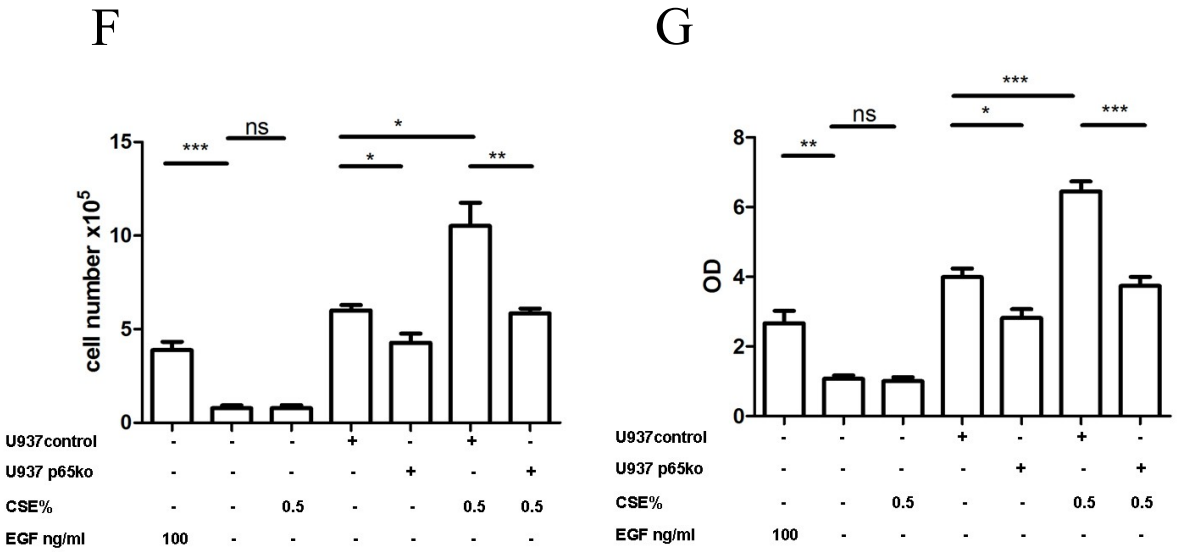
Control vector
p65 clone1
p65 clone2
Control vector
p65
β-actin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G
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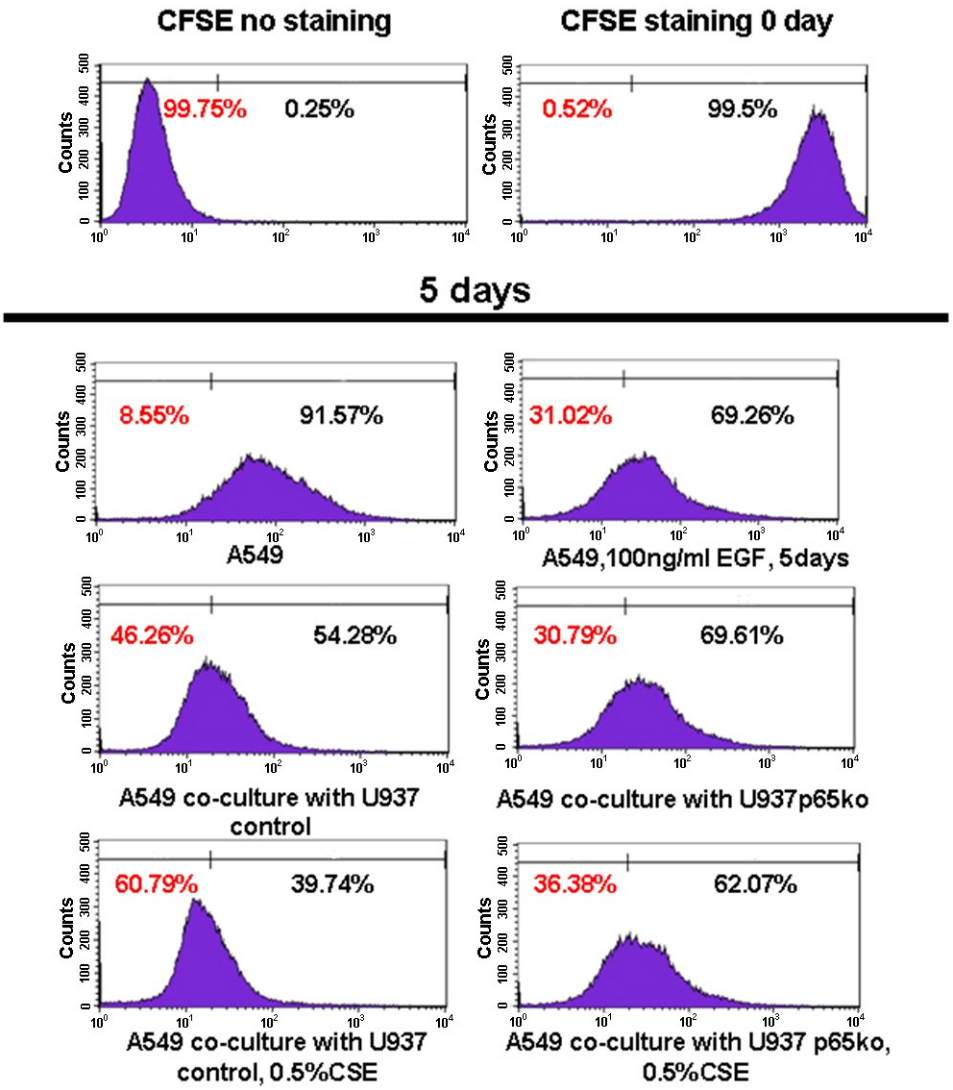
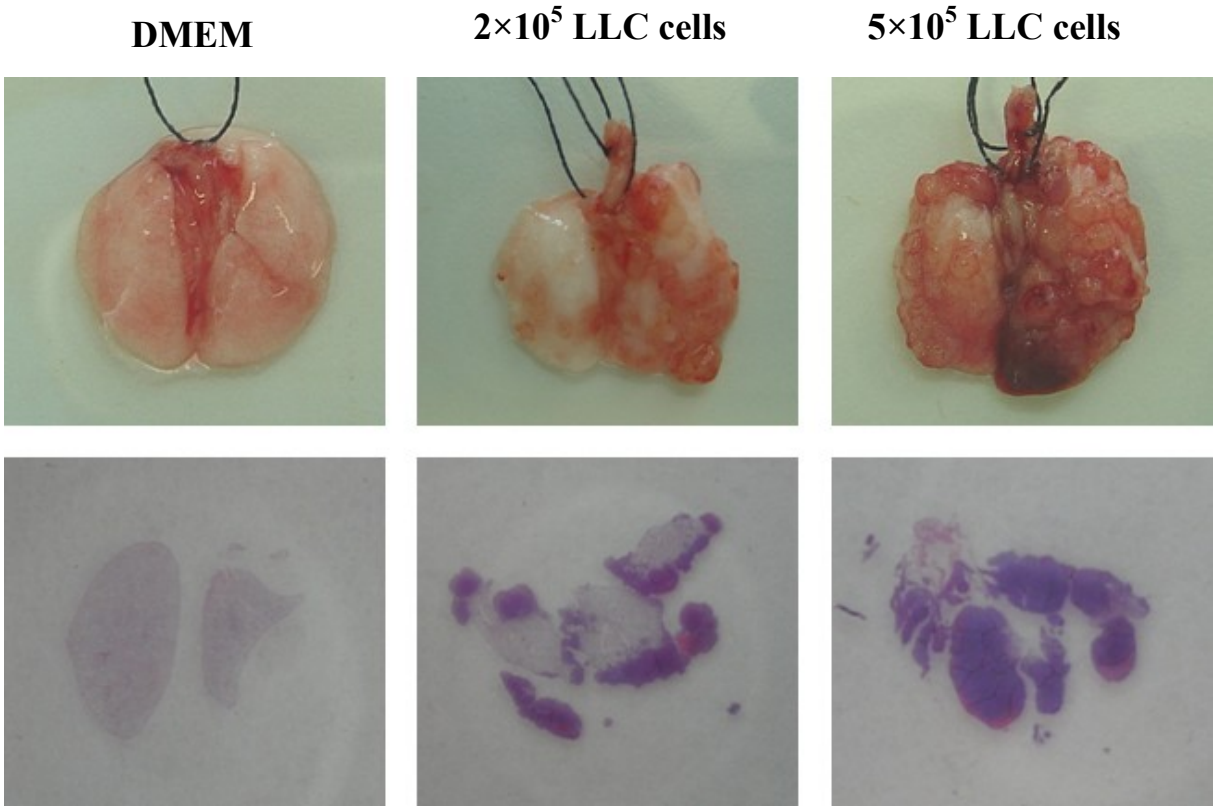


Figure 9: Inhibition of NF-κB activation reduces proliferation of A549 cells.
A549 cells and human macrophages were treated for 1-2 hours with Bay 11-7085 (10uM) or QNZ (50nM) before co-culture and/or CSE stimulation. Cells were exposed to 0.5 % CSE for 5 days. EGF was used as control. Proliferation of A549 cells was analyzed by (A) cell number count, (B) BrdU assay, and (C) CFSE assay after 5 days co-culture and/or CSE exposure (D) A549 cells were co-cultured with human macrophages and were exposed to 0.5 % CSE or air for 5 days in NF-κB inhibitor (10uM Bay 11-7085, 50nM QNZ, 20uM PDTC) contained medium. A549 cell lysates were analyzed by immunoblotting for expression of CyclinD1 protein. (E) U937 cells were transfected with p65 shRNA plasmid or with a nonspecific control shRNA plasmid. After selection, expression of p65 was analysed by immunoblot. Proliferation of A549 cells induced by U937 control cells, U937 p65ko cells and/or CSE was analyzed by (F) cell number count, (G) BrdU assay and (H) CFSE assay after 5 days of co-culture and/or CSE exposure. Results are means ± SEM, significant difference, * $p<0,05$; ** $p<0,01$; *** $p<0,001$; ns, not significant.

3.4 Generation of a lung cancer metastasis model in mice

A lung cancer metastasis model in mouse was established by intravenous injection of LLC cells. LLC cells were derived from mouse Lewis lung carcinoma. To evaluate the success of the model, LLC cells were injected through the tail vein into female C57/BL6 mice at a concentration of 2 or 5×10^5 cells in 200 µl DMEM or DMEM alone per mouse. 15 days later, mice were sacrificed, their lungs were weighed and the tumor nodules on the lung surface were counted. Tumor formation was confirmed by light microscopy using hematoxylin and eosin (H&E) staining (Fig. 10.A). In contrast to controls, lung weights and tumor nodules increased in a LLC cell number dependent manner (Fig. 10B). Mice survival times were assessed for a period of 8 weeks and showed that the life span of tumor-bearing mice were shortened when given a higher LLC inoculum (Fig. 10C).

A



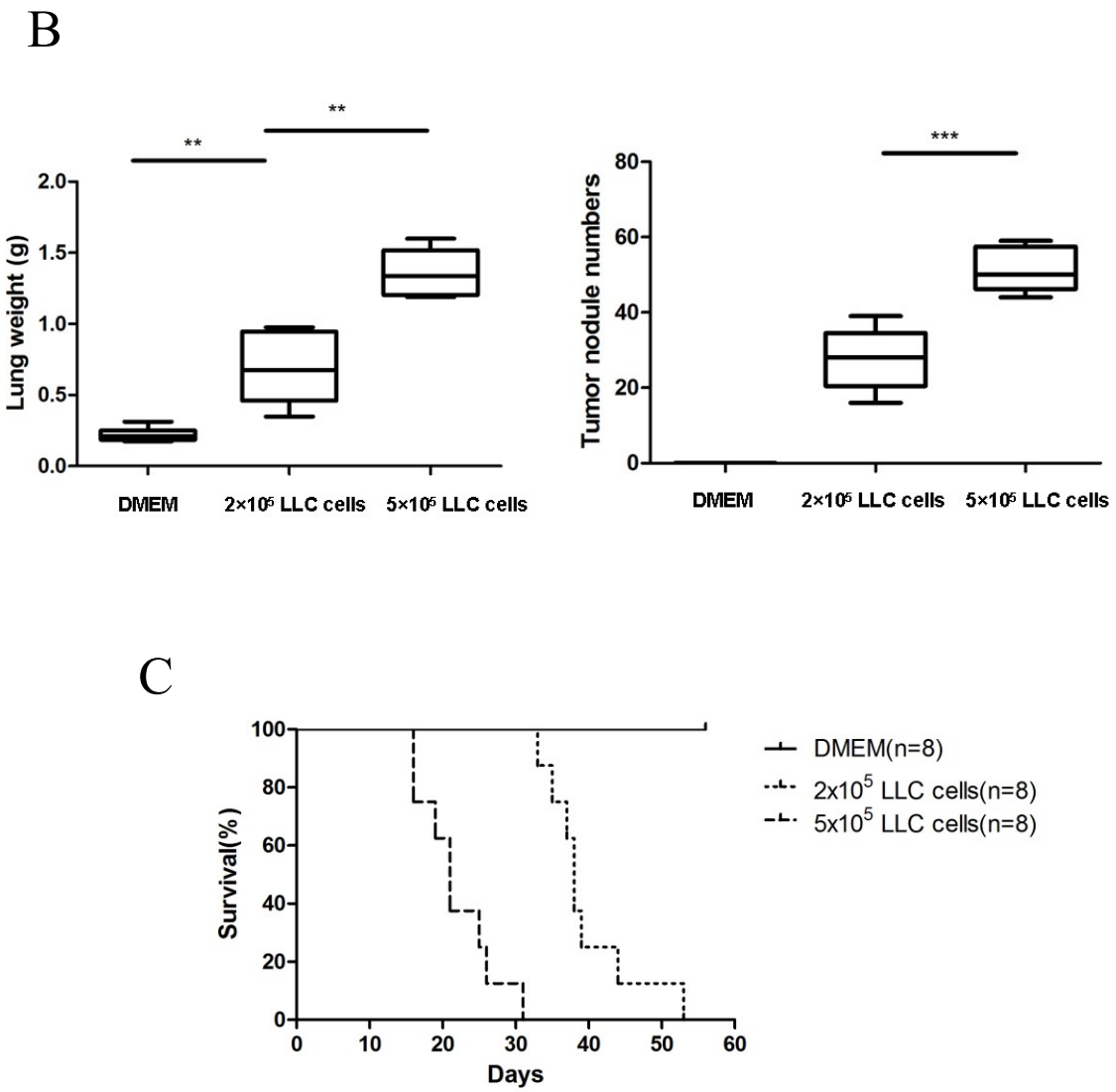


Figure 10: Evaluation of mouse lung cancer metastasis model
(A) Lung appearance (up) and histology (H&E stain; down) in C57/BL6 mice 15 days after LLC inoculation with indicated cell numbers or DMEM. (B) Lung weight and the numbers of tumour nodules detectable on the lung surface were determined and statistically analyzed 15 days after 2 or 5×10^5 LLC cells were injected into C57/BL6 mice. Results are means ± SEM, n=6, ** p<0,01; *** p<0,001. (C) Survival curves of C57/BL6 mice injected with indicated cell numbers or DMEM via the tail vein (p<0,001; Log-rank test for statistic analysis; n=8).

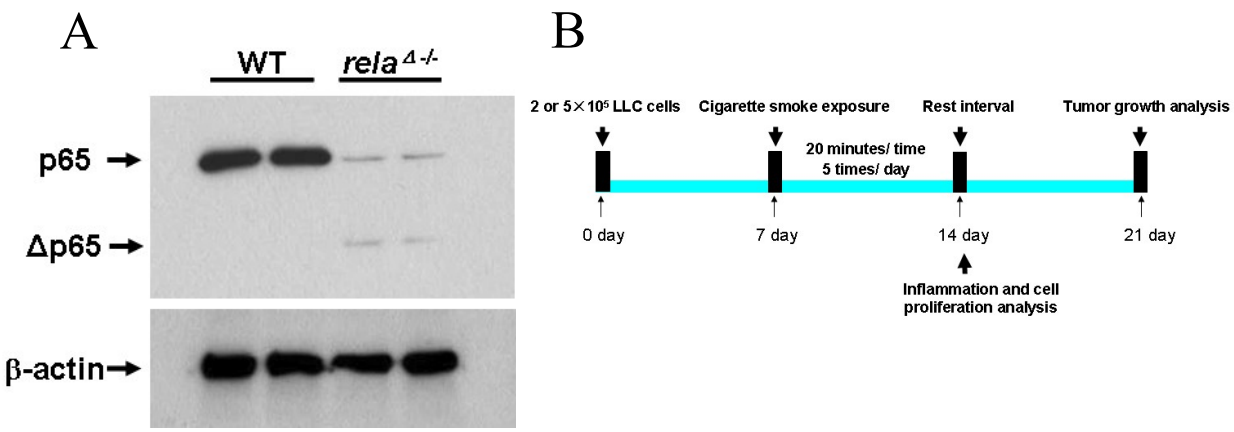
3.5 Smoke induces inflammation dependent on myeloid RelA/p65

NF-κB/RelA plays a key role in inflammatory and immune responses. In immune cells, NF-κB is a critical regulator for the expression of cytokines and chemokines (Perkins and Gilmore, 2006; Grivennikov and Karin, 2010a). To assess the contribution of RelA/p65 to CS induced lung inflammation, *rela*$^{\Delta-/-}$ mice were used that specifically lack RelA/p65 in the myeloid lineage. The initiation and progression of inflammatory responses require

inflammatory factors which are primarily derived from myeloid cells including alveolar macrophages. Western blot analysis of proteins of alveolar macrophages obtained from *rela*$^{\Delta -/-}$ mice showed the p65 deletion in these cells and displayed a truncated form of RelA/p65 (Δp65) compared to *rela*$^{wt/wt}$ LysMCre (WT) mice (Fig. 11A).

To examine the role of myeloid RelA/p65 in CS-induced lung tumor proliferation, 7 days after intravenous injection of 2 or 5×10^5 LLC cells into 8-10week-old sex-matched *rela*$^{\Delta -/-}$ mice and WT mice via the tail vein, the mice were exposed to mainstream cigarette smoke (MCS) generated from burning cigarettes for 20 minutes each time, 5 times per day for 7 days. 24h after the last CS exposure, mice were sacrificed and their lungs were removed for inflammation and cell proliferation analysis. In addition, after a 7 day rest interval, mice were sacrificed for lung tumor growth analysis. Control groups were incubated with filtered air (Fig. 11B).

