Aus der Fakultät für Biologie der
Julius-Maximilians-Universität Würzburg

# Immunity, Inflammation and Cancer: The role of Foxp3, TLR7 and TLR8 in gastrointestinal cancer

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**Grimmig T**, Kim M, Germer CT, *et al*. The role of FOXP3 in disease progression in colorectal cancer patients. Oncoimmunology. 2013;2(6):e24521.

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# I. INTRODUCTION

## 1. Increasing global burden of cancer

Cancer is one of the leading causes of death worldwide and the global cancer burden is expected to rise significantly.[1] In 2012, the worldwide burden of cancer rose to an estimated 14 million new cases per year. This number is expected to rise to 22 million annually within the next two decades with cancer deaths predicted to rise from an estimated 8.2 million annually to 13 million per year.[2] In Germany counting 100.687 cases among females (22.4%) and 118.202 case among males (28.9%) cancer was found to be the second most cause of death besides malignancies of the circulatory system in 2010 (figure 1).[3]

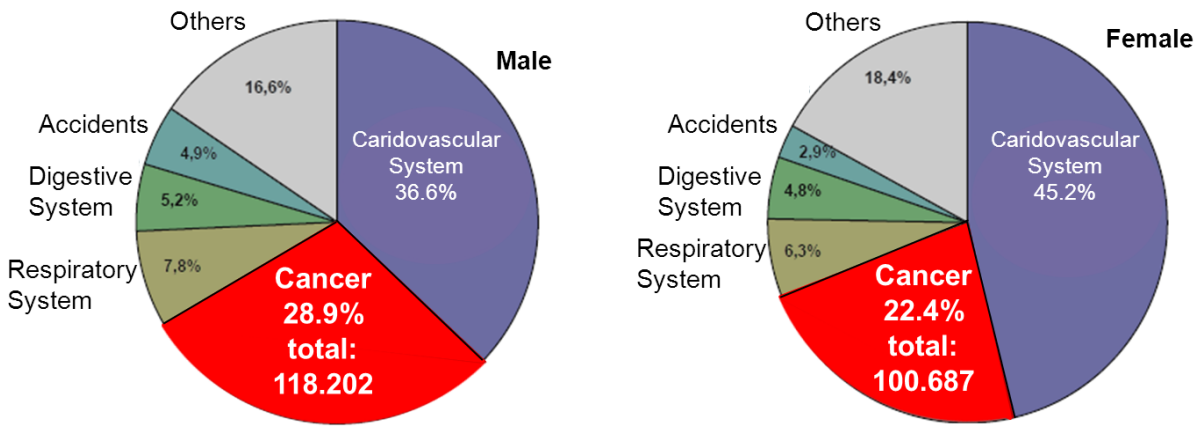


**Figure 1: The most frequent causes of death in Germany in 2010. (modified from Becker *et al*)** [3]

Besides diseases of the cardiovascular system cancer was found to be the second most common cause of death in 2010 in Germany among females (right diagram) and males (left diagram).[3]

## 2. Gastrointestinal cancer

Gastrointestinal (GI) cancer comprises a group of neoplastic disorders that affect the gastrointestinal tract. Types of GI cancer include esophageal, stomach, gallbladder, liver, pancreatic and colorectal tumors (figure 2).[4] According to Ferlay *et al*, GI cancer accounts for more than 37% of cancer related deaths worldwide.[5] In Germany in 2010, taken together tumors of the GI tract were the most frequent causes of cancer related deaths both within females and males (figure 2).[4] Among the group of GI tumors colorectal and pancreatic cancer amount to the most common

causes of cancer deaths. In 2010, colorectal cancer made the third (female) and second (male), pancreatic cancer the forth (female and male) most frequent cause of cancer deaths in Germany (figure 2).[4]

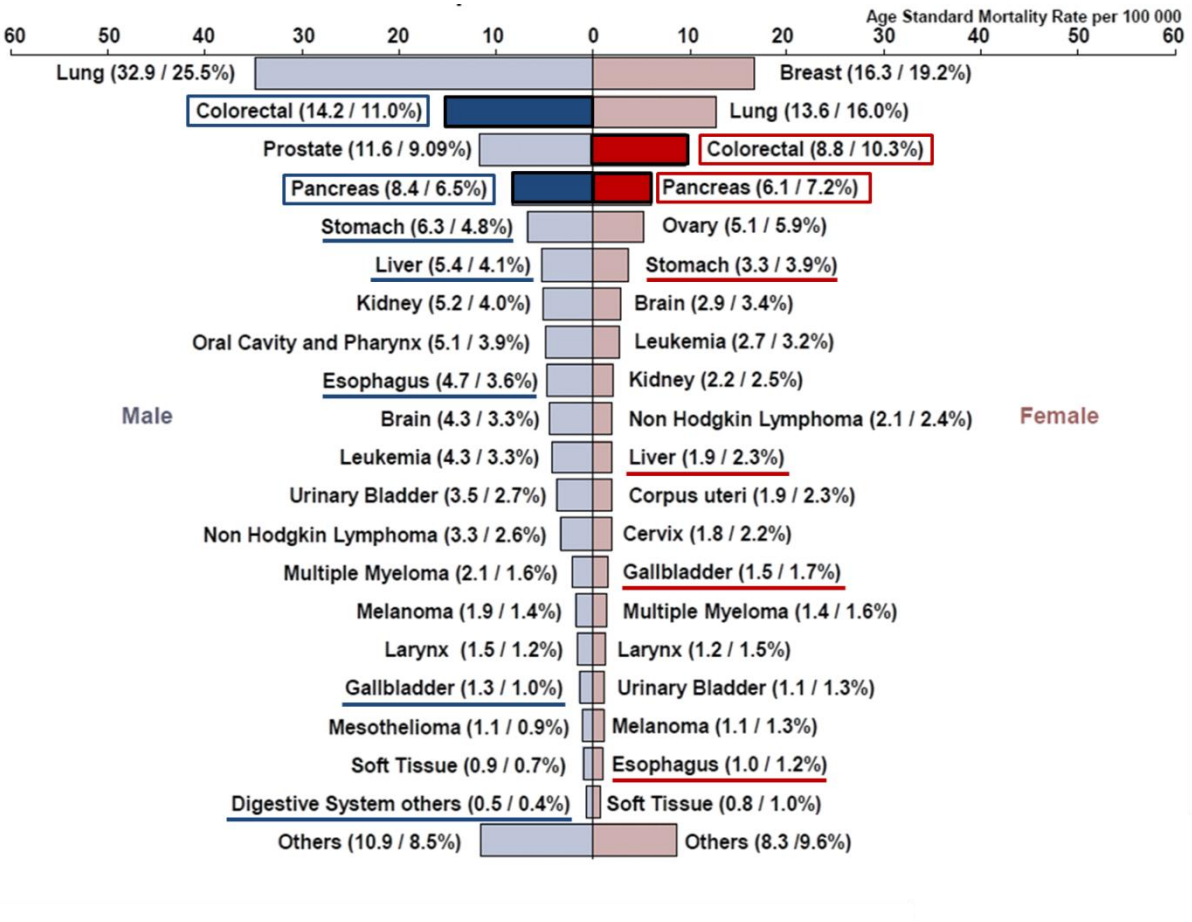


**Figure 2: The 20 most frequent causes of cancer deaths in 2010. (modified from Becker *et al*)** [4]

Gastrointestinal cancer includes esophageal, stomach, gallbladder, liver, pancreatic and colorectal tumors. Among both females and males it accounted for the most common causes of cancer related deaths in Germany in 2010 (red and blue selections) with especially colorectal and pancreatic cancer as the most frequent causes (red and blue boxes).[4]

## 2.1 Colorectal Cancer

Colorectal cancer (CRC) is a major cause of morbidity and mortality throughout the world. It accounts for over 9% of all cancer incidences and is the third most common cancer worldwide and the fourth most common cause of death.[6,7] In developed countries, CRC is the second most diagnosed type of cancer and the second most common cause of cancer-related death.[8] In Germany, among the 20 most frequent

causes of cancer deaths in 2010 CRC made 10.3% of all cases among females and 11.0% among males (figure 2).[4] The 5-year survival rate of patients diagnosed with advanced stage IV disease is less than 16%.[9] Surgery was a long-established treatment for CRC and could be curative for patients whose cancer had not spread. But close to half the patients that undergo curative resection ultimately die of metastatic or recurrent disease due to residual microscopic disease that was not evident at the time of surgery.[10-12]

Several risk factors are associated with the incidence of colorectal cancer. Next to age (in the U.S. more than 90% of colorectal cancer cases occur in people aged 50 or older)[13] and hereditary factors especially environmental and lifestyle risk factors play an important role in the development of colorectal cancer.[6] Those factors include nutritional practices, physical activity and obesity, cigarette smoking and heavy alcohol consumption.[6 14] Furthermore, inflammatory malignancies such as Ulcerative colitis and Crohn disease turned out to support the development of CRC.[6] For instance, the relative risk of CRC in patients with inflammatory bowel disease has been estimated between 4- to 20-fold.[14]

## 2.2 Pancreatic cancer

Worldwide, over 200.000 people die annually of pancreatic cancer. It is one of the most fatal cancers with the highest incidence and mortality rates found in developed countries.[15] In Germany in 2010, pancreatic cancer was the fourth most cause of cancer related deaths (figure 2).[4] Over 16.000 people were diagnosed with pancreatic cancer with a relative five-year survival rate of 8%.[16] Even with early diagnosis, mortality rates are high making neoplasms of the pancreas one of the few cancers where the mortality rate approaches 100%.[17]

Tobacco smoking is one of the most common risk factors for pancreatic cancer, as smokers have a two-fold increased risk of developing the disease compared with nonsmokers.[17] Familial pancreatic cancer is also well documented. Several inherited mutations are associated with pancreatic cancer, but only about 10% or less of pancreatic cancers are caused by an inherited factor.[17] Next to additional potential risk factors including physical inactivity, aspirin use, occupational exposure to certain pesticides and dietary factors such as carbohydrate or sugar intake, some studies

support the hypothesis that glucose intolerance and hyperinsulinemia are important in the development of pancreatic cancer.[15] Beyond that more recent data have now linked inflammatory processes within chronic pancreatitis with an increased risk of pancreatic cancer.[17,18]

## 3. Human immune system

The human immune system is a remarkably adaptive defense system that protects the host from invading microorganism and cancer by using a variety of cells and molecules which recognize and eliminate potential pathogens. Next to physical barriers that prevent most bacteria and viruses from entering the body, the immune system comprises two different response components: innate and adaptive immunity (figure 3).[19,20] The nonspecific innate immunity provides the first line of host defense against pathogens until a specific immune response has developed.[19,21] The specific adaptive or acquired immunity requires the activation of a functional immune system, involving lymphocytes and their products.[19,22]

### 3.1 Innate (nonspecific) immunity

The innate immunity presents an inherited first line defense that recognizes and eliminates pathogens that manage to cross the physical barriers of the human body.[23] Defense mechanisms of the innate immunity usually result in the initiation of inflammatory reactions induced by either the complement cascade or a cellular system (figure 3, left).[24] The complement cascade is a system of several small plasma proteins that circulate as inactive precursors in the circulating system. Once activated by protease induced cleavage complement proteins can initiate proteolytic reactions on the surface of invading microorganisms and mark them for the cellular components of innate immunity.[25] The cellular system consists of various cell types such as phagocytes, dendritic cells and natural killer (NK) cells (figure 3, left).[24] Microorganisms are identified by pattern recognition receptors (PRR) on the surface of these innate immune cells which recognize and bind to pathogen associated molecular patterns (PAMPs). These PAMPs include common molecules of bacterial

carbohydrates and peptides, lipopolysaccharide, bacterial and viral RNA (ribonucleic acid) and DNA (deoxyribonucleic acid) and other microbial molecules.[26]

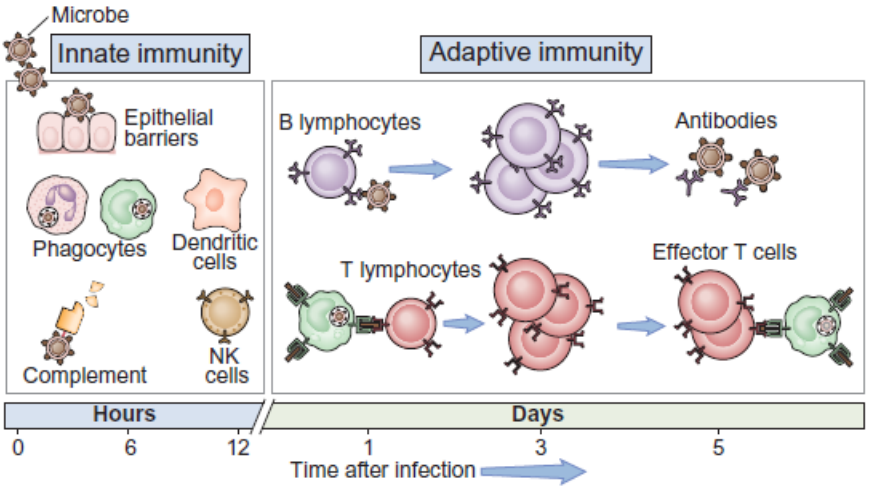


**Figure 3: The principle mechanisms of innate and adaptive immunity.**[24]

The immune system comprises innate and adaptive immunity. The nonspecific innate immunity serves as a first line defense and consists of the complement cascade and a cellular system including phagocytes, dendritic cells and natural killer (NK) cells. The specific adaptive immunity depends on the activation B and T lymphocytes and their products.[24]

## 3.2 Adaptive (specific) immunity

Although the innate immune system serves as an effective defense mechanism to sense and eliminate a variety of pathogens, host defense is not entirely covered by its strategies. The immense quantity of immunogenic structures, as well as the ability of pathogens to use mutations for evasion from host detection, has driven the evolution of the adaptive immune system.[27]

Adaptive immunity is subdivided into humoral and cell-mediated immunity. Humoral immunity is mediated by antibodies which are produced by B lymphocytes. By binding specific pathogen associated structures secreted antibodies can contribute to eliminate extracellular microbes (figure 3, right and figure 4, left).[25] Defense against intracellular targets is provided by T lymphocytes (T cells) of the cell-mediated immunity (figure 4, right). While CD4+ (cluster of differentiation 4) T helper lymphocytes ($T_H$ cells) activate phagocytes such as macrophages to eliminate pathogens, CD8+ cytotoxic T lymphocytes (CTLs) kill infected host cells directly to eliminate reservoirs of infection (figure 4, right).[24]

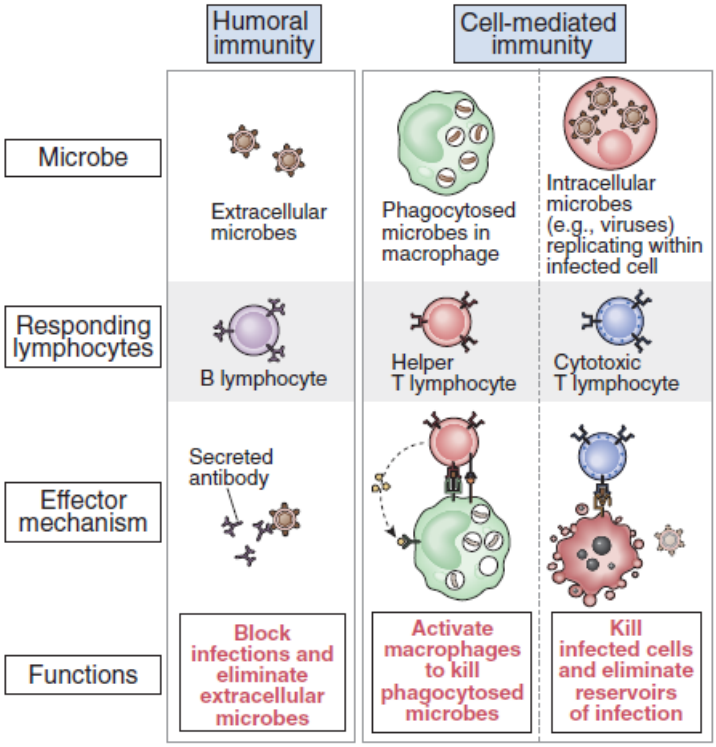


**Figure 4: Types of adaptive immunity.**[24]

The adaptive immunity consists of the humoral and cell-mediated systems. In humoral immunity, B lymphocytes secrete antibodies that target extracellular pathogens. In cell-mediated immunity, T cells either activate macrophages to destroy phagocytized microbes (T helper lymphocytes) or directly eliminate infected host cells (cytotoxic T lymphocytes).[24]

## 4. Regulatory T cells (Treg)

Although the previously described responses of immunity help to eliminate or slow down the spread of pathogens within a host, if they are not tightly controlled, they can result in severe inflammation and collateral tissue damage.[28,29] Therefore, immune response and inflammatory processes need to be closely regulated by various host suppressor mechanisms.

Among the population of T lymphocytes Sakaguchi *et al* identified a class of CD4+/CD25+ T cells which modulate the immune system, maintain tolerance to self-antigens and abrogate autoimmune disease.[30,31] These suppressor T cells, later renamed as regulatory T (Treg) cells, were originally reported as cells that suppress CD4+ T cell-induced organ-specific autoimmune disease, but recently they were also shown to suppress immune responses against foreign antigens and pathogens.[31,32] To date, CD25 is the most specific cell surface marker for such Treg cells.[33]

Additionally, the transcription factor forkhead box protein 3 (Foxp3) turned out to be of particular importance for Treg cells. It serves as crucial key molecule for genetically controlling their development and function.[34-36]

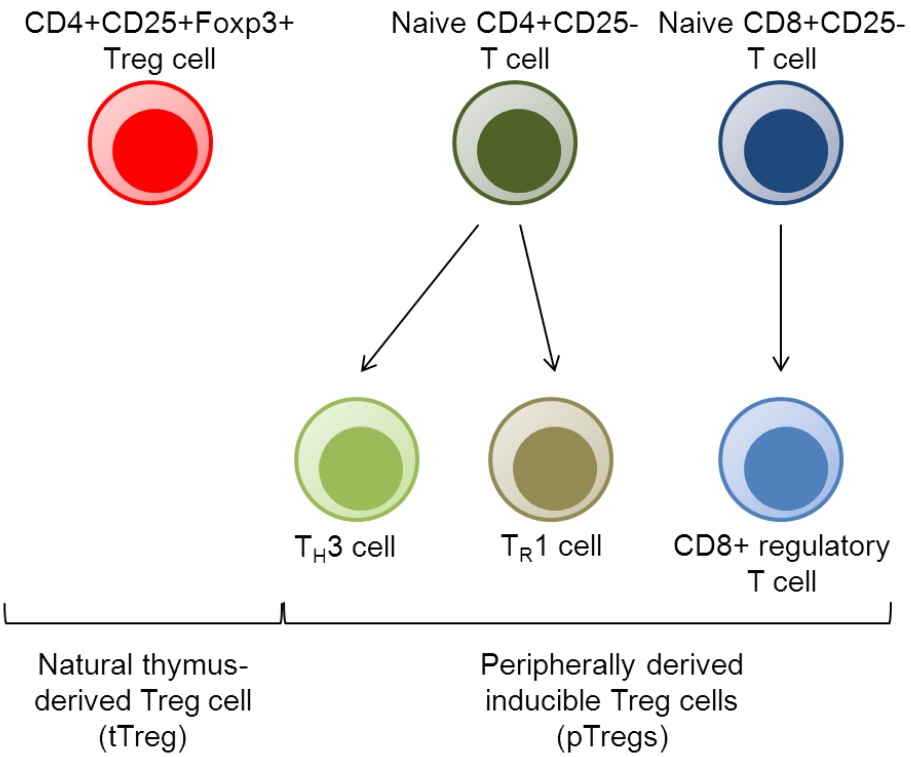


**Figure 5: Natural and inducible regulatory T cells.**

Natural regulatory T (Treg) cells derive from the thymus and are of a CD4+CD25+Foxp3+ subtype. The inducible populations include distinct subtypes of naive CD4+ and CD8+ T cells: T regulatory 1 ($T_R1$) cells, T helper 3 ($T_H3$) cells and CD8+ regulatory T cells.[29,37]

It is now firmly established that there are two distinct populations of Foxp3+ Treg cells: one natural (constitutive) and one inducible (adaptive) population (figure 5). The population of naturally occurring Treg cells is derived from the thymus and is self-reactive. These cells are referred to as thymus-derived Treg (tTreg) cells. The inducible population is peripherally generated from naive CD8+CD25- cells (CD8+ regulatory T cell) or CD4+CD25- cells (T helper 3, $T_H3$, cells and T regulatory 1, $T_R1$, cells). They are referred to as peripherally derived Treg (pTreg) cells.[29,37] Current data suggest that Foxp3 expression in the tTreg cell subset is stable, whereas its expression in the pTreg cell subset needs to be activated.[37-39]

This is still an ongoing area of research; however several studies showed that nuclear factor kappa B (NF-κB) can regulate Foxp3 expression.[37,40]

## 4.1 Foxp3 regulation and function

It is now well-established that Foxp3 is required for development of Treg cells both in human and mice; however, recent studies raise the question whether Foxp3 expression alone is sufficient to program conventional T cells into Treg cells. There may be other important factors required along with Foxp3.[34,35,41]

The induction of Foxp3 expression in CD4+CD25- cells has been shown to be extrinsically initiated by the cytokine transforming growth factor beta (TGF-β).[42] The downstream signaling cascade leading to Foxp3 induction is not yet clearly established; however several key players have been identified. In compliance with conventional TGFβ signaling, Smad3 (mothers against decapentaplegic homolog 3) has been identified as a necessary parameter for Foxp3 induction.[41,43] Additionally, Stat5 (signal transducer and activator of transcription 5) which is induced downstream of IL2RB (interleukin-2 receptor subunit beta) and NFAT (nuclear factor of activated T-cells) that is activated after TCR (T cell receptor) triggering, can initiate Foxp3.[41,44,45] Signaling through the Notch receptor/trancription factor pathway may also be involved in Foxp3 expression and the signaling protein Akt (protein kinase B) which plays a key role in cellular survival has been established as a repressor of novel Foxp3 induction.[41,43,46]

## 4.2 Regulatory T cells, Foxp3 and cancer

Human tumors are often infiltrated by immune cells, predominantly T lymphocytes and myeloid cells, which are recruited to the site by chemokines and cytokines secreted by the various cells in the tumor milieu.[47,48] Among those T cells CD4+CD25+Foxp3+ Treg cells are of particular interest.

As Foxp3+ Treg cells are mainly supposed to eliminate self-reactive lymphocytes, they can be unfavorable to the immune response against tumors. Since most of the

tumor-associated antigens (TAA) are recognized as self, they are more likely to activate Treg cells rather than effector T cells. In addition, tumor cells often acquire the ability to secrete cytokines such as TGF-β, which induces Foxp3 expression in naive T cells. Indeed, high levels of Foxp3+ Treg cells have been detected in the tumor environments of many cancers.[41,47,49,50]

These findings brought up the question whether the observed increase in Treg cells can be linked to the clinical outcome of tumor patients.[47] The majority of the data indicates that increased Treg cell frequency can be generally considered as a marker of poor prognosis in cancer, presumably due to Treg cell mediated suppression of anti-tumor immunity, which benefits the tumor.[47,51-53] A high density of tumor infiltrating Treg cells in tumor specimen has been associated with poor outcome in ovarian, pancreatic, and hepatocellular carcinoma.[54-56] This might be expected, as Treg cells are able to inhibit anti-tumor immunity and mediate immune tolerance, thus favoring tumor growth. In this context, Treg cells could be viewed as the major component of tumor escape from the host immune system.[47] Yet there are some studies which actually link increased Treg cell frequency to a favorable prognosis, for example in lymphomas and colorectal cancer.[57,58] However some results were more heterogeneous depending on the cancer entity and in some studies, no Treg increase was observed.[59,60]

Additionally to the significance of Foxp3 expressing Treg cells in human solid tumors, more recent clinical data from lung, breast, pancreatic, hepatocellular and urinary bladder cancer as well as melanoma provided first evidence for Foxp3 expression to be mediated also by tumor cells themselves which therefore may have an impact on possible anti-tumor immune responses as well.[61-66]

## 5. Toll-like receptors

Although the innate immune system is very effective in dealing with the vast majority of the infections, it has been long believed to be nonspecific to the invading pathogen. This idea of the nonspecific nature of the innate immunity has been challenged by the discovery of a certain class of receptors, the Toll-like receptors (TLRs).[26] TLRs are an evolutionarily conserved family of molecules that recognize conserved patterns of microbial structures. In this role, they are part of a class of host

receptors known as PRRs. PRRs such as TLRs are best known for their ability to recognize conserved bacterial structures named PAMPs.[25,26,67,68]

TLRs can be subdivided into three families, depending on the type of macromolecular ligand recognized (nucleic acid, protein, lipid). TLRs 1, 2, 4, 6 and 10 are involved in lipid recognition, TLR 5 recognizes proteins and TLRs 3, 7, 8 and 9 sense nucleic acids, although there are exceptions to this trend.[69] Additionally, TLRs can be discriminated by their location: TLR1, TLR2, TLR4, TLR5 and TLR6 are located on the outer membrane of the cells, whereas TLR3, TLR7, TLR8 and TLR9 are found on endosomes (figure 6).[70,71]

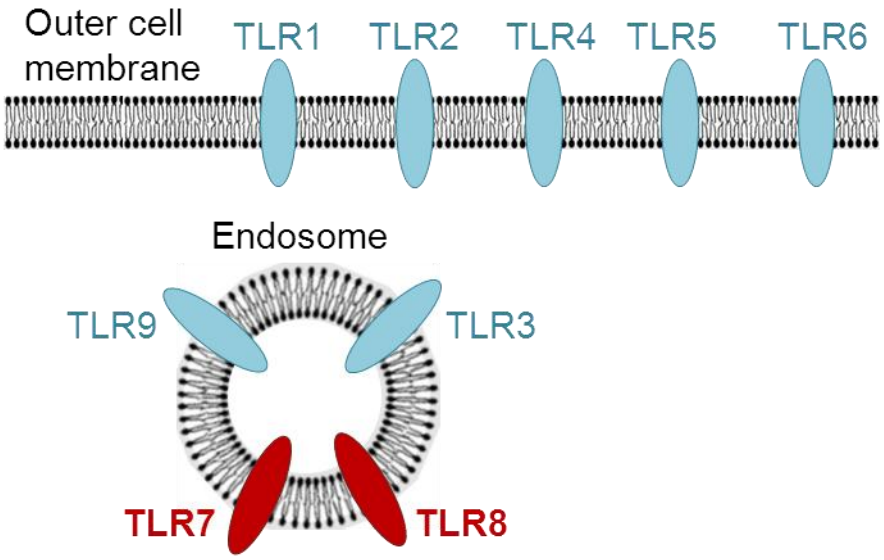


**Figure 6: Cellular localization of Toll-like receptors.**

Toll-like receptor (TLR) 1, TLR2, TLR4, TLR5 and TLR5 are located on the outer membrane of cells, whereas TLR3, TLR7, TLR8, and TLR9 are located on the endosomal membrane.[70,71]

Additionally to PAMPs of bacterial origin, TLRs can also be stimulated by so-called damage-associated molecular patterns (DAMPs) which arise from inflammation and cellular injury. This implies that even in the absence of pathogens, disrupted or injured cells can recruit innate inflammatory cells and DAMPs can activate TLR signaling when released outside the cell following tissue injury.[72,73]

Upon ligation, all TLRs form homo- and heterodimers and transmit signals throughout the cell via TIR (Toll/interleukin-1 receptor)-TIR homotypic binding with one or a

combination of four adaptor proteins: myeloid differentiation factor 88 (MyD88), TICAM1 (Toll-like receptor adaptor molecule 1), TIRAP (TIR domain containing adaptor protein) and TICAM2. All TLRs (except for TLR3) signal through MyD88 while TLR3 signals through TICAM1. TLR4 signals through both the MyD88 and the TICAM1 pathway.[69,71]

## 5.1 Toll-like receptors and cancer

Recently, enhanced expression of TLRs has been described in a variety of different tumors.[74-76] Depending on the tumor entity TLR expression and signaling in cancer cells can be linked to either favorable or poor outcome.

### 5.1.1 Anti-cancer effects of TLRs

One of the main mechanisms underlying the antitumor activity of TLRs is their capability to activate the development of a tumor-specific immune response. The activation of TLRs stimulates (directly or indirectly) the migration of NK cells, cytotoxic T cells and type I T helper cells into the tumor, which causes the lysis of tumor cells via secretion of various effectors (perforin and IFN-γ, interferon gamma) and results in the secretion of type I IFNs (IFN-α, β).[77-79] Another possible mechanism underlying the antitumor effect of TLRs is the TLR-dependent transition of tumor-stimulating macrophages (M2 type) into the tumor-suppressing type M1. Type M2 macrophages are characterized by the expression of cytokines, such as TGF-β and IL-10 (interleukin 10). TGF-β mediates tumor cell proliferation, while IL-10 directs the development of the immune response to $T_H2$, thus blocking the development of the cellular antitumor immune reactions. Additionally, type M1 macrophages stimulate the development of the cellular antitumor ($T_H1$) immune response via the expression of IL-1, IL-6, IL-12, TNF-α and IFN-γ.[77,80]

Currently, several TLR agonists are in clinical trials as anti-tumor agents. Particularly, both natural (e.g. single stranded RNA, ssRNA) and synthetic (e.g. Imiquimod) agonists of TLR7 and TLR8 have demonstrated high activity against chronic lymphocytic leukemia and tumors of the skin. Furthermore, the TLR9 ligand CpG can suppress the growth of lymphomas and tumors of the brain, kidney and skin.

In addition, the TLR3 activator poly(IC) has also been demonstrated to possess pro-apoptotic effects on tumor cells.[77,81-83]

### 5.1.2 TLRs as positive regulators of cancer

Chronic inflammations are crucial factors in the development of some malignant neoplasms. In particular, stomach cancer can be associated with chronic inflammation induced by Helicobacter pylori. Additionally, chronic inflammation of the digestive tract is often associated with colorectal cancer.[77,84,85] Mechanisms of inflammatory response are known to significantly associate with TLR signaling. Multiple studies proposed mechanisms to explain TLR implication in the stimulation of tumor formation and development, including the stimulation of angiogenesis and tumor cell proliferation as well as induction of chemoresistance and Treg activation.[69,71,77]

Not only various infection associated pattern can stimulate carcinogenesis via interaction with TLRs. DAMPs, the nuclear and cytoplasmic proteins of necrotic cells are known to serve as TRL ligands. DAMPs released from damaged cells can be recognized by various TLRs on the surface of immune cells with subsequent activation of TLR-dependent signals resulting in the suppression of the antitumor immune response and, as a consequence, in the stimulation of tumor progression.[73,77]

TLR ligation and stimulation induces recruitment of MyD88, leading to activation of the NF-κB and MAPK (mitogen-activated protein kinases) signaling pathways. NF-κB activation initiates the up regulation of anti-apoptotic factors such as Bcl-2 (B cell lymphoma 2), cIAP1 (baculoviral IAP repeat-containing protein 1) and cIAP2 in tumor cells and can induce chronic inflammation by producing COX-2 (cyclooxygenase-2) and several pro-inflammatory cytokines.[74,86,87] COX-2 together with TLR expression is known to play a crucial role in transformation of normal cells to cancer cells and in angiogenesis, reduced apoptosis and immunosuppression of malignant tumors.[88,89] Additionally, our previous studies indicated that endosomally expressed TLR7 and TLR8 is associated with tumor progression in colorectal cancer and reduced tumor-specific survival amongst patients with high TLR7 and TLR8 expression in colorectal cancer cells.[74]

## 5.2 TLR7 and TLR8

TLR7 and TLR8 were found to be closely related, sharing their intracellular endosomal location, as well as their ligands (figure 6 and 7).[90] TLR7 or TLR8 ligation activates intracellular pathways that result in the expression of pro-inflammatory cytokines, chemokines and type I interferons (IFNs). All TLRs are type I membrane proteins, composed of an external domain responsible for ligand recognition (ligand recognition region, LRR), a transmembrane region (TMR) and the cytoplasmic TIR domain, which mediates downstream signaling.[90,91] After ligand binding by TLR7 or TLR8 the TIR domain-containing adaptor molecule MyD88 is recruited. The association of TLR7/8 and MyD88 stimulates the recruitment of members of the IL-1 receptor-associated kinase (IRAK) family resulting in the downstream activation of MAPKs and the IκB kinase (IKK) complex via the induction of transforming growth factor-activated kinase 1 (TAK1). Members of the MAPK family phosphorylate and activate the transcription factor activator protein 1 (AP-1), whereas the IKK complex is involved in the nuclear translocation of the transcription factor NF-κB. Both AP-1 and NF-κB control the expression of pro-inflammatory cytokine genes. Furthermore, members of the interferon regulatory factor (IRF) family are activated resulting in type I IFN induction (figure 7).[70,90,91]

To date, several ligands have been characterized as TLR7 and/or TLR8 ligands, classified in synthetic compounds and natural nucleoside structures. Some synthetic compounds were already produced and used as immune activators before they were characterized as TLR7/TLR8 ligands. Imidazoquinolines are nucleoside analogue structures that are known to initiate immune cells to produce pro-inflammatory and regulatory cytokines.[90] TLR7 agonist Imiquimod is a TLR7 agonist which is licensed for the therapy of basal cell carcinoma. Another synthetic agonist is Resiquimod (R848), a selective ligand for murine TLR7 and for TLR7 and TLR8 in humans.[92,93]

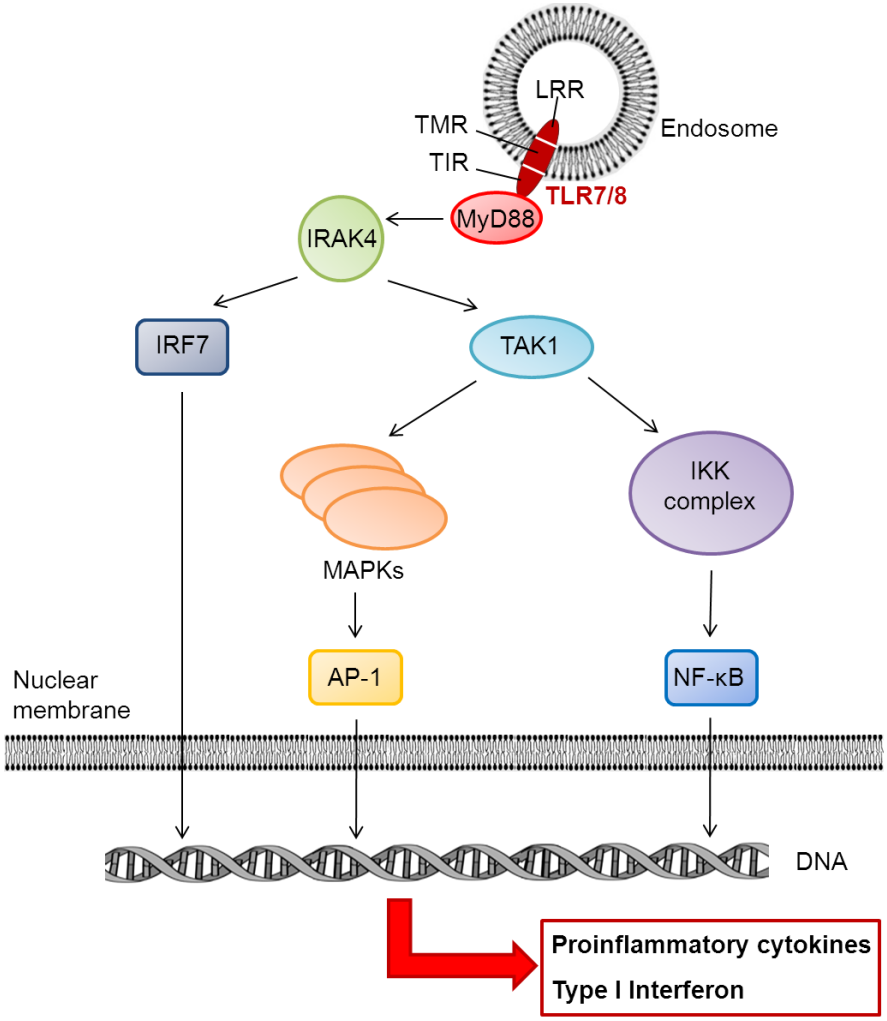


**Figure 7: TLR7 and 8 mediated signaling pathway.**

TLR7 or TLR8 ligation activates intracellular pathways that result in the expression of pro-inflammatory cytokines, chemokines and type I IFNs. The association of TLR7/8 and MyD88 stimulates the recruitment of IL-1 receptor-associated kinases (IRAK, e.g. IRAK1) resulting in the activation of MAPKs (mitogen-activated protein kinases) and the IκB kinase (IKK) complex via the induction of transforming growth factor-activated kinase 1 (TAK1). Members of the MAPK family phosphorylate and activate the transcription factor activator protein 1 (AP-1), whereas the IKK complex is involved in the nuclear translocation of the transcription factor NF-κB. Both AP-1 and NF-κB control the expression of pro-inflammatory cytokine genes. Additionally, members of the interferon regulatory factor (IRF) family are activated resulting in type I IFN (interferon) induction[90]

### 5.2.1 TLR7/8 and cancer

Data from previous studies indicated that TLR7 and TLR8 expression is associated with tumor progression in colorectal cancer and reduced tumor-specific survival amongst patients with high TLR7 and TLR8 expression in colorectal cancer cells.[74] Small molecule agonists acting at TLR7 and TLR8 have sparked a great interest in cancer research owing to their profound anti-tumoral activity. The predominant

anti-tumoral mode of action of these agents is TLR7 and TLR8 mediated activation of NF-κB.[94] Building on the promising data with Imiquimod in clinical trials for the treatment of cutaneous tumors, other synthetic TLR7 and TLR8 agonists like R848 were developed. R848 is a selective ligand for murine TLR7 and for TLR7 and TLR8 in humans. Unfortunately, it has been shown that TLR agonists can also promote cancer cell survival and migration as well as tumor progression. For example, TLR7 and TLR8 agonists have been demonstrated to increase tumor viability and metastasis of human lung cancer cells.[95,96] Interestingly, these ligands are under investigation in clinical trials for breast cancer and melanoma.[92,93]

# 6. Aim of the studies

## 6.1 The influence of Foxp3 expressing Treg and cancer cells in gastrointestinal cancer

The frequency of tumor infiltrating Treg cells can in some cases be connected to the clinical outcome of cancer patients. While in several entities increased Treg cell frequency can be considered as a marker of poor prognosis, there are studies which actually link Treg cell increase to a favorable outlook. Additionally, the transcription factor Foxp3 that is responsible for Treg cell function can be found in tumor cells of several entities and possibly perform immunosuppressive functions. Therefore, the purpose of this study was to determine Foxp3 expression in tumor cells of pancreatic and colorectal cancer *ex vivo* and *in vitro*. In addition, the prognostic significance of Foxp3 expression in cancer cells and tumor infiltrating Treg cells was to be determined also.

## 6.2 The impact of TLR7 and TLR8 expression and signaling in gastrointestinal cancer

Since TLR signaling can have tumor promoting as well as inhibiting effects depending on tumor entity and target receptor this study focused on the impact of TLR7 and TLR8 expression and signaling in gastrointestinal cancer. Next to the analysis of TLR7 and 8 expression in human tumor tissue of pancreatic cancer at different UICC (union internationale contre le cancer) stages and human pancreatic

and colon cancer cell lines, the effects of TLR7/8 signaling via the agonist R848 on tumor proliferation and chemoresistance *in vitro* were of particular interest. Additionally, R848 induced activation of NF-κB and the possible increase of the expression of pro-inflammatory parameters were to be determined.

## II. MATERIALS AND METHODS

### 1. Patients and controls

### 1.1 Ethics statement

Ethical approval for this research was obtained from the Human Research Ethics Committee of the University of Wuerzburg. All patients providing tumor tissue as well as normal tissue samples signed a consent form prior to surgical removal of the intestinal cancer to allow for these researches to be undertaken.

### 1.2 Colorectal cancer tissues for Foxp3 analysis

Sixty-five patients with histologically confirmed CRC undergoing curative surgical resection in our department between 01/2001 and 06/2004 were included in the study. The histological stage of the tumor was determined according to the UICC staging system.[97,98] Tumors were evaluated for localization, tumor stage and their differentiation grade in our Department of Pathology. Data concerning age, gender, level of wall infiltration and lymph node metastasis were collected in a database. Patients, who underwent any neoadjuvant treatment or R1 resection, were excluded from analysis.[98] Tumor tissue samples as well as normal colon tissue samples from the patients were frozen instantly in liquid nitrogen and stored at -80°C until analyzed. Normal colon tissues from healthy individuals served as controls (n=10). The mucinous phenotype of CRC could be associated with false positive subcellular reactions by immunohistochemistry and was therefore excluded from our study. Clinicopathological characteristics of the study population are summarized in table 13. All patients completed at least a 60 months follow-up after resection.[98]

### 1.3 Pancreatic cancer tissues for TLR7/8 studies

In a retrospective analysis, 48 out of 112 patients with a mean age of 69 ± 5.2 years and histologically confirmed pancreatic cancer of the exocrine pancreas were evaluated in the study. Only consecutive patients from whom appropriate tumor material for further analysis (tumor border and tumor center) was available in a period from 06/2003 to 05/2005 in our surgical department were included. Patients were followed up in our Comprehensive Cancer Center (completeness index 0.96).[99]

The classification of pancreatic cancer was asserted in criterion of the UICC for determination of the tumor stage[97]. Cancer specimens were instantly acquired in liquid nitrogen and stored at -80°C until analyzed. Tumors were evaluated for localization, tumor stage, and their differentiation grade in our Department of Pathology. Tumor samples of UICC II (n=12) and UICC III (n=12) were compared with specimens of chronic pancreatitis (n=8) and normal tissue of healthy controls (n=8). Normal tissue of healthy patients and specimens of chronic pancreatitis were kindly provided by Prof. Helmut Friess (Department of General Surgery, University of Munich, Germany).[99]

## 2. Animals

Balb/c nude mice were maintained under defined conditions in accordance with institutional guidelines and experiments were performed according to approved experimental protocols.

## 3. Materials

### 3.1 Established colon and pancreatic cancer cell lines

The human colon cancer cell lines HT-29, SW480 and SW620 as well as the human pancreatic cancer cell line PANC1 and the human fetal normal colonic mucosa line FHC were obtained from ATCC (Manassas, VA) und cultivated as described in 4.1.2.

### 3.2 Primary pancreatic cancer cell lines

The primary pancreatic cancer cell lines PaCa DD 135, PaCa DD 159 and PaCa DD 185 were kindly provided by PD Dr. Felix Rückert (Department of Surgery, University of Mannheim, Germany) and cultivated as described in 4.1.2.

## 3.3 Consumtion items

**Table 1: Consumption items.**

| **Product** | **Company** |
|---|---|
| Cell culture flasks Cell Star ($25\ cm^2$, $75\ cm^2$, $175cm^2$) | Greiner Bio-One (Frickenhausen, Germany) |
| Cell scratcher | Sarstedt AG & Co. (Nuembrecht, Germany) |
| Cellometer SD100 Counting Chamber | Peqlab (Erlangen, Germany) |
| Combitips advanced (2, 5, 10 mL) | Eppendorf (Hamburg, Germany) |
| Cryo vials | Greiner Bio-One (Frickenhausen, Germany) |
| Cuvette polystyrol 10 x 4 x 45 mm | Sarstedt AG & Co. (Nuembrecht, Germany) |
| Falcon tubes Cell Star (15, 50 mL) | Greiner Bio-One (Frickenhausen, Germany) |
| Flow cytometry tubes | Sarstedt AG & Co. (Nuembrecht, Germany) |
| Fuji Medical X-RayFilm 100 NIF | Fujifilm Cooperation (Tokyo, Japan) |
| iBlot Gel Transfer Stacks | Life Technologies (Carlsbad, CA) |
| Microscope slides | Paul Marienfeld GmbH (Lauda-Koenigshofen, Germany) |
| Microspcope cover slips | Paul Marienfeld GmbH (Lauda-Koenigshofen, Germany) |
| Pipette filter tips (10, 200 µL) | Sarstedt AG & Co. (Nuembrecht, Germany) |
| Pipette filter tips (1000 µL) | Biozym Scientific GmbH (Hessisch Oldendorf, Germany) |
| Pre-Separation Filters, 30 µm | Milteny Biotech (Bergisch Gladbach, Germany) |
| Safeseal micro tubes (1.5, 2 mL) | Sarstedt AG & Co. (Nuembrecht, Germany) |
| Scalpel blade | PFM medical (Koeln, Germany) |
| Serological pipettes (2, 5, 10, 25 mL) | Greiner Bio-One (Frickenhausen) |
| Tissue culture test plates 96-well | TPP (Trasadingen, Switzerland) |

## 3.4 Media, buffers, solutions

### 3.4.1 Kits, ready-to-use buffers, reaction solutions and reagents

**Table 2: Kits, ready-to-use buffers, reaction solutions and reagents.**

| **Product** | **Company** |
|---|---|
| 3,39-Diaminobenzidine (DAB) | Biogenex (San Ramon, CA) |
| 49,6-Diamidino-2-phenylindoldihydrochlorid (DAPI) | Sigma-Aldrich (Steinheim, Germany) |
| Acetone | Sigma-Aldrich (St.Louis, MO) |
| Blue/Orange Loading Dye, 6X | Promega (Mannheim, Germany) |
| Boric acid | Sigma-Aldrich (St.Louis, MO) |
| Bovine serum albumin (BSA) | Sigma-Aldrich (St.Louis, MO)) |
| Cell titer AQueous One Solution Cell Proliferation Assay | Promega (Mannheim, Germany) |
| Dimethylsulfoxide (DMSO) | Sigma-Aldrich (St.Louis, MO) |
| Dithiothreitol (DTT) | Sigma-Aldrich (St.Louis, MO) |
| EnVision K1395 double-stain Block | Dako (Glostrup, Denmark) |
| Ethanol | Sigma-Aldrich (St.Louis, MO) |
| Ethylendiamintetraacetate (EDTA) | Sigma-Aldrich (St.Louis, MO) |
| Ficoll 400 | Sigma-Aldrich (St.Louis, MO) |
| Fluoromont | SouthernBiotech (Birmingham, USA) |
| Foxp3 Staining Buffer Set | Miltenyi Biotec (Bergisch Gladbach, Germany) |
| Glycergel | Dako (Glostrup, Denmark) |
| Haemalaun | Sigma-Aldrich (St.Louis, MO) |
| HiMark Prestained High Molecular Weight Protein Standard | Life Technologies (Carlsbad, CA) |
| IGEPAL CA-630 | Sigma-Aldrich (Steinheim, Germany) |
| ImProm-II Reverse Transcription System | Promega (Mannheim, Germany) |
| Intraprep Kit | Bechman Coulter (Krefeld, Germany) |

| | |
|---|---|
| iScript cDNA Synthesis Kit | Promega (Mannheim, Germany) |
| K1395 Fast Red | Dako (Glostrup, Denmark) |
| LightCycler-DNA Master SYBR Green I Mix | Applied Biosystems (Darmstadt, Germany) |
| Lysis buffer Cytobuster | Merck (Darmstadt, Germany) |
| MESA GREEN qPCR Mastermix Plus for SYBR Assay | Eurogentec Deutschland GmbH (Koeln, Germany) |
| Methanol | Sigma-Aldrich (St.Louis, MO) |
| Non-fat dried milk powder | AppliChem (Darmstadt, Germany) |
| Normal mouse serum | Biomeda (Burlingame, CA) |
| NuPAGE Antioxidant | Life Technologies (Carlsbad, CA) |
| NuPage MOPS running buffer | Life Technologies (Carlsbad, CA) |
| NuPAGE Novex 4-12% Bis-Tris Gel | Life Technologies (Carlsbad, CA) |
| Nupage reducing agent (10x) | Life Technologies (Carlsbad, CA) |
| NuPage SDS sample buffer (4x) | Life Technologies (Carlsbad, CA) |
| Phenylmethylsulfonyl fluoride (PMSF) | AppliChem (Darmstadt, Germany) |
| Polyvinyl-alcohol mounting medium | Sigma-Aldrich (St.Louis, MO) |
| Ponceau S 0.1 % (w/v) in 5 % acetic acid | Sigma-Aldrich (St.Louis, MO) |
| RNAlater | Sigma-Aldrich (St.Louis, MO) |
| RNeasy Mini Kit | Qiagen (Hilden, Germany) |
| Roti-Quant | Carl Roth (Karlsruhe, Germany) |
| SeabluePlus2 Pre-Stained Standard | Life Technologies (Carlsbad, CA) |
| SIGMAFAST Protease Inhibitor Tablets | Sigma-Aldrich (St.Louis, MO) |
| Sodium chloride (NaCl) | Sigma-Aldrich (St.Louis, MO) |
| Sodium deoxycholat (NaDOC) | Sigma-Aldrich (St.Louis, MO) |
| Sodium dodecyl sulfate (SDS) | Sigma-Aldrich (St.Louis, MO) |

| | |
|---|---|
| Sodium fluoride (NaF) | Sigma-Aldrich (St.Louis, MO) |
| SuperSignal West Femto Maximum Sensifity Substrate | Thermo Scientific (Waltham, MA) |
| SuperSignal West Pico Chemiluminescent Substrate | Thermo Scientific (Waltham, MA) |
| Taqman Gene Expression Mastermix | Life Technologies (Carlsbad, CA) |
| Tissue-Tek | Sakura (Torrance, CA) |
| Trisaminomethane hydrochloride (Tris-HCl) | Sigma-Aldrich (St.Louis, MO) |
| Tween 20 | AppliChem (Darmstadt, Germany) |

### 3.4.2 Cell culture media, solutions and reagents

**Table 3: Cell culture media, solutions and reagents.**

| **Product** | **Company** |
|---|---|
| Accutase | Sigma-Aldrich (St.Louis, MO) |
| Cholera toxin | Sigma-Aldrich (St.Louis, MO) |
| Dulbeccos Modified Eagles Medium (DMEM) | ATCC (Manassas, VA) |
| DMEM:F12 Medium | ATCC (Manassas, VA) |
| Dulbecco's Phosphate Buffered Saline (DPBS) | Life Technologies (Carlsbad, CA) |
| Enzyme free cell dissociation solution | Merck Millipore (Billerica, MA) |
| Fetal bovine serum (FBS) | Life Technologies (Carlsbad, CA) |
| Geneticin selective antibiotic (G418) | Life Technologies (Carlsbad, CA) |
| Hydrocortisone | Sigma-Aldrich (St.Louis, MO) |
| 2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethansulfonicacid (HEPES) | Sigma-Aldrich (St.Louis, MO) |
| Insulin | Sigma-Aldrich (St.Louis, MO) |
| L-glutamine | Biochrom AG (Berlin, Germany) |
| Penicillin/streptomycin (pen/strep) | Biochrom AG (Berlin, Germany) |

| | |
|---|---|
| RPMI 1640 medium | Life Technologies (Carlsbad, CA) |
| Transferrin | Sigma-Aldrich (St.Louis, MO) |
| Trypanblue solution (0.4 %) | Sigma-Aldrich (St.Louis, MO) |
| Trypsin/EDTA solution (0.05 %/0.02 %) | Biochrom AG (Berlin, Germany) |
| Resiquimod (R848) | InvivoGen (San Diego, CA) |
| 5-Fluorouracil (5-FU) | Medac (Wedel. Germany) |

### 3.4.3 Self-made buffers

RIPA buffer:
20 mM Tris-HCl (pH 7.5)
150 mM NaCl
1 mM EDTA
1 % (v/v) IGEPAL CA-630
1 % (m/v) NaDOC
1 % (m/v) SDS
5 mM (m/v) NaF

1 tablette protease inhibitor cocktail (Sigma-Aldrich) per 100 mL
Added fresh before use: 1 mM DTT, 1 mM PMSF

0.05% TBS-Tween (TBS-T):
500 mM Tris-HCl
1.5 M NaCl.
0.05 % (v/v) Tween 20
pH 7.4

Blocking solutions:
5 % (m/v) non-fat dried milk powder in TBS-T
or
5 % (m/v) BSA in TBS-T

10x TBE buffer: 1 M Tris

100 mM EDTA

0.83 M boric acid

pH 8.3

## 3.5 Antibodies

### 3.5.1 Primary antibodies

**Table 4: Primary antibodies.**

| Target | Clonality | Host | Conjugate | Company |
|---|---|---|---|---|
| CD25 | mAb (monoclonal antibody) | mouse | - | Dako (Glostrup, Denmark) |
| CD34 | mAb | goat | - | AbD Serotec (Duesseldorf, Germany) |
| CD34 | mAb | mouse | PE (Phycoerythrin) | Beckman Coulter (Krefeld, Germany) |
| CD4 | mAb | mouse | - | Dako (Glostrup, Denmark) |
| CD8 | mAb | goat | - | Dako (Glostrup, Denmark) |
| CK (cytokeratin)-20 | mAb | mouse | - | Dako (Glostrup, Denmark) |
| CK-7 | mAb | mouse | - | Dako (Glostrup, Denmark) |
| COX-2 | mAb | mouse | - | Santa Cruz Biotechnology (Dallas, TX) |
| COX-2 | mAb | mouse | - | Novus Biologicals (Littleton, CO) |
| EpCAM (Epithelial cell adhesion molecule) | mAb | mouse | FITC (Fluorescein isothiocyanate) | Miltenyi Biotec (Bergisch Gladbach, Germany) |
| Foxp3 | mAb | mouse | PE | Miltenyi Biotec (Bergisch Gladbach, Germany) |
| Foxp3 | mAb | mouse | - | Abcam (Cambridge, UK) |
| Foxp3 | pAb (polyclonal antibody) | goat | - | Abcam (Cambridge, UK) |
| IL-10 | mAb | mouse | - | R&D Systems (Minneapolis, MN) |

| | | | | |
|---|---|---|---|---|
| TGF-β | mAb | mouse | - | AbD Serotec (Duesseldorf, Germany) |
| TLR7 | pAb | rabbit | - | IMGENEX (San Diego, CA) |
| TLR7 | pAb | rabbit | - | ProSci (Poway, CA) |
| TLR8 | pAb | mouse | - | ProSci (Poway, CA) |
| β-actin | mAb | mouse | - | Santa Cruz Biotechnology (Dallas, TX) |

### 3.5.2 Secondary antibodies and isotype controls

**Table 5: Secondary antibodies.**

| Secondary antibodies | Conjugate | Company |
|---|---|---|
| AffiniPure donkey anti-goat IgG (immunoglobulin G) | FITC | Jackson ImmunoResearch (West Grove, PA) |
| AffiniPure donkey anti-goat IgG | HRP (horseradish peroxidase) | Jackson ImmunoResearch (West Grove, PA) |
| AffiniPure donkey anti-goat IgG | Cy (cyanine) 5 | Jackson ImmunoResearch (West Grove, PA) |
| AffiniPure donkey anti-mouse IgG | Cy3 | Jackson ImmunoResearch (West Grove, PA) |
| AffiniPure donkey anti-mouse IgG | Cy5 | Jackson ImmunoResearch (West Grove, PA) |
| AffiniPure donkey anti-mouse IgG | HRP | Jackson ImmunoResearch (West Grove, PA) |
| AffiniPure donkey anti-rabbit IgG | Cy3 | Jackson ImmunoResearch (West Grove, PA) |
| AffiniPure donkey anti-rabbit IgG | HRP | Jackson ImmunoResearch (West Grove, PA) |
| Donkey anti goat IgG | HRP | Santa Cruz Biotechnology (Dallas, TX) |
| Goat anti mouse IgG | FITC | Beckman Coulter (Krefeld, Germany) |
| Goat anti mouse IgG | HRP | GE Healthcare Life Sciences (Little Chalfont, UK) |
| Goat anti rabbit IgG | FITC | Beckman Coulter (Krefeld, Germany) |
| Goat anti rabbit IgG | AP (alkaline phosphatase) | Dako (Glostrup, Denmark) |
| Rabbit anti mouse IgG | HRP | Dako (Glostrup, Denmark) |
| Goat Anti-Mouse IgG | Alexa Fluor 488 | Abcam (Cambridge, UK) |