To investigate the role of myeloid cells in the CS induced pulmonary inflammation, inflammatory cells and mediators in lungs of mice were characterized. CS exposure significantly increased the total cell number and absolute numbers of macrophages, neutrophils, and lymphocytes in BALFs of WT and myeloid RelA/p65 deficient mice as compared to air exposed mice (Fig. 11C). The concentrations of various inflammatory factors, such as IL-6, KC, and TNF-α, were significantly increased after CS exposure in the lungs of WT mice but not in lungs of myeloid RelA/p65 deficient mice (Fig. 11D). These data show that while depletion of myeloid RelA/p65 does not impact the influx of inflammatory cells into the lung after smoke challenge, myeloid RelA/p65 mediates the release of inflammatory mediators such as pro-inflammatory cytokines in the inflamed lung.



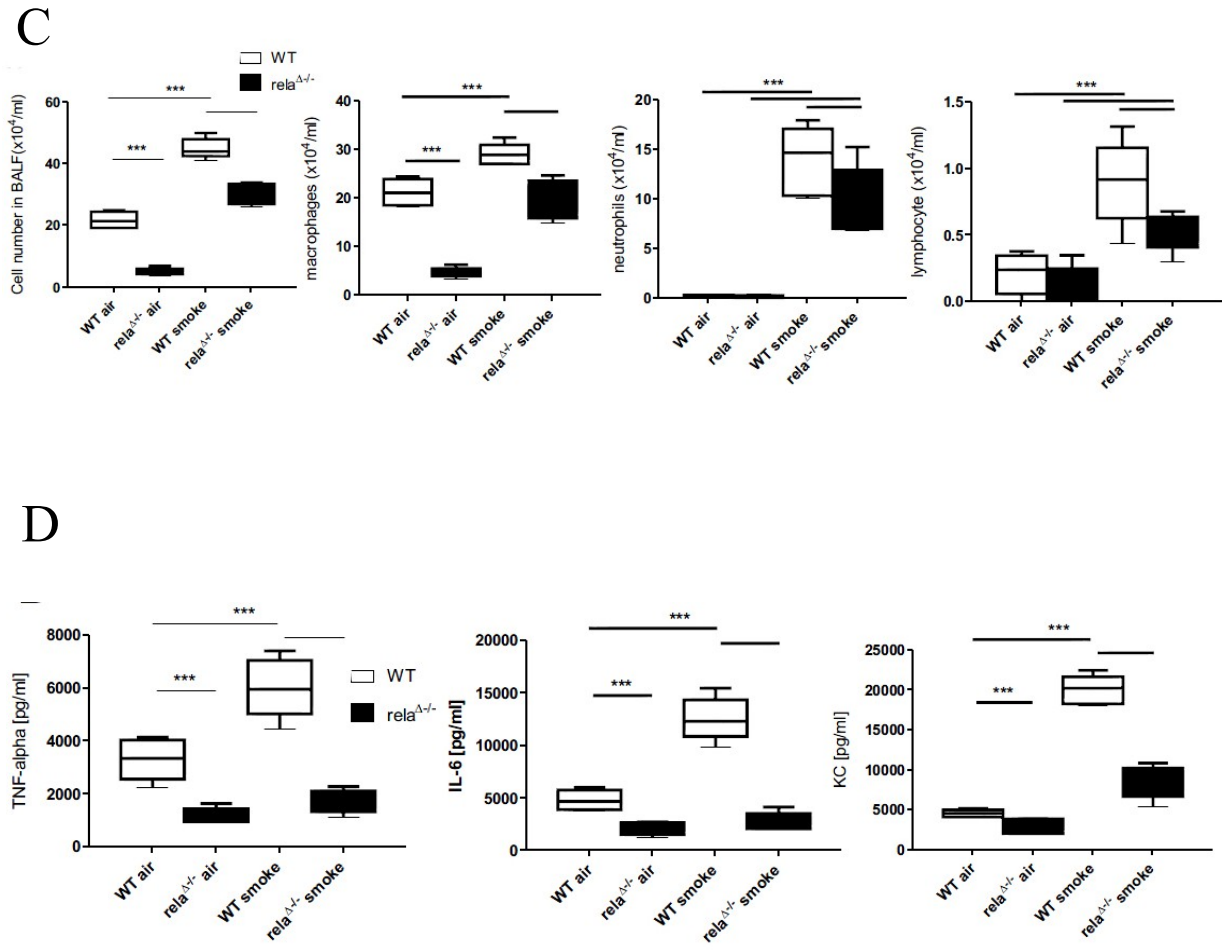
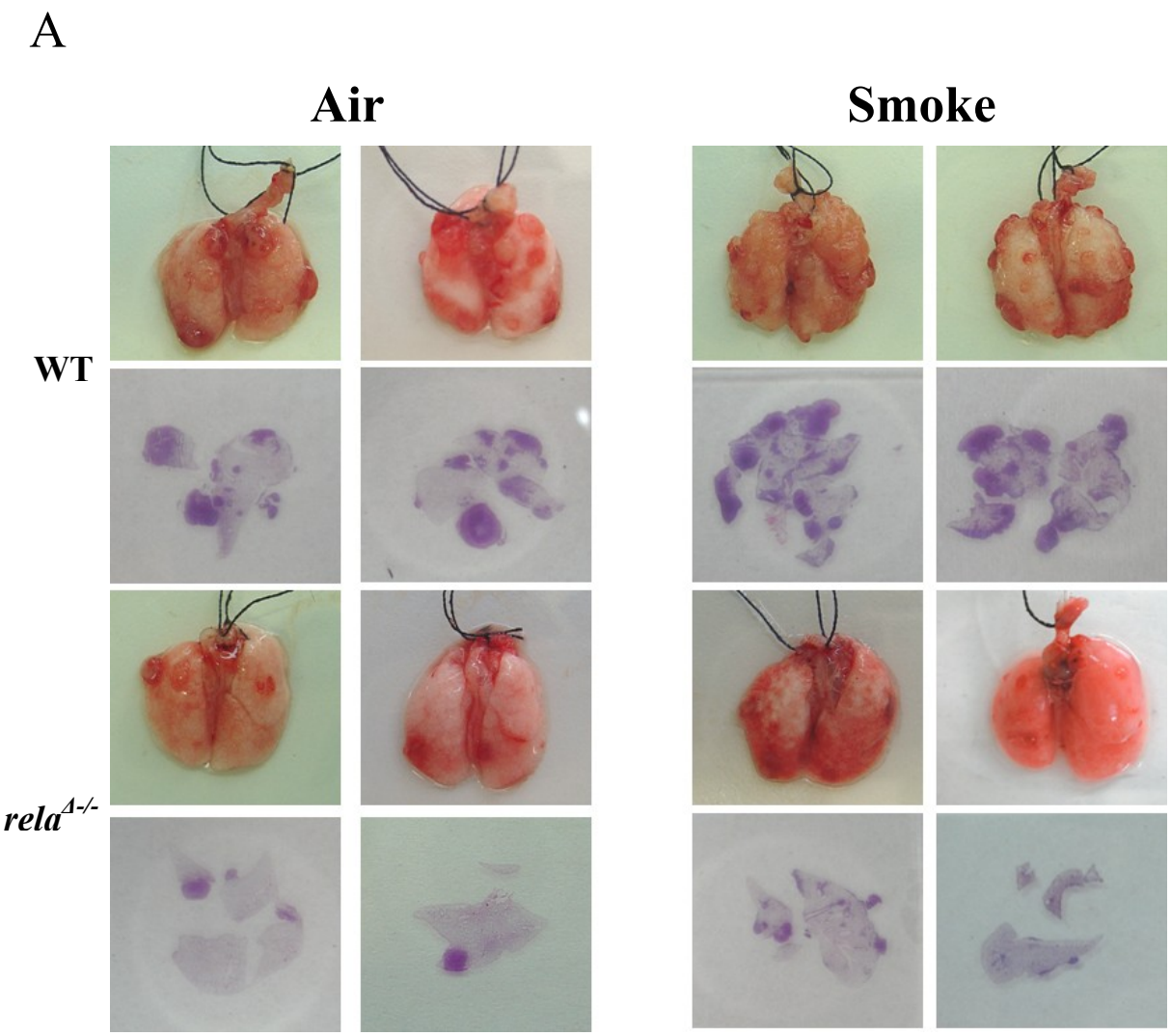


Figure 11: CS-induced inflammation is dependent on myeloid cell RelA/p65
(A) Expression of p65 and truncated p65 (Δp65) in alveolar macrophages of WT mice and *rela*$^{\Delta-/-}$ mice. The expression of β-actin was used as internal control for the amount of proteins. (B) Schema of experimental protocol of CS-induced inflammation or growth of tumor. (C) BALF of WT mice and *rela*$^{\Delta-/-}$ mice (inoculated with 5×10^5 LLC cells through the tail vein) were collected 24h after last CS or air exposure. Cellular composition was evaluated using cytospin preparations. (D) Secretion of inflammatory cytokines in tumour-bearing lungs of WT mice and *rela*$^{\Delta-/-}$ mice (5×10^5 LLC cells inoculation) 24h after the last CS-exposure was analyzed by ELISA. Fresh lungs were homogenized, centrifuged and supernatants were analyzed. Results are means ± SEM, n=5, significant difference, *** $p<0,05$.

3.6 Myeloid RelA/p65 is necessary for metastatic lung tumor growth

Next, it was investigated whether the CS induced inflammatory response contributes to lung tumor growth. Tumors were generated by injection of 2×10^5 LLC cells in WT mice and *rela*$^{\Delta-/-}$mice, followed by 7 days of CS exposure and a 7 day rest interval (Fig. 11B). Lung weights

and the numbers of tumor nodules were determined at day 21. WT mice exposed to CS exhibited significantly increased lung nodule numbers and lung weights as compared to the air exposure control animals (Fig. 12A-C). In contrast, deletion of RelA/p65 in myeloid cells significantly reduced lung tumor nodule numbers and lung weights in air and smoke exposed mice (Fig.2A-C). H&E staining confirmed the massive increase of tumor load in WT mice exposed to CS and the significant reduction of tumor growth in *rela*$^{\Delta-/-}$mice (Fig.12A). In addition, the survival rate of tumor injected WT and *rela*$^{\Delta-/-}$ mice exposed to air or CS was examined. Deletion of p65 in myeloid cells resulted in increased survival (Fig.12D). There was no difference in the survival rate of air- or CS-exposed WT mice or *rela*$^{\Delta-/-}$ mice (Fig. 12D). Taken together, RelA/p65 in myeloid cells is required for lung tumor growth.



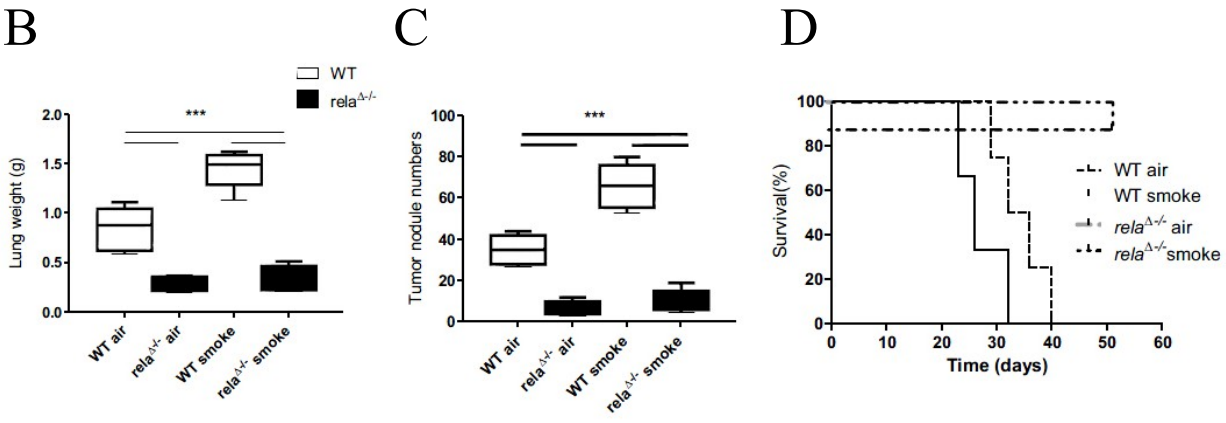


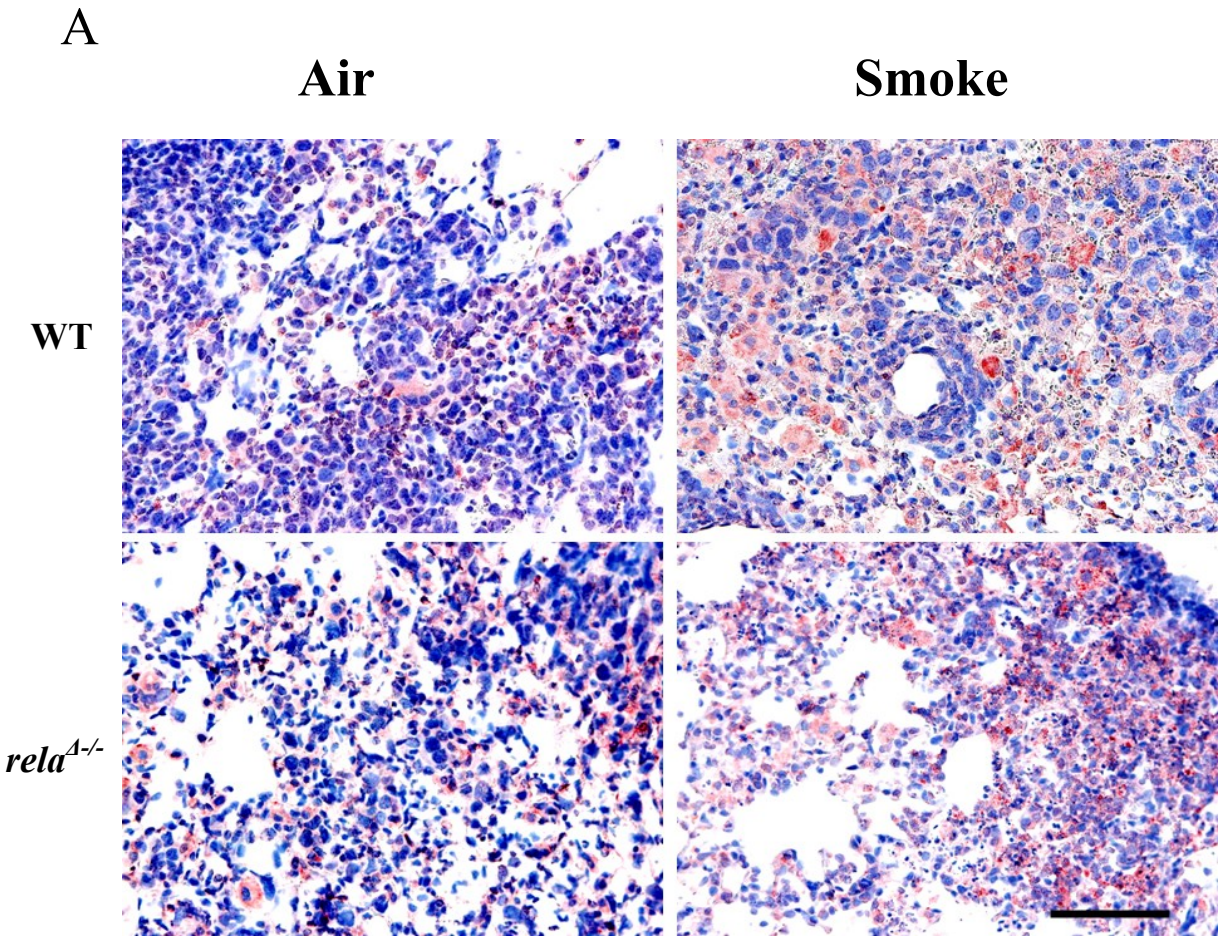
Figure 12: Myeloid cells-specific RelA/p65 truncation inhibits CS-promoted growth of lung cancer
(A) Macroscopic and microscopic pathology (H&E stain) of lung after LLC inoculation in WT or *rela*$^{\Delta-/-}$mice with or without MCS exposure. 2×10^5 LLC cells were intravenously injected into sex-matched WT mice and *rela*$^{\Delta-/-}$mice, 7 days later, mice were smoked for 7 days, at day 21, the lungs were removed. (B,C) Lung weights and the numbers of tumor nodules detectable on the lung surface were determined. Results are means ± SEM, n=5, significant difference, *** $p<0,05$. (C) Survival curves of mice ($p<0,001$; Log-rank test for statistic analysis; n=8).

3.7 Myeloid depletion of RelA/p65 results in decreased secretion of TNF-alpha from alveolar macrophages

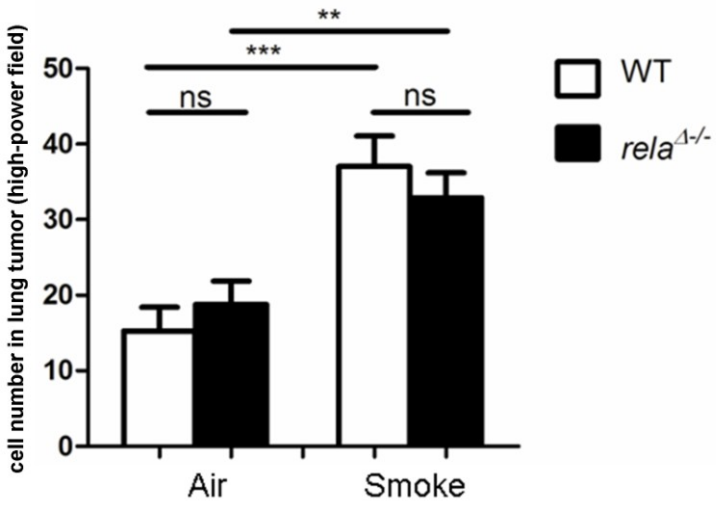
Macrophage infiltration is an important characteristic of tumorigenesis and there is correlation between macrophages abundance and poor prognosis (Murdoch et al., 2008). To investigate whether recruitment of myeloid cells into tumors depends on RelA/p65, the infiltration of macrophages in tumor nodules was examined by immunostaining using CD68 as macrophage marker. CS exposure was associated with increased numbers of macrophages in the tumor areas (Fig. 13A,B). RelA/p65 ablation did not significantly alter the recruitment of macrophage as compared to WT mice (Fig. 13A,B).

TNF-α is a major pro-inflammatory cytokine released by macrophages in a NF-κB dependent manner and is implicated in the regulation of tumor growth (Takahashi et al., 2010; Oguma et al., 2008). As TNF-α was reduced in the lungs of *rela*$^{\Delta-/-}$mice (Fig. 11D), immunostaining was used to determine whether TNF-α expression in myeloid cells is altered in mice with dysfunctional RelA/p65. In mice exposed to CS, expression of TNF-α in myeloid cells was increased, while *rela*$^{\Delta-/-}$mice revealed much weaker TNF-α levels (Fig. 13C). These data suggest that cytokine released from myeloid cells regulate tumor growth.