**Table 6: Isotype controls.**

| Isotype controls | Conjugate | Company |
|---|---|---|
| Goat IgG1 | - | Pharmigen (Heidelberg, Germany) |
| Mouse IgG | - | eBioscience (San Diego, CA) |
| Mouse IgG1 | - | Pharmigen (Heidelberg, Germany) |
| Mouse IgG1 | FITC | eBioscience (San Diego, CA) |
| Mouse IgG1 | PE | Beckman Coulter |
| Mouse IgG1 | PE | Miltenyi Biotec (Bergisch Gladbach, Germany) |
| Rabbit IgG | - | eBioscience (San Diego, CA) |

## 3.6 Primer pairs, primer assays and gene expression assays

**Table 7: Primer pairs.**

| Target | Primer pairs | Company |
|---|---|---|
| 18S rRNA | forward: TCA AGA ACG AAA GTC GGA GGT TCG<br>reverse: TTA TTG CTC AAT CTC GGG TGG CTG | Biomers (Ulm, Germany) |

**Table 8: QuantiTect primer assays.**

| Target | Primer assays | Company |
|---|---|---|
| CD25 | Hs_IL2RA_1_SG QuantiTect primer assay | Qiagen (Hilden, Germany) |
| CD4 | Hs_CD4_1_SG QuantiTect primer assay | Qiagen (Hilden, Germany) |
| Foxp3 | Hs_FOXP3_1_SG QuantiTect primer assay | Qiagen (Hilden, Germany) |
| GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) | Hs_GAPDH_1_SG QuantiTect primer assay | Qiagen (Hilden, Germany) |
| IL-10 | Hs_IL10_1_SG QuantiTect primer assay | Qiagen (Hilden, Germany) |
| TGF-β | Hs_TGFB1_1_SG QuantiTect primer assay | Qiagen (Hilden, Germany) |

| | | |
|---|---|---|
| TLR7 | Hs_TLR7_1_SG QuantiTect primer assay | Qiagen (Hilden, Germany) |
| TLR8 | Hs_TLR8_1_SG QuantiTect primer assay | Qiagen (Hilden, Germany) |
| β-actin | Hs_ACTB_1_SG QuantiTect primer assay | Qiagen (Hilden, Germany) |

**Table 9: TaqMan gene expression assays.**

| Target | Taqman gene expression assay | Assay ID | Company |
|---|---|---|---|
| COX-2 | PTGS2 | Hs00153133_m1 | Life Technologies (Carlsbad, CA) |
| GAPDH | GAPDH | Hs02758991_g1 | Life Technologies (Carlsbad, CA) |
| GUSB (Beta-glucuronidase) | GUSB | Hs00939627_m1 | Life Technologies (Carlsbad, CA) |
| HPRT1 (hypoxanthine phosphoribosyltransferase 1) | HPRT | Hs02800695_m1 | Life Technologies (Carlsbad, CA) |
| IL-1B | IL-1B | Hs01555410_m1 | Life Technologies (Carlsbad, CA) |
| IL-8 | IL-8 | Hs00174103_m1 | Life Technologies (Carlsbad, CA) |
| Ki-67 | MKI67 | Hs01032443_m1 | Life Technologies (Carlsbad, CA) |
| NF-κB | NF-kappaB | Hs00765730_m1 | Life Technologies (Carlsbad, CA) |
| TLR7 | TLR7 | Hs01933259_s1 | Life Technologies (Carlsbad, CA) |
| TLR8 | TLR8 | Hs00152972_m1 | Life Technologies (Carlsbad, CA) |
| TNF-α (Tumor necrosis factor alpha) | TNFA | Hs01113624_g1 | Life Technologies (Carlsbad, CA) |
| β-actin | ACTB | Hs01060665_g1 | Life Technologies (Carlsbad, CA) |

## 3.7 Lab devices

**Table 10: Lab devices.**

| Device | Company |
|---|---|
| BioPhotometer | Eppendorf (Hamburg, Germany) |
| Biorad CFX96 Touch Real-Time PCR Detection System | Biorad (Hercules, CA) |
| Cellometer Auto T4 Plus | Peqlab (Erlangen, Germany) |
| Centrifuge Biofuge fresco | Kendro (Langenselbold, Germany) |
| Centrifuge Multifuge 1 S-R | Thermo Scientific (Waltham, MA) |
| Cryostat Leica CM3050 S | Leica Biosystems (Wetzlar, Germany) |
| Cytospin4 Cytocentrifuge | Thermo Scientific (Waltham, MA) |
| DNA Engine Opticon 2 System | MJ Research (Waltham, MA) |
| Dynatech MRX microplate reader | Dynatech (Houston, TX) |
| Elektophoresis Power Supply E835 | Consort (Turnhout, Belgium) |
| Flow Cytometer Coulter EPICS XL | Beckman Coulter (Brea, CA) |
| iBlot dry blotting system | Life Technologies (Carlsbad, CA) |
| Incubator Function Line | Thermo Scientific (Waltham, MA) |
| Inverted microscope Axiovert 40C | Zeiss (Jena, Germany) |
| Laminar flow hood Hera Save | Kendro (Langenselbold, Germany) |
| Mastercycler Gradient | Eppendorf (Hamburg, Germany) |
| Multipette plus | Eppendorf (Hamburg, Germany) |
| Nanodrop 2000c | Thermo Scientific (Waltham, MA) |
| Pipette controller Accu Jet pro | Brand GmbH (Wertheim, Germany) |
| Pipettes (10, 200, 1000 µL) | Eppendorf (Hamburg, Germany) |
| QIAcube | Qiagen (Hilden, Germany) |

| | |
|---|---|
| QIAshredder | Qiagen (Hilden, Germany) |
| Scale CP 4201 | Sartorius (Göttingen, Deutschland) |
| Scanner CanonScan9000F | Canon (Tokyo, Japan) |
| Shaker Unimax 1010 | Hedolph Instruments GmbH (Schwabach, Germany) |
| Thermo Max Mikrolate Reader | MWG Biotech (Ebersberg, Germany) |
| Thermomixer | Eppendorf (Hamburg, Germany) |
| Vortex Mixer Gene2 | Scientific Instruments (West Palm Beach, FL) |
| Water bath | Memmert (Schwabach, Germany) |
| XCell Sure Lock chamber | Life Technologies (Carlsbad, CA) |
| Zeiss camera | Zeiss (Oberkochen, Germany) |

## 3.8 Software

**Table 11: Software.**

| **Software** | **Company** |
|---|---|
| Opticon Monitor Analysis Software Version 2.02. | MJ Research (Waltham, MA) |
| Metamorph software package | Visitron Systems (Puchheim, Germany) |
| Microsoft Office 2010 | Microsoft Corporation (Redmond, WA) |
| Biorad CFX manager analysis software | Biorad (Hercules, CA) |
| Canon EP Navigator EX | Canon (Tokyo, Japan) |
| Softmax Pro 4.8 | Molecular Devices (Sunnyvale, CA) |
| Nanodrop 2000/2000C | Thermo Scientific (Waltham, MA) |
| SAS 9.2 | SAS (Cary, NC) |
| SPSS | SPSS (Munich, Germany) |
| Coulter, Epics XL-MCL, System II | Beckman Coulter (Krefeld, Germany) |

# 4. Methods

## 4.1 Cell culture

All cell culture procedures were performed in a laminar flow hood and proper aseptic techniques were applied.

### 4.1.1 Thawing frozen cells

Frozen cells were taken from liquid nitrogen and put in a 37°C water bath for thawing (< 60 seconds). After that, cells were diluted slowly, using pre-warmed growth medium and transferred to a 50 mL falcon tube. A centrifugation step at 300 x g (FHC cells: 125 x g) was performed for 10 minutes to spin cells down. Afterwards, supernatant was discarded, the pellet was resuspended in 1000 µL of pre-warmed growth medium and transferred to a culture flask containing pre-warmed growth medium.

### 4.1.2 Maintaining and passaging adherent cells

Colon cancer cells were cultured using RPMI 1640 medium supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) penicillin/streptomycin (pen/strep). Transduced PANC1 cells were cultured using DMEM medium supplemented with 10% (v/v) FBS, 1% (v/v) G418 and 1% (v/v) pen/strep. FHC cells were cultivated using DMEM:F12 medium containing 25 mM HEPES, 10 ng/mL cholera toxin, 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 100 ng/mL hydrocortisone and 10% (v/v) FBS. Medium for primary pancreatic cancer cells PaCa DD 135, PaCa DD 159 and PaCa DD 185 was assembled by mixing two parts of DMEM medium supplemented with 20% (v/v) FBS with one part of Keratinocyte-SFM. All cell lines were incubated at 37°C in 5% $CO_2$.

For passaging growth medium was taken off, cells were washed with 10 mL DPBS and incubated with 5 mL trypsin for five to seven minutes (depending on cell line and density) at 37°C. Afterwards 10 mL of medium was added, cells were transferred to a 50 ml falcon tube and spun down for 10 minutes at 300 x g (FHC cells: 125 x g). Subsequently supernatant was discarded, the pellet was resuspended in 1000 µL of

pre-warmed growth medium and filled to a total volume of 10 mL. To seed a determined number of cells trypan blue staining and counting was performed as described in 4.1.3. For passaging 1:10 1000 µL of the cell suspension were transferred to a new culture flask containing pre-warmed growth medium.

### 4.1.3 Trypan blue staining and counting of cells

After detaching and washing, cells were mixed 1:1 with trypan blue solution and 20 µL of the mix were transferred to a Cellometer SD100 Counting Chamber. Counting of cells was performed on a Cellometer Auto T4 Plus. Viable cells exclude trypan blue, while dead cells stain blue due to trypan blue uptake.

### 4.1.4 Cryopreservation of cells

For freezing cells were trypsinized and centrifuged as described in 4.1.2. After removing the supernatant the pellet was resuspended by adding 1500 µL freezing medium (growth medium supplemented with 10% (v/v) DMSO). Cells were transferred to a 2 mL cryo vial and put at -80°C in a cryo box containing isopropanol overnight. The following day vials were transferred to liquid nitrogen for long-term storage.

### 4.1.5 Lentiviral transduction of PANC1 cells

In contrast to the tumor tissues from patients with pancreatic cancer or from patients with chronic pancreatitis established tumor cell lines express only very low levels of TLR7 and TLR8. For *in vitro* studies it was necessary to overexpress both receptors in those cells.[99] The most common established pancreatic cell line PANC1 was transduced with lentivirus vectors encoding for human TLR7 (pLV-Ubiqc-TLR7-IRES-Neo) and human TLR8 (pLV-Ubiqc-TLR8-IRES-Neo) under the control of the ubiquitin promoter. Cells transduced with an empty vector served as a control. The vector design and transduction of TLR7 and TLR8 PANC1 cells was performed by Sirion Biotech (Martinsried, Germany). Cells were subjected to antibiotic selection of geneticin (G418)-resistant cells. The transduced PANC1 cells

(TLR7+, TLR8+ and empty vector PANC1 cells) were cultured as described in 4.1.2 containing additionally 1% G418.

## 4.2 Ficoll density gradient separation of PBMCs from human blood samples

A total volume of 10 mL of blood was transferred from the collection vial to a 50 mL falcon tube containing 15 mL of Ficoll. Centrifugation without the brake was conducted at 860 x g for 10 minutes. Upper plasma layer was drawn off carefully not to disturb the lower PBMC (peripheral blood mononuclear cells) interface. Afterwards the PBMC layer was transferred to a new 50 mL falcon tube and RPMI 1640 medium was added at a total volume of 50 mL. After centrifugation for 10 minutes at 300 x g supernatant was discarded, the pellet was resuspended in 1000 µL medium and filled to a total volume of 10 mL for cell counting. Counting was performed as described in 4.1.3. For a second washing step medium was added at a total volume of 50 mL and centrifugation was carried out at 300 x g for 10 minutes. Subsequently, the supernatant was discarded and the pellet was resuspended in 1.5 mL freezing medium (RPMI 1640 medium supplemented with 10% (v/v) DMSO) per 5 x $10^6$ cells. For cryopreservation 1.5 mL of the cell suspension were transferred to a 2 mL cryo vial and put at -80°C overnight. The following day vials were transferred to liquid nitrogen for long-term storage.

## 4.3 Animals

Balb/c nude mice were maintained under defined conditions in accordance with institutional guidelines and experiments were performed according to approved experimental protocols. For *in vivo* growth studies 2 x $10^6$ transduced PANC1 cells were injected subcutaneously into both flanks of recipient Balb/c nude mice (TLR7+ PANC1 n=5, TLR8+ PANC1 n=5, empty vector PANC1 n=4). 40 days post tumor cell inoculation the mice were sacrificed, tumor growth was documented and tumor volume was determined ($V=\pi/6 \times a \times b \times c$; a=length, b=width, c=height).[99]

## 4.4 Immunological methods

### 4.4.1 Immunohistochemical and immunofluorescent stainings

#### 4.4.1.1 Cryosections

Colon and pancreatic tumor tissue samples as well as normal colon tissue samples from the patients were snap-frozen instantly upon surgical removal in liquid nitrogen using Tissue Tek and suitable cryo molds. Samples were transferred to -80°C until analyzed. Normal colon tissues of healthy individuals were prepared as controls. Normal pancreas tissue of healthy patients (n=8) and specimens of chronic pancreatitis were kindly provided by Prof. Helmut Friess (Department of General Surgery, University of Munich, Germany). Serial cryostat sections (5 mm) were made using a Cryostat Leica CM3050 S and mounted on microscope glass slides.

#### 4.4.1.2 Cytospin preparations

For cytospin preparations colorectal and pancreatic tumor cells from the patients were cultured in short-term primary cultures. Established human colon cancer cell lines and pancreatic PANC1 cells were harvested at an exponential growth phase using Enzyme free cell dissociation or Accutase solution. After washing with DPBS twice, cells were adjusted at a final concentration of $2 \times 10^5$ cells/mL. Cytospin preparations were performed with 50 µL cell suspension at 550 rpm for one minute in a Cytospin4 Cytocentrifuge.

#### 4.4.1.3 Staining procedures

Staining of tissue sections was performed on serial cryostat sections. All colon tumors stained positive for cytokeratin (CK)-20 and negative for CK-7, a pattern characteristic for colonic adenocarcinoma.[100] Additionally HE (hematoxylin and eosin) stainings from each tumor tissue were assessed to differentiate between cancer cell areas, stromal areas and infiltrating immune cells.

For immune staining procedures serial cryostat sections were fixed in acetone for 10 minutes and then dried for 5 minutes. For immunofluorescence staining, the slides were incubated with the primary antibody or isotype control antibody diluted in TBS

plus 0.5% (m/v) bovine serum albumine (BSA) overnight at 4°C in a humidified chamber and with secondary fluorochrome conjugated antibody for 30 minutes at room temperature in a humidified chamber. Then the slides were incubated with the second primary antibody diluted in TBS plus 0.5% (m/v) BSA overnight at 4°C in a humidified chamber followed by incubation with secondary fluorochrome conjugated antibody for 30 minutes at room temperature in a humidified chamber. For sequential immunofluorescence triple staining, after washing, the slides were incubated with the third primary antibody diluted in TBS plus 0.5% (m/v) BSA overnight at 4°C in a humidified chamber followed by incubation with a secondary fluorochrome conjugated antibody for 30 minutes at room temperature. Slides were counterstained with DAPI, covered with Polyvinyl-alcohol mounting medium and analyzed using a Zeiss camera.

For immunohistochemical staining, the slides were incubated as described previously with a primary antibody or isotype control antibody. After incubation with the primary antibody, HRP-conjugated AffiniPure Donkey anti-mouse or a Donkey anti-rabbit or a Donkey anti-goat IgG were used as secondary antibodies. Slides were subsequently incubated for 5 minutes in DAB, counterstained with Haemalaun and mounted with Glycergel. The quantification of each immunoenzymatic staining of tumor cells in six individual magnified fields for each staining sample was scored by cell counting performed by two independent investigators blinded for the underlying disease. The magnified fields were representative for the whole tumor section. The result of the staining was expressed in percentages (%) positivity. All values were expressed as mean ± SD.

### 4.4.2 Flow cytometry (FACS)

Human colon cancer cells SW620 and SW480 were harvested at an exponential growth phase using enzyme free cell dissociation solution. After washing with DPBS twice, 5 x $10^5$ cells were treated with Foxp3 staining buffer kit according to the manufacturer's instructions and incubated with PE-conjugated antibody against Foxp3 or IgG1 isotype control for 30 minutes at 4°C in the dark. The fluorescence of Foxp3 was measured and analyzed with a Coulter EPICS XL flow cytometer.[98] Cells derived from patients of normal pancreas, chronic pancreatitis and pancreatic cancer

were analyzed for the expression of TLR7, TLR8 and CD34. The total suspension of 5 x $10^6$ cells was pelleted and resuspended in DPBS. For intracellular staining Beckman Coulter Intraprep-Kit was used. Cells were incubated with PE-conjugated antibody CD34 or isotype control antibody for 20 minutes. After washing, cells were incubated with TLR7 and TLR8 primary antibody or IgG isotype control antibody for 20 minutes followed by subsequent incubation of FITC conjugated secondary antibody for 20 minutes after a washing step. The cells were analyzed with a Coulter EPICS XL flow cytometer.[99]

### 4.4.3 Western blot

#### 4.4.3.1 Preparation of tissue and cell lysates

Protein extracts from tissue samples (250 µg) were performed using lysis buffer Cytobuster. Tumor tissues were cut in small pieces and 250 µg were homogenized for 15 minutes in lysis buffer using QIAshredder before centrifugation (full speed, 4°C, 20 minutes). The supernatant was collected and stored at -80°C.[99]

PANC1 cell lysates were prepared using RIPA buffer. Adherent cells were detached using a cell scratcher, washed with DPBS and pelleted at 300 x g for 10 minutes. After resuspension in RIPA buffer containing DTT and PMSF, cells were incubated on ice for 10 minutes, centrifuged at 13.000 x g and 4°C for 15 minutes. Supernatant was collected and stored at -80°C.

#### 4.4.3.2 SDS polyacrylamide gel electrophoresis (SDS-Page)

Protein concentration of lysates was determined by Bradford assay using Roti-Quant solution diluted 1:5 with water. To perform SDS-Page, NuPAGE SDS Buffer and NuPAGE Novex Mini Gels were used according to the manufacturer's instructions. Samples were prepared by adding NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent followed by incubation at 70°C for 10 minutes to denature the proteins. After loading the samples, SDS-Page was performed at 4°C, 200 V and 113 mA for 90 to 120 minutes. As a reference an appropriate protein ladder depending on the size of the proteins of interest was used.

#### 4.4.3.3 Western blotting

Western blot of separated proteins on nitrocellulose was carried out using iBlot dry Blotting System and iBlot Gel Transfer Stacks according to the manufacturer's instructions. To survey the successful transfer the membrane was stained with Ponceau S and subsequently destained with TBS-T. Afterwards the membrane was either used for immunodetection (4.4.3.4) or stored for subsequent analysis. For storage, the membrane was placed between two filters papers soaked with TBS-T and kept at -20°C in a suitable dish.

#### 4.4.3.4 Immunodetection

Membranes were blocked with 5% non-fat dried milk powder or 5% BSA in TBS-T for 60 minutes at room temperature before probing with primary antibodies to TLR7, TLR8, β-actin and COX-2 by incubation overnight at 4°C. After washing three times for 10 minutes with TBS-T, secondary HRP-conjugated antibody was incubated for 60 minutes at room temperature. Following three washing steps with TBS-T, ECL (enhanced chemoluminescence) detection was performed using SuperSignal West Pico Chemiluminescent Substrate or SuperSignal West Femto Chemiluminescent Substrate according to the manufacturer's instructions.

### 4.5 MTS cell proliferation assay

The CellTiter 96 Aqueous One Solution Assay is a colorimetric method to determine the number of viable cells in proliferation or cytotoxicity assays. The containing MTS (3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. The quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture.

### 4.5.1 Determination of the median lethal dose ($LD_{50}$) for 5-FU in PANC1 cells

Empty vector PANC1 cells were cultured at a concentration of 5 x $10^3$ cells per well in 96-well plates. After 24 hours medium was changed and 5-FU (5-fluorouracil, working concentration, 10 – 10.000 µmol/L) was added to each well. The cells were incubated for 48 hours, then the medium (without 5-FU) was renewed and the cells were incubated for another period of 24 hours at 37ºC in 5% $CO_2$. 20 µL CellTiter 96 Aqueous One Solution Assay was added to the cells and one hour later the cells were measured at 490 nm in a Dynathech MRX microplate reader. All samples were run in triplicates; and every assay was performed twice. Untreated cells served as a control. The median lethal dose was defined as amount of drug resulting in 50% killing within two days.

### 4.5.2 Proliferation and cytotoxicity assays

To analyze the effect of 5-FU on transduced TLR7+ and TLR8+ PANC1 cells were treated as described above using 100 µmol/L and 1000 µmol/L 5-FU. Untreated cells served as a control. MTS assay was performed as described previously.

To investigate the effect of stimulation with R848 on tumor cell proliferation 2 x $10^6$ PANC1 cells were seeded in cell culture flasks preincubated for 24 hours following daily stimulation using 10 µg/mL R848 for three days. Then cells were detached using Accutase solution and seeded 6000 cells/well in 96 well plates. After additional incubation time of 24 hours and 72 hours MTS assay was performed as described above. The relative proliferation was calculated as the values after 72 hours divided by the corresponding values after 24 hours multiplied by 100%.[99]

Afterwards the effect of R848 stimulation on the chemosensitivity of transduced PANC1 cells was analyzed. 4000 cells/well were seeded in 96 well plates, preincubated for 48 hours and then treated with R848 (10 µg/mL). After an additional incubation of 48 hours cells were treated with 500 µmol/L 5-FU and after another 48 hours MTS assay was performed as described previously.[99] Tumor cell proliferation was determined relative to untreated cells.

## 4.6 Gene expression analysis

### 4.6.1 Extraction of total RNA from tissue and adherent cells

Cancer tissues stored in RNAlater were thawed and subsequently cut in small pieces. Afterwards samples were homogenized on a Tissue Lyser in Buffer RLT (component of RNeasy Mini Kit). Following centrifugation for 3 minutes at full speed and 4°C supernatant was transferred to a new vial and RNA extraction was performed on QIACube platform using RNeasy Mini Kit according to the manufacturer's instruction.

For isolation of total RNA from established cancer cell lines, cells were detached using a cell scratcher, washed with DPBS and pelleted at 300 x g for 5 minutes. Supernatant was discarded and cells were resuspended in Buffer RLT. Extraction of RNA was performed on QIACube platform using RNeasy Mini Kit according to the manufacturer's instruction.

After extraction of total RNA, samples were stored at -80°C for further analysis. Prior to analysis in RT-qPCR RNA concentration was determined using the NanoDrop 2000c.

### 4.6.2 Real-time quantitative reverse transcription-PCR

Gene expression was analyzed using reverse transcription following quantitative real-time PCR (RT-qPCR). Reverse transcription from RNA to cDNA was carried out by using iScript cDNA Synthesis Kit or ImProm-II™ Reverse Transcription System according to the manufacturer's instructions.

For analysis of pancreatic and colon tissues and colon cancer cell lines all PCR reactions were carried out with a DNA Engine Opticon 2 System. Each PCR reaction was performed in 25 µL volumes containing the LightCycler-DNA Master SYBR Green I mix, forward and reverse primers or Quatitect primer assays and 100 ng template cDNA. Initial denaturation at 95°C for 15 minutes was followed by 40 cycles of a denaturation step at 95°C for 15 seconds, an annealing step at 57.5°C for 30 seconds, and an extension step at 72° C for 30 seconds.

For the experiments using the human pancreatic cancer cell line PANC1 and human fetal colon mucosa cells FHC gene quantification was performed using Taqman Gene Expression Master Mix and specific Taqman Gene Expression Assays according to the manufacturer´s instructions on a Biorad CFX96 Touch Real-Time PCR Detection System. Quantification data were analyzed with the Biorad CFX Manager Analysis software and Microsoft Excel 2010.

Housekeeping genes β-actin, GAPDH (glyceraldehyde 3-phosphate dehydrogenase), GUSB (beta-glucuronidase), 18S rRNA (ribosomal RNA) and HPRT1 (hypoxanthine phosphoribosyltransferase 1) were used for relative quantification. Reproducibility was confirmed by three independent PCR runs. The average threshold cycle (Cq) value was calculated as the cycle number at which the fluorescence of the reporter reaches a fixed threshold. The difference (ΔCq) between the average Cq values of the samples in the target wells and those of the housekeeping genes was assessed, followed by the calculation of the difference between the average ΔCq values of the samples for each target and the ΔCq value of the control for that target (ΔΔCq). The relative quantification value, fold difference, is expressed as $2^{-\Delta\Delta Cq}$.

### 4.6.3 Agarose gel electrophoresis

To visualize the successful gene expression of TLR7 and TLR8 in transduced PANC1 cells agarose gel electrophoresis of RT-qPCR products was carried out using 2 % (m/v) agarose TBE gels. Samples were mixed with 6 x Loading Dye and applied on the gel wells. Electrophoresis was performed in TBE buffer at 150 V for 40 minutes. PANC1 cells transduced with empty vector and PBMCs were used as controls.

## 4.7 Statistical analysis

### 4.7.1 Foxp3 Studies

Statistical analysis was performed using SAS 9.2 and was kindly supported by Mr. Dipl.-Math. Mathias Brosz. Overall survival was defined as the time period between randomization and death of any cause. Patients, who were lost to follow-up, were censored at the date of last contact. The overall survival was evaluated by

means of PROC PHREG (Cox Proportional Hazards Model). The parameters of prognostic potential, identified in a stepwise procedure, have been further investigated by Kaplan-Meier method (PROC LIFETEST). For univariate analysis mean cut-off value for either high or low expression was set at 12% for Foxp3 in tumor infiltrating Treg and 16% for Foxp3 in cancer cells.[98] Univariate analysis of significance for Foxp3 expression of tumor infiltrating Treg and cancer cell expression differences in survival curves were evaluated by Log-rank test. In the same way survival curves were compared for N and T categories as well as primary tumor. Two independent groups of patients were analyzed using Student's t test (Satterthwaite). More than two groups were analyzed applying PROC GLM (analysis of variances) with posthoc testing (Tukey). Frequency distributions were compared using kxm tables (Chi-quadrat). Pearson's correlation coefficient was used to describe and to test bivariate correlations. A p-value of less than 0.05 was considered statistically significant.[98]

### 4.7.2 TLR7/8 studies

Results were expressed as mean ± SEM in groups of patients with normal pancreatic tissue, chronic pancreatitis and pancreatic cancer. Comparisons were performed by ANOVA or paired and unpaired t-test when appropriate. Bonferroni's correction for multiple comparisons was used to determine the level of significance of p. A p-value of less than 0.05 was considered statistically significant.[99]

## III. RESULTS

### 1. The role of Foxp3 in human gastrointestinal cancer

Since Foxp3 is a parameter usually to be found in Treg cells, established pancreatic and colon cancer cell lines were first analyzed for the expression of Foxp3 and its downstream targets IL-10 and TGF-β. Additionally, expression levels in primary pancreatic cancer cell lines were determined.

#### 1.1. Expression of Foxp3, IL-10 and TGF-β in pancreatic cancer cell lines

The established pancreatic cancer cell line PANC1 and primary pancreatic cancer cell lines PaCa DD 135, PaCa DD 159 and PaCa DD 185 were analyzed for Foxp3, IL-10 and TGF-β expression.

**A**

| Cell line | Cq value (mean) Foxp3 | Cq value (mean) β-actin |
|---|---|---|
| PANC1 | 27.71 | 18.03 |
| PaCa DD 135 | 24.67 | 19.39 |
| PaCa DD 159 | 24.60 | 19.86 |
| PaCa DD 185 | 24.66 | 18.57 |

**B**

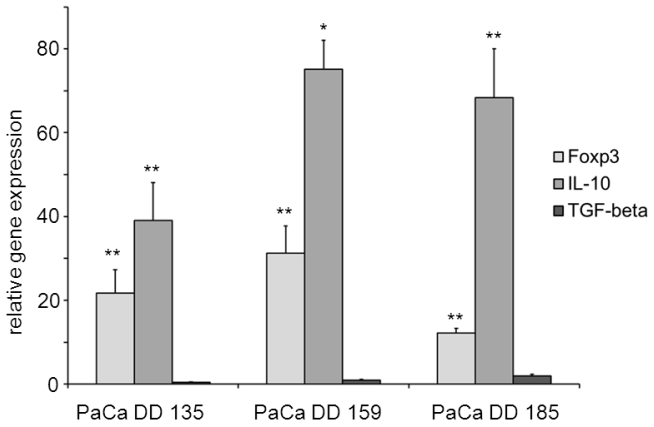


**Figure 8: Gene expression of Foxp3, IL-10 and TGF-β in pancreatic cancer cell lines.**

**(A)** Mean Cq values in RT-qPCR demonstrate Foxp3 gene expression in PANC1, PaCa DD 135, PaCa DD 159 and PaCa DD 185 cells. Mean Cq values for β-actin were used as reference. **(B)** Relative gene expression of Foxp3 and IL-10 was significantly increased in primary pancreatic cell lines compared to PANC1 cells. No significant differences were observed in TGF-β expression. The normalization was performed with PANC1 cells. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$. *p<0.0001, **p<0.005

In RT-qPCR mean Cq values of about 28 in PANC1 and 24 in PaCa DD 135, PaCa DD 159 and PaCa DD 185 cells demonstrated Foxp3 gene expression. β-actin served as a reference (figure 8A). Additionally, in primary pancreatic cancer cell lines relative gene expression of Foxp3 and IL-10 was significantly increased compared to PANC1 cells (figure 8B). Fold differences (relative gene expression) for Foxp3 were found at 22 in PaCa DD 135 cells ($p<0.005$), 31 in PaCa DD 159 cells ($p<0.005$) and 12 in PaCa DD 185 cells ($p<0.005$) while values for IL-10 were 39 in PaCa DD 135 cells ($p<0.005$), 75 in PaCa DD 159 cells ($p<0.0001$) and 68 in PaCa DD 185 cells ($p<0.005$). No significant differences were observed in TGF-β expression in comparison to PANC1 cells (figure 8B).

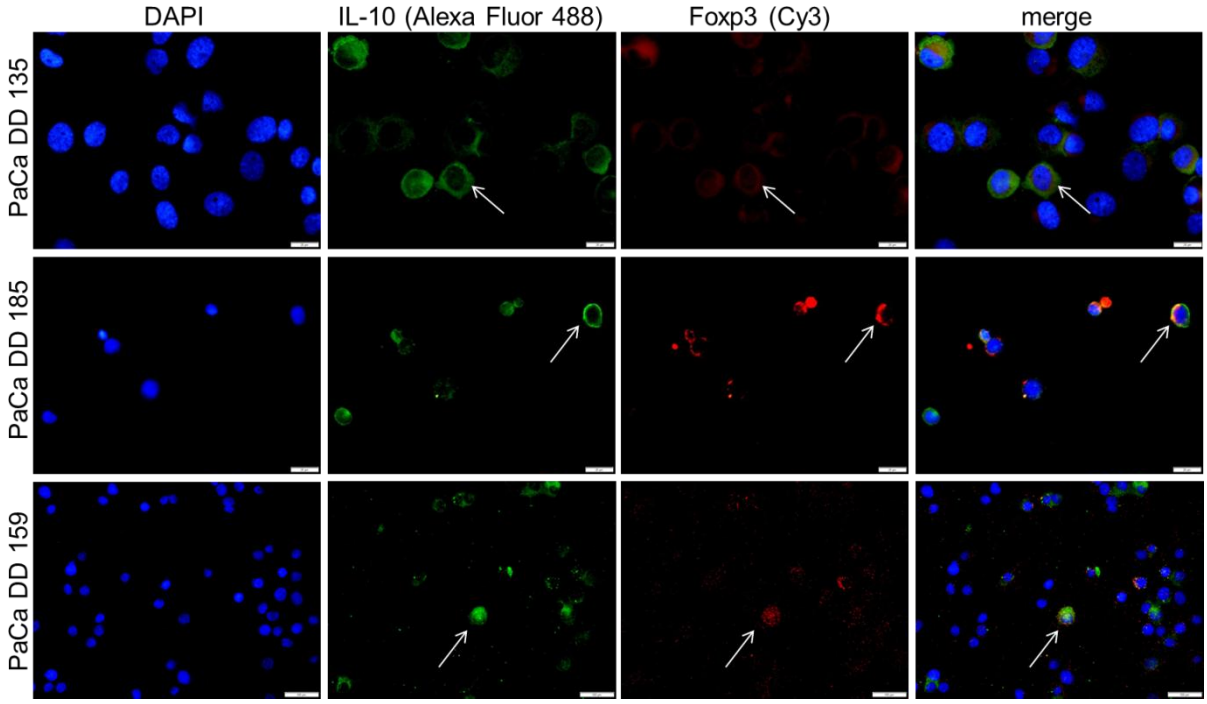


**Figure 9: Co-expression of Foxp3 and IL-10 in primary pancreatic cancer cell lines PaCa DD 135, PaCa DD 159 and PaCa DD 185.**

Representative examples of immunofluorescence double staining of Foxp3 and IL-10 in PaCa DD 135, PaCa DD 159 and PaCa DD 185 cells. Co-expression of both antigens was observed in all investigated cell lines. Alexa Fluor 488 green, Cy3 red and DAPI blue (nuclear counterstaining).

Next, protein expression analysis of Foxp3 and IL-10 in primary pancreatic cancer cell lines was performed by immunofluorescence double staining. Foxp3 and IL-10 protein expression was observed in all investigated primary pancreatic cancer cell

lines PaCa DD 135, PaCa DD 159 and PaCa DD 185. Additionally, a strong co-expression of both antigens was found (figure 9).

### 1.2. Expression of Foxp3, IL-10 and TGF-β in colon cancer cell lines

Human colon cancer cell lines SW480 and SW620 were analyzed for Foxp3, IL-10 and TGF-β gene and protein expression.

Significantly increased gene expression of Foxp3 and IL-10 was observed in both cancer cell lines compared to FHC cells (figure 10A). SW480 showed 4 times elevated Foxp3 and 33 times raised IL-10 mRNA levels ($p<0.05$) while in SW620 values were found at 2 for Foxp3 and 9 for IL-10 ($p<0.05$ and $p<0.005$). No significant changes were observed in TGF-β expression compared to FHC cells (figure 10A).

Additionally, in immunofluorescence double staining co-expression of Foxp3 and IL-10 could be demonstrated (figure 10B). These finding correspond with those made in pancreatic cancer cell lines.

To determine the amount of Foxp3 expressing colon cancer cells FACS analysis was performed with cell lines SW480 and SW620. Compared to isotype control 5.3% and 6.1% of colon cancer cells were found to express transcription factor Foxp3 (figure 11).

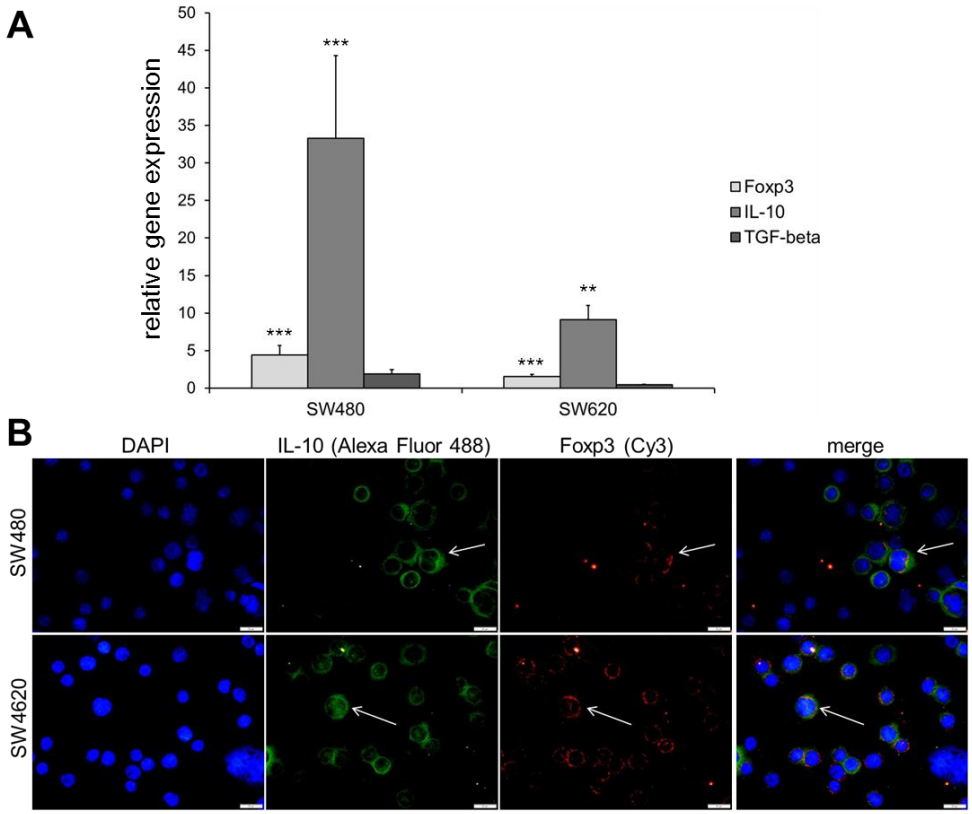


**Figure 10: Expression of Foxp3 and IL-10 in colon cancer cell lines SW480 and SW620.**

**(A)** Relative gene expression of Foxp3 and IL-10 was significantly increased in primary pancreatic cell lines compared to PANC1 cells. No significant differences in TGF-β expression. The normalization was performed with FHC cells. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$. **p<0.005, ***p<0.05 **(B)** Representative examples of immunofluorescence double staining of Foxp3 and IL-10 in SW480, and SW620 cells. Co-expression of both proteins was observed in both cell lines. Alexa Fluor 488 green, Cy3 red and DAPI blue (nuclear counterstaining).

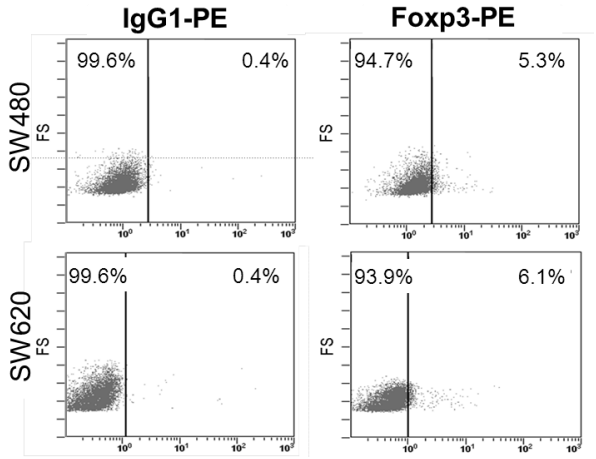


**Figure 11: Protein expression of Foxp3 in colon cancer cell lines by flow cytometry.**[98]

FACS analysis of Foxp3 expression in SW480 and SW620 colon cancer cell lines compared to isotype control. 5.3% and 6.1% of colon cancer cells express Foxp3; PE: phycoerythrin; FS: forward scatter.[98]

## 1.3. Stage-dependent expression of Foxp3 in colorectal cancer tissue

### 1.3.1. Gene expression analysis of CD4, CD25, Foxp3, IL-10, and TGF-β

To analyze whether CD4, CD25, Foxp3, IL-10 and TGF-β expression in CRC may be associated with clinical tumor progression tumors of limited disease (UICC I/II) and advanced disease (UICC III/IV) were investigated. RT-qPCR analysis showed significantly increased gene expression of CD4 and CD25 in limited disease tumors (UICC I/II, relative gene expression of 4 and 5, $p<0.005$) compared to tumors of advanced disease (UICC III/IV, relative gene expression of 3, $p<0.005$).[98] In accordance to this finding, gene expression of Foxp3 and immunosuppressive cytokines IL-10 and TGF-β was significantly decreased in limited disease tumors (UICC I/II, values of 3 for Foxp3 and 1 for IL-10 and TGF-β, $p<0.005$) compared to those of advanced disease (UICC III/ IV, 4 for Foxp3 and 3 for IL-10 and TGF-β, $p<0.005$) (figure 12).[98]

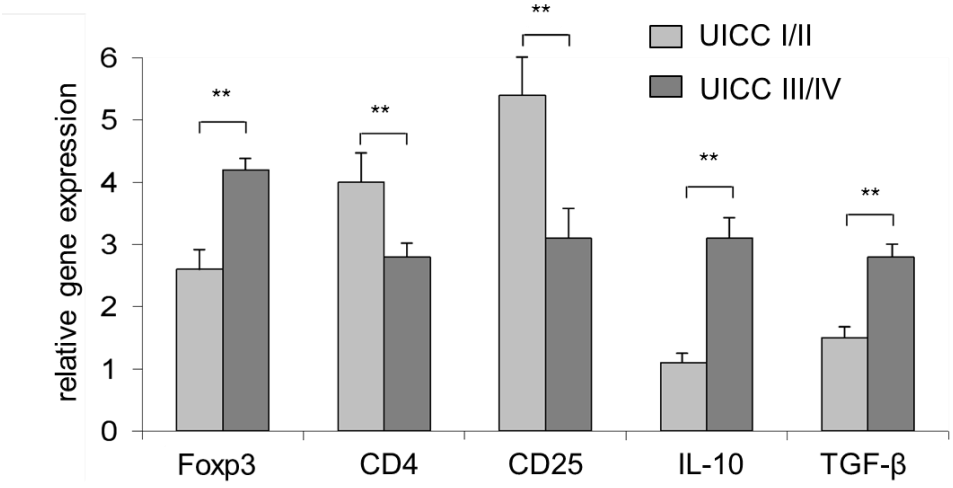


**Figure 12: Gene expression analysis of CD4, CD25, Foxp3, IL-10, and TGF-β in early (UICC I/II) and late stage (UICC III/IV) CRC.[98]**

Significantly increased gene expression of CD4 and CD25 at stages UICC I/II compared to tumors at stage UICC III/IV. Gene expression of Foxp3, IL-10 and TGF-b was significantly reduced at stages I/II compared to UICC III/IV. The normalization was performed with normal tissue. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$. **$p < 0.005$.[98]

### 1.3.2. Protein expression analysis of CD4, CD25, Foxp3, IL-10, and TGF-β

#### 1.3.2.1. Treg cell mediated protein expression

Next, Treg cells were examined for protein expression of CD4, CD25, Foxp3, IL-10 and TGF-β by immunohistochemistry. As shown in Figure 13A, increased expression of all investigated parameters were observed in limited disease tumors (UICC I/II) compared to advanced disease tumors (UICC III/IV) and normal tissue (control). Altogether infiltrated Foxp3+ Treg cells were found in 61 out of 65 tumors of the patients (n= 61/65, 93.8%). Immunofluorescence double staining demonstrated that Foxp3+ Treg cells were mainly of CD4+ T cell phenotype (figure 13B).[98]

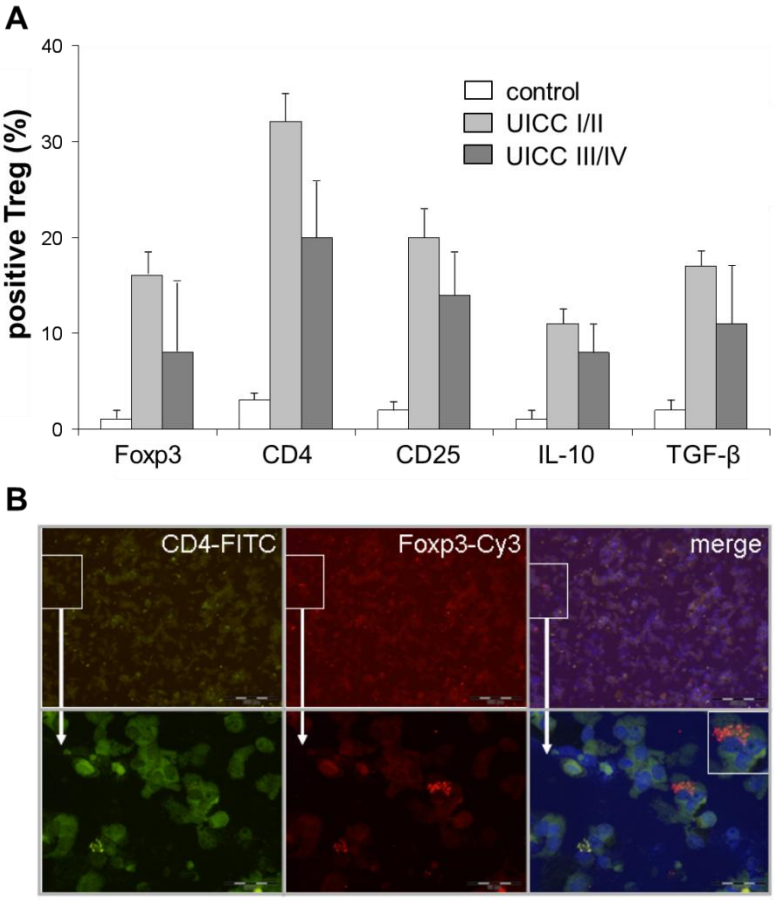


**Figure 13: Immunohistochemical and immunofluorescent analysis tumor infiltrating immune cells for characteristics of Treg cells in primary colorectal cancer.[98]**

**(A)** Increased CD4, CD25, Foxp3, IL-10, and TGF-β expression at stage UICC I/II compared to UICC III/IV. The result of the staining was expressed in percentages (%) positivity. All values were expressed as mean ± SD. All pairwise tests (Turkey) result in $p<0.001$ with three exceptions: Foxp3, control vs. UICC III/IV, $p = 0.091$; IL-10, UICC I/II vs. UICC III/IV, $p = 0.021$; TGF-ß, UICC I/II vs. UICC III/IV, $p = 0.020$. **(B)** Representative example of an immunofluorescence double staining of Foxp3 and CD4 in Treg. Foxp3 expression was mainly observed on CD4+ T cells (x100 magnification above; x400 magnification below). FITC green, Cy3 red and DAPI blue (nuclear counterstaining).[98]

### 1.3.2.2. Cancer cell mediated protein expression

The expression of Foxp3 and immunosuppressive cytokines IL-10 and TGF-β in cancer cells was examined by immunohistochemical and immunofluorescent staining. As shown in figure 14A, Foxp3, IL-10, and TGF-β expressing cancer cells increased from early to late stages of disease compared to normal tissue (control). Overall, Foxp3 expressing cancer cells were found in 60 out of 65 tumor cases (n=60/65, 92.3%). Additionally, Foxp3 expression in cancer cells of patients with CRC was demonstrated using immunofluorescence double staining (figure 14B).[98]

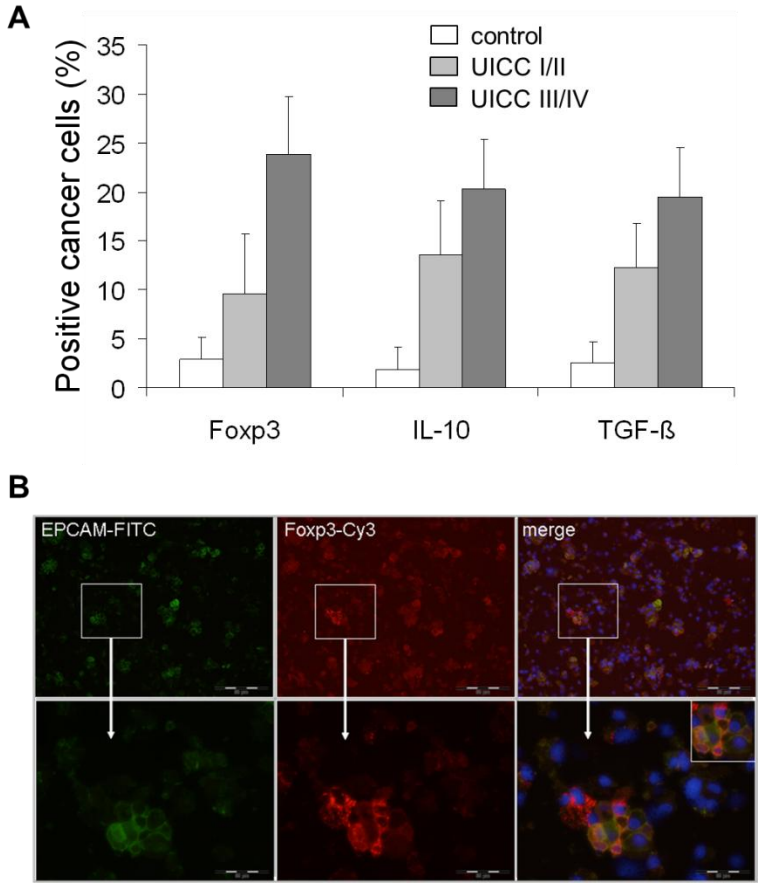


**Figure 14: Immunohistochemical and immunofluorescent analysis of Foxp3, IL-10 and TGF-β expression in tumor cells from patients with CRC.[98]**

**(A)** Foxp3, IL-10 and TGF-β expressing cancer cells increased from UICC I/II to UICC III/IV compared to normal tissue. The result of the staining was expressed in percentages (%) positivity. All values were expressed as mean ± SD; all pairwise tests (Tukey) result in $p<0.001$ with exception of control vs. UICC I/II in Foxp3+ ($p<0.05$). **(B)** Representative example of an immunofluorescence double staining, showing Foxp3 expression and EPCAM co-staining in cancer cells of patients with CRC (x100 magnification above; x400 magnification below). FITC green, Cy3 red and DAPI blue (nuclear counterstaining).[98]

## 1.4. Correlation of Foxp3 expression

### 1.4.1. Correlation of Foxp3+ cancer cells with the expression of IL-10 and TGF-β

To examine whether the expression of the immunosuppressive cytokines IL-10 and TGF-β corresponded with the Foxp3 expressing cancer cells two different groups were stratified according to the percentages of expression in the immunohistochemical analysis. Considering Foxp3 expression in cancer cells as a continuous variable, regression analysis showed that Foxp3 cancer cell expression had a weak but significant direct correlation with the expression of the immunosuppressive cytokines IL-10 (R2=0.23, p<0.001, n=65; r=0.48) and TGF-β (R2=0.33, p<0.001, n=65; r=0.57) (figure 15A and B). Additionally, immunofluorescence double staining demonstrated protein expression IL-10 and TGF-β in Foxp3 expressing cancer cells (figure 15C).[98]

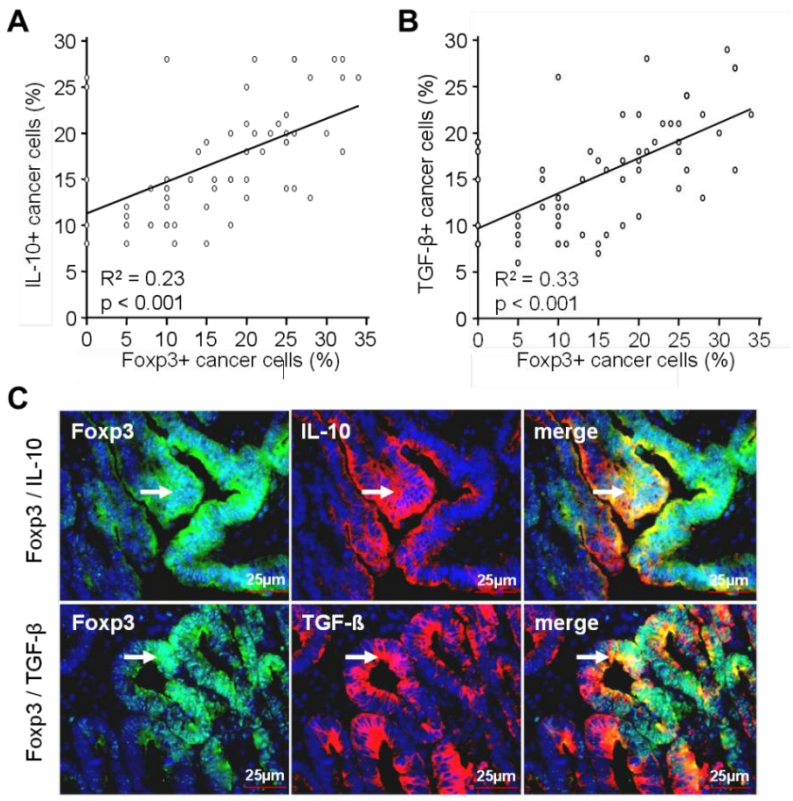


**Figure 15: Correlation of cancer cell mediated Foxp3 expression with immunosuppressive cytokines IL-10 and TGF-β.[98]**

**(A/B)** Significant correlation of Foxp3 cancer cell expression with the expression of IL-10 **(A)** and TGF-β **(B)**. Regression analysis; R2, coefficient of determination, p<0.001 **(C)** Representative example of an immunofluorescence double staining of IL-10 and TGF-β in Foxp3+ cancer cells. FITC green, Cy3 red and DAPI blue (nuclear counterstaining).[98]

### 1.4.2. Correlation of Foxp3 Treg with Foxp3 cancer cells

To examine whether Foxp3 Treg cell expression corresponded with the Foxp3 cancer cell expression, the same procedure as describe previously was used. Considering Foxp3 cancer cell expression as a continuous variable, regression analysis showed that Foxp3 cancer cell expression had a weak but significant inverse correlation with the Foxp3 Treg cell expression (R2=0.17, p=0.01, n=65; r=20.41) (figure 16A). Immunohistochemistry showed increased Foxp3+ Treg cells in Foxp3 negative cancer stromal tissue (black arrow) (figure 16B). In contrast, there were no or negligible Foxp3+ Treg cells found in Foxp3 positive cancer tissue (black arrow) (figure 16C).[98]

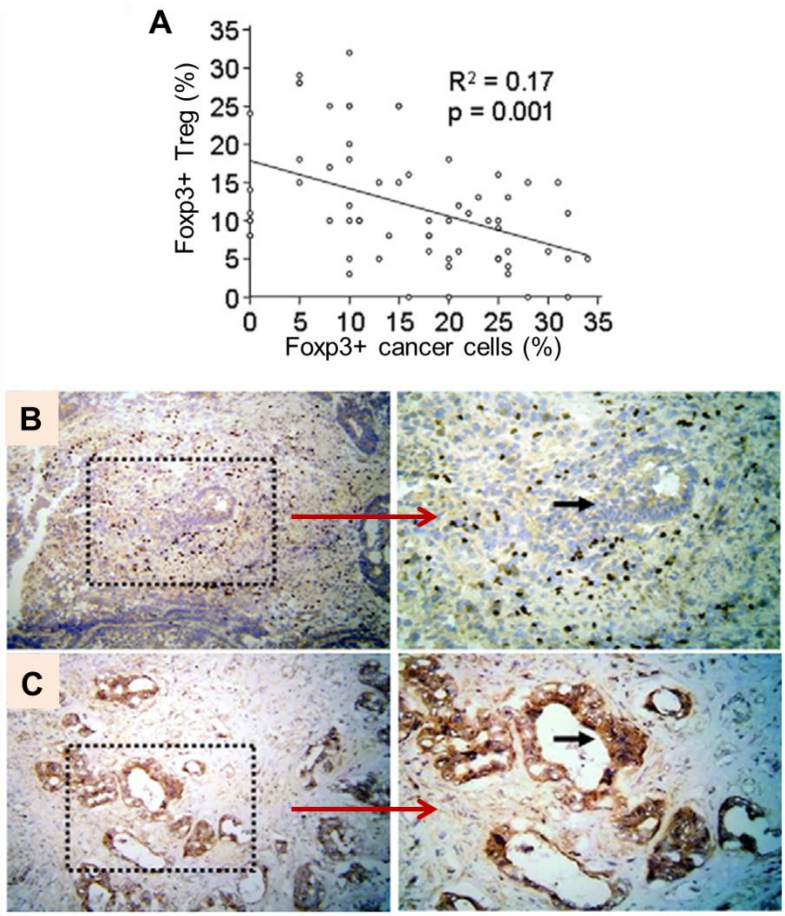


**Figure 16: Correlation of Foxp3+ Treg cells with Foxp3 expressing cancer cells.**[98]

**(A)** Significant inverse correlation of cancer cell mediated Foxp3 expression with the Foxp3+ Treg cells. Regression analysis; R2, coefficient of determination, p=0.001. **(B)** Increased numbers of Foxp3+ Treg cells in Foxp3 negative cancer tissue found by immunohistochemical staining (black arrow). **(C)** Only occasionally or no Foxp3+ Treg cells in Foxp3 positive cancer tissue detected by immunohistochemistry (black arrow). DAB brown, Haemalaun blue (nuclear counterstaining), magnifications x100 (left) and x200 (right).[98]