A


Air
Smoke
WT
rela Δ-/-

B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40
30
20
10
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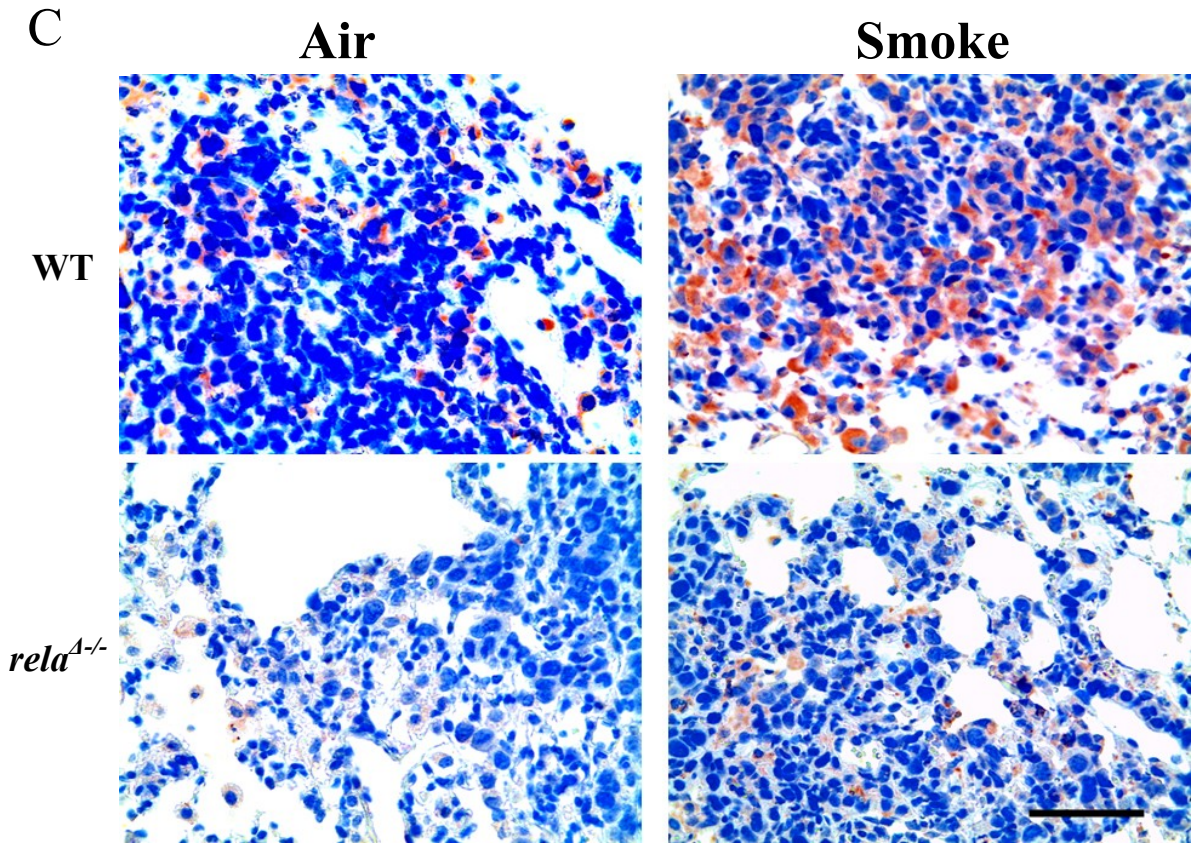
WT
rela Δ-/-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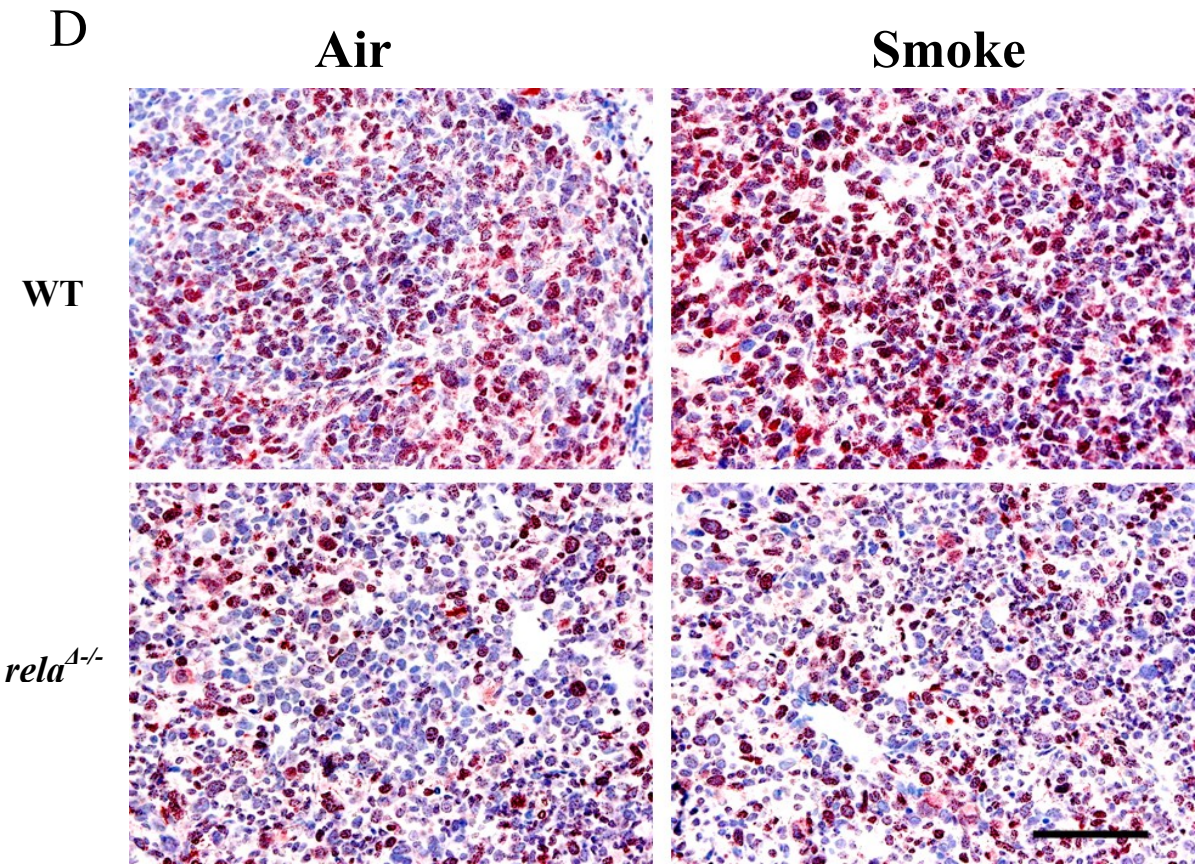
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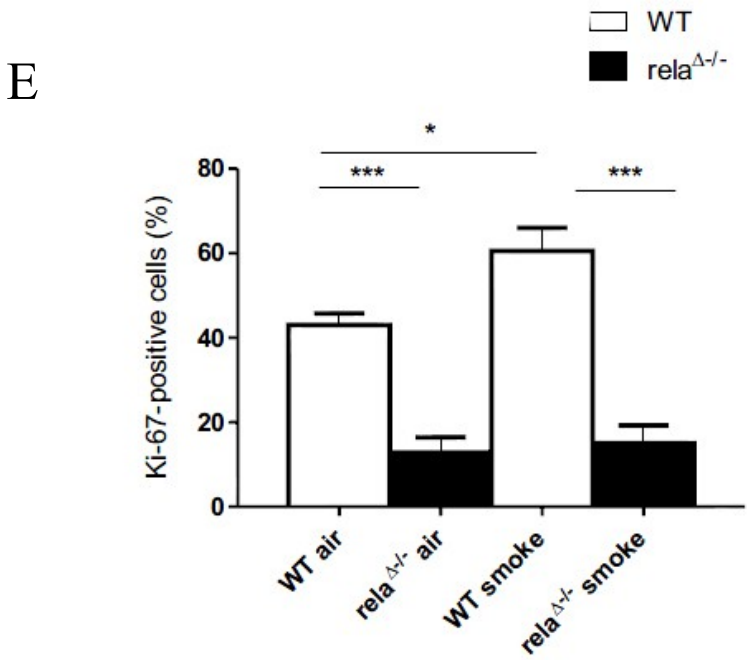


Figure 13: Myeloid cell RelA/p65 is required for TNF-α expression in macrophages and proliferation of tumor cells.
(A) Tumor bearing mice were exposed to air or MCS and lungs were removed, fixed and paraffin embedded 24h later of last MCS exposure. Paraffin-embedded tumour-bearing lung sections were analyzed by anti-CD68 antibody immunohistochemical analysis. Scale bars= 100µm. (B) The numbers of CD68-positive cells in randomly selected lung tumour were counted. Results are means ± SEM of 5 high-power fields, n=5, significant difference, ** p<0,01; *** p<0,001; ns, not significant. (C) Immunohistochemical analysis of TNFα expression in MCS or air-treated lung tumours of WT mice and *rela*$^{\Delta-/-}$ mice. Scale bars= 100µm. (D) Tumors were examined by immunostainingwith anti-Ki-67-antibodies to detect proliferating cells. Bar = 100 µm. (E) Percentage of Ki-67-positive tumor cells. Results are means ± SEM, n=5, significant difference,* p<0.05; *** p<0,001.

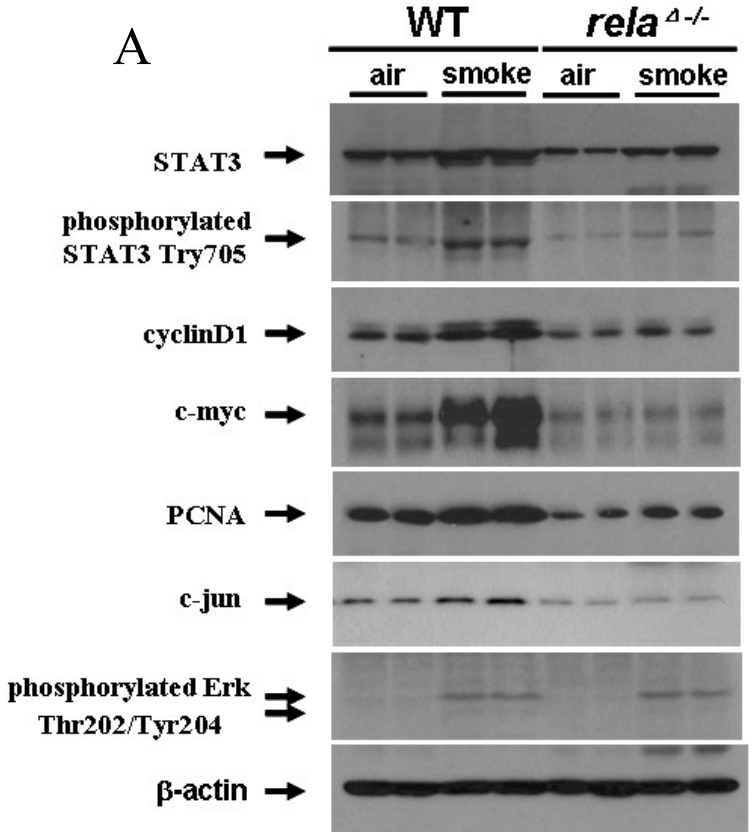
3.8 Myeloid cell RelA/p65 activates a proliferation program in cancer cells

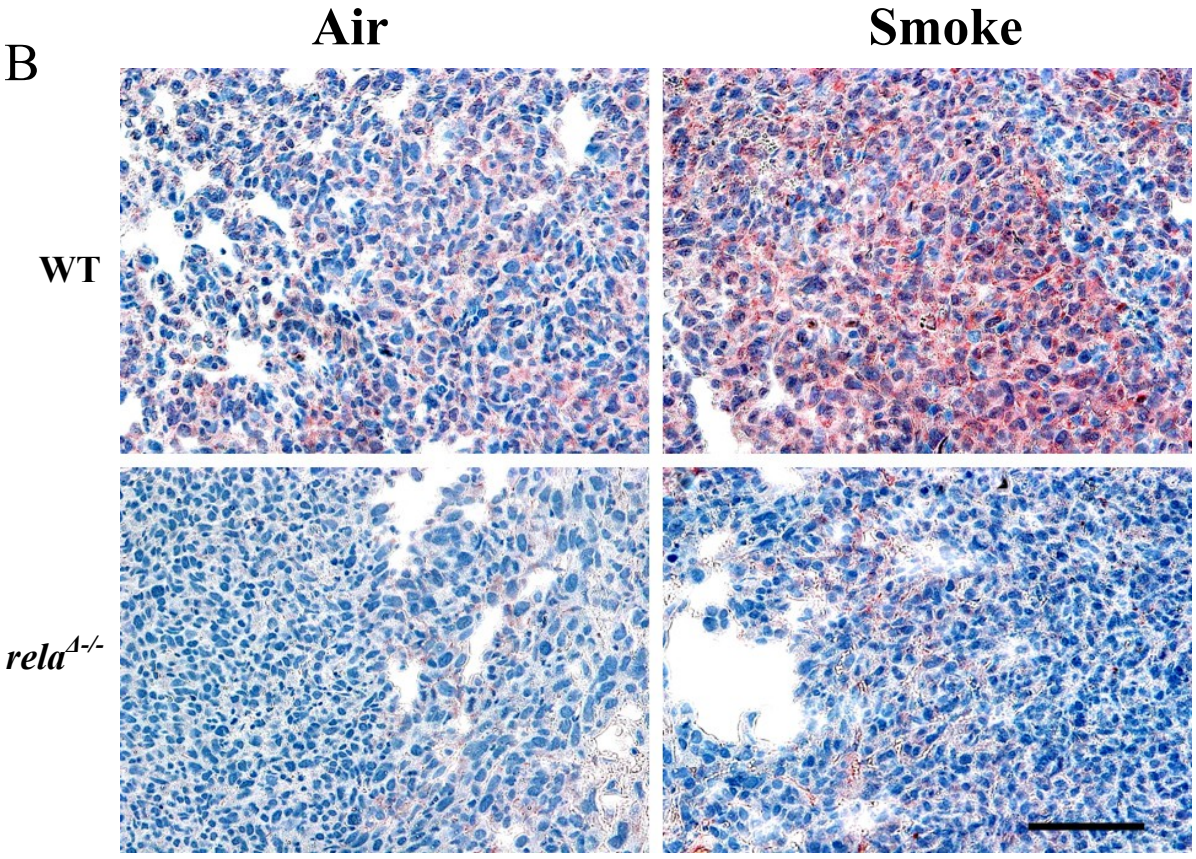
To investigate whether CS-induced inflammation impacts on tumor cell biology, tumor cell proliferation was examined by immunohistochemical analysis of the proliferation markers Ki-67. The numbers of Ki-67-positive cells were higher in CS-exposed tumors as compared to tumors of air exposed animals (Fig. 13D,E). Staining for Ki-67 in tumors of *rela*$^{\Delta-/-}$ mice was reduced as compared to WT mice (Fig. 13D,E).

To follow up the results of the pathway analysis, we investigated whether these pathways are activated dependent on myeloid NF-κB. Tumors were generated by injection of 5×10^5 LLC cells and mice were sacrificed 24h later of last CS exposure. Tumors were carefully microdissected using needles from lungs, lysed, and examined by immunoblotting. In contrast to the air exposure group, expression of cyclin D1 and c-*myc*, which regulate the progression from G1 to S phase, were induced in response to CS treatment in lung tumors of WT mice

(Fig.14A). CS exposure also increased the expression of PCNA (Fig. 14A). Immunoblot analysis of tumor lysates of WT mice revealed upregulation of c-jun, ERK phosphorylation, STAT3 and STAT3 phosphorylation in lung tumors after CS exposure (Fig.14A). Truncation of RelA/p65 in myeloid cells significantly reduced the expression of cyclin D1, c-Myc, and PCNA as well as the activation of STAT3 and c-jun in tumor cells (Fig.14A). Interestingly, no changes on ERK phosphorylation in lung tumor upon ablation of RelA/p65 in myeloid cells was observed (Figure 14.A) indicating that CS accelerates Erk phosphorylation in lung tumor independent of the myeloid NFκB signalling pathway.

Wnt/β-catenin signaling plays important roles in embryonic development and tumorigenesis (Oguma et al., 2008; Reya and Clevers, 2005). Therefore, it was investigated whether myeloid RelA/p65 is involved in the regulation of Wnt/β-catenin signaling in lung tumors. Unphosphorylated β-catenin was examined in lung tumors of WT mice and *rela*$^{\Delta-/-}$ mice by immunostaining. There was a strong staining in the tumor cells of WT mice after CS exposure (Fig.14B). *rela*$^{\Delta-/-}$ mice showed decreased staining for unphosphorylated β-catenin in the tumors of both the air exposure and CS groups (Fig.14B). These results were confirmed by immunoblot analysis for unphosphorylated β-catenin in tumor lysates (Fig.14C). These data show that myeloid RelA/p65 regulates tumor cell proliferation and activates growth signaling cascades.





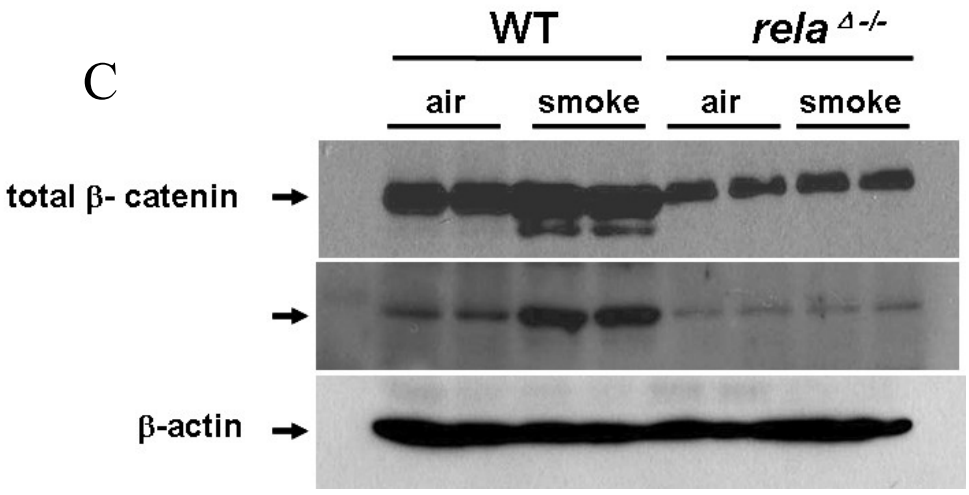


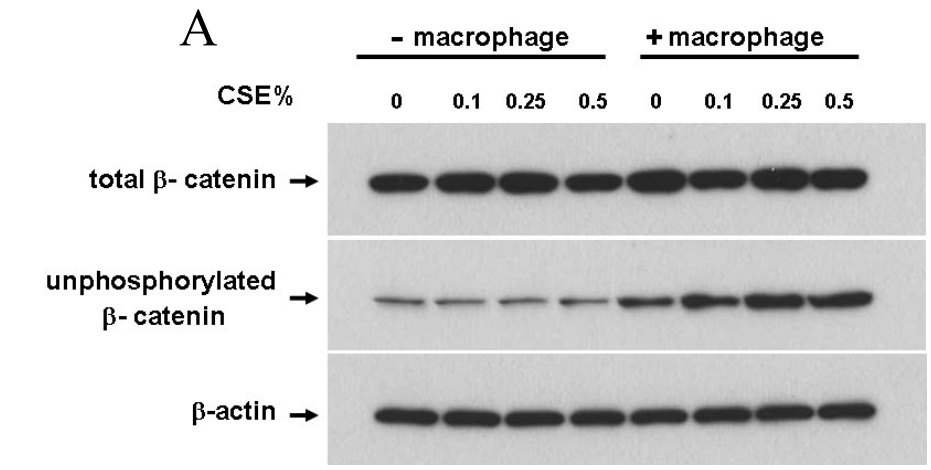
Figure 14: Myeloid cell RelA/p65 is necessary for induction of proliferation and of survival signaling pathways in tumor cells.
Tumours bearing mice were exposed to air or CS. Lungs were removed, fixed and paraffin embedded 24h after the last CS exposure. (A) Excised lung tumors were analyzed for the expression and phosphorylation of the indicated proteins by immunoblot analysis. (B) Expression of unphosphorylated β-catenin was examined by immunohistochemical analysis in tumor-bearing lung sections of WT mice and *rela*$^{\Delta-/-}$mice. Bar=100 µm. (C) Isolated lung tumors were analyzed for expression and unphosphorylation of β-catenin.