## 1.5. Kaplan-Meier and multivariate analysis

Multivariate Cox regression analysis was performed stepwise including age, gender, primary tumor (colon or rectum), UICC (I/II or III/IV), depth of tumor invasion (T category 1/2 or 3/4), differentiation (1/2 or 3/4), lymph node metastasis (N category), Foxp3 (%), Treg (%), TGF-ß (%) and IL-10 (%). The stepwise procedure kept in the model the N category and Foxp3 expression in colon cancer cells as prognostic parameters (Chi-quadrat statistics, $p<0.01$, table 12).[98]

**Table 12: Multivariate analysis of prognostic factors of the study population**.[98]

| | Unfavorable factor | Hazard ratio (HR) | 95% CI of HR | p-values (Chi-Quadrat) |
|---|---|---|---|---|
| Lymph nodes metastasis | Positive | 8.97 | 2.28 to 35.31 | 0.002 |
| Foxp3+ cancer cells | high (>16%) | 1.09 | 1.02 to 1.14 | 0.006 |

The identified prognostic factors from Cox regression model are presented in figures 17A and C. The mean value of Foxp3 cancer cell expression by immunohistochemical analysis for all studied tissue samples of the 65 tumors was determined at 16%. Among patients with CRC, those with high Foxp3 cancer cell expression (>16%) had a poorer prognosis than those with low Foxp3 expression levels (<16%) ($p<0.001$, Log-Rank test) (figure 17A and table 13).[98]

Considering immunohistochemical analysis of the samples for Foxp3 Treg cell expression the mean value was calculated at 12%. There was no significant difference in the overall survival comparing patients with low and high Foxp3 Treg expression levels (>12% or <12%) ($p =0.204$, Log-Rank test) (figure 17B and table 13). In patients without lymph node metastasis were significant differences in overall survival compared to patients with lymph node metastasis ($p<0.001$, Log-Rank test) (figure 17C).[98]

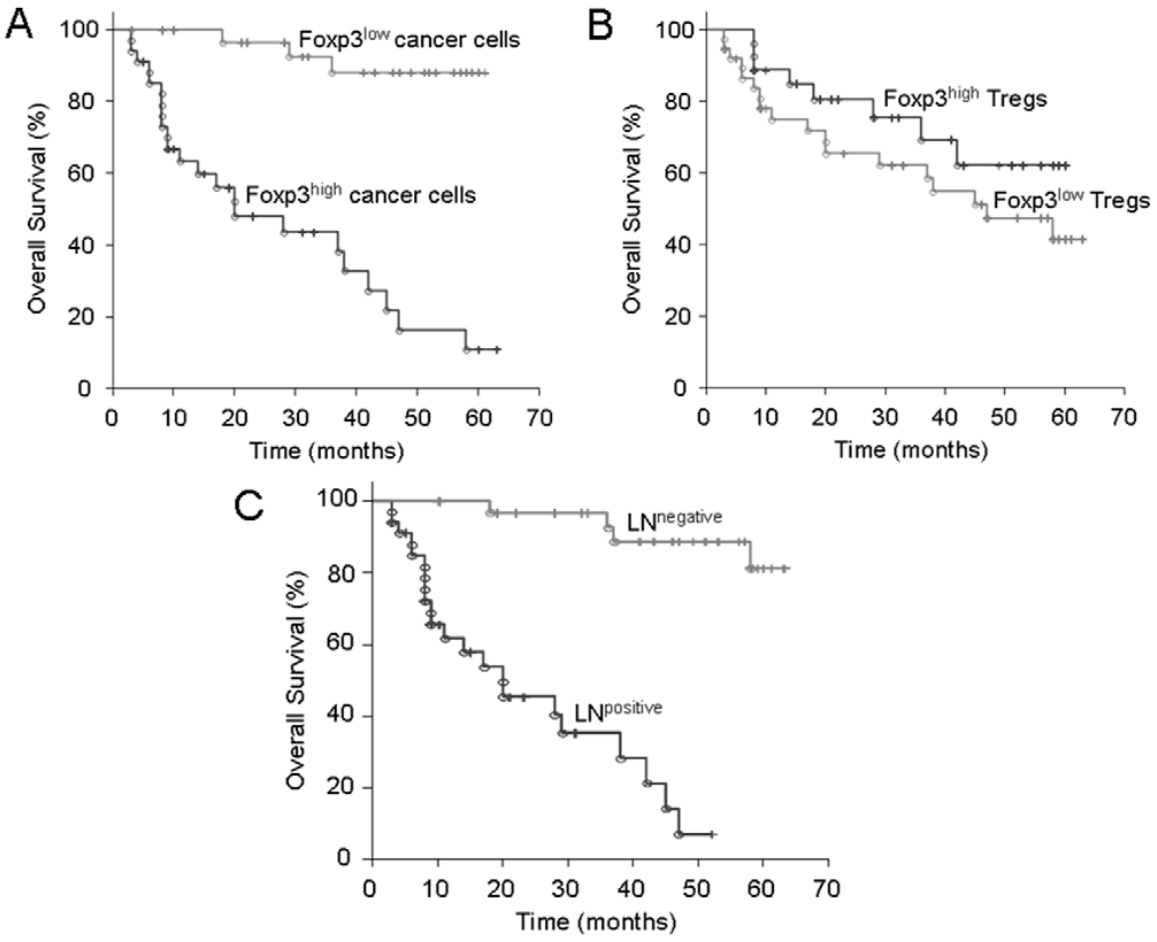


**Figure 17: Overall survival of CRC patients with cancer cell mediated Foxp3 compared with the overall survival of those with infiltrated Foxp3+ Treg in their tumors.**[98]

**(A)** Patients with high Foxp3 cancer cell expression (>16%, as mean cut-off) had a poorer prognosis than those with low Foxp3 cancer cell expression profiles (<16%; mean cut-off: 16%), ($p<0.001$, Log-Rank test). **(B)** No significant difference in the overall survival comparing patients with low and high Foxp3 Treg cell expression profiles (mean cut-off: 12%), ($p = 0.202$, Log-Rank test). **(C)** Patients with lymph node metastasis had a poorer prognosis than those without lymph node metastasis ($p<0.001$, Log-Rank test). The times of the censored data are indicated by short vertical lines.[98]

Other parameters such as TGF-ß, IL-10, UICC and T category showed additionally significant differences in overall survival for the corresponding lower expression and grading, respectively ($p<0.001$, Log-Rank tests). Age, gender, primary tumor and histological differentiation were not associated with prognosis in univariate analysis (table 13).[98]

**Table 13: Clinicopathological characteristics and Foxp3 expression profiles of CRC patients.**[98]

| | Total | Foxp3 (cancer cells) | | | Foxp3 (Treg) | | |
|---|---|---|---|---|---|---|---|
| | | Low | High | Test | Low | High | Test |
| **Cases (n)** | 65 (100%) | 31 (100%) | 34 (100%) | | 38 (100%) | 27 (100%) | |
| **Age (y; mean ± sd)** | 64.0±5.8 | 63.9±5.7 | 64.2±5.9 | n.s. | 64.2±6.0 | 63.8±5.6 | n.s. |
| **Gender** | | | | | | | |
| Male | 37 (57%) | 18 (58%) | 19 (56%) | n.s. | 21 (55%) | 16 (59%) | n.s. |
| Female | 28 (43%) | 13 (42%) | 15 (44%) | | 17 (45%) | 11 (41%) | |
| **Primary tumor** | | | | | | | |
| Colon | 26 (40%) | 12 (39%) | 14 (41%) | n.s. | 16 (42%) | 10 (37%) | n.s. |
| Rectum | 39 (60%) | 19 (61%) | 20 (59%) | | 22 (58%) | 17 (63%) | |
| **Differentiation** | | | | | | | |
| G1 | 12 (18%) | 7 (22%) | 5 (15%) | n.s. | 4 (11%) | 8 (30%) | n.s. |
| G2 | 31 (48%) | 16 (52%) | 15 (44%) | | 21 (55%) | 10 (37%) | |
| G3/4 | 22 (34%) | 8 (26%) | 14 (41%) | | 13 (34%) | 9 (33%) | |
| **Depth of invasion** | | | | | | | |
| pT1 | 14 (22%) | 10 (32%) | 4 (12%) | <0.001 | 8 (21%) | 6 (22%) | n.s. |
| pT2 | 23 (35%) | 14 (45%) | 9 (27%) | | 13 (34%) | 10 (37%) | |
| pT3 | 17 (26%) | 7 (23%) | 10 (29%) | | 10 (26%) | 7 (26%) | |
| pT4 | 11 (17%) | - | 11 (32%) | | 7 (19%) | 4 (15%) | |
| **Lymph node metastasis** | | | | | | | |
| pN0 | 31 (48%) | 25 (81%) | 6 (18%) | <0.001 | 14 (37%) | 17 (63%) | 0.038 |
| pN1–3 | 34 (52%) | 6 (19%) | 28 (82%) | | 24 (63%) | 10 (37%) | |
| **UICC stage** | | | | | | | |
| UICC I | 15 (23%) | 13 (42%) | 2 (6%) | <0.001 | 8 (21%) | 7 (26%) | n.s. |
| UICC II | 19 (29%) | 16 (52%) | 3 (9%) | | 7 (18%) | 12 (45%) | |
| UICC III | 22 (34%) | 1 (3%) | 21(62%) | | 16 (43%) | 6 (22%) | |
| UICC IV | 9 (14%) | 1 (3%) | 8 (23%) | | 7 (18%) | 2 (7%) | |
| **Mean OS (m)** | | 35 | 27 | | 38 | 35 | |
| **Median OS (m)** | | n.a. | 20 | | 47 | n.a. | |
| **Log-Rank test** | | p<0.001 | | | p=0.204 | | |

Y, years; G, grading; UICC, International Union against Cancer; R, residual tumor; OS, overall survival; m, months; n.s., not significant; n.a., not applicable.

## 2. The role of TLR7 and TLR8 in human gastrointestinal cancer

Since TLR7 and TLR8 are parameter usually to be found in cells of the immune system, established pancreatic and colon cancer cell lines were first analyzed on the expression of both receptors and its downstream targets. Additionally, expression levels in primary pancreatic cancer cell lines as well as colon and pancreatic cancer tissues were determined.

### 2.1. TLR7 and TLR8 in colon cancer

#### 2.1.1. TLR7 and TLR8 in colon cancer cell lines

The established colon cancer cell lines SW480, SW620 and HT-29 were analyzed for TLR7 and TLR8 gene expression by RT-qPCR. Additionally, immunohistochemical staining was performed to demonstrate TLR protein expression.

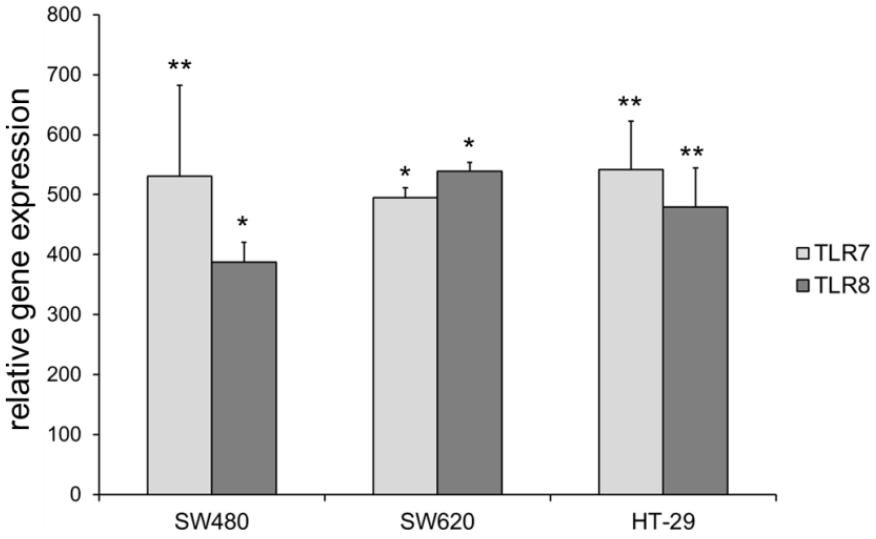


**Figure 18: Gene expression of TLR7 and TLR8 in colon cancer cell lines.**

Relative gene expression of TLR7 and TLR8 was significantly increased in SW480, SW620 and HT-29 colon cancer cell lines compared to FHC cells. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$. *p<0.0001, **p<0.005.

In RT-qPCR relative gene expression was significantly increased. Compared to FHC cells investigated colon cancer cell lines showed 531 (SW480, p<0.005),

495 (SW620, p<0.0001) and 542 (HT-29, p<0.005) times elevated TLR7 and 387 (SW480, p<0.0001), 539 (SW620, p<0.0001) and 479 (HT-29, p<0.005) times elevated TLR8 mRNA levels (figure 18). Moreover, exemplary immunohistochemical staining confirmed protein expression of TLR7 and TLR8 in HT-29 cells (figure 19).

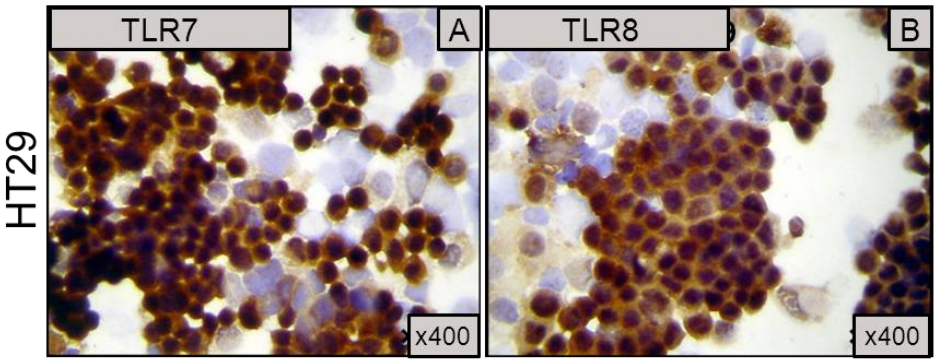


**Figure 19: Protein expression of TLR7 and TLR8 in HT-29 colon cancer cell line.**

Representative example of TLR7 **(A)** and TLR8 **(B)** protein expression in HT-29 cells by immunohistochemical staining. DAB brown, Haemalaun blue (nuclear counterstaining), x400 magnification.

### 2.1.2. TLR7 and TLR8 in colon cancer tissue

As described previously by former collaborators of the work group cancer cell mediated TLR7 and TLR8 expression was found in tumor tissue of patients with CRC.[74] In addition, Grimm *et al* showed that TLR7 and TLR8 expression were associated with tumor progression and reduced tumor-specific survival amongst patients with high TLR7 and TLR8 expression in cancer cells.[74] To demonstrate activation of TLR signaling in colon cancer tissue immunofluorescence staining for MyD88 and NF-κB was performed. As shown in figure 20, increased MyD88 and NF-κB expression was observed in advanced disease tumors (UICC IV) as compared to limited disease tumors (UICC II). No or occasionally low expression was found in normal tissue.

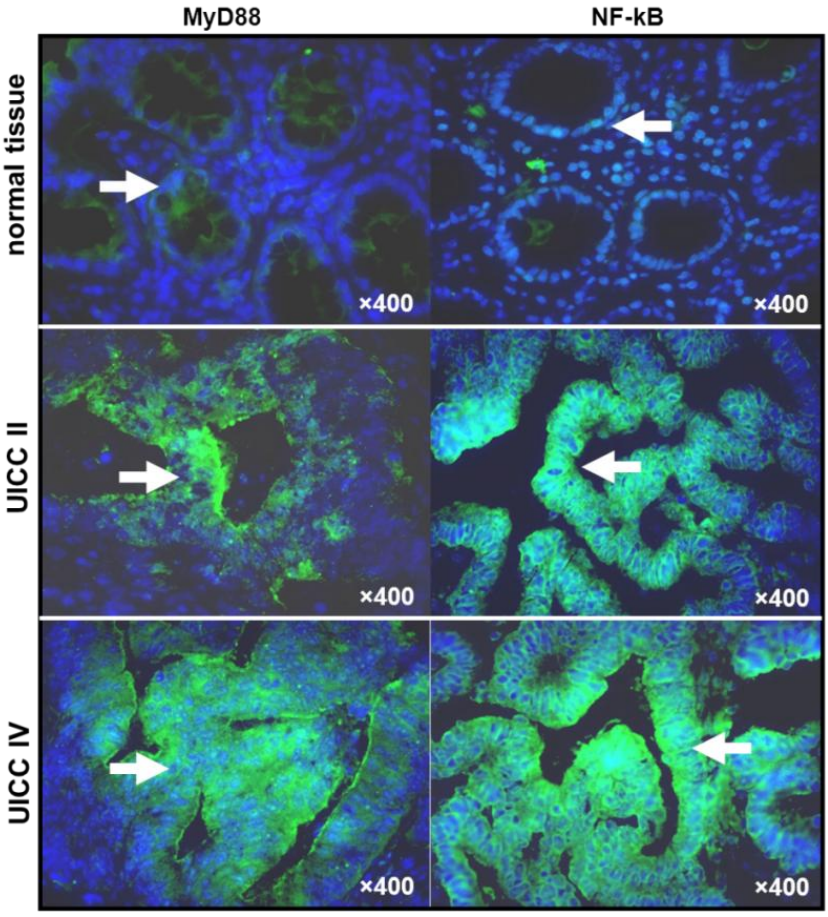


**Figure 20: Expression of MyD88 and NF-κB in colon cancer and normal tissue.**

Representative example of an immunofluorescence double staining, showing MyD88 (left) and NF-κB (right) staining in tissues of patients with CRC and normal tissue. FITC green and DAPI blue (nuclear counterstaining), x400 magnification.

## 2.2. TLR7 and TLR8 in pancreatic cancer

### 2.2.1. TLR7 and TLR8 in pancreatic cancer cell lines

The expression of TLR7 and TLR8 was characterized in several human pancreatic cancer cell lines by RT-qPCR. While there was no or occasionally low expression of both receptors observed in established pancreatic cancer cell line PANC1, primary pancreatic cancer cells PaCa DD 135, PaCa DD 159 and PaCa DD 185 demonstrated significantly increased gene expression of TLR7 and TLR8. Compared to PANC1 cells primary pancreatic cancer cell lines showed 83 (PaCa DD 135, $p<0.0001$), 65 (PaCa DD 159, $p<0.005$) and 16 (PaCa DD 185, $p<0.05$) times elevated TLR7 and 250 (PaCa DD 135, $p<0.05$), 122 (PaCa DD 159, $p<0.05$) and 34 (PaCa DD 159, $p<0.005$) times elevated TLR8 mRNA levels (figure 21).

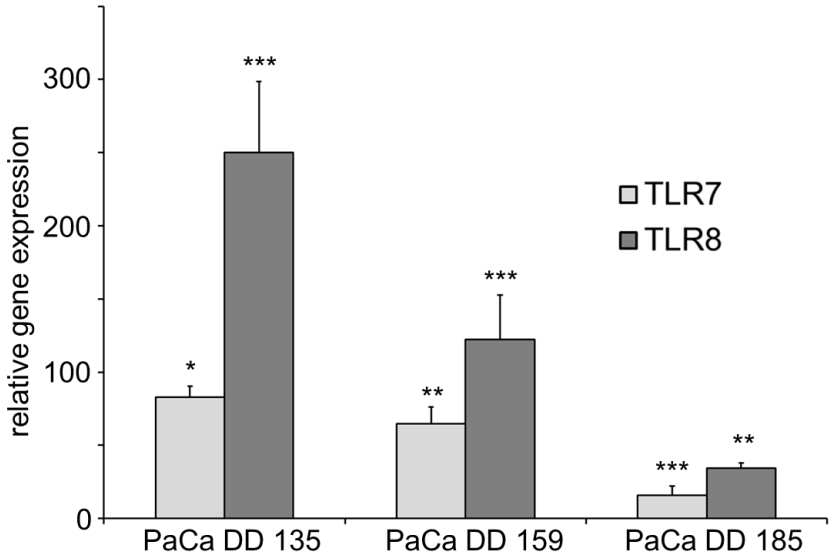


**Figure 21: Gene expression of TLR7 and TLR8 in primary pancreatic cancer cell lines.**

Relative gene expression of TLR7 and TLR8 was significantly increased in PaCa DD 135, PaCa DD 159 and PaCa DD185 primary pancreatic cancer cell lines compared to PANC1 cells. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$. *p<0.0001, **p<0.005, ***p<0.05.

### 2.2.2. Stage-dependent expression of TLR7 and TLR8 in pancreatic cancer tissue

TLR7 and TLR8 expression by pancreatic cancer, chronic pancreatitis and normal pancreatic tissue was analyzed by immunohistochemistry in tissue of 16 patients with pancreatic cancer (UICC II and UICC III, n=16) or chronic pancreatitis (n=8) or normal pancreas (n=8). In general, TLR7 expression was found at a higher level in all chronic pancreatitis and pancreatic cancer patients than TLR8 (figure 22C, left bars). Figures 22A and B show examples of positive TLR7 and TLR8 tumor cell expression in pancreatic cancer (figure 22A, B, below left for UICC II and below right for UICC III). In contrast, no or occasionally low TLR7 or TLR8 expression was detected in normal pancreatic cells (figure 22A and B, top left), whereas strong expression of TLR7 and TLR8 was detected in cells from chronic pancreatitis (figure 22A, B, top right).[99]

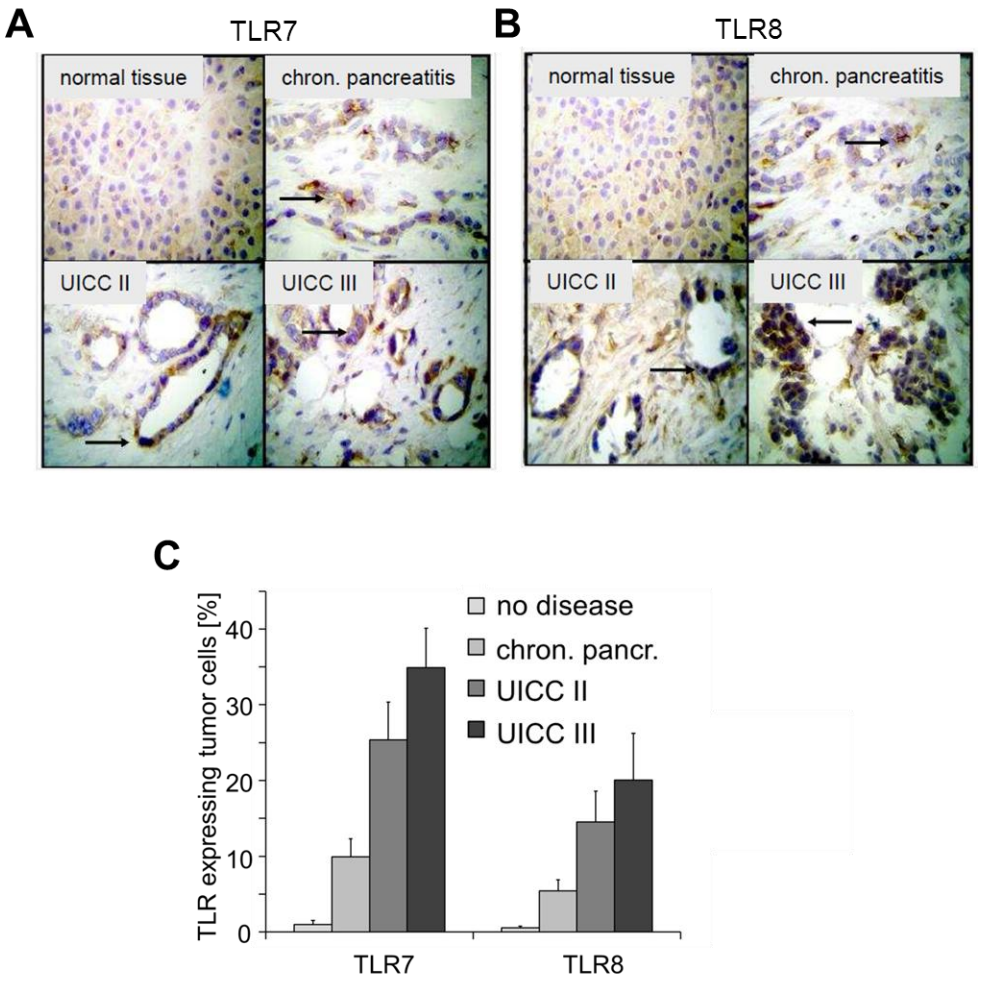


**Figure 22: Immunohistochemical detection of TLR7 and TLR8 in pancreatic cancer, chronic pancreatitis and normal pancreatic tissue.**[99]

**(A)** and **(B)** Strong expression (UICC II, below left) and very strong expression of TLR7 and TLR8 (UICC III, below right) was observed in pancreatic cancer. Increased expression of TLR7 and TLR8 was detected in chronic pancreatitis (top right). No or occasionally low expression in normal pancreas was observed (top left). DAB brown, Haemalaun blue (nuclear counterstaining). Magnification top x100 and below x200 **(C)** Quantification of TLR7 and TLR8 expressing pancreatic cells in normal pancreatic tissue (no disease), chronic pancreatitis, pancreatic cancer from UICC II and UICC III patients.[99]

Interestingly, the same results were also observed by western blot analysis and RT-qPCR of the tumor tissues. Increased TLR7 and TLR8 protein expression was found in pancreatic cancer (UICC III) compared to normal pancreatic tissue (figure 23A). Additionally, mRNA for both receptors was significantly over-expressed in late stage pancreatic cancer (UICC III) comparison to tissue of chronic pancreatitis and early stage pancreatic cancer (UICC II) (figure 23B, $p<0.005$).[99]

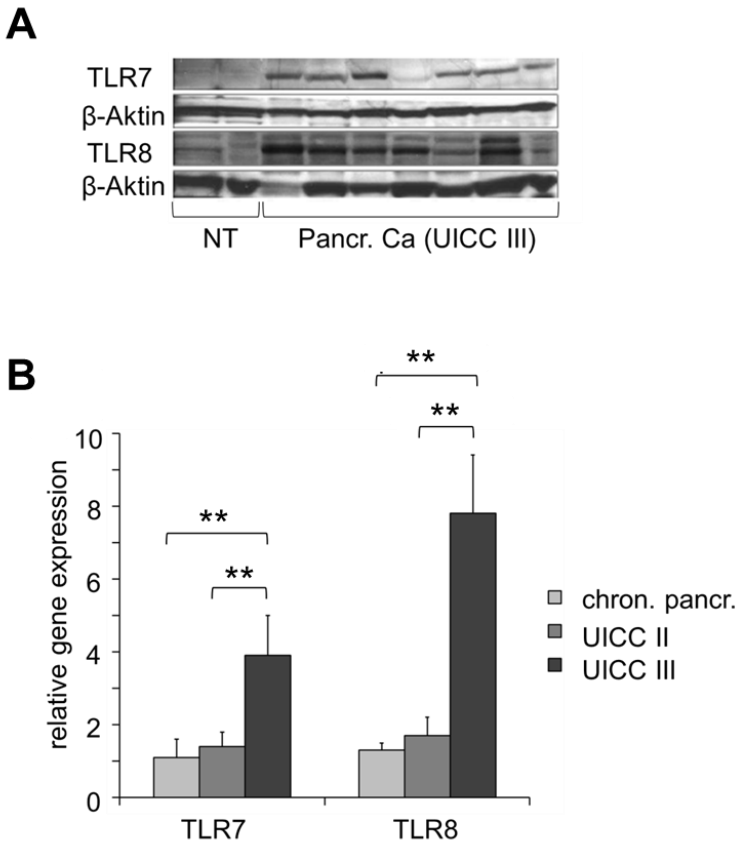


**Figure 23: Increased TLR7 and TLR8 expression in advanced pancreatic cancer.[99]**

**(A)** Increased TLR7 and TLR8 protein expression in pancreatic cancer (UICC III) compared to normal demonstrated by western blot. ß-actin probe was used as a control for protein loading. NT normal tissue **(B)** Significant elevation in gene expression of TLR7 and TLR8 in advanced tumor stages (UICC III, $p<0.005$). Increased gene expression of TLR7 and TLR8 in low tumor stages (UICC II) and chronic pancreatitis. Normal pancreatic tissue was standardized to baseline. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$. chron. pancr. chronic pancreatitis.[99]

Moreover, TLR7 and TLR8 expression was analyzed in dissociated cells derived from the same patient tissues together with CD34, a marker for endothelial cells and known to be expressed by cancer cells with neoangiogenetic potential, by FACS and immunohistochemical analysis (cytospins). Indeed TLR7, TLR8 and CD34 were positively expressed in pancreatic cancer cells and cells from chronic pancreatitis (figure 24A and B), but not in normal pancreatic cells (figure 24C). Comparison of the precise cellular co-localization of TLR7 or TLR8 with CD34 that was analyzed by immunofluorescence double staining revealed increased co-expression of TLR7 or TLR8 with CD34 in tumor cells (figure 24D), indicating that those cells were indeed cancer cells expressing the angiogenic surface molecule.[99]

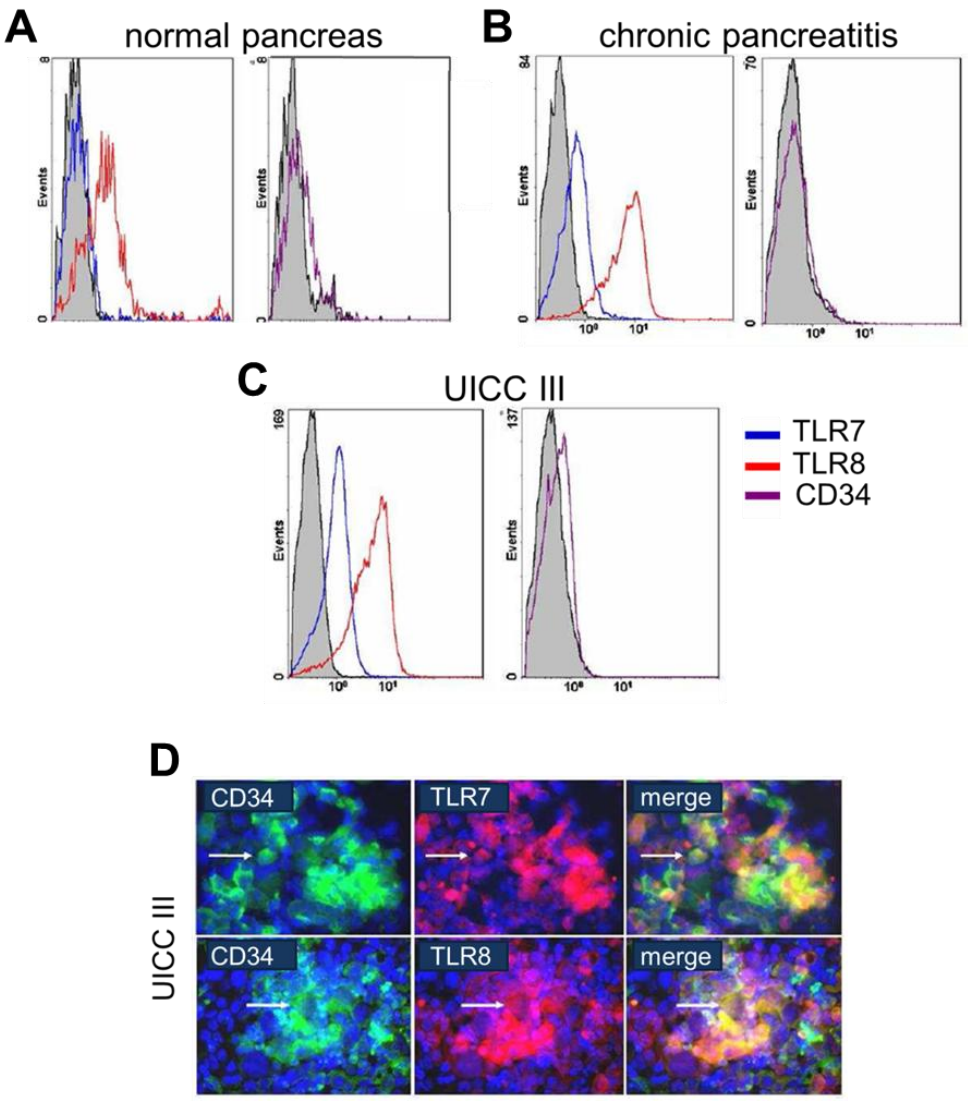


**Figure 24: Detection of TLR7 and TLR8 expression in dissociated pancreatic cancer cells, chronic pancreatitis, and normal pancreatic cells.**[99]

**(A)** FACS analysis of TLR7 (blue line), TLR8 (red line) and CD34 (purple line) expression in normal pancreatic cells showed no positivity for TLR7 and CD34. Little expression of TLR8 was observed. IgG control black line. **(B)** FACS analysis of TLR7 (blue line), TLR8 (red line), and CD34 (purple line) expressing cells derived from chronic pancreatitis showed also increased expression of TLR7 and TLR8. CD34 was not detected. IgG control black line **(C)** FACS analysis of TLR7 (blue line), TLR8 (red line) and CD34 (purple line) expression in pancreatic cancer cells (UICC III) showed increased expression of TLR7, TLR8, and CD34. IgG control black line. **(D)** Immunofluorescence double staining in pancreatic cancer cells (UICC III, cytospins) showed increased co-expression of CD34 with TLR7 and TLR8. FITC green, Cy3 red and DAPI blue (nuclear counterstaining).[99]

To analyze whether inflammation in pancreatic cancer was associated with TLR7 and TLR8 expressing cancer cells the expression of COX-2 in pancreatic cancer cells was analyzed by immunohistochemical staining and western blot. Indeed, increased COX-2 expression together with TLR7 and TLR8 positivity was detected (figure 25A,

top and below right, and B). No positivity was observed in normal pancreatic cells (figure 25A, top and below left, and B). These data indicate inflammation in pancreatic cancer in association with TLR7 and TLR8 expressing cancer cells.[99]

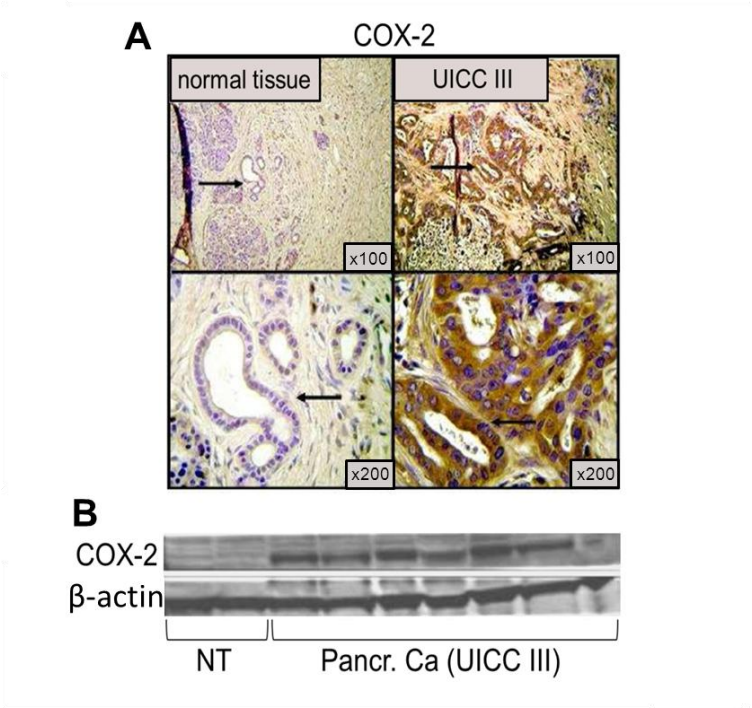


**Figure 25: Detection of COX-2 expression in pancreatic cancer, and normal pancreatic tissue.[99]**

**(A)** Increased COX-2 expression in pancreatic cancer compared to normal tissue was detected in immunohistochemical staining. DAB brown, Haemalaun blue (nuclear counterstaining). Magnification top x100 and below x200. **(B)** Confirmation of increased COX-2 expression in pancreatic cancer (Pancr. Ca UICC III) compared to normal tissue (NT) by western blot. ß-actin probe was used as a control for protein loading.[99]

## 2.3. TLR7 and TLR8 in human PANC1 pancreatic cancer

In contrast to tumor cells derived from patients with pancreatic cancer and observed primary pancreatic cancer cell lines, established tumor cell line PANC1 expressed no or only very low levels of TLR7 and TLR8. Therefore, for further *in vitro* studies both receptors were successfully transduced in the most common pancreatic cancer cell line, PANC1, using a Lentivirus-mediated stable gene expression as described in 4.1.5. PANC1 cells transduced with empty vector construct served as a control for all *in vitro* and *in vivo* experiments. Peripheral blood mononuclear cells (PBMC) were used as positive control for TLR7 and TLR8 expression (figure 26B). Indeed significantly increased gene expression of TLR7 and TLR8 was observed in

transduced PANC1 cells (TLR7+ PANC1 cells, p<0.005 and TLR8+ PANC1 cells, p<0.0001) by RT-qPCR and following agarose gel electrophoresis (figure 26A and B). In figure 26C successful protein expression of TLR7 or TLR8 by transduced PANC1 cells is demonstrated using western blot.[99]

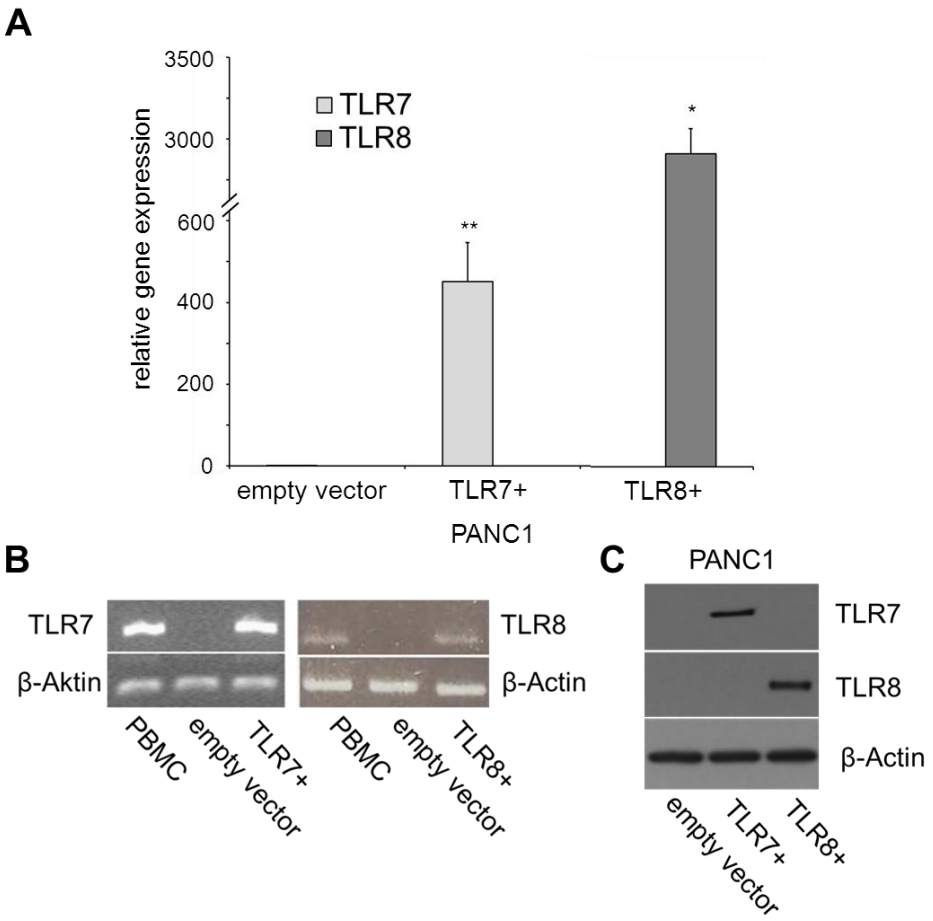


**Figure 26: Depiction of the successful transduction of TLR7 and TLR8 in PANC1 cells.[99]**

**(A)** Increased gene expression of TLR7 and TLR8 was detected in TLR7+ and TLR8+ PANC1 cells by RT-qPCR compared to empty vector PANC1 cells. Empty vector PANC1 cells were standardized to baseline. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$. *p<0.0001, **p<0.005. **(B)** Agarose gel electrophoresis of RT-qPCR products. PANC1 cells transduced with empty vector and PBMCs were used as controls. ß-actin probe was used as internal control for RT-qPCR. **(C)** Confirmation of increased TLR7 and TLR8 protein expression in transduced PANC1 cells by western blot. PANC1 cells transduced with empty vector were used as controls. ß-actin probe was used as a control for protein loading.[99]

## 2.4. Xenogenic tumor growth of TLR7+ and TLR8+ PANC1 cells in Balb/c nude mice

Tumor cell proliferation *in vivo* in Balb/c nude mice was examined by xenotransplantation experiments of transduced PANC1 cells. 40 days after subcutaneous injection tumor growth caused by TLR7+ (n=5) or TLR8+ (n=5) PANC1 cells was found to be enhanced compared to empty vector PANC1 cells (n=4) (figure 27A). Furthermore, determination of the tumor volume showed a significant increase of TLR7+ PANC1 induced tumors in contrast to empty vector PANC1 induced ones (figure 27B, $p<0.005$).

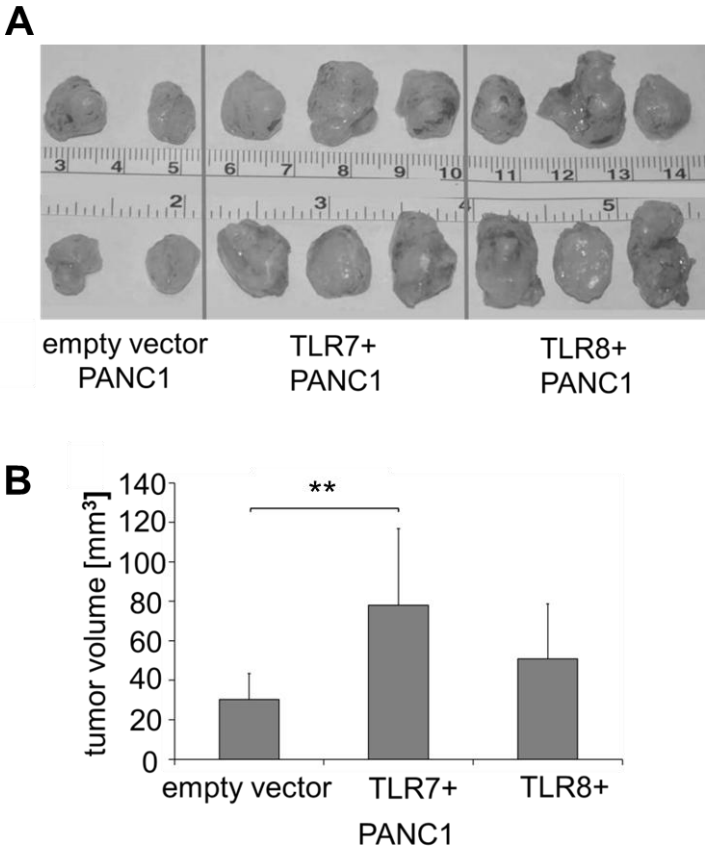


**Figure 27: Increased tumor growth of TLR7+ and TLR8+ PANC1 xenografts in Balb/c nude mice.**[99]

**(A)** Increased tumor size in subcutaneously injected Balb/c nude mice triggered by TLR7+ (n=5) and TLR8+ (n=5) PANC1 cells compared to empty vector PANC1 cells (n=4) **(B)** Significant increase in tumor volume caused by TLR7+ PANC1 cells in Balb/c nude mice ($p<0.005$) and accelerated growth of TLR8+ (n=5, $p<0.06$) PANC1 cell induced tumors compared to empty vector PANC1 cells (n=4).**$p<0.005$.[99]

## 2.5. TLR7 and TLR8 stimulatory effects on tumor growth

To analyze the promoting effect of TLR7 or TLR8 expression and stimulation on PANC1 tumor cell proliferation *in vitro* MTS proliferation assays were performed.

### 2.5.1. Effects in PANC1 human pancreatic cancer cells

72 hours after seeding untreated TLR7+ and TLR8+ PANC1 cells showed significantly elevated proliferation rates of 181% and 182% compared to untreated empty vector PANC1 cells (153%, $p<0.005$) (figure 28A). Additional stimulation with TLR7 and TLR8 agonist R848 induced a relative proliferation rate of 206% in TLR7+ PANC1 cells and 251% in TLR8+ PANC1 cells compared to 170% in empty vector PANC1 cells (figure 28C, $p<0.05$ and $p<0.0001$). Furthermore, RT-qPCR analysis of R848 treated TLR7+ and TLR8+ PANC1 cells showed a significant increase in the expression of proliferation marker Ki-67 compared to empty vector PANC1 cells (figure 28B, $p<0.0001$ and $p<0.0005$).

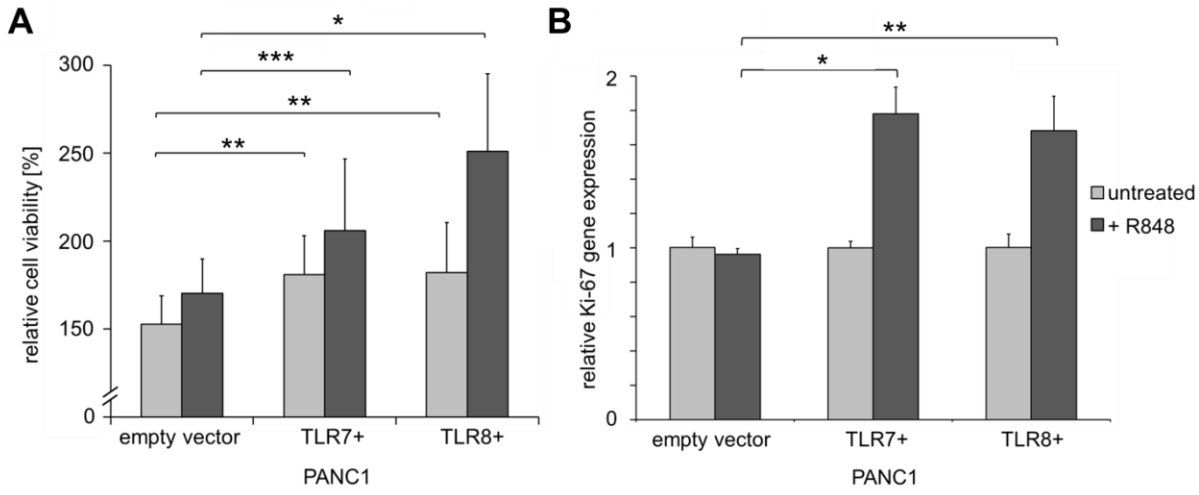


**Figure 28: Promoting effect of TLR7 and TLR8 expression and stimulation on PANC1 tumor cell proliferation *in vitro*.**[99]

**(A)** Significantly accelerated proliferation of TLR7+ and TLR8+ PANC1 cells without and with R848 stimulation compared to empty vector PANC1 cells analyzed by MTS assay **(B)** Increased gene expression of Ki-67 in R848 stimulated TLR7+ and TLR8+ PANC1 cells compared to empty vector PANC1 cells. Untreated PANC1 cells were standardized to baseline. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$.*p<0.0001, **p<0.005, ***p<0.05.[99]

### 2.5.2. Effects on NF-κB target gene expression

To determine whether TLR7 and TLR8 stimulation activates intracellular signaling pathways and the synthesis of pro-inflammatory cytokines gene expression levels of NF-κB, COX-2, IL-1β, IL8 and TNF-α were analyzed in response to stimulation of TLR7+ and TLR8+ PANC1 cells with R848.

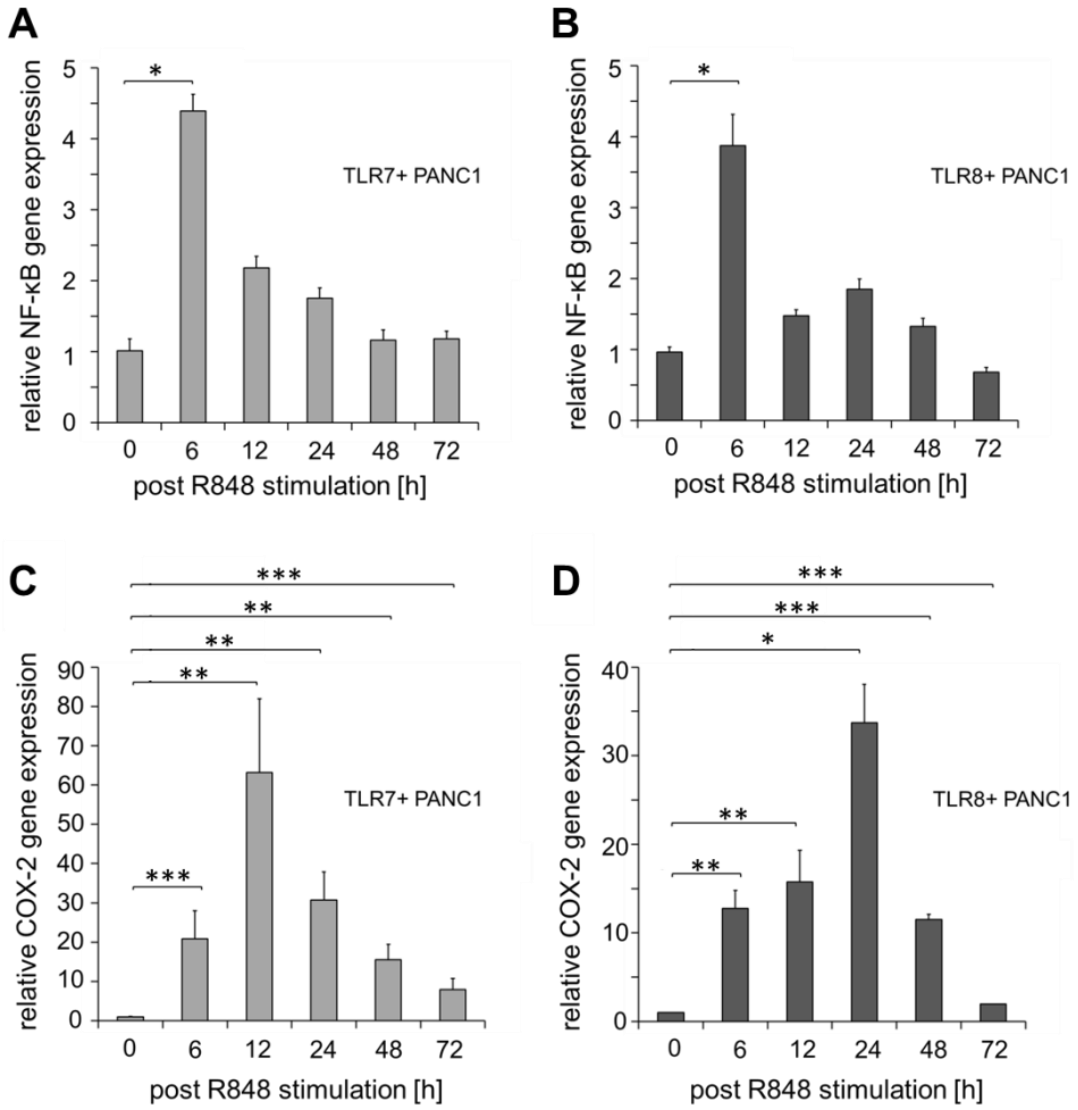


**Figure 29: Increased NF-κB and COX-2 gene expression in response to R848 stimulation of TLR7+ and TLR8+ PANC1 cells.[99]**

**(A)** and **(B)** Stimulation of TLR7+ and TLR8+ PANC1 cells with R848 resulted in significantly increased gene expression levels of NF-κB 6 hours post stimulation. **(C)** and **(D)** Significantly escalated COX-2 gene expression levels 6h to 72h after stimulation with maximum expression for TLR7+ PANC1 cells at 12h and for TLR8+ PANC1 cells at 24h. Untreated PANC1 cells were standardized to baseline. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$. *p<0.0001, **p<0.005, ***p<0.05.[99]

Six hours past TLR activation with R848 about 4-fold increased gene expression of NF-κB in TLR7+ and TLR8+ PANC1 cells had been observed (figure 29A and B, $p<0.0001$) compared to untreated cells. 72 hours after R848 stimulation NF-κB was down regulated in TLR7+ and TLR8+ PANC1 cells and reached the initial gene expression levels.

Additionally, R848 stimulation induced about a 60-fold increased gene expression level of COX-2 in TLR7+ PANC1 cells (12 hours) and about a 34-fold increased level in TLR8+ PANC1 cells (24 hours) compared with untreated cells (figure 29C and D, $p<0.005$ and $p<0.0001$). COX-2 expression levels remain significantly elevated in comparison to untreated cells even 72 hours after stimulation.

Concerning IL-1β, stimulation with R848 resulted in 40 and 32 fold gene expression 12 and 24 hours post stimulation in TLR7+ PANC1 cells and 14 to 4 fold expression 6 to 72 hours in TLR8+ PANC1 cells with maximum expression at 24 hours in comparison to untreated PANC1 cells (figure 30A and B, $p<0.0001$ and $p<0.005$).

Additionally, IL-8 mRNA levels were found 53 and 134 fold elevated 12 and 24 hours post stimulation in TLR7+ PANC1 cells and 5 to 52 fold 6 to 72 hours in TLR8+ PANC1 cells compared to untreated PANC1 cells. Maximum expression in TLR8+ PANC1 was demonstrated 24 hours post R848 treatment (figure 30C and D, $p<0.0001$).

TNF-α (TNFA) gene expression levels were found 44 and 119 fold increased 12h and 24h after stimulation in TLR7+ PANC1 cells and 11 to 67 fold 6 to 72 hours in TLR8+ PANC1 cells with maximum expression at 24 hours (figure 30E and F, $p<0.0001$ and $p<0.005$).