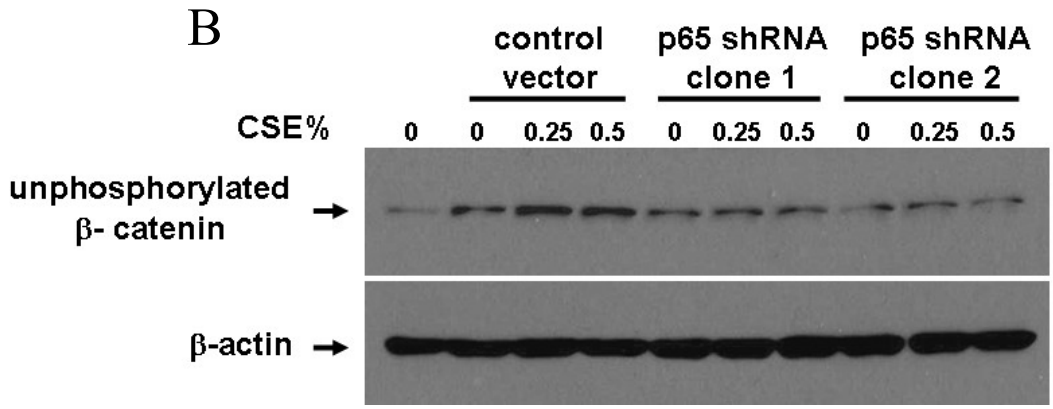
3.9 Macrophage TNF-alpha activates Wnt/β-catenin signaling through GSK3β and Akt

To dissect the cellular pathways involved in myeloid RelA/p65 induced Wnt/ß-catenin signaling in cancer cells, in vitro tissue coculture models were performed. We examined the presence of unphosphorylated β-catenin using human peripheral blood monocyte-derived macrophage and A549 cells. Consistent with in vivo results, macrophages mediated the activation of β-cateninin in cancer cells in response to CSE (Fig.15.A), whereas CSE did not show a significant increase of unphosphorylated β-cateninin levels in A549 cells that were not co-cultured with macrophages (Fig. 15.A). Konckdown of p65 in U937 cells by shRNA reduced accumulation of unphosphorylated β-cateninin in A549 cells (Fig. 15.B). These findings suggest RelA/p65 in macrophages is required for promotion of Wnt/β-catenin signalling in cancer cells.

To evaluate whether macrophage released TNF-α is responsible for Wnt/β-catenin activation, lung cancer cells (A549) were directly stimulated with TNF-α. Administration of TNF-α significantly increased the accumulation of unphosphorylated β-cateninin in A549 cells after 4 hours (Fig.15C). Addition of a neutralizing antibody against TNF-α significantly suppressed macrophage-induced accumulation of unphosphorylated β-cateninin in A549 cells (Fig. 15D). These results show that TNF-α release from macrophages is required and sufficient for the activation of the Wnt/β-catenin pathway in cancer cells.

GSK3β has been shown to phosphorylate β-catenin leading to its degradation via the ubiquitin pathway (Oguma et al., 2008, Kaler et al., 2009). Furthermore, Akt has been reported to be involved in Wnt signaling by phosphorylating GSK3β at Ser9 (Oguma et al., 2008, Sharma et al., 2002). Therefore, it was analyzed whether TNF-α phosphorylates GSK3β or Akt. A549 cells were treated with TNF-α and proteins were analyzed for GSK3β phosphorylation on Ser9 and Akt phosphorylation on Ser473. TNF-α exposure resulted in phosphorylation of GSK3β and Akt in A549 cells (Fig. 15C). These results suggest that TNF-α promotes the Wnt/β-catenin pathway by phosphorylation of GSK3β and the activation of the Akt pathway.


A
- macrophage
+ macrophage
CSE%
0 0.1 0.25 0.5 0 0.1 0.25 0.5
total β- catenin
unphosphorylated β- catenin
β-actin


B
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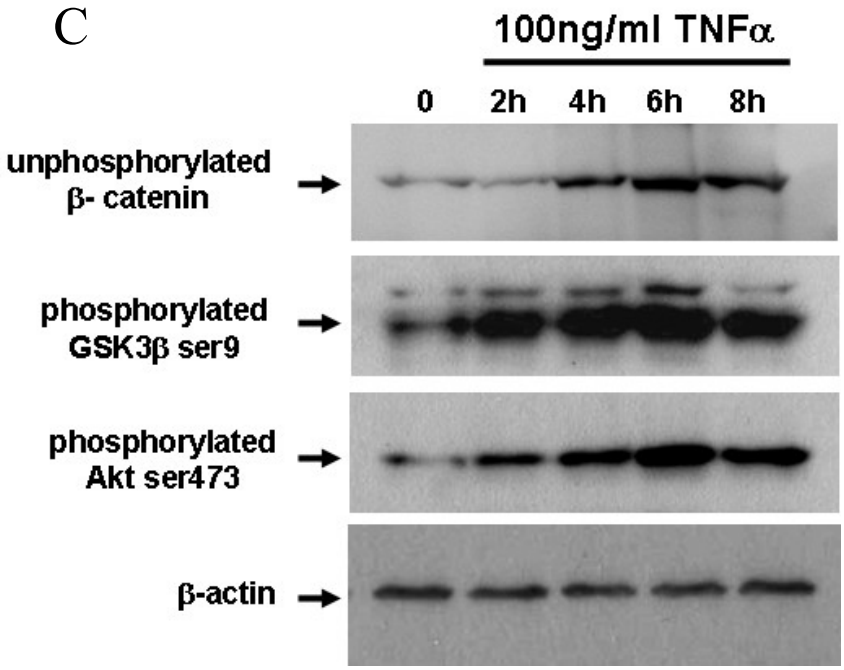
p65 shRNA clone 2
CSE%
0 0 0.25 0.5 0 0.25 0.5 0 0.25 0.5
unphosphorylated β- catenin
β-actin


C
100ng/ml TNFα
0 2h 4h 6h 8h
unphosphorylated β- catenin
phosphorylated GSK3β ser9
phosphorylated Akt ser473
β-actin

D

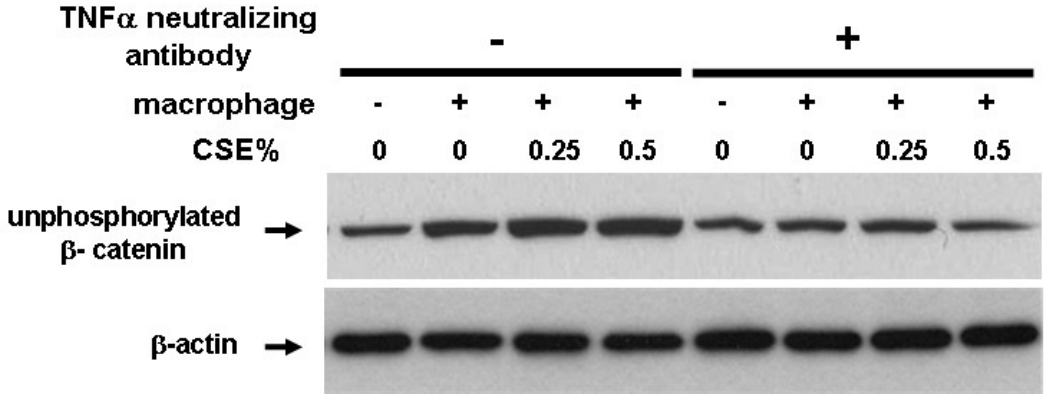


Figure 15: TNF-α released from macrophages activates β-cateninin through GSK3β and Akt pathways.
(A) A549 cells were co-cultured with human peripheral blood monocyte-derived macrophage and were exposed to 0-5%CSE for 72h. A549 cell lysates were analyzed by immunoblotting for the abundance of unphosphorylated β-catenin. (B) P65 was knocked down in U937 cells by shRNA and the effect on A549 cells was determined by detection of unphosphorylated β-catenin by immunoblotting. (C)A549 cells were treated by 100 ng/ml TNF-α for the indicated times. Expression of the indicated protein was detected by immunoblotting analysis. (D) TNF-α blocking antibody (10μg/ml) was applied to CSE-exposed A549 cells incoculture with human peripheral blood monocyte-derived macrophage. After 72h, the indicated proteins in A549 cells were analyzed by immunoblotting.

3.10 The Wnt/β-catenin signaling pathway is active in human NSCLC cells

To investigate whether the described mechanisms and pathways are relevant in patients, we analyzed human lung cancer samples by immunohistochemical detection of macrophages (CD68), TNF-α, and unphosphorylated β-catenin. Sections of healthy lung tissue, adenocarcinomas, and squamous cell carcinomas were stained. While sections of healthy lung tissue showed weak signals for unphosphorylated β-catenin, LC sections revealed positive staining for unphosphorylated β-cateninin in tumor cells toghether with the presence of CD-68 positive cells (Fig. 16). Staining for TNF-α revealed a strong expression in myeloid cells in the tumor stroma. These data shows that the principal pathways described in the present study are active in human disease and are likely relevant for disease progression.

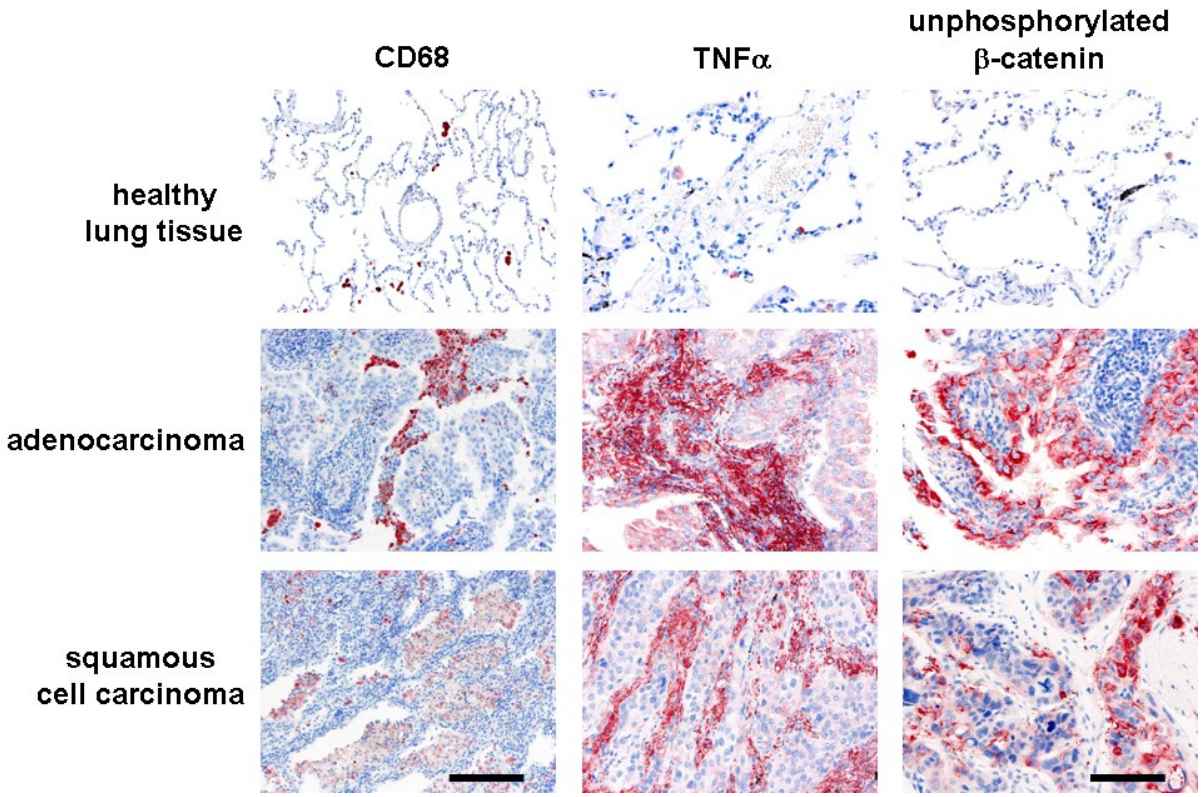
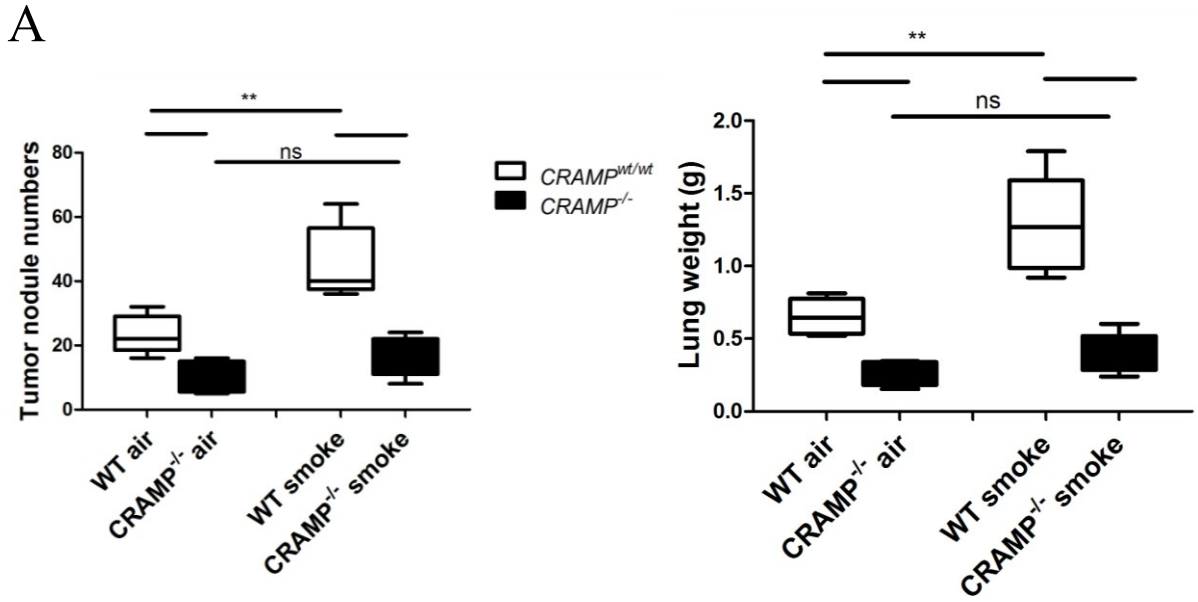


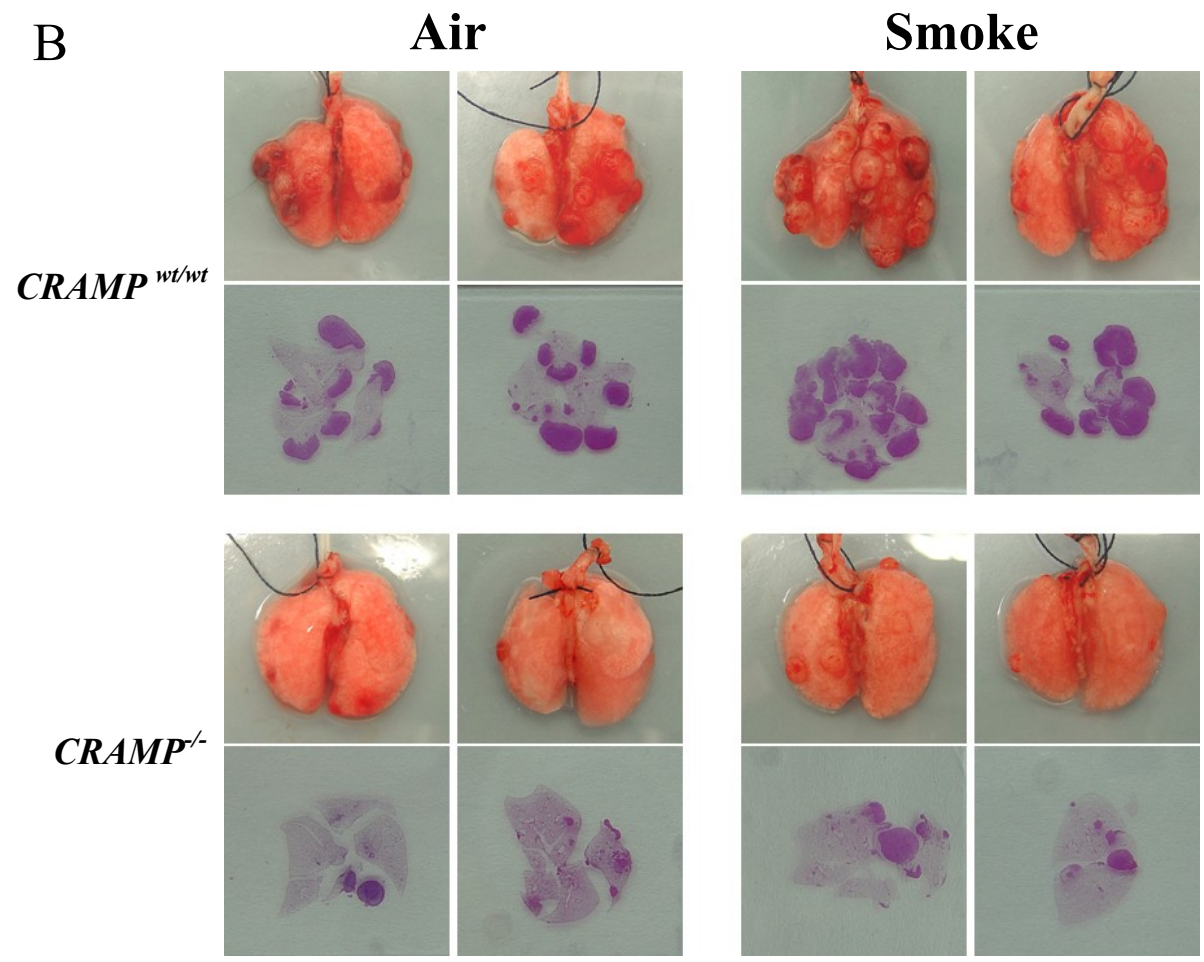
Figure 16: NSCLCs in humans reveal macrophage infiltrations and abundant β-catenin accumulation. Tissue section of healthy lung tissue, adenocarcinomas, and squamous cell carcinomas were stained with antibodies against CD68, TNF-α and unphosphorylated β-catenin. CD68, Bar = 200 µm; TNF-α and unphosphorylated β-catenin, Bar = 100 µm.

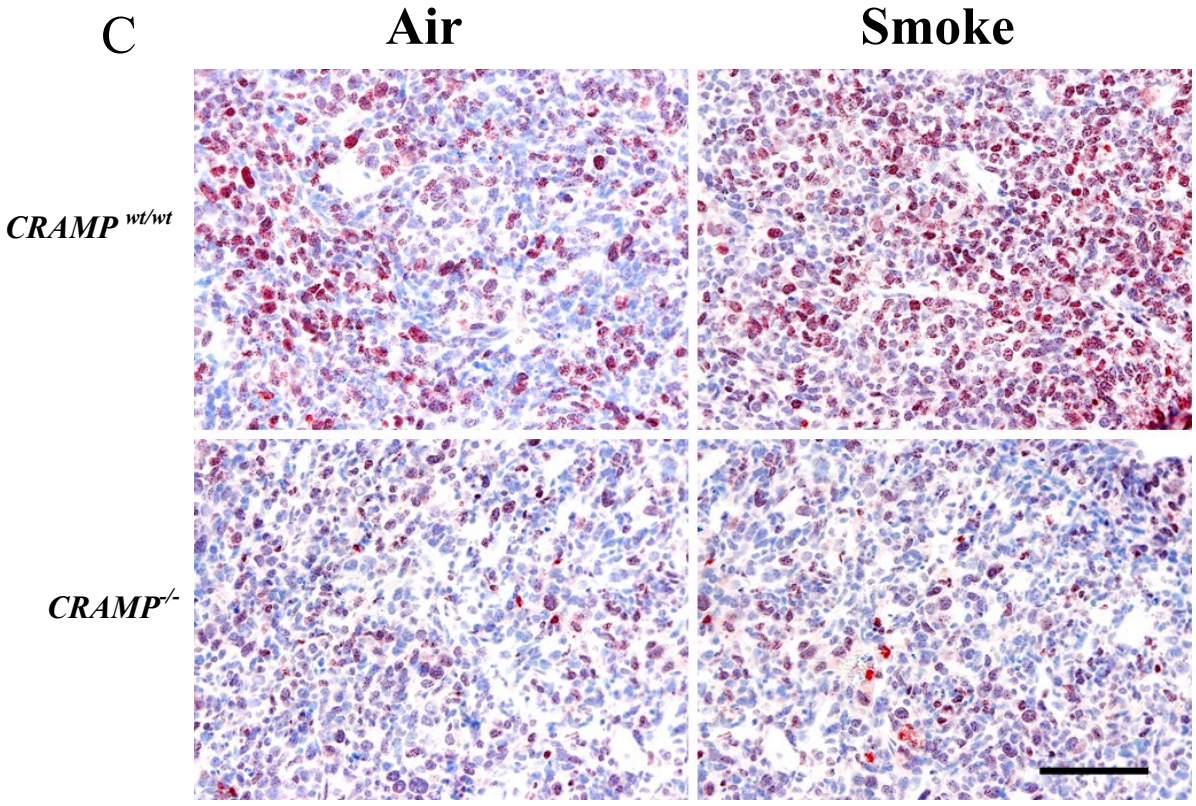
3.11 The cathelicidin CRAMP promotes metastatic lung tumor growth

The previous results indicate that the human cathelicidin LL-37 acts as a growth factor for human lung cancer cells and promotes tumor cell growth (Von Haussen, et al., 2008). To investigate the role of CRAMP in lung metastatic carcinomas and CS-induced tumor promotion, the metastatic lung cancer model was used in which tumors were generated by injection of 2×10^5 LLC cells in CRAMP knockout mice (*CRAMP*$^{-/-}$) and in littermate control mice (*CRAMP* $^{wt/wt}$) followed by 7 days CS exposure and a 7 days' rest interval. Control groups were incubated with filtered air. Lung weights and number of tumor nodules were determined at day 21. There was a dramatic increase in lung nodule number and lung weight

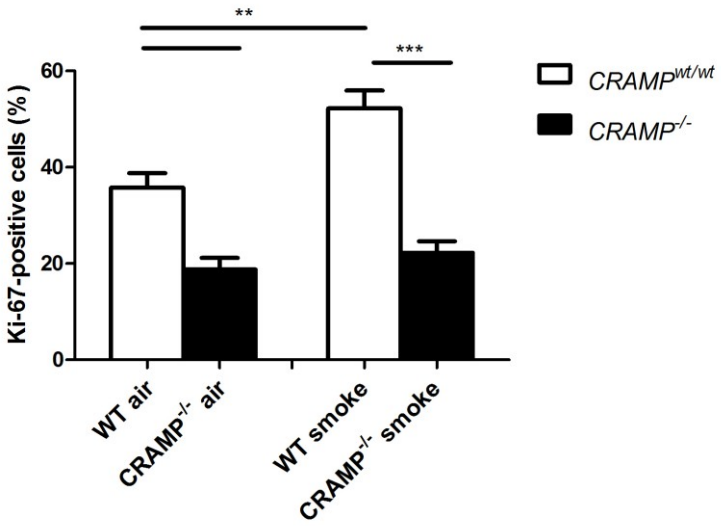
(Fig. 17A) in lungs of *CRAMP* $^{wt/wt}$ mice that were exposed to CS relative to air exposure control animals. Furthermore, CRAMP deficiency resulted in reduced lung tumor nodule numbers and lung weight. There was no enhanced tumor growth in *CRAMP*$^{-/-}$ mice exposed to CS (Fig. 17A). H&E staining showed that the tumor load of lungs of CS-exposed *CRAMP* $^{wt/wt}$ mice were much higher compared to lungs of air exposed mice and *CRAMP*$^{-/-}$ mice (Fig. 17B). In addition, immunohistochemical staining for the proliferation marker Ki-67 showed that the number of Ki-67-expressing cells was higher in CS-exposed *CRAMP* $^{wt/wt}$ tumors compared to air exposed *CRAMP* $^{wt/wt}$ mice and *CRAMP*$^{-/-}$ mice indicating that CS does not induce proliferation of tumor cells in *CRAMP*$^{-/-}$ mice (Fig. 17C, D). Furthermore, CS exposure shortened the life time of *CRAMP* $^{wt/wt}$ mice and *CRAMP*$^{-/-}$ mice exhibited longer survival times than *CRAMP* $^{wt/wt}$ mice (Fig. 17E). There was no difference in the survival rates of air- or CS-exposed *CRAMP*$^{-/-}$ mice (Fig. 17E). These data show that ablation of CRAMP in mice results in reduced smoke induced tumor proliferation and tumor promotion.




B
Air
Smoke
CRAMP wt/wt
CRAMP-/-


C
Air
Smoke
CRAMP wt/wt
CRAMP-/-

D



E

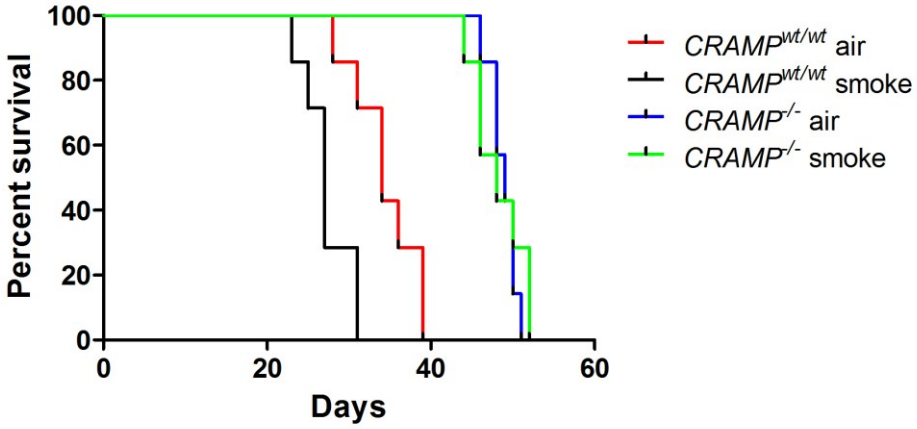
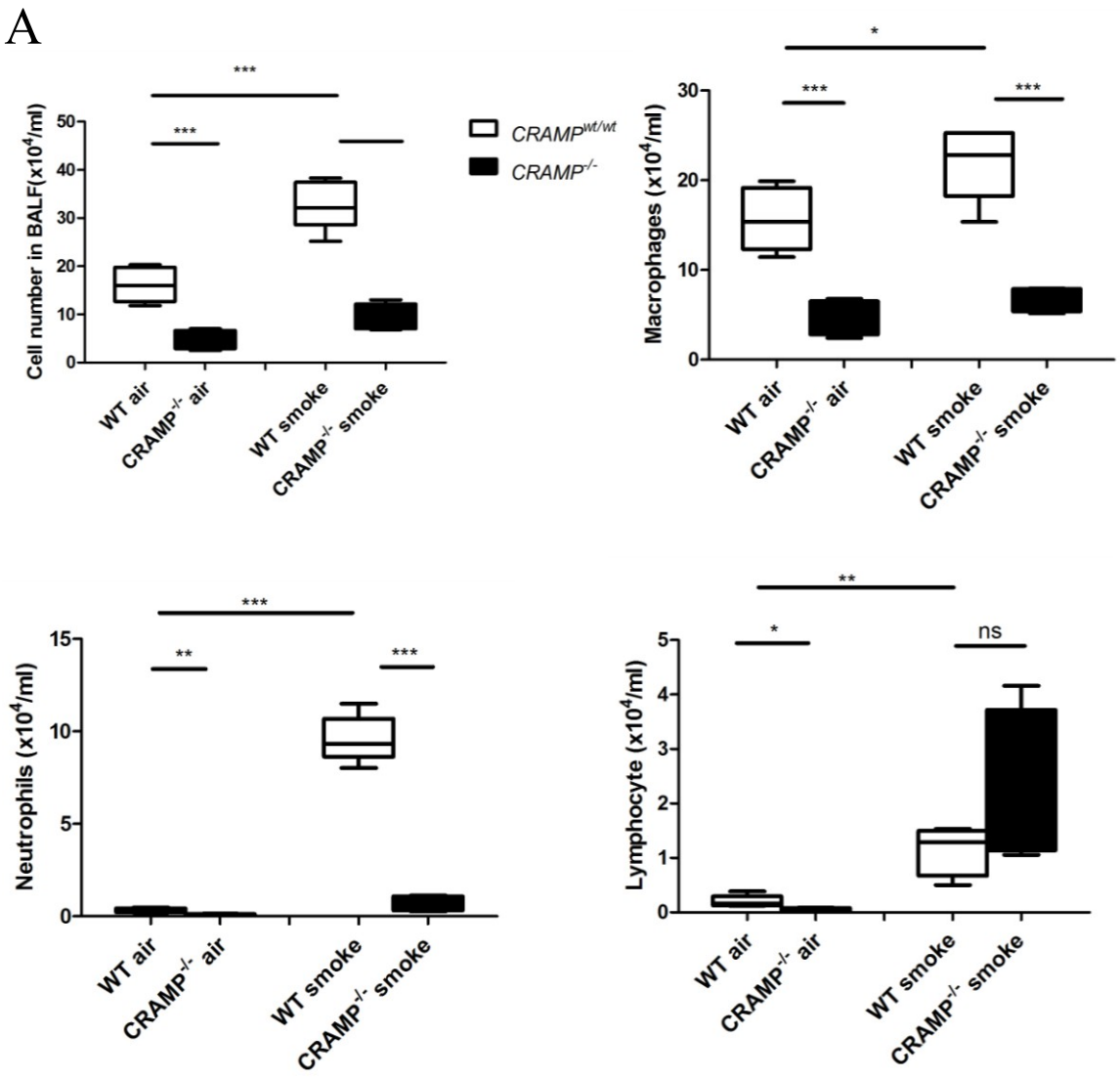


Figure 17: CRAMP deletion decreases MCS-promoted growth of lung cancer
(A) 2×10^5 LLC cells were intravenously injected into sex-matched *CRAMP* $^{wt/wt}$ mice and *CRAMP*$^{-/-}$ mice. At day 7, mice were smoked for 7 days, at day 21, the lungs were removed. The numbers of tumor nodules detectable on the lung surface and lung weight were determined. (B) Lungs of *CRAMP* $^{wt/wt}$ and *CRAMP*$^{-/-}$ 21 days after LLC cell inoculation (2×10^5 cells) with or without MCS exposure. Lung appearance (up) and histology (H&E stain; down). (C) Tumor-bearing lungs were removed, fixed and paraffin embedded 24h later of last MCS exposure. Proliferating cells were examined in lung tumors of *CRAMP* $^{wt/wt}$ mice and *CRAMP*$^{-/-}$ mice by immunostaining with anti-Ki-67-antibodies in paraffin-embedded sections. Scale bars= 100µm. (D) The percentage of Ki-67-positive tumour cells among all tumour cells. Results are means ± SEM, n=5, significant difference,** $p<0,01$; *** $p<0,001$. (E) Kaplan-Meier survival analysis of the indicated mouse strains injected with 2×10^5 LLC cells via the tail vein exposed to either CS or air ($p<0,001$; Log-rank test for statistic analysis; n=7).

3.12 CRAMP is required for CS induced influx of myeloid cells into the lung

It is well established that cathelicidins function as chemoattractants for immune cells (Coffelt et al., 2009; Kurosaka et al., 2005). It was hypothesized that cathelicidins play a role in the CS induced recruitment and infiltration of immune cells into the lung and into tumor tissue thereby promoting tumor proliferation and progression. The total cell number as well as the number of macrophages, neutrophils, and lymphocytes was increased after CS exposure in BALFs of *CRAMP* $^{wt/wt}$ mice compared to air exposed mice, whereas CS exposure did not induce the influx of myeloid cells into the lungs of *CRAMP*$^{-/-}$ mice (Fig. 18A). In addition, the infiltration of macrophages into lung tumors was increased in *CRAMP* $^{wt/wt}$ mice but not in *CRAMP*$^{-/-}$ mice after CS exposure as shown by immunohistochemistry (Fig. 18B). These data show that CRAMP plays a critical role in directing myeloid cells into the lung and into tumot tissue after smoke exposure.



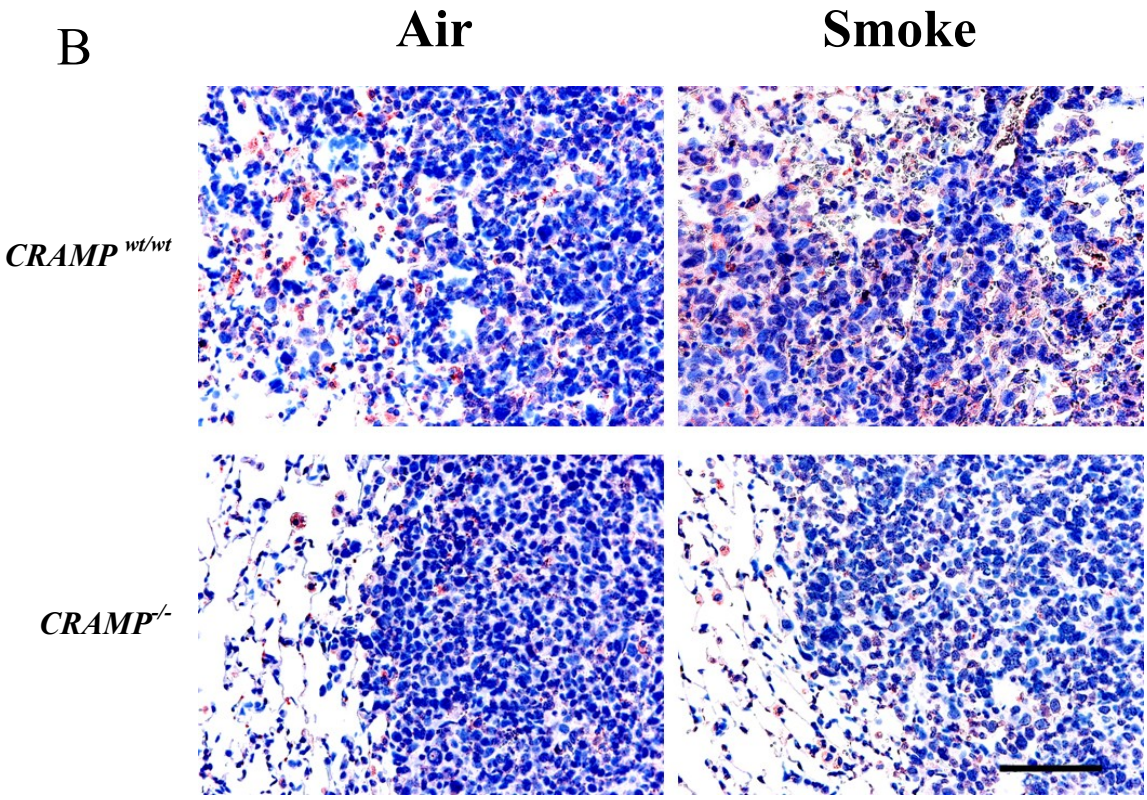
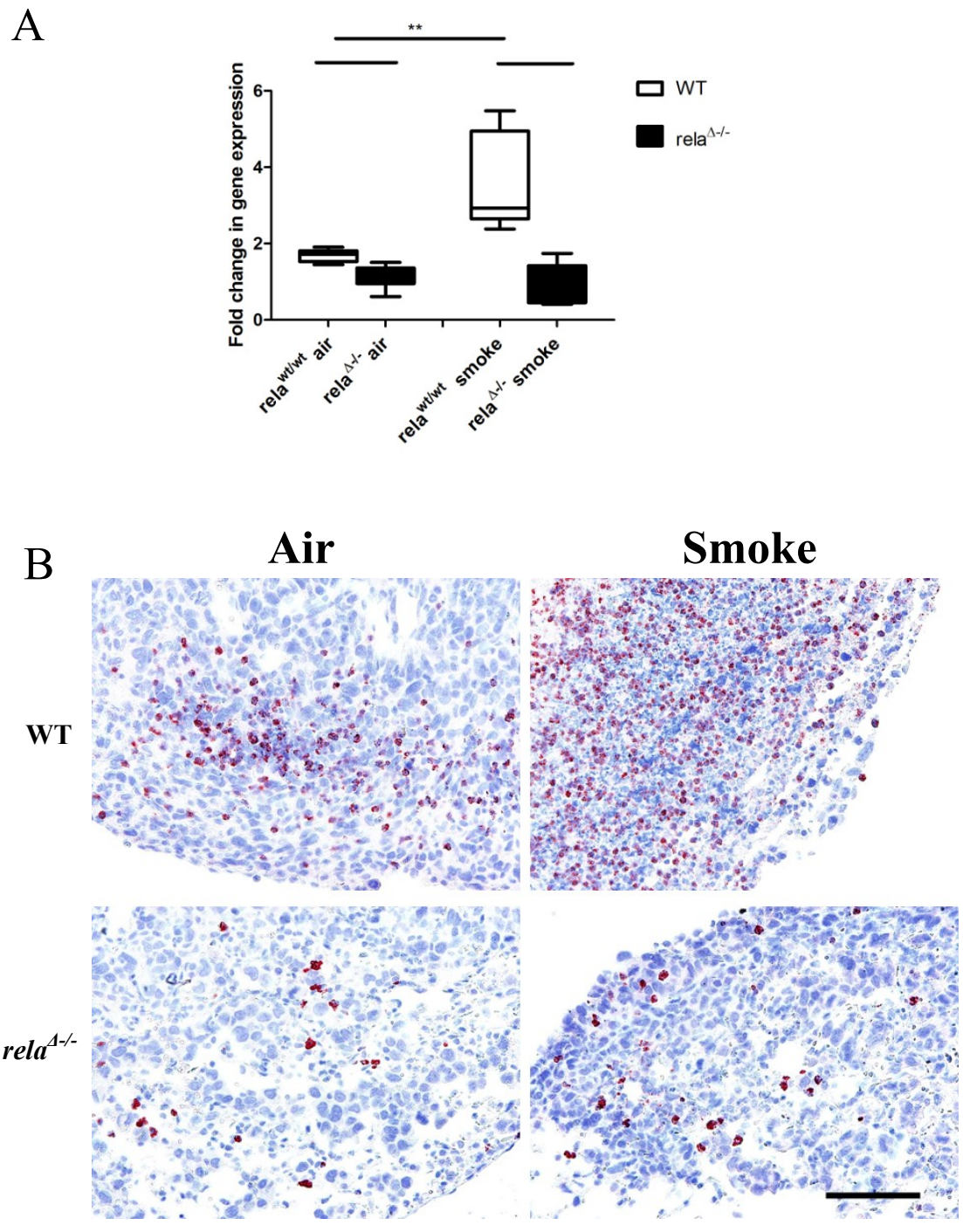