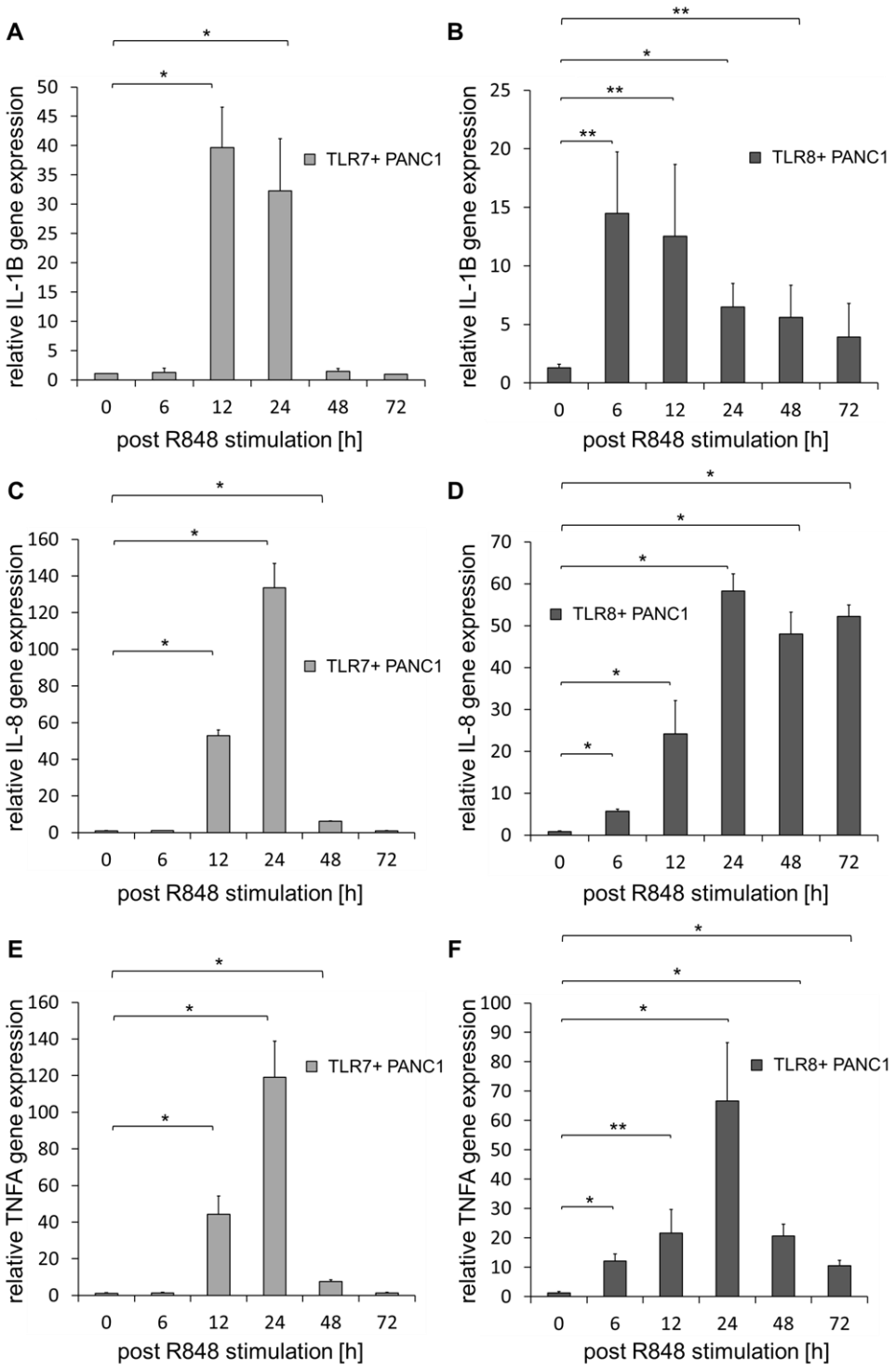


**Figure 30: Increased gene expression of IL-1β, IL-8 and TNF-α in response to R848 stimulation of TLR7+ and TLR8+ PANC1 cells.**

**(A)** and **(B)** Stimulation with R848 resulted in significantly increased gene expression levels of IL-1β (IL-1B) 12 and 24 hours post stimulation in TLR7+ PANC1 cells and 6 to 72 hours in TLR8+ PANC1 cells. **(C)** and **(D)** Significantly elevated IL-8 gene expression levels 12 and 24 hours post stimulation in TLR7+ PANC1 cells and 6 to 72 hours in TLR8+ PANC1 cells. **(E)** and **(F)** Significant increase in TNF-α (TNFA) gene expression levels 12 and 24 hours after stimulation in TLR7+ PANC1 cells and 6 to 72 hours in TLR8+ PANC1 cells with maximum expression at 24 hours. Untreated PANC1 cells were standardized to baseline. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$. *p<0.0001, **p<0.005.

### 2.5.3. Effects on chemotoxicity

To analyze the chemoresistance of stimulated and non-stimulated TLR7+ and TLR8+ PANC1 cells 5-FU was used. 5-FU is amongst other chemotherapeutics used as treatment for pancreatic cancer.[101] At first the $LD_{50}$ (median lethal dose) concentration for 5-FU was determined using non-stimulated empty vector PANC1 cells in MTS assay. $LD_{50}$ is defined as the dose required to kill half the members of a tested study population.[99] As shown in figure 31 for empty vector PANC1 cells $LD_{50}$ concentration was found at 500 µmol/L.

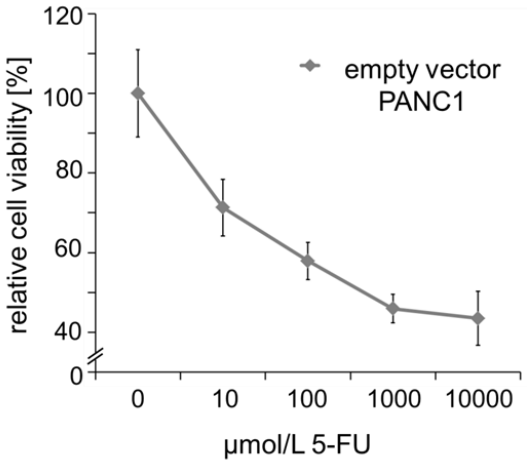


**Figure 31: $LD_{50}$ concentration of 5-FU for non-stimulated empty vector PANC1 cells.[99]**

Decreasing relative cell viability in connection to increasing 5-FU concentration determined by MTS assay. The $LD_{50}$ concentration of 5-FU for empty vector PANC1 cells was found at 500 µmol/L.[99]

To investigate the effects of TLR7 and TLR8 overexpression in PANC1 cells on chemosensitivity transduced cells were treated with two different concentrations of 5-FU (100 µmol/L and 1000 µmol/L). For both concentrations moderate decrease of chemosensitivity of unstimulated TLR7+ and TLR8+ PANC1 cells was demonstrated when compared to empty vector PANC1 cells. Using 100 µmol/L 5-FU relative cell viability of TLR7+ and TLR8+ cells was reduced to 62% and 73% in contrast to 58% for empty vector cells (figure 32A, $p<0.05$ and $p<0.0001$) while at a concentration of 1000 µmol/L 5-FU TLR7+ and TLR8+ cells showed values of 49% and 56% compared to 46% of empty vector cells (figure 32A, $p<0.05$ and $p<0.0001$).

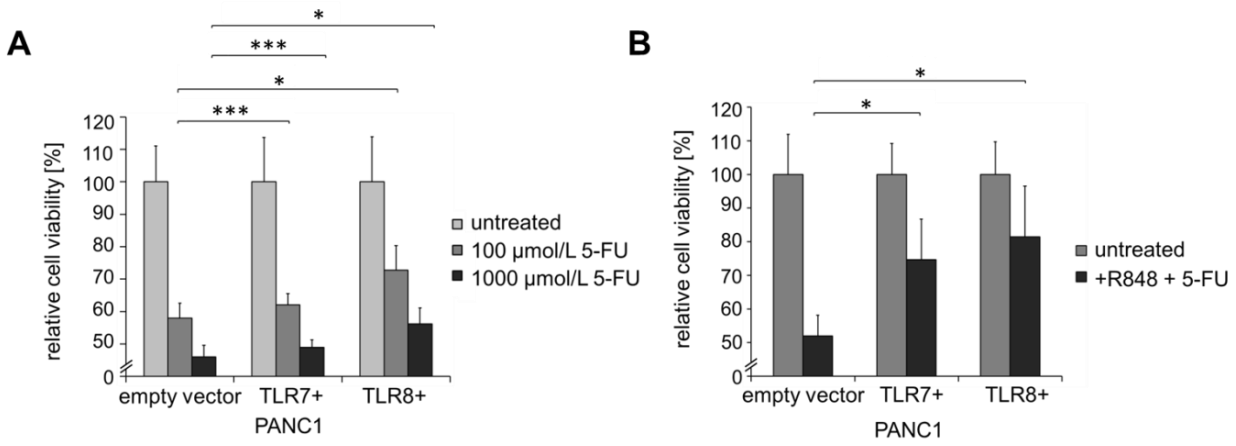


**Figure 32: Reduced chemosensitivity of TLR7+ and TLR8+ PANC1 cells.**[99]

**(A)** Significantly decreased sensitivity of TLR7+ PANC1 cells and TLR8+ PANC1 cells to different concentrations of 5-FU (100 µmol/L and 1000 µmol/L) compared to empty vector PANC1 cells analyzed by MTS assay. **(B)** Significant reduction of chemosensitivity to 5-FU (500 µmol/L) in R848 treated TLR7+ PANC1 cells and TLR8+ PANC1 cells compared to empty vector PANC1 cells. Untreated PANC1 cells were standardized to baseline.*p<0.0001, ***p<0.05.[99]

Stimulation of TLR7+ and TLR8+ PANC1 cells for 48 hours with the agonist R848 prior to treatment with 500 µmol/L 5-FU ($LD_{50}$ for empty vector PANC1 cells) increased the surviving fraction of TLR7+ and TLR8+ cells in contrast to empty vector PANC1 cells. While empty vector PANC1 cells demonstrated a reduced relative cell viability of 52%, viability of TLR7+ and TLR8+ cells was merely decreased to 75% and 81% (figure 32B, p<0.0001).

# IV. DISCUSSION

## 1. Cancer immunosurveillance and immunoediting

Cell transformation is a frequent event, which does not necessarily lead to tumor development due to the presence of an immune system capable of patrolling and eliminating damaged and potentially dangerous cells (figure 33, top). This process is named the cancer immunosurveillance theory.[102] Originally, cancer immunosurveillance was thought to be carried out solely by the adaptive immune system and only at the earliest stages of cellular transformation. By now, it is known that both the innate and adaptive immune system take part in the process and not only protect the host from tumor development but also edit the immunogenicity of tumors that may eventually form. In 2004, Dunn *et al* considered the term “cancer immunosurveillance” no longer sufficient to accurately describe the complex interactions that occur between a developing tumor and the immune system of the host and proposed the term “cancer immunoediting”, to emphasize that immunity is not only preventing but also shaping neoplastic diseases.[103,104]

Cancer immunoediting consists of three phases: elimination, equilibrium and escape.[105] During the elimination phase the immune system is capable to overcome and eliminate the tumor before it can progress to a clinically relevant disease. Cells and molecules of innate and adaptive immunity may eradicate the developing tumor cells and protect the host from tumor formation. However, if this process is not successful, the tumor cells enter the equilibrium phase, where due to genetic instability and immune selection new populations of tumor cells are produced. At that point elimination of the tumor by the immune system is no longer possible but it is still able to control tumor growth. The escape phase is initiated when the tumor has overcome the immune system. Tumor cells may eventually evade the immune responses by a variety of mechanisms and progress to a clinically apparent disease.[105,106] It is now recognized that tumors can either directly or indirectly impede the development of antitumor immune responses e.g. through the elaboration of immunosuppressive cytokines (such as TGF-β and IL-10) or via mechanisms involving T cells with immunosuppressive activities (i.e., CD4+CD24+ Treg cells).[103]

## 2. Immunosuppressive mechanisms of Foxp3+ Treg cells

Among the mechanisms that take effect during the escape phase of cancer immunoediting, Treg cells are of particular interest since they may down-regulate anti-tumor immune responses due to their inhibitory functions. Foxp3 is a crucial parameter for the development and function of Treg cells.[37] The mechanism underlying the suppressive function of Treg cells is still debated, but there is evidence that the suppressive activity is mediated either through secretion of immunosuppressive cytokines or cell–cell contact (figure 34).[29]

Natural CD4+CD25+Foxp3+ Treg cells express the surface protein cytotoxic T lymphocyte antigen 4 (CTLA4). This co-stimulatory molecule can interact with CD80 and/or CD86 on the surface of antigen-presenting cells (APCs) such as DCs. This interaction can transmit inhibitory signals on effector T cell activation and proliferation (figure 34, left).[29,107] Via the secretion of TGF-β and/or IL-10 both natural and inducible Treg cells such as $T_R1$, $T_H3$ and CD8+ regulatory T cells may exert their suppressive functions (figure 34, right).[29] These immunosuppressive cytokines inhibit the proliferation and cytokine production of effector T cells, including $T_H1$ cells, $T_H2$ cells and CD8+ cytotoxic T lymphocytes (CTLs), either directly or through their inhibitory influence on the maturation and activation of DCs or other APCs. Additionally, IL-10 inhibits the production of TNF and IL-12 by DCs and macrophages while TGF-β can induce the expression of Foxp3 in CD4+CD25- cells and thereby increase the number of induced Treg cells to further amplify immunosuppressive effects.[29,42,108-110]

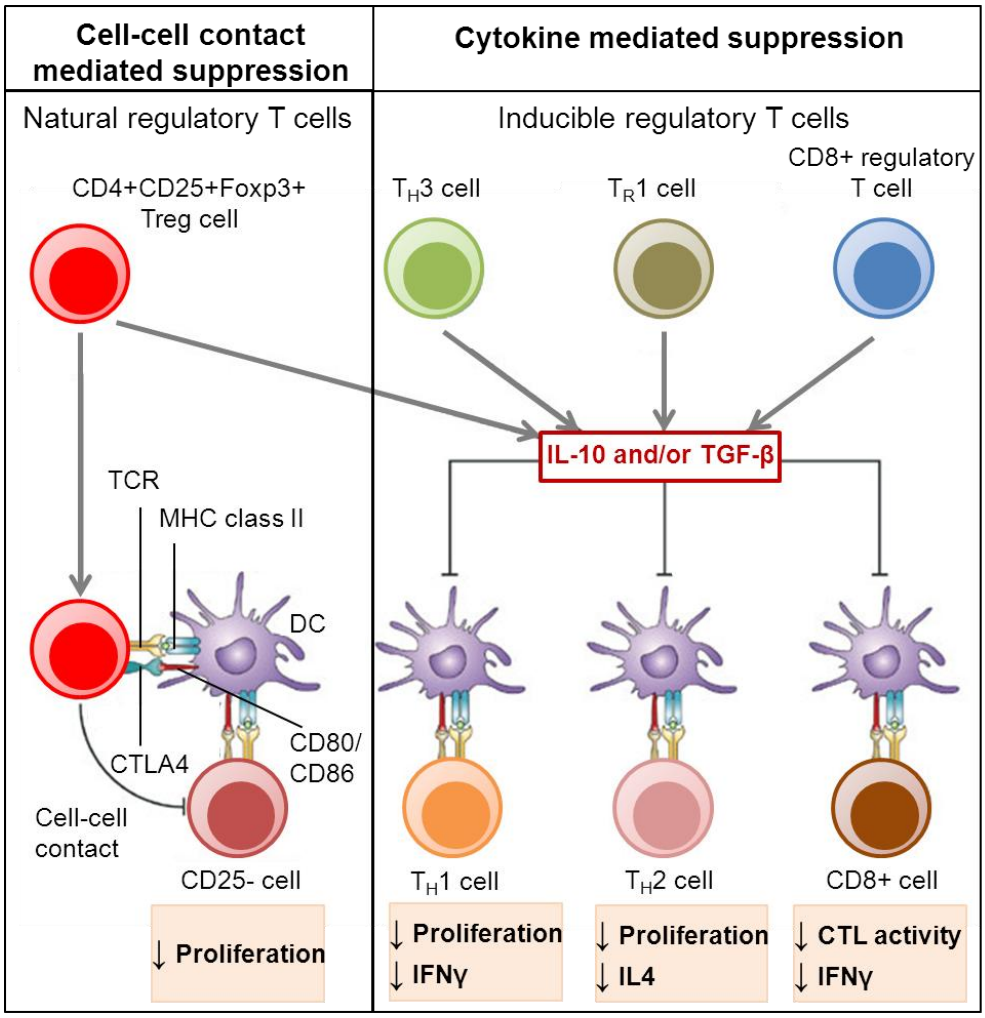


**Figure 33: Immunosuppressive mechanisms of Treg cells. (modified from Mills *et al*)**[29]

Immunosuppressive effects of Treg cells are mediated either through secretion of immunosuppressive cytokines (right) or cell–cell contact (left). CTLA4 can interact with CD80 and/or CD86 on the surface of DCs to transmit inhibitory signals on effector T cell activation and proliferation. IL-10 and TGF-β inhibit the proliferation and cytokine production of effector T cells, including $T_H1$ cells, $T_H2$ cells and CD8+ cytotoxic lymphocytes. CTL: cytotoxic lymphocyte, CTLA4: cytotoxic T lymphocyte antigen 4, DC: dendritic cell, MHC: major histocompatibility complex, TCR: T cell receptor [29]

## 3. Foxp3 in gastrointestinal cancer

As described in I.4.2 high levels of Foxp3+ Treg cells have been detected in the tumor microenvironment of many cancers including GI cancer and brought up the question whether these findings can be linked to the clinical outcome of tumor patients.[98] In addition to the significance of Foxp3+ Treg cells, Foxp3 expressing tumor cells turned out to be of special interest regarding tumor development and progression.

## 3.1. Foxp3 in pancreatic cancer

In pancreatic cancer patients, it has been well documented that Foxp3+ Treg cell levels are elevated both in peripheral blood and in the tumor microenvironment.[111] In addition, Treg cell infiltration correlates directly with tumor stage and prognosis.[55,112,113] In 2007, Hinz *et al* described the expression and function of Foxp3 in pancreatic ductal adenocarcinoma cells. They demonstrated that cancer cell mediated Foxp3 expression was induced by TGF-β2 and that co-culturing of Foxp3 expressing tumor cells with naive T cells could inhibit T cell proliferation.[63] In accordance with these findings, as shown in III.1.1, Foxp3 gene and protein expression could be demonstrated in the established human pancreatic cancer cell line PANC1 as well as in primary human pancreatic cancer cell lines PaCa DD 135, PaCa DD 159 and PaCa DD 185. Additionally, increased expression of the immunosuppressive cytokine IL-10 was observed in analyzed primary pancreatic cancer cell lines. These findings together with data provided by Hinz *et al* suggest that pancreatic cancer cells may exert immunosuppressive effects on T cells by mimicking Treg function to evade anti-tumor immune responses.[63]

## 3.2. Foxp3 in colorectal cancer

In CRC, data on the connection of tumor infiltrating Treg cells with patients' outcome is not that clear. To date, few studies have analyzed infiltrating Treg cells in CRC using Foxp3+ staining. A recent study demonstrated that Treg cell density was higher in locally limited than in metastatic disease but was not associated with the survival of CRC patients.[114] Salama *et al* reported an improved survival for patients whose tumors had a high density of infiltrated Foxp3+ Treg cells and this finding was confirmed by Frey *et al*.[115,116] Thus, studies of the prognostic value of Foxp3+ Treg cells in CRC have led to highly discrepant findings. Based on recently described clinical findings on elevated Foxp3 expression in lung, hepatocellular, pancreatic cancer and urinary bladder cancer as well as melanoma, it has been suggested that Foxp3 expression was not necessarily associated with Treg cells alone but also with cancer cells.[61,64,65,98] Expressing Foxp3 would enable the tumor to down-regulate effector T cell responses directed against the tumor. By discriminating Foxp3 expression of cancer cells from infiltrating Treg cells and correlation with overall

survival in patients, new insights could be gained in its prognostic significance for CRC.[98]

### 3.2.1. Treg cell mediated expression of Foxp3 in colorectal cancer patients

As shown in III.1.3 Foxp3+ expressing T cells were found in different amounts in more than 90% of the patient tumors. In accordance with previous studies in various solid tumors, significantly higher numbers of infiltrating CD4+CD25+Foxp3+ T cells were found in all CRC samples compared with normal colon tissues.[54-56,117,118] Gene expression analysis of CRC tissue showed significantly increased gene expression of CD4 and CD25 in limited disease tumors compared to tumors of advanced disease indicating elevated numbers of tumor infiltrating T cells at early stages. Protein expression analysis of these T cells demonstrated increased expression of CD4, CD25, Foxp3, IL-10 and TGF-β in limited disease tumors compared to advanced stages. These data suggest that Foxp3+ Treg cells were mainly found in early stage tumors. However, the association of Foxp3+ Treg cells and their impact on overall survival remains controversial:[98] in the here presented data no significant correlation was observed between the expression pattern of Foxp3 on tumor-infiltrating Treg cells and prognosis. Improved survival and the potentially protective role of Treg cells described in some studies might be explained by their capacity of reducing the development of an aggressive and cytotoxic, potentially proliferation promoting cytokine milieu, which is the basis for an inflammation-driven progress of malignant diseases.[98,119,120] Additionally, previous studies have examined the suppressive capacities of CD8+CD25+Foxp3+ T cells in CRC tissues.[121] In CRC specimens that were analyzed in this study, Foxp3+ T cells were mainly of a CD4+ subtype while the absolute number of CD8+CD25+Foxp3+ T cells was low.[98]

### 3.2.2. Cancer cell mediated expression of Foxp3 in colorectal cancer patients

As described in III.1.2, cancer cell mediated gene and protein expression of Foxp3 was found in the two human colon cancer cell lines SW480 and SW620. Additionally, increased IL-10 expression was demonstrated by RT-qPCR and immunohistochemistry, indicating potential immunosuppressive effects.

Gene expression analysis of CD4, CD25, FoxP3, IL-10 and TGF-β in CRC tissue showed significantly lower expression levels of Foxp3 and immunosuppressive cytokines IL-10 and TGF-β in limited disease tumors compared to those of advanced stages, while CD4 and CD25 were found at higher levels in early stages. These data indicate that Foxp3 gene expression is mediated not only by T cells alone but also by CRC cells.[98] Immunohistochemical stainings showed increased percentage of Foxp3 expressing cancer cells in late stage tumors compared to earlier stage tumors which confirmed this assumption. These findings together with the *in vitro* results provide evidence of a significantly increased tumor-related expression of the transcription factor Foxp3 in colorectal cancer cells. [98]

Moreover, cancer cell mediated Foxp3 expression could be linked to an adverse prognosis. Patients with high a Foxp3+ expression profile in their cancer cells were associated with a poorer prognosis than patients with low expression profile of Foxp3+ in their cancer cells.[98] This correlation of high Foxp3 expression pattern with poor prognosis was not observed for infiltrating Treg in the tumor as described in III.1.4.2. Next to lymph node metastasis, multivariate Cox regression analysis demonstrated Foxp3 expression in colon cancer cells as a significant prognostic parameter of survival in human CRC.[98] In addition, cancer cell mediated Foxp3 expression appears as an independent prognostic factor for CRC patients. Cancer cell expression of Foxp3 followed by secretion of immunosuppressive cytokines such as IL-10 and TGF-β into the tumor's microenvironment may give the tumor a powerful tool to inhibit anti-tumor immune responses hence resulting into tumor progression (figure 35).[98,122]

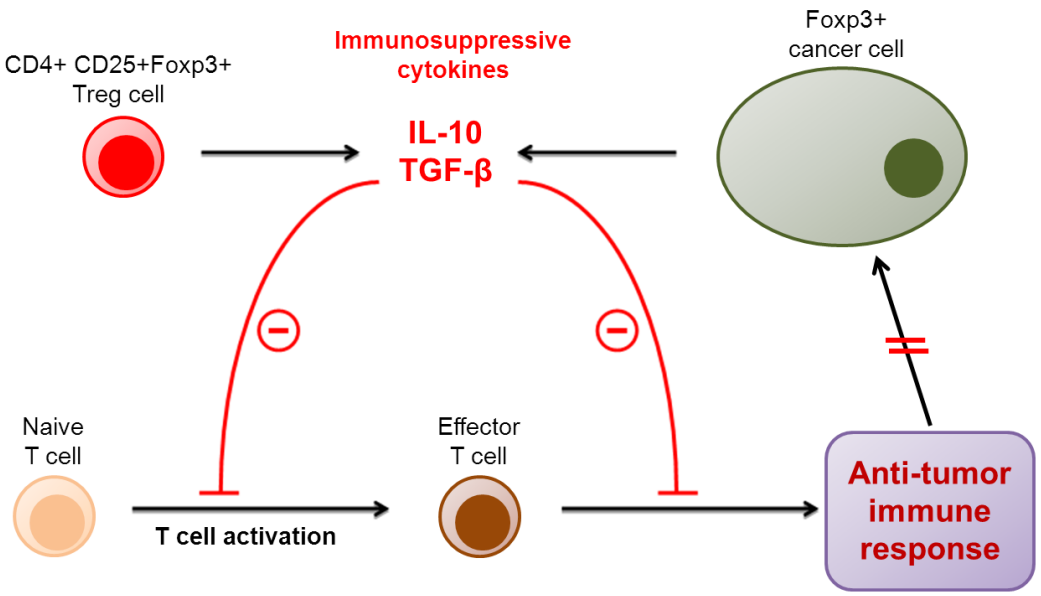


**Figure 34: Impact of Foxp3 on oncogenesis and tumor progression. (modified from Grimmig *et al*)**[122]

Immunosuppressive cytokines such as IL-10 and TGF-β released by either FOXP3+ Treg cells or FOXP3+ cancer cells inhibit the activation of naive T cells, hence limiting antitumor immune responses and favoring oncogenesis and tumor progression.[122]

## 3.3. Targeting Foxp3 related immunosuppression for prognosis and cancer immunotherapy in gastrointestinal cancer

Since the prospect of targeting transcription factors like Foxp3 is generally avoided because of the widespread and often unforeseen activities of transcriptional regulators, therapeutic strategies involving Foxp3 related immunosuppression either aim at Treg cell depletion or inhibition of Foxp3 induced immunosuppressive cytokines.