Figure 18: CRAMP ablation reduces MCS-induced recruitment of immune cells into mice lung.
(A) Tumors bearing *CRAMP* $^{wt/wt}$ and *CRAMP*$^{-/-}$ mice were exposed to air or MCS. BALFs were collected 24h after the last MCS or air exposure. Cellular composition was evaluated using cytospin preparations. Results are means ± SEM, n=5, significant difference,* p<0,05;** p<0,01; *** p<0,001; ns, not significant. (B) Tumour-bearing lungs were removed, fixed and paraffin embedded 24h later of last MCS exposure. Paraffin-embedded tumour-bearing lung sections were analyzed by anti-CD68 antibody immunohistochemical analysis. Scale bars= 100µm.

3.13 CS-induced recruitment of CRAMP-positive immune cells depends on myeloid RelA/p65

As indicated above, CS induces lung cancer proliferation through activation of myeloid NF-κB and subsequent release of inflammatory factors that promote tumor growth. Furthermore, it has been shown that NF-κB signalling pathway is involved in cathelicidin regulation and function (Li et al., 2009). To examine whether NF-κB/RelA mediates the influx of CRAMP-positive immune cells into the lung and into tumor tissue we exposed *rela*$^{\Delta-/-}$ mice to CS. Litter mates (WT) were used as control. The relative *CRAMP* mRNA expression in metastatic carcinomas was compared between air and CS-exposed mice. CS exposure resulted in an increased expression of CRAMP in the lungs of WT mice compared to air exposed mice, whereas CS exposure did not result in enhanced expression levels of CRAMP in *rela*$^{\Delta-/-}$ mice (Fig. 19.A). Furthermore, immunohistochemistry showed that expression levels of CRAMP in

tumor cells was considerably weak, almost un-measurable, whereas myeloid cells infiltrated into the tumor tissue expressed remarkably higher levels of CRAMP (Fig. 19.B). The number of CRAMP-positive myeloid cell was significantly increased in the tumor tissue of WT mice after CS exposure, but not in *rela*$^{\Delta-/-}$ mice (Fig. 19.B). We also found that CS exposure increased the number of CRAMP-positive immune cell in noncancerous lesions region (Fig. 19.C). These results indicate that RelA/p65 is required for the influx of CRAMP-positive immune cells into tumor tissue.



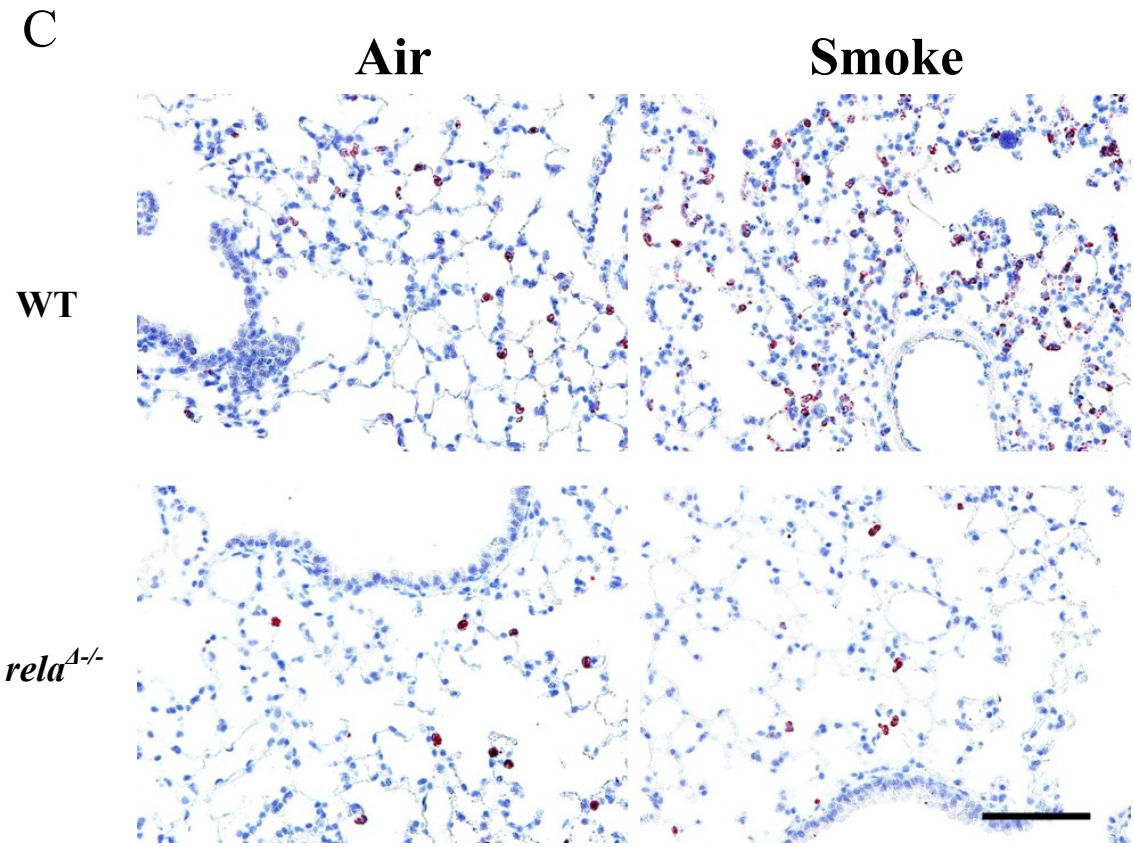


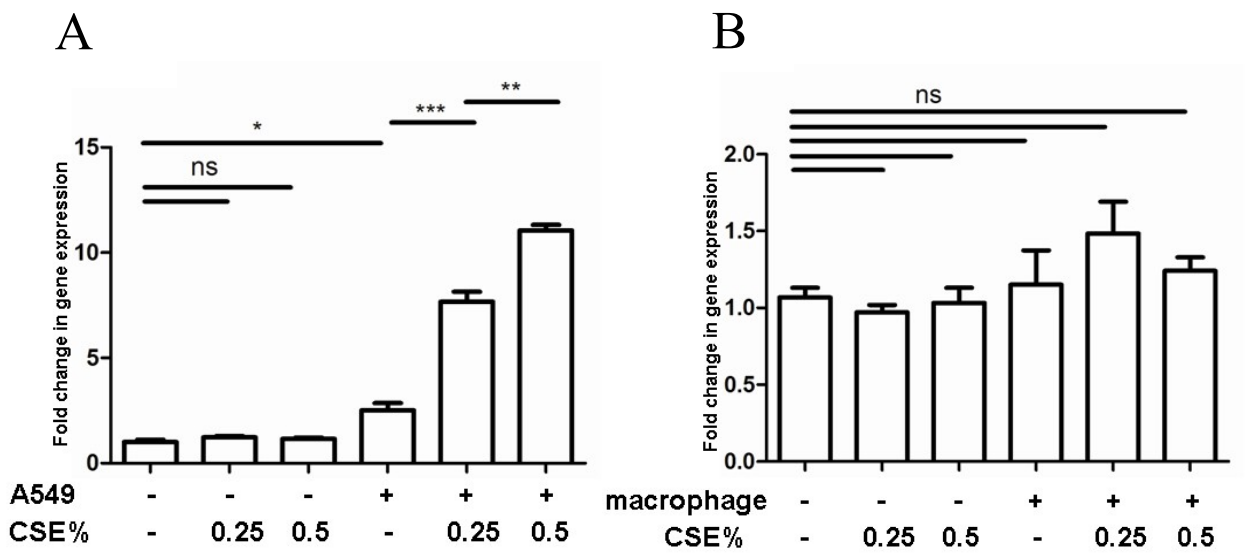
Figure 19: Expression of CRAMP and CRAMP-positive immune cells infiltration in WT mice and *rela*$^{\Delta-/-}$ mice.
(A) CS exposure increases the expression of *CRAMP* gene whereas myeloid cell RelA/p65 deletion decreases CS-induced *CRAMP* induction. Lung total RNA was isolated 24h after last CS exposure and analyzed by real-time PCR. Mean fold change ± SEM, n=5, significant difference, ** p<0,01 versus air control. (B,C) Presence of CRAMP-positive immune cells in lungs of air- and MCS-exposed tumor-bearing mice. Lung sections prepared 24 h after last CS exposure of mice were analyzed by immunostaining for CRAMP. (B) tumor. (C) Noncancerous lesions region. Scale bar = 100 µm.

3.14 Cancer cells mediate the CS induced expression of the human cathelicidin LL-37/hCAP-18 in macrophages

To demonstrate that CS induces the expression of cathelicidin in human tissue, human macrophages derived from peripheral blood monocytes were co-cultured with A549 cells. After 48 hours co-incubation with A549 cells, the induction of human cathelicidin LL-37/hCAP18 mRNA was increased in macrophages and was further enhanced by CSE in a dose dependent manner (Fig. 20.A). In A549 cells, the expression of LL-37/hCAP18 mRNA remained constant even when the cells were exposed to CSE, co-culture with macrophages or

co-culture with macrophages in combination with CSE (Fig. 20.B). Interestingly, macrophages that were treated with CSE alone did not show elevate expression of LL-37/hCAP18 mRNA suggesting soluble factors produced by A549 cells mediating the expression of this peptide.

It has been shown that the vitamin D receptor (VDR) mediates the expression of LL-37/hCAP18 mRNA in human macrophages (Liu, et al., 2006, Gombart, et al., 2005). Therefore, the regulation of the VDR was investigated in human macrophages upon co-incubation with A549 cells. In agreement with expression of LL-37/hCAP18 mRNA, an increase of VDR mRNA in macrophages was observed when co-cultured with A549 cells and exposed to CSE (Fig. 20.C). Likewise, Cyp27B1 mRNA levels were up-regulated in co-cultured and CSE exposed macrophages (Fig. 20.D). Cyp27B1 catalyzes the conversion of inactive provitamin D_3 hormone (25-hydroxyvitamin D_3; $25(OH)D_3$) into the bioactive form (1,25-dihydroxyvitamin D_3; $1,25(OH)_2D_3$). Like LL-37/hCAP18 expression, no observable up-regulation of VDR and Cyp27B1 mRNA was seen in CSE-treated macrophages without the presence of A549 cells. In addition, macrophages were treated with various concentrations of $1,25(OH)_2D_3$ and LL-37/hCAP18 mRNA expression was assessed. $1,25(OH)_2D_3$, strongly induced LL-37/hCAP18 gene expression in dose-dependent manner (Fig. 20.E). Taken together, these results suggest that the CSE induced expression of LL-37/hCAP18 is mediated by lung tumor cells involving vitamin D activated signalling pathways.



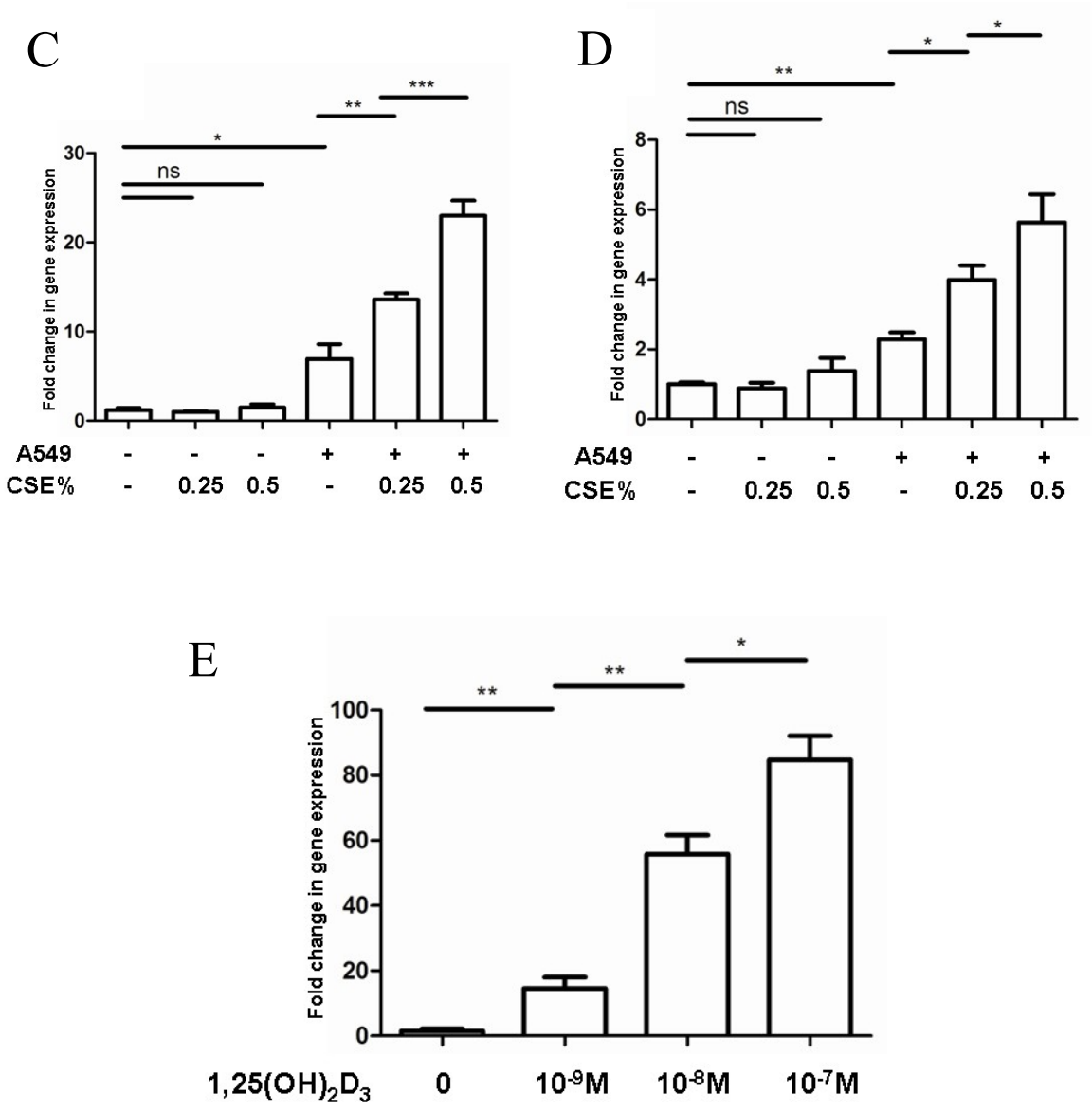
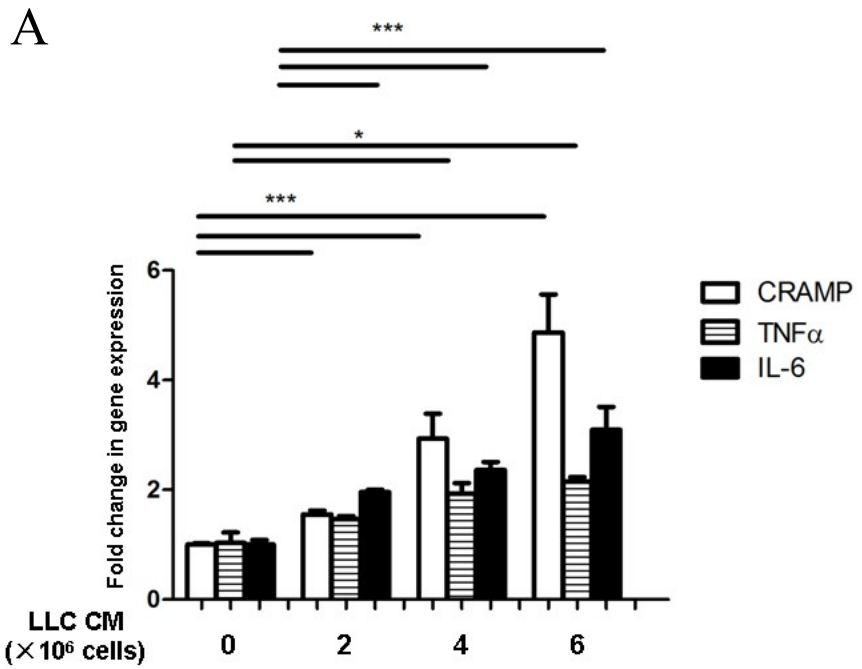


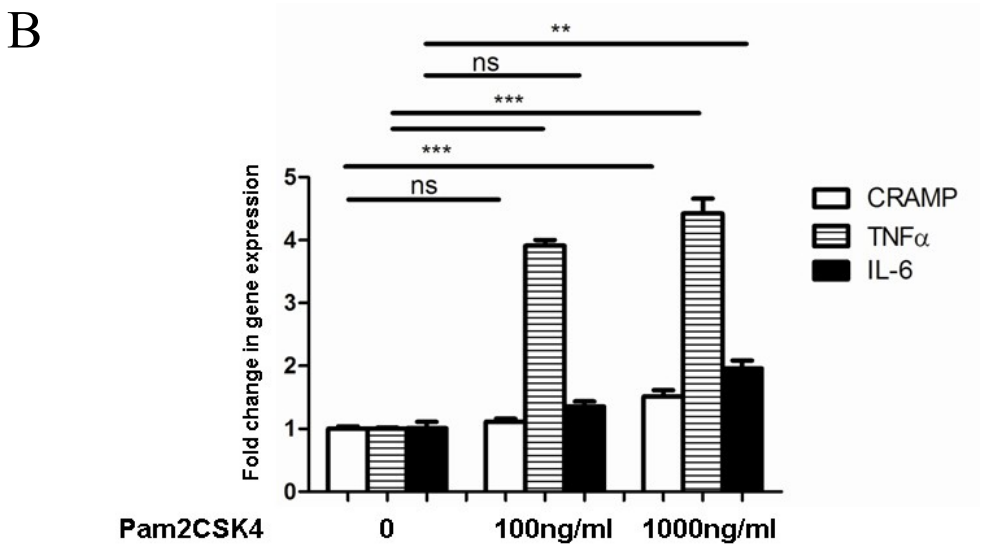
Figure 20: A549 cells in combination with CSE induce LL-37/hCAP18 and relative genes expression in macrophages and A549 cells.
(A,B) A549 cells co-culture with human peripheral blood monocyte-derived macrophages and then were exposed to 0-0.5%CSE for 48h in 6-well plates. Total RNA were isolated from macrophages and A549 cells, and gene expression was assessed by real-time PCR. Expression of LL-37/hCAP18 mRNA in macrophages (A) and A549 cells (B). Expression of VDR (C) and Cyp27B1 (D) mRNA in macrophages were assessed by real-time PCR. (E) Regulation of LL-37/hCAP18 mRNA on stimulation with $1,25(OH)_2D_3$ for 24h. Mean fold change ± SEM, * p<0,05;** p<0,01; *** p<0,001; ns, not significant.

3.15 Regulation of CRAMP expression

In contrast to LL-37/hCAP18, the transcriptional mechanism that regulates CRAMP gene expression is not clear. Since TLR2 and biglycan, a small leucine-rich proteoglycan, have been shown to mediate host innate immunity (Schaefer et al., 2005; Kim et al., 2009), it was

hypothesized that these extracellular signals might induce CRAMP gene expression. The mouse macrophage cell line RAW264.7 was treated with different concentrations of serum-free conditioned medium from LLC cells (LLC CM), biglycan, and TLR2-TLR6 ligand Pam2CSK4. As determined by real-time PCR, conditioned medium from LLC induced a significantly high expression of the CRAMP gene in RAW264.7 cells in a LLC cell number-dependent manner (Figure 21A). Biglycan did not induce the expression of CRAMP (Fig. 21C). In addition, Pam2CSK4 induced up-regulation of the CRAMP gene in RAW264.7 cells. The effect was slight and rather high doses of Pam2CSK4 were required indicating the induction of this gene is likly independent in TLR2 or TLR6 (Fig. 21B). It was also found that induced expression of TNFα and IL-6 mRNA in RAW264.7 cells (Fig. 21A). In Pam2CSK4 treated macrophages, a strong increase of TNFα mRNA expression was observed as well as a weak induction of IL-6 mRNA (Fig. 21B). Additionally, the induction of *CRAMP* was not observed after treatment with inflammatory factors such as TNFα, IL-1β, and INFγ for 24 hours (Fig. 21C). Taken together, these data suggest that tumor-derived inflammatory factors mediate expression of CRAMP in macrophages; however, which factor or factors mediate the regulation of CRAMP needs to further inestigated in the future.





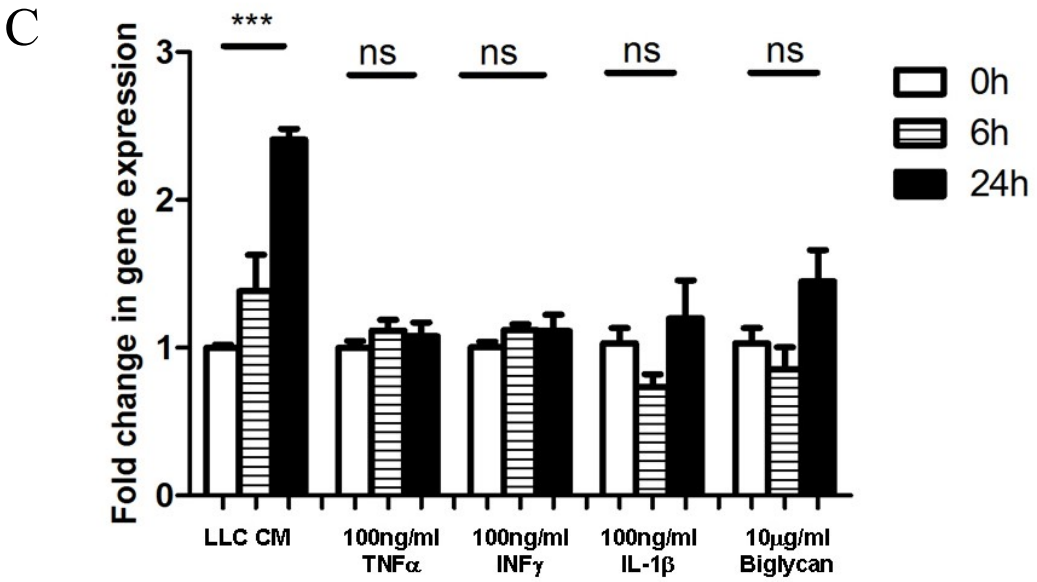


Figure 21: Induction of CRAMP mRNA expression by conditioned medium from LLC cells and various inflammatory factors.

(A) RAW264.7 cells were treated with LLC cells conditioned medium for 24 hours. CRAMP, TNFα and IL-6 mRNA expression were analyzed by Real-time PCR. Medium were collected from 2×, 4×, and 6×10^6 cells/10cm dish. (B) RAW264.7 cells were cultured with Pam2CSK4 (100ng /ml and 1000ng/ml) for 24 hours, CRAMP, TNFα and IL-6 mRNA expression were measured. Mean fold change ± SEM, ** p<0,01; *** p<0,001; ns, not significant. (C) RAW cells were treated with the indicated compounds for 6 and 24 hours, and CRAMP mRNA expression was analyzed by Real-time PCR. Mean fold change ± SEM, ns, not significant.

4 Discussion

The main finding of the present study was: (i) smoke-induced pulmonary inflammation increases lung cancer proliferation *in vivo* and *in vitro* and that RelA/p65 of myeloid cells contributes to promotion of lung tumor cells by increasing cell proliferation; (ii) One crucial pathway comprises RelA/p65-derived TNFα induces and subsequent activation of Wnt/β-catenin signalling in tumor cells; (iii) cathelicidin promote cigarette smoke-induced lung carcinoma proliferation, it is associated with the recruitment of immune cells and activation of myeloid NF-κB.

A strong association of inflammation with cancer has been supported by clinical and experimental observations (Coussens and werb, 2002, Yoshimura, et al., 2006). Inflammation is suggested to cause DNA alterations and induces oncogenic mutations by the production of reactive oxygen and nitrogen species, which result in tumor initiation (Grivennikov, et al., 2010, Takahashi, et al., 2010). Furthermore, inflammation promotes tumor progression via inflammatory cytokines produced by infiltrating immune cells such as macrophages and neutrophils (Grivennikov, et al., 2010). It has been shown that interaction between neoplastic cells and their close surrounding that includes macrophages, granulocytes, dendritic cells and lymphocytes are crucial to each step of tumorigenesis (Coussens and werb, 2002, Yoshimura, et al., 2006). These immune cells have a remarkable ability to produce pro-inflammatory mediators, such as TNFα and IL-6, which can serve as mitogens and survival factors for tumorigenesis and tumor promotion (Grivennikov, et al., 2010).

It has been reported that chronic inflammation induced by cigarette smoke, asbestos or silica is clearly linked to development of cancer (Yoshimura, et al., 2006). Pulmonary disorders such as COPD, a chronic airway inflammatory disease induced by microbial infection or cigarette smoke, are associated with the greatest risk for lung cancer (Walser et al., 2008; Punturieri et al., 2009). Particulate material from cigarette smoke and other irritants induce injury and chronic lung inflammation and contribute to the development of lung cancer (Grivennikov et al., 2010; Punturieri et al., 2009). However, most of the research focuses on the role of CS and chronic inflammation as tumor initiators. Whether CS or CS induced inflammatory responses are tumor promoters remains poorly understood.

4.1 Cigarette smoke enhances lung tumor promotion by inducing pulmonary inflammation

Cigarette smoke is the most important risk factor for lung cancer, which is associated with about 85-90% of cases (Jemal et al., 2003). Even though the carcinogenicity of CS has been successfully shown in several animal experiments, it is still poorly understood whether this complex mixture also has tumor-promoting effects (D'Agostini et al., 2001; Witschi et al., 2002). In this work, a mouse model is described in which CS exposure has a strong tumor-promoting effect on metastatic lung cancer cells. It is shown that cigarette smoke increases lung cancer promotion in mice inoculated with LLC cells. The in vivo results are supported by in *vitro* experiments. It is shown that cigarette smoke remarkably enhances macrophages induced growth of the lung cancer cell line A549 cells. The data of this study clearly proof that co-culture of cancer cells with human macrophages leads to an increased growth of A549 cells. Macrophages which are activated in response to CS are considered to be a major source of inflammatory cytokine (Yang et al. 2006b). The increased growth of tumor cells was be correlated with an induction of pro-inflammation cytokines.

Several factors can contribute to tumor promotion, whereas inflammation induced elevation of inflammatory cytokines that promote proliferation and survival of malignant cells is likely to be a major tomor-promoting mechanism (Karin, 2006). It is currently thought that pro-inflammatory effects induced by cigarette smoke contribute to the development of COPD and lung cancer (Walser et al., 2008). In this study, the presence of a subacute inflammatory pulmonary response in mice exposed to CS is demonstrated. It is shown that cigarette smoke challenge results in an increase of total cells in BALF, including macrophages, neutrophils and lymphocytes. Especially macrophages are associated with tumor promotion. CS induces the induction and release of TNFα, IL-6, and KC in mice lung tissues. Previous reports showed that macrophages of the lung have an important role in the inflammatory responses seen in smoker and COPD patients and their number correlates with disease severity (Barnes, et al., 2003; Yang, et al., 2008; Vlahos et al., 2006). Macrophages can be activated by cigarette smoke or tumor derived factors, such as versican, resulting in the production of inflammatory mediators, such as TNFα, IL-6 and IL-8 (Yang et al. 2006b; Kim et al., 2009). Here, it is shown that both cigarette smoke exposure and co-culture with A549 cells increases TNFα, IL-6, and IL-8 releases from macrophages. However, what is noticeable is that the treatment of combination of CS and A549 cells resulted in a higher release of TNFα, IL-6, IL-8 from macrophage than stimulation of CSE or A549. Therefore, cigarette smoke and lung

cancer cells synergistically induce the expression of pro-inflammatory factors in macrophage. Interestingly, the tumor cell line A549 also showed increased expression of TNFα, IL-6, and IL-8, suggesting that tumor cells itself are involved in inflammation induced tumor promotion within the tumor microenvironment.

In this study, we evaluated macrophage infiltration into lung cancer tissue. It is shown that mouse lung tumors had more infiltrating macrophages after CS exposure compared with tumors of air control groups indicating that macrophage infiltration might contribute to CS-induced lung tumor growth. It is further shown that the presence of infiltrating macrophages is remarkable higher in human NSCLC than healthy lung. Previous reports have shown a close association between macrophage infiltration and poor prognosis in cancer (Ono, 2008, Moghaddam, et al., 2008). However, a correlation between extensive TAM infiltration and poor prognosis in lung cancer is contradictory (Pollard, 2004). For example, Koukourakis et al. found that high TAM infiltration were associated with a poor prognosis in 141 cases of early stage I-II NSCLC (Koukourakis et al., 1998), whereas Kerr et al. showed that there is a better correlation between tumor regression and high TAM number from comparison of 28 regressing NSCLC cases and 67 control cases (Kerr et al., 1998). Different approaches used in TAM assessment may contribute to these contradictory results in the mentioned lung cancer studies (Bingle et al., 2002).

4.2 NF-κB activation is crucial in inflammation promoted lung tumor proliferation

Activation of NF-κB is responsible for the induction of a variety of target genes that are important for tumorigenesis (Yoshimura et al., 2006). NF-κB activation in inflammatory cells controls the production of pro-inflammatory cytokines, including TNFα, IL-1, IL-6, and IL-23, which mediate tumor promotion and progression, as well as NF-κB activation in tumor cells (Karin and Greten, 2005; Grivennikov and Karin, 2010a). NF-κB activation also is found in tumor cells where it regulates cell proliferation, survival, angiogenesis, invasion, and metastasis (Luo et al., 2004; Grivennikov and Karin, 2010b; Naugler and Karin, 2008). The results shown in this study report that NF-κB activation is present in macrophages and lung cancer cells in an artifical tumor microenvironment. Tumor cells can induce NF-κB activation in macrophage and macrophage also activate NF-κB in tumor cell indicating that both cell types contribute to cancer development. Furthermore, cigarette smoke enhances the activation of NF-κB in macrophages and cancer cells, which is needed for tumor promotion.

RelA/p65 is an important component of classical NF-κB pathway and forms a dimer with p50. Upon activation of the classical NF-κB pathway, the RelA/p65:p50 dimer translocates to the nucleus resulting in the transcription of many genes encoding cytokines, chemokines, pro-angiogenic factors, adhesion molecules, and antiapoptotic proteins (Karin and Greten, 2005; Yoshimura, 2006). Here, a murine model with $rela^{F/F}$ LysMCre ($rela^{\Delta-/-}$) mice that have a truncated myeloid RelA/p65 gene was used (deletion of exons 7-10 of *rela* gene) (Algul et al., 2007; Hess C et al., 2010; Clausen et al., 1999) to demonstrate that the activation of RelA/p65 in myeloid cells is required for CS-induced promotion of lung cancer. It is shown that myeloid truncation of RelA/p65 in $rela^{\Delta-/-}$ mice resulted in a nearly complete abrogation of CS induced tumor promotion as well as in strongly decreased expression of NF-κB dependent proliferative genes in tumor cells, such as PCNA, CyclinD1 and c-Myc. It is further shown that a reduced release of pro-inflammatory cytokines, including TNFα, IL-6 and IL-8, in $rela^{\Delta-/-}$ mice. Interestingly, RelA/p65 deletion did not perturb recruitment of macrophages to the tumors suggesting that factors released by macrophages and not the influx of macrophages into tumor tissue itself, stimulate the proliferation of tumors. Additionally, it there was a decreased STAT3 activation in lung tumor cells of CS or air exposed $rela^{\Delta-/-}$ mice, which is likely to be a major effect of declining release of IL-6 from lung. This finding is supported by earlier reports showing that IL-6 produced by lamina propria myeloid cells enhances proliferation of tumor-initiating cells and protects premalignant intestinal epithelial cells from apoptosis through transcription factor STAT3 mediation (Grivennikov et al., 2009). These results strongly suggest that RelA/p65 dependent production of inflammatory cytokines in myeloid cells, such as TNFα and IL-6, enhance lung tumor proliferation.

4.3 The role of Wnt/β-catenin signalling in cigarette smoke promoted lung cancer proliferation

It has been shown that elevation of inflammatory factors from myeloid cells enhances the proliferation and survival of cancer cells, which is a major tumor promoting mechanism (Grivennikov et al., 2010, Takahashi et al., 2010). However, the mechanism how inflammatory mediators derived from myeloid cells promote tumor proliferation is not entirely clear (Kim et al., 2009). In this study, target oncogenes of Wnt/β-catenin signalling, such as CyclinD1 and c-Myc, were activated in CS-induced lung tumor promotion, which attracted our attention. This study shows that Wnt/β-catenin signalling is activated in cancer cells of NSCLC patients indicating that activation of this signalling pathway is most likely

involved in tumor growth. The animal experimental results clearly show that cigarette smoke promotes activation of Wnt/β-catenin signalling in tumor cells of WT mice. A number of studies have shown that dysregulation of the Wnt/β-catenin pathway is implicated in tumorigenesis (Reya and Clevers, 2005). In the lung, aberrant activation of Wnt/β-catenin pathway is involved in pulmonary fibrosis and cancer transformation (Konigshoff and Eickelberg, 2010; Licchesi et al., 2008). Aberrant Wnt1/-catenin expression is an independent poor prognostic marker of non-small cell lung cancer after surgery (Xu et al., 2011) and Wnt1 overexpression is associated with the expression of tumour-associated Wnt-targets, tumour proliferation, angiogenesis and a poor prognosis in NSCLCs (Huang et al., 2008). Increasing evidence suggests that promotion of the Wnt/β-catenin pathway has an important role in tumor growth and progression (Fodde et al., 2007). Basbaum´s research group reported that cigarette smoke induced malignant transformation of human bronchial epithelial cells is mediated by the activity of Wnt/β-catenin and sonic hedgehog pathways (Lemjabbar-Alaoui et al., 2006). In our study, it was not found that cigarette smoke directly promotes Wnt/β-catenin signalling in the lung cancer cell line A549. The cigarette smoke induced activity of Wnt/β-catenin signalling may be a cell type-specific response.

Moreover, the results of this study show that the presence of macrophages increases CSE induced activation of Wnt/β-catenin signalling. The findings also suggest that RelA/p65 in myeloid cells (particularly in macrophages) is required for promotion of Wnt/β-catenin signalling in cancer cells. Therefore, some soluble factor(s) released by macrophages in a NF-κB dependent manner are likely to be responsible for Wnt/β-catenin activity in lung cancer cells. Accumulating evidence indicates that TNFα is linked with inflammation-associated tumors (Greten, et al., 2004; Pikarsky, et al., 2004; Kim, et al., 2009). Importantly, in gastric tumor cells, Wnt/β-catenin signalling is involved in TNFα regulated tumorigenesis (Oguma, et al., 2008). Here it is shown that TNFα stimulation promotes activation of Wnt/β-catenin in A549 cells. Moreover, inhibition of Wnt/β-catenin signalling by addition of TNFα neutralizing antibody in CSE-stimulated co-cultures confirmed the function of TNFα. GSK3β is a key player of the inhibition of the Wnt/β-catenin pathway. GSK3β phosphorylates a set of conserved Ser and Thr residues of β-catenin, leading to its proteasomal degradation (Reya and Clevers, 2005; Moon, et al., 2004). Several experiments indicate that Akt can activate the transcriptional activity of β-catenin by phosphorylation of GSK3β (Oguma et al., 2008; Sharma et al., 2002). Here it is demonstrated that TNFα activates the Akt pathway and inactivates GSK3β in lung tumor cells. This may

contribute to promotion of Wnt/β-catenin pathway activity. Accordingly, the present results suggest that cigarette smoke induced release of TNFα from myeloid cells mediated by NF-κB pathway activation increases Wnt/β-catenin signalling in tumor cells, which is responsible for lung tumor promotion.

4.4 LL-37/hCAP18 is required for cigarette smoke induced lung carcinoma progression

It has been characterized that LL-37 is secreted by immune and epithelial cells and acts as a pro-inflammatory peptide which links inflammation, host defence, tissue repair, and cell growth (Bucki et al., 2010). As described above, inflammation plays a pivotal role in tumor development and many of the immune cell-derived inflammatory molecules mediate this procession (Grivennikov et al., 2010). Antimicrobial activity is a major function of LL-37. It shields, for instance, mice from skin and urogenital infection and protects from tuberculosis (Nizet et al., 2001; Chromek et al., 2006; Liu et al. 2006). Considering that disrupting microbial cell membranes and/or inducing autolysis are mechanism of the antimicrobial activity of LL-37, it was suggested that similar action can be used to kill tumor cells (Bucki, et al., 2010). However, several studies found that LL-37 is over-expressed in human lung, breast, ovarian, and prostate cancer and a tumor-promoting effect of this peptide has been demonstrated (Von Haussen et al. 2008; Heilborn et al., 2005; Coffelt et al., 2008; Hensel et al., 2010). Previous reports from our laboratory showed that LL-37 is strongly expressed in human lung cancer cells and that the peptide promotes tumor growth by direct interaction with cancer cells (Von Haussen et al. 2008). This study further confirms the tumor promoting ability of CRAMP (the murine homolog of human LL-37) by using *CRAMP* deficient mice. In CRAMP deficient mice, the growth of lung tumors induced by the injection of LLC cells was inhibited. Moreover, this study shows that CS exposure increases the induction of CRAMP in mice lungs and demonstrates that the tumor-promoting effect of CS is decreased as a result of targeted deletion of CRAMP. These results indicate that cathelicidin has a close association with CS induced tumor promotion.

Although the biologic role of LL-37 in tumor promotion has been partly elucidated, it remains unclear which pathway mediates the function of this peptide. The angiogenic effect of LL-37 is likely to be one mechanism of the cathelicidin dependent tumor growth. Previous studies showed that LL-37 induces functionally important angiogenesis by binding to the peptide to FPRL1 of endothelial cells (Koczulla et al., 2003; Steinstraesser et al., 2006). PLC-g/PKC/NF-κB, the Erk-1/2 MAPK, and the PI3K/Akt pathway are involved in the endothelial

activation elicited by LL-37 (Koczulla et al., 2003). Furthermore, the peptide regulates the vascularization in ovarian tumors and prostate cancer (Coffelt et al., 2009; Hensel et al., 2010). LL-37 may also affect tumor cells directly by stimulating cell growth. Previous reports showed that LL-37 treatment of lung cancer cell lines augments cell growth and indicate that involvement of EGFR pathway (Von Haussen et al. 2008). LL-37 treatment of lung cancer cells results in phosphorylation of EGFR and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK), principal factors of signalling pathways involved in tumor pathogenesis (Von Haussen et al. 2008). Additionally, activation of ERK1/2 and Akt pathway is also implicated in angiogenesis and cellular proliferation of prostate cancer (Hensel et al., 2010). LL-37 stimulates growth of ovarian cancer cell lines depending on the presence of serum suggesting this peptide needs accessory or carrier proteins to execute its effects (Coffelt et al., 2008). Release and activation of MMP2 and MMP9 induced by LL-37 may reflect an invasion advantage for tumor cells, as these 2 MMPs are up-regulated in ovarian and prostate cancer cells (Coffelt et al., 2008; Hensel et al., 2010).

It is currently thought that infiltration of tumors by inflammatory cells is important to tumor development. These cells regulate tumor growth by means of production of cytokines and chemokines that act in autocrine and paracrine manners (Grivennikov et al., 2010). The data presented here showed that CS exposure enhances the number of macrophages infiltrating into tumors, especially tumor margin, which serve to facilitate proliferation of lung tumor cells. Notably, deletion of CRAMP in mice reduces macrophage migration into tumors, and CS did not promote engraftment of these cells in CRAMP deficient mice. LL-37 has been shown to be a chemotactic factor which mediates multipotent mesenchymal stromal cells (MSCs) migration to tumors mediated by FPRL1 (Coffelt et al., 2009). Like LL-37, CRAMP also exhibits a direct effect on the migration of immune cells including monocytes, neutrophils, and macrophages (Kurosaka et al., 2005). Thus, it is a possible explanation that LL-37 contributes to the promotion of lung tumor progression through recruitment and infiltration of inflammatory cells into tumor tissue in where these cells provide pro-angiogenic and growth factors that support tumor growth and progression.

Previous reports showed that LL-37 is expressed in various human lung cancer cell lines and in human lung cancer including large cell carcinoma, squamous cell carcinoma, and adenocarcinoma (Von Haussen et al. 2008). In this study, immunostaining for CRAMP in mouse lung tumor sections proved that CRAMP expression was much higher in inflammatory cells than tumor cells and that the number of cells positive for CRAMP increased in tumors after CS exposure. The *in vitro* studies demonstrated that the induction of LL-37/hCAP18 in

macrophages is up-regulated upon co-culture with A549 cells and that addition of CSE further enhances this peptide expression. These results indicate that CS exposure induces inflammatory cells contributing cathelicidin to the tumor microenvironment. This increase in peptide may then lead to further recruitment of immune cells into the tumor microenvironment. Additionally, high LL-37 levels secreted by inflammatory cells into a cytokine milieu may also influence tumor cell angiogenesis, proliferation, and invasion.

Cytokines and growth factors, such as LPS, IL-1α, and insulin-like growth factor-1 in keratinocytes, have been reported to induce LL-37/hCAP18 expression (Erdag and Morgan, 2002; Sorensen et al., 2003). However, most of inflammatory mediators including TNFα, IL-6, IL-8, and INF-γ do not induce expression of LL-37/hCAP18 (Gombart et al.,2005). Gombart and co-works reported that VDR mediates a strong up-regulation of LL-37/hCAP18 gene in response to treatment of cells with $1,25(OH)_2D_3$ and its analogs (compound I ($1,25R,26-(OH)_3$-22-ene-D_3), KH1060 (20-epi-22oxa-24a,26a,27a-trihomo-$1,25(OH)_2D_3$) and EB1089 (1,25-dihydroxy-22,24-diene, 24,26,27-trihomo)) (Gombart et al.,2005). As VDR is expressed a wide range of tissues, including monocytes, neutrophils, and cell lines from lung as well as head and neck squamous cell carcinomas, it is likely that LL-37/hCAP18 can be induced in all of these tissues (Wang et al., 2004; Gombart et al.,2005). $1,25(OH)_2D_3$ and its analogs have been observed to induce expression of LL-37/hCAP18 in myeloid cells, acute myeloid leukaemia (AML), keratinocytes, and colon cancer cells (Gombart et al.,2005). TLR2/1 activation of human macrophages induces expression of VDR and Cyp27B1genes and is required for downstream LL-37/hCAP18 production (Liu et al., 2006). The data of this study show that lung cancer cells induce LL-37/hCAP18, VDR, and Cyp27B1gene expression in macrophages and that the combination with CSE results in a synergistic induction of these factors. It is further shown that CSE alone does not induce the expression of these 3 genes in macrophages. The results presented here provide a possible indication that tumor derived inflammatory mediators activate VDR and Cyp27B1 in macrophages and that Cyp27B1 catalyzes the synthesis of $1,25(OH)_2D_3$ from the precursor $25(OH)D_3$ leading to the induction of the antimicrobial peptide cathelicidin LL-37/hCAP18 via VDR. It is the role of CSE in this process to induce inflammation resulting in the up-regulation of inflammatory factors expression in macrophages and tumor cells.

The induction of CRAMP by vitamin D_3 does not occur in mice because of the absence of the VDRE in the murine CRAMP promoter (Gombart et al., 2005). The present data report that versican, an aggregating chondroitin sulphate proteoglycan, is up-regulated in lung cancer cells. Versican activates pro-inflammatory responses and induces TNFα secretion by myeloid

cells in a TLR2, TLR6, and CD14 dependent manner leading to tumor metastatic growth (Kim et al., 2009). Furthermore, another extracellular matrix proteoglycan biglycan has been reported to act in macrophages as an endogenous ligand of TLR4 and TLR2, which mediate innate immunity and induce release of inflammatory mediator (Schaefer et al., 2005). Therefore, versican and biglycan potentially induce the CRAMP gene. Unfortunately, increased CRAMP expression after biglycan treatment in macrophages could not be observed. As versican is not available from commercial company, the regulation effect to CRAMP was not studied. Additionally, high doses of the TLR2-TLR6 ligand Pam2CSK4 only induced a weak up-regulation of CRAMP gene expression in macrophages suggesting that the induction of this gene is possibly independent of TLR2 or TLR6. In agreement with the regulation of the human cathelicidin LL-37/hCAP18, these data also show that TNFα, IL-1β, and INFγ fail to up-regulate mRNA expression of CRAMP in mouse macrophages. Analysis of the *CRAMP* gene sequence showed that a NF-κB site is present at its promoter region, indicating NF-κB might be implicated in regulation of CRAMP (Pestonjamasp et al., 2001). In support of this notion, the *in vivo* studies offer evidence that NF-κB activation is involved in inflammation induced CRAMP up-regulation using a murine model of RelA/p65 deletion in myeloid cell. The binding sites of GM-CSF (granulocyte-monocyte colony stimulating factor) and NF-IL6 (nuclear factor for IL-6) have been identified in the CRAMP gene. These sites might be candidates for regulation of expression (Pestonjamasp et al., 2001). The exact transcriptional mechanisms that regulate CRAMP gene expression need to be examined in future studies.

5 Conclusions

To investigate the role of myeloid NF-κB cells in lung cancer proliferation, an in vitro co-culture model was established that mimicked the microenvironment of tumors. The impact of myeloid NF-κB on tumor promotion was further studied in a murine lung cancer model which was established by intravenous injection of lewis lung carcinoma cells into mice that specifically lack RelA/p65 in the myeloid lineage. Furthermore, an experimental smoking protocol was developed to study cellular mechanisms underlying cigarette smoke induced promotion of lung cancer.

In this study, it is demonstrated that cigarette smoke exposure results in an inflammatory response of the lung characterized by elevated pro-inflammatory factors and the recruitment of macrophages and neutrophils into the lung. Cigarette smoke and lung cancer cells synergistically induced the expression of pro-inflammatory factors in macrophages. Moreover, cigarette smoke-induced inflammation remarkably enhanced lung tumor promotion and decreased the survival rate of mice due to increased proliferation in tumors. The role of myeloid NF-κB in tumor promotion was investigated. Deletion of RelA/p65 in myeloid cells of mice impaired CS-induced inflammation in tumor-bearing lungs. Furthermore, $rela^{\Delta-/-}$ mice showed a longer survival and a nearly complete abrogation of CS-induced tumor promotion, as well as strongly decreased expression of NF-κB-dependent proliferative genes such as c-Myc, cyclin D1, and PCNA in tumor cells. In the *in vitro* co-culture model, inhibition of the NF-κB pathway in macrophages also decreased inflammation-induced A549 cells growth.

In addition, immunohistochemistry showed that the Wnt/β-catenin signalling pathway is activated in cancer cells of NSCLC patients. This is in line with results obtained from animal experiments that showed that cigarette smoke promotes the activation of Wnt/β-catenin signalling in tumor cells in mice. Furthermore, RelA/p65 in myeloid cells (particularly in macrophages) was required for promotion of Wnt/β-catenin signalling in cancer cells. Cigarette smoke exposure increased the expression of TNFα from myeloid cells in a NF-κB dependent manner. TNFα suppressed the phosphorylation of β-catenin by phosphorylation of GSK3β and the activation of the Akt pathway leading to Wnt/ β-catenin promotion.

Cigarette smoke induced the expression and secretion of the cathelicidin CRAMP in mice lungs, especially in macrophages, and its action required the activation of myeloid RelA/p65. Deletion of CRAMP inhibited immune cell migration into the tumor microenvironment and

decreased lung tumor metastatic growth and CS tumor-promoting effects. Inflammation-induced expression of LL-37/hCAP-18 in macrophages involves vitamin D receptor and Cyp27B1 activation shown in the in vitro co-culture model.

Accordingly, the study presented here suggests that cigarette smoke-induced pulmonary inflammation increases lung cancer promotion and that RelA/p65 in myeloid cells contributes to the inflammation-induced promotion of lung tumor cells by increasing cell proliferation. The main findings of the present study are: (i) smoke-induced pulmonary inflammation increases lung cancer proliferation *in vivo* and *in vitro* and RelA/p65 of myeloid cells contributes to promotion of lung tumor cells by increasing cell proliferation; (ii) One crucial pathway comprises RelA/p65-induced TNFα and subsequent activation of Wnt/β-catenin signalling in tumor cells; (iii) cathelicidin promotes cigarette smoke-induced lung carcinoma proliferation, which is associated with the recruitment of immune cells and activation of myeloid NF-κB.

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List of Abbreviations

AID	activation-induced cytidine deaminase
AMPs	antimicrobial peptides
AOM	azoxymethane
APC	adenomatous polyposis coli
Arg-1	arginase-1
BALF	bronchoalveolar lavage fluid
Bcl-2	B-cell lymphoma 2
BCL-xL	B-cell lymphoma xL
BrdU	bromodeoxyuridine
CAC	colitis-associated cancer
cFLIP	cellular caspase-8 (FLICE)-like inhibitory protein
CFSE	carboxyfluorescein succinimidyl ester
cIAPs	inhibitor of apoptosis protein
CK1	casein kinase1
COPD	chronic obstructive lung disease
COX-2	cyclooxygenase-2
CRAMP	cathelicidin-related antimicrobial peptid
CS	cigarette smoke
CSE	cigarette smoke extract
CSF1	colony-stimulating factor 1
CYP27B1	25-hydroxyvitamin D-1-a-hydroxylase
DEN	diethylnitrosamine
DMBA	7,12-dimethylbenz(a)anthracene
DMEM	Dulbeccos's modified Eagles medium
Dsh	Dishevelled
DSS	dextran sodium sulphate
EBV	Epstein-Barr virus
EGFR	epidermal- growth-factor receptor
ELAM-1	endothelial leukocyte adhesion molecule 1
EMT	epithelial mesenchymal transition

ERK	extracellular signal-regulated kinase
EU	European Union
FCS	fetal calf serum
FGF	fibroblast growth factor
FPRL1	formyl peptide receptor-like 1
GM-CSF	granulocyte-monocyte colony stimulating factor
GSK3β	glycogen synthase kinase 3β
hBD	human β-defensin
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HGF	hepatocyte growth factor
HIF1α	hypoxia-inducible factor1 alpha
ICAM-1	inter-cellular adhesion molecule 1
iDC	immature dentritic cells
IFNγ	interferon γ
IκBα	inhibitor of κBα
IKK	IκB kinase
IL-1	interleukin 1
IL-1β	interleukin 1 β
IL-6	interleukin 6
IL-8	interleukin 8
JAKs	Janus kinases
LLC	Lewis lung carcinoma cells
LMP	latent membrane protein
LPS	lipopolysaccharide
LRP	lipoprotein receptor-related protein
MAPK	mitogen-activated protein kinase
mBD-29	murine β-defensin 29
MCP	monocyte chemoattractant protein
MCH	major histocompatibility complex
MCS	mainstream cigarette smoke
Mgl2	macrophage galactose-type C-type lectin–2
MMP	matrix metalloproteases
MR	mannose receotor

MSCs	multipotent mesenchymal stromal cells
nAChRs	nicotinic acetylcholine receptors
NF-κB	factor kappa-light-chain-enhancer of activated B cells
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N'-nitrosonornicotine
NSCLC	non-small cell lung cancer
PAHs	polycyclic aromatic hydrocarbons
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PDTC	pyrrolidine dithiocarbamate
PI3K	phosphoinositid-3-kinasen
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol-12myristat-13acetat
QNZ	6-Amino-4-(4-phenoxyphenylethylamino) quinazoline
RHD	Rel homology domain
RNAi	RNA interference
RNI	reactive nitrogen intermediates
RNS	reactive nitrogen species
ROS	reactive oxygen species
RT	room temperature
SCLC	small cell lung carcinoma
SNCG	synuclein-γ
STAT	signal transducers and activators of transcription
TAMs	tumor-associated macrophages
TCF/LEF	T cell factor/lymphocyte enhancer factor
TGFβ	transforming growth factor
TLR	toll-like receptor
TNFα	tumour necrosis factor α
TRAIL	TNF-related-apoptosis-inducing-ligand
Treg	T regulatory
TSP	total suspended particulate
UC	ulcerative colitis
uPA	urokinase type of plasminogen activator

VCAM-1	vascular cell adhesion molecule 1
VDR	vitamin D receptor
VDRE	vitamin D response element
VEGF	vascular endothelial growth factor
VEGF-A	vascular endothelial growth factor-A
VEGFR-1TK	VEGFR-1/Flt-1 tyrosine kinase
WT	wild type mouse strain

Publication and Presentations

Parts of this work have been revealed in the following publication:

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1. ATS International Conference (Denver, USA, May, 2011)
2. 52. Kongress der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin e.V. (Dresden, Germany, April, 2011)
3. 51. Kongress der Deutschen Gesellschaft für Pneumologie und Beatmungsmedizin e.V. (Hannover, Germany, March, 2010)
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