One new approach to cancer therapy is based on the combination of adoptive transfer of tumor-specific CTLs and Treg cell depletion using anti-CD25 antibodies.[123] Notably, anti-CD25 treatment alone would be less effective because anti-CD25 mAb may reduce not only CD25+CD4+ Treg cells but also CD25+ activated CD4+ and CD8+ effector T cells.[33,124]

As described previously, TGF-β can induce the differentiation of peripheral CD4+CD25- precursors into functional CD4+CD25+ Treg cells through the induction of Foxp3.[123,125] Additionally, TGF-β is one of the factors involved in suppression of

T cell proliferation and function.[123,126] Consequently, inhibition of TGF-β function may be a promising target to benefit anti-tumor immunity. Indeed, several studies have demonstrated that suppression of TGF-β functions may overcome TGF-β related immunosuppressive effects and restore anti-tumor immune responses.[123,127,128] SB-431542 is a small ATP-mimetic inhibitor of the kinase activity associated with members of the Activin Receptor-like Kinase family involved in TGF-β signaling.[123,129] This drug has been shown to inhibit tumor growth and motility by blocking TGF activity.[123,130] Another strategy to suppress TGF-β function *in vivo* is based on the use of adenoviral vectors, inducing the expression of Smad-7, an inhibitor of the TGF-β pathway, which was demonstrated to inhibit metastatic tumor growth in nude mice.[123,131]

Next to blockade of TGF-β, inhibition of the immunosuppressive cytokine IL-10 may be a promising approach. In a mouse myeloma model, inhibition of IL-10 by anti-IL-10 antibodies allowed to overcome tumor-induced immunosuppression.[123,132]

Additionally, the TIM (T cell Ig and mucin) gene family has recently been identified as being potentially involved in the regulation of effector T cell response. As demonstrated by Degauque *et al*, Foxp3 expression was downregulated *in vitro* in the presence of the agonist anti-TIM-1 mAb.[123,133]

## 4. Inflammation and cancer

Inflammatory conditions in cancer tissues are known to play a significant role in disease progression. Inflammation is linked to cancer through two pathways: extrinsic inflammation induced by non-transformed cells (e.g. invading pathogens), and intrinsic inflammation induced by transformed cells.[134,135] Via the activation of inflammation-linked transcription factors such as NF-κB, STAT3 or HIF1α, increasing amounts of pro-inflammatory mediators can be released contributing to further enhancement of inflammatory conditions (figure 36).[84,135] As a result, this so-called tumor-associated inflammation leads to changes in tumor cell proliferation, transformation and survival as well as alteration in migration, invasion and metastasis. Moreover inhibitory effects on adaptive immunity and altered responses to anticancer agents may also be observed (figure 36).[135] Additional to data confirming that chronic inflammatory processes are relevant factors in the

development of some malignant neoplasms, recent epidemiological studies indicate that chronic inflammation may even have a causal relationship to the formation and development of tumors.[135-138]

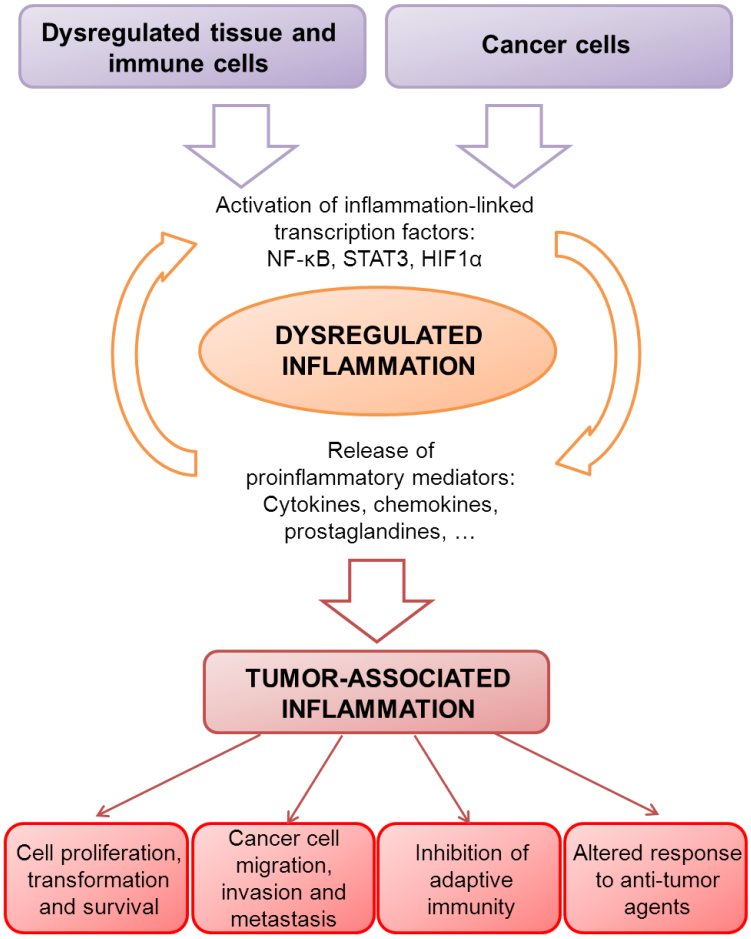


**Figure 35: Tumor-associated inflammation. (modified from Muller-Hubenthal *et al*)** [135]

Dysregulated inflammation caused by either the tumor or immune cells leads to activation of inflammation-linked transcription factors such as NF-κB, STAT3 or HIF1α, increasing amounts of pro-inflammatory mediators and further enhancement of inflammatory conditions. This tumor-associated inflammation may influence tumor cell proliferation, transformation and survival and cause alterations in migration, invasion and metastasis. Moreover there can be inhibitory effects on adaptive immunity and altered responses to anticancer agents, [135]

## 5. The role of inflammation in gastrointestinal cancer

It is known for several GI tract cancers that localized infections and inflammation exists before disease occurrence. For example ulcerative colitis can be linked to colitis-associated CRC; helicobacter pylori infection is associated with gastric cancer and Hepatitis B Virus (HBV) or Hepatitis C Virus (HCV) infections can be connected with hepatocellular carcinoma (HCC).[139]

## 5.1. Inflammation in colorectal cancer

In patients with CRC, tumor-associated inflammation is known to influence proliferation, invasion, and angiogenesis promoting signaling in cancer cells and the tumor's microenvironment. For instance, patients with colonic manifestation of Crohn's disease or ulcerative colitis show significantly increased risk for CRC. Additionally, inflammatory bowel disease (IBD) has long been identified as risk factor for CRC that is connected with dismal prognosis.[140,141] Moreover, the risk of malignancy correlates with the duration of IBD.[140,142]

Recently, the role of intestinal pathogens in carcinogenesis has attracted increasing attention when colonization by intracellular E. coli was detected at tumor sites.[140,143] PAMP mediated activation of TLRs such as TLR9, has been demonstrated to initiate inflammation and consequently could provide a link between pathogens and transformed cells.[144] Next to TLR9, TLR4 has been shown to play a role in CRC since high expression levels of TLR4 and related signaling via MyD88 was correlated with poor prognosis.[145] Besides, deregulated activation of the inflammation associated transcription factors STAT3 and NF-κB is found in several GI tract cancers and correlates with poor prognosis.[146] Consequently, induced TLR activation results in NF-κB activation and signaling that leads to the expression of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6. This may sustain further tumor growth and progression by inducing the expression of pro-inflammatory mediators.[134,146]

## 5.2. Inflammation in pancreatic cancer

Epidemiological and experimental evidence indicates that inflammation plays a significant role in the development and progression of pancreatic cancer.[147-149] For instance, sporadic chronic pancreatitis is connected with an increased risk of pancreatic cancer that correlates with the duration of inflammation. Moreover, patients with chronic pancreatitis are 17 times more likely to develop pancreatic cancer.[17,150,151] Farrow *et al* suggest, that this chronic inflammatory state could create a so-called ''landscaper defect''.[152] In the "landscaper theory" an abnormal microenvironment makes epithelial cells more susceptible to malignant transformation via factors that provoke either genomic damage or increased growth

which would provide transformed cells with proliferation advantages.[152,153] In pancreatic cancer, chronic inflammation leads to damage of stromal cells and up-regulation of pro-inflammatory mediators. Subsequent healing allows these damaged cells to be exposed to growth factors which result in growth stimulation and inhibition of apoptosis. This combination of cell damage and proliferation may lead to a defective cell population that, further exposed to an abnormal microenvironment and triggered by inflammatory processes, encourages the production of transformed cells and may finally result in the development of cancer (figure 37).[152] Inflammatory mediators, such as COX-2, NF-κB and STAT3 play a key role in inflammation induced epithelial cell damage and increased proliferation.[154]

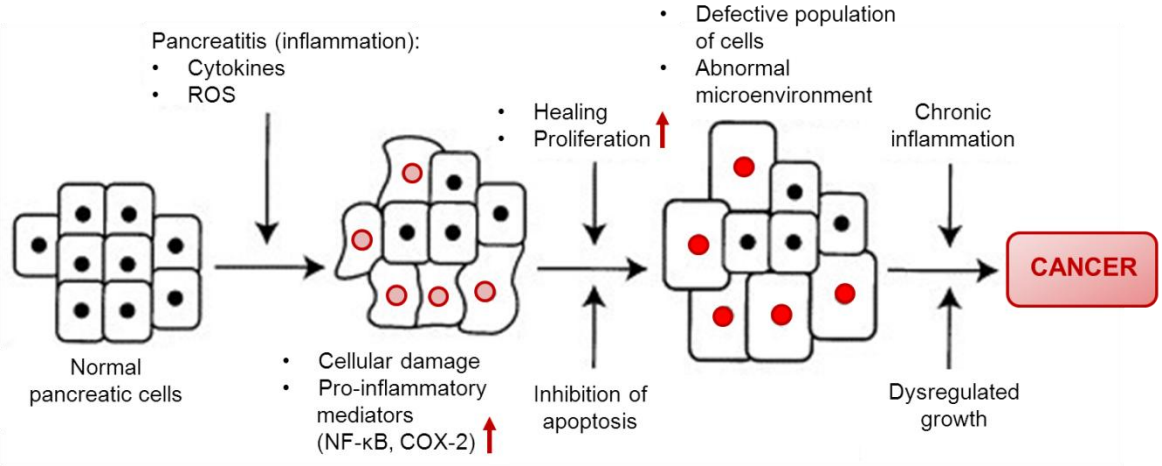


**Figure 36: Pancreatic cancer: inflammation and the landscaper theory. (modified from Farrow *et al*)**[152]

Chronic inflammation causes damage of stromal cells and subsequent healing allows these damaged cells to be exposed to growth factors. This combination of cell damage and proliferation may lead to a defective cell population that encourages the production of transformed cells and may finally result in the development of cancer.[152]

## 6. TLR7/8 expression and signaling in colorectal cancer

Analysis of the human colon cancer cell lines SW480, SW620 and HT-29 by RT-qPCR and immunohistochemical staining gave evidence for the expression of TLR7 and TLR8 in colon cancer. Additionally, cancer cell mediated TLR7 and TLR8 expression in CRC tissues has already been described by former collaborators of the own working group.[74] Grimm *et al* showed that increased expression of TLR7 and TLR8 in colon cancer cells was associated with tumor progression and reduced tumor-specific survival.[74,99]

Immunofluorescence double staining for MyD88 and NF-κB showed increased expression of both targets in advanced disease tumors compared to limited disease tissue, suggesting that inflammation responses through TLR signaling via MyD88 followed by activation of NF-κB may potentially mediate resistance to apoptosis and promote further tumor progression.

## 7. TLR7/8 expression and signaling in pancreatic cancer

Recent findings by Ochi *et al* demonstrated that TLR7 expression is upregulated in human and murine pancreatic cancer pointing to the hypothesis that TLR7 signaling might be involved in pancreatic carcinogenesis.[155] Moreover, mice lacking TLR7 exclusively within their inflammatory cells were protected from neoplasia which supports the idea that TLR mediated inflammation plays a crucial role in tumor developement.[155] In the human pancreatic cancer cell line PANC1, no significant TLR7 or TLR8 expression was detected whereas the primary pancreatic cancer cell lines PaCa DD 135, PaCa DD 159 and PaCa DD 185 demonstrated significantly increased gene and protein expression of both receptors. Conditions of long-term cell culture with the lack of a tumor-specific microenvironment may result in a down-regulation of TLR7 and TLR8.[99]

### 7.1. Stage dependent cancer cell mediated expression of TLR7 and TLR8

As described in III.2.2.1 and III.2.2.2, TLR7 and TLR8 were highly expressed by primary human pancreatic cancer cells, both in tumor tissue and primary pancreatic cancer cell lines PaCa DD 135, PaCa DD 159 and PaCa DD 185. In pancreatic cancer, high levels of TLR7 and TLR8 were associated with advanced stage of disease. Increased TLR expression that correlates with a disease progress suggests that TLR7 and TLR8 expression and signaling contributes to inflammation-mediated tumor progression, particularly since elevated levels of COX-2 were detected together with TLR7 and TLR8 positivity in pancreatic cancer tissue. In accordance to this hypothesis, normal pancreatic cells expressed neither TLR7 nor TLR8.

### 7.2. TLR7/8 signaling leads to the induction of NF-κB, COX-2 and pro-inflammatory cytokines

As shown in III.2.5.2, R848 mediated TLR7 and TLR8 signaling led to increased gene expression of NF-κB, pointing to the activation of the NF-κB pathway. NF-κB signaling is known to be involved in cellular promotion, transformation and progression of several human cancers by regulating many genes implicated in cell proliferation, survival, migration, tumorigenesis and metastasis.[156-158] Targets of NF-κB connected to cell proliferation and survival include oncogenes such as cyclin D, Bcl-2, Bcl-XL, and inhibitor of apoptosis protein (IAP).[156,159] Moreover, the expression of angiogenesis factors and adhesion molecules, such as GRO1, IL-8 and VEGF (vascular endothelial growth factor), are linked to NF-κB activation.[156,160]

Additionally, the expression of COX-2 can be initiated by NF-κB signaling.[88,161] Invasion and angiogenesis of gastric cancer cells was described to be mediated by COX-2 after TLR2 and TLR9 activation, leading to inflammation and cancer progression.[162] Additionally, COX-2 has been demonstrated to play a key role in inflammation-induced epithelial cell damage and increased proliferation within the landscaper theory of pancreatic cancer.[154] All these events are linked to COX-2 driven prostaglandin (PGE) 2 biosynthesis.[89,99,161] Recent data showed that TLR8 signaling strongly promotes inflammatory mediator biosynthesis of PGE2 and thromboxane A2 (TXA2) through the COX-2 pathway.[163] Interestingly, COX-2 expression was found to be up-regulated in the investigated patient tumors as well, and was associated with TLR7 and TLR8 positivity in the investigated specimens of pancreatic cancer.[99]

Besides the expression of COX-2, TLR7 and TLR8 stimulation caused the induction of the inflammatory cytokines IL-1β, IL-8 and TNF-α, most likely through the activation of the NF-kB pathway.

IL-1β has been shown to be implicated in the pathogenesis of inflammatory diseases and to contribute to tumor growth and metastasis.[164,165] Furthermore, in pancreatic cancer IL-1β has been demonstrated to be linked to promotion of cancer cell invasiveness and IL-1β induced activation of NF-κB and up-regulation of COX-2 contributes to chemoresistance of pancreatic cancer cells *in vitro*.[147,166-168] In further studies, IL-1β was connected with increased migratory potential in human pancreatic

cancer cells and in murine pancreatic tumors high levels of IL-1β were found in the tumor microenvironment that promoted recruitment of proangiogenic macrophages.[147,169,170] Additionally, IL-1β was found to be significantly elevated in the serum of pancreatic cancer patients compared to healthy controls.[147,171]

Elevated levels of the pro-inflammatory cytokine IL-8 have been observed in various solid tumors, including colon and pancreatic cancer.[171,172] Especially in pancreatic cancer IL-8 has become of major interest since it was found to play a crucial role in tumor progression.[147,173] IL-8 has been shown to promote angiogenesis by the induction of VEGF, neuropilin (NRP) 2 and VEGF receptors. Additionally, IL-8 leads to the phosphorylation of ERK (extracellular-signal regulated kinase), which indicates the activation of the MAPK signaling pathway that contributes to cell proliferation and survival and is associated with tumorigenesis.[173,174] Tumor aggressiveness has also been linked to the expression of IL-8 in pancreatic cancer. By regulating the activity of matrix metalloproteinase (MMP) 2, IL-8 can enhance the invasiveness of tumor cells and support formation of metastasis.[147,175] Elevated levels of IL-8 in the serum of pancreatic cancer patients have been found to be associated with poor clinical outcome and have been suggested as a prognostic marker.[147,176,177]

Although TNF-α is known to have cytotoxic effects on tumor cells at high doses, a tumor-promoting role has also been demonstrated by several pre-clinical studies.[147,178] Increased levels of TNF-α have been found in the serum of pancreatic cancer patients compared to patients with chronic pancreatitis and healthy controls.[179] By up-regulation of the epidermal growth factor (EGF) receptor TGF-α has been shown to contribute to the promotion of cancer cell proliferation.[180] Moreover, TNF-α has been demonstrated to increase the invasiveness of human pancreatic cancer cells *in vitro* and tumor growth and metastatic potential in mice. Using anti-TNF-α therapy a significant reduction in tumor growth and metastases could be observed in these mice.[181]

### 7.3. Increased tumor cell proliferation and chemoresistance related to TLR7 and TLR8 expression and signaling

As shown in xenograft models in Balb/c nude mice as well as in *in vitro* proliferation assay TLR7 or TLR8 expression was associated with accelerated tumor growth and

cell proliferation, respectively. Moreover, additional stimulation of pancreatic cancer cells with the TLR7 and TLR8 agonist R848 led to further increase in tumor cell proliferation. As described previously several targets of NF-κB, which is induced by TLR7 and TLR8 signaling, are connected to cell proliferation and survival. Among these are anti-apoptotic mediators such as cyclin D, Bcl-2, Bcl-XL, and IAP.[156,159] Cyclin D is involved in the regulation of cell cycle progression and in particular cyclin D-dependent kinases CDK4 and CDK6 have been identified as major oncogenic drivers.[182] Overexpression of Bcl-2 and Bcl-XL has been demonstrated to inhibit cell death induced by various treatments including growth factor deprivation, hypoxia, oxidative stress and cytotoxic therapy.[183,184] Next to members of the Bcl-2 family, IAP is also implicated in cell death regulation, including inhibition of apoptosis and necrosis, regulation of cell cycle and inflammation.[185] Moreover, TLR7/8 mediated induction of IL-8 and TGF-α can activate several growth factors and their receptors and lead to the activation of the MAPK signaling pathway, which contributes to tumor cell proliferation.[173,174,180]

Interestingly, stimulation of TLR7 or TLR8 induced not only an increase in tumor cell proliferation but also a strong chemoresistance of PANC1 tumor cells against 5-FU. The induction of anti-apoptotic Bcl-2-family proteins that possess the ability to suppress cell death induced by cytotoxic anticancer drugs may explain this observation.[184] Additionally, TLR7 and TLR8 signaling has been shown to induce the expression of the canonical Notch target genes HES1 (hairy and enhancer of split) and HEY1 (enhancer of split with YRPW motif).[186] This interaction indicates that activation of Notch target genes is a common feature of TLR response. In addition, Güngör *et al* demonstrated that activated Notch signaling could up-regulate NF-κB and lead to increased chemoresistance in pancreatic cancer.[187]

## 8. Targeting TLR7 and TLR8 mediated inflammation for tumor therapy

Targeting TLRs does not present a novel tool in cancer therapy. In fact, a considerable number of studies suggest that the induction of innate immunity by targeted TLR activation has beneficial effects to reduce tumor growth. TLR7 activation is currently used for the treatment of various malignancies, such as melanoma, breast cancer and basal cell carcinoma, and TLR3 and TLR9 ligands

have been shown to reduce growth of renal cell carcinoma and metastatic colorectal cancer.[99,188,189] Despite those data that would point on favorable effects of TLR activation, the fact that about 15% of human tumors are associated with chronic inflammation remains confusing.[189] Moreover, it has been shown that TLR ligands can promote cancer cell survival, migration and tumor progression. For example, TLR agonists have been demonstrated to increase tumor viability and metastasis of human lung cancer cells, proliferation in human myeloma cells (TLR3), adhesion and metastasis in human colorectal cancer cells (TLR4) and migration in human gliobastoma (TLR4) or human breast cancer cells (TLR2).[95,99,190-192] Interestingly, in the present study we also observed that TLR7 or TLR8 stimulation increased tumor cell proliferation and resistance to the cytostatic agent 5-FU in pancreatic cancer.[99]

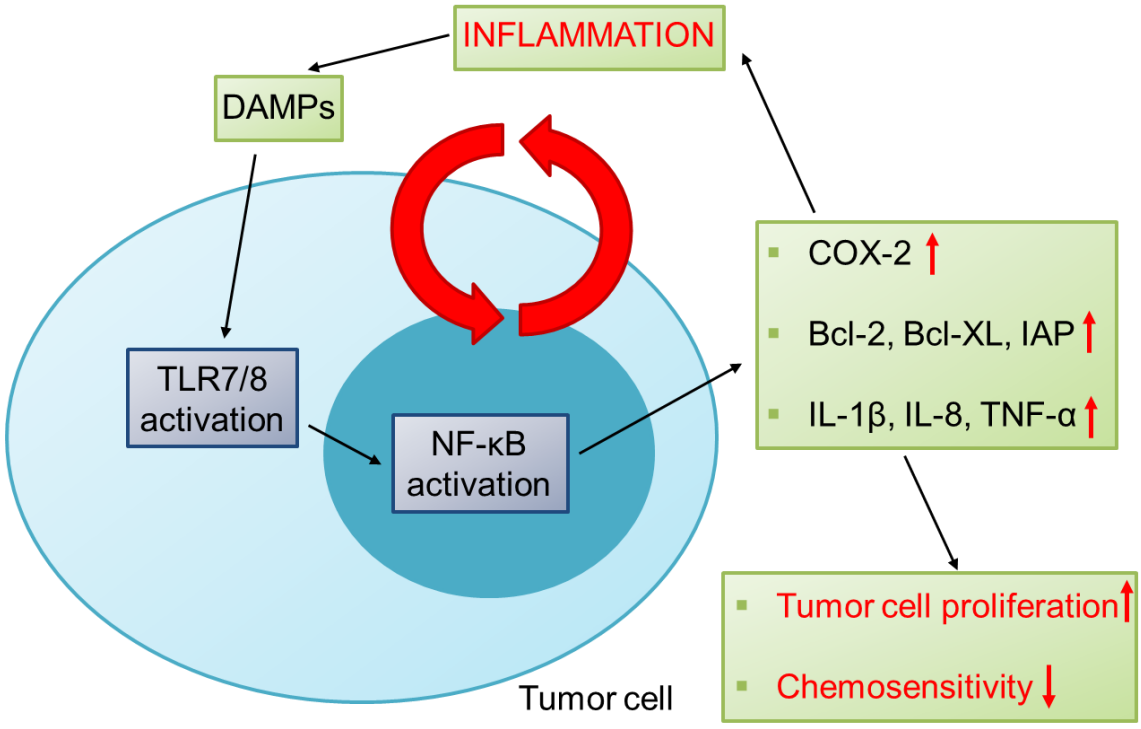


**Figure 37: Tumor cell mediated TLR7 and TLR8 signaling in inflammatory-linked cancers.**

Inflammation-induced formation of DAMPs can induce TLR7 and TLR8 signaling within the tumor cell, which may lead to the activation of NF-κB pathway. This results in the up-regulation of COX-2, pro-inflammatory cytoklines such as IL-1β, IL-8 and TNF-α and anti-apoptotic mediators like Bcl-2, Bcl-XL and IAP. These components on the one hand contribute to further enforcement of an inflammatory microenvironment and on the other hand lead to increased tumor cell proliferation and reduced chemosensitivity.

These data are also supported by the work of Ochi *et al*. They demonstrated that normal pancreatic tissue of mice and human do not express TLR7. In contrast to this, in both a mouse p48Cre;KrasG12D pancreatic cancer model as well as in human pancreatic cancer specimen TLR7 was found to be strongly up-regulated. Moreover, they showed that treatment with cancer progression accelerator caerulein in Kras mutant mice in the presence of an inhibitory oligonucleotide for TLR7 could prevent malignant progression.[155] This strong pro-carcinogenic effect of TLR7 signaling was indeed surprising since TLR7 has been shown to mediate anti-neoplastic effects in other cancer models as described above.[189] To explain this contradiction, Ochi *et al* suggest that in cancers without primary inflammation, activation of TLRs induces innate immunity which might break self-tolerance of the immune system towards the tumors cells and thus promote anti-tumor immune responses. In tumor entities that are known to be connected with inflammatory processes such as colon and pancreatic cancer TLR activation might rather accelerate carcinogenesis via the induction of NF-κB and STAT3 signaling.[155,189]

Together with the data presented here on TLR7 and TLR8 signaling in pancreatic cancer, these observations point to the hypothesis that in inflammatory-linked cancers tumor progression, survival, metastatic potential and mediation of chemoresistance are closely associated with TLR7 and TLR8 expressing pancreatic cancer cells.[99] By the formation of DAMPs an inflammatory microenvironment can induce TLR7 and TLR8 within the tumor cell, which leads to the activation of NF-κB signaling. NF-κB mediated up-regulation of COX-2, pro-inflammatory cytokines such as IL-1β, IL-8 and TNF-α and anti-apoptotic mediators like Bcl-2, Bcl-XL and IAP contribute to further enforcement of an inflammatory microenvironment and lead to increased tumor cell proliferation and reduced chemosensitivity (figure 38). Therefore, inhibition of TLR7 and TLR8 signaling in inflammation driven cancers such as pancreatic or colon cancer might be a potential novel mechanism to reduce tumor growth, chemoresistance and COX-2 induced carcinogenesis.

## V. SUMMARY

Regulatory T cells (Treg) expressing the transcription factor forkhead-box protein P3 (Foxp3) have been demonstrated to mediate evasion from anti-tumor immune responses during tumor progression. Moreover, Foxp3 expression by tumor cells themselves may allow them to counteract effector T cell responses, resulting in a survival benefit of the tumor. For gastrointestinal cancers, in particular pancreatic and colorectal cancer (CRC), the clinical relevance of Foxp3 is not clear to date. Therefore the aim of this study was to analyze its impact in CRC and pancreatic cancer. To determine the relevance of Foxp3 for tumor progression and patient survival, gene and protein analysis of human pancreatic and colon cancer cell lines as well as tumor tissues from patients with CRC was performed. The results derived from the patients with CRC were correlated with clinicopathological parameters and patients' overall survival. Cancer cell mediated Foxp3 expression *in vitro* was demonstrated in human pancreatic cancer cell lines PANC1, PaCa DD 135, PaCa DD 159 and PaCa DD 185 as well as in human colon cancer cell lines SW480 and SW620. Additionally, Foxp3 expressing cancer cells were found in *ex vivo* tumor tissue samples of patients with CRC. The percentage of Foxp3+ cancer cells increased from stages UICC I/II to UICC III/IV compared to normal tissue. Moreover, high tumor cell mediated Foxp3 expression was associated with poor prognosis compared to patients with low Foxp3 expression. In contrast, low and high Foxp3 level in tumor infiltrating Treg cells demonstrated no significant differences in patients' overall survival. Correlation analysis demonstrated a significant association of Foxp3 cancer cell expression with the expression of immunosuppressive cytokines IL-10 and TGF-β. These findings suggest that Immunosuppressive cytokines such as IL-10 and TGF-β released by rather Foxp3+ cancer cells than Foxp3+ Treg cells may inhibit the activation of naive T cells, hence limiting antitumor immune responses and favoring tumorigenesis and progression.

Chronic inflammation has been shown to be an important epigenetic and environmental factor in numerous tumor entities. Recent data suggest that tumorigenesis and tumor progression may be associated with inflammation-triggered activation of Toll-like receptors (TLR). In this study, the specific impact of both TLR7 and TLR8 expression and signaling on tumor cell proliferation and chemoresistance is analyzed in inflammation linked CRC and pancreatic cancer. By gene and protein

expression analysis of human pancreatic and colon cancer cell lines TLR7 and TLR8 expression was determined *in vitro*. Additionally, expression of TLR7/TLR8 in UICC stage I-IV pancreatic cancer, chronic pancreatitis and normal pancreatic tissue was examined. For in *vitro/in vivo* studies TLR7/TLR8 overexpressing PANC1 cell lines were generated and analyzed for effects of TLR expression and stimulation on tumor cell proliferation and chemoresistance. Cancer cell mediated TLR7 and TLR8 expression *in vitro* was demonstrated in human colon cancer cell lines SW480, SW620 and HT-29 as well as in primary pancreatic cancer cell lines PaCa DD 135, PaCa DD 159 and PaCa DD 185. Additionally, TLR7 and TLR8 expressing tumor cells were found in *ex vivo* tissue samples of patients with pancreatic cancer and chronic pancreatitis. Significantly elevated expression levels of TLR7 and TLR8 were found in advanced tumor stages (UICC III) compared to early tumor stages (UICC II) and chronic pancreatitis. No or occasionally low expression was detected in normal pancreatic tissue. In contrast to the tissues from patients with pancreatic cancer or chronic pancreatitis, established pancreatic tumor cell lines express only very low levels of TLR7 and TLR8. Therefore, for *in vitro* and xenograft studies TLR7 or TLR8 overexpressing PANC1 cells were generated. Proliferation promoting effects of TLR7 and TLR8 expression and stimulation with R848 were detected *in vitro*. Additionally, increased tumor growth of TLR expressing PANC1 cells was demonstrated in subcutaneously injected Balb/c nude mice. Interestingly, activation of TLR7 or TLR8 induced not only an increase in tumor cell proliferation but also a strong chemoresistance of PANC1 cells against 5-fluorouracil (5-FU). Moreover, treatment with R848 resulted in elevated expression levels of NF-κB, COX-2 and inflammatory cytokines IL-1β, IL-8 and TNF-α, suggesting TLR7/8 signaling to contribute to an inflammatory, anti-apoptotic and proliferation promoting tumor microenvironment. These findings emphasize the particular role of TLR7 and TLR8 in inflammation related cancers and their relevance as potential targets for cancer therapy.

## VI. ZUSAMMENFASSUNG

In jüngerer Vergangenheit wurde regulatorischen T-Zellen, die den Transkriptionsfaktor forkhead-box protein P3 (Foxp3) exprimieren, wiederholt die Fähigkeit zugesprochen, Antitumorimmunreaktionen während der Tumorentwicklung und –progression abzuschwächen. Daneben sind Tumorzellen selbst befähigt Foxp3 zu exprimieren. Sie können damit der Effektor-T-Zell-Antwort entgegen wirken und so Tumorwachstum begünstigen. Die klinische Bedeutung der Foxp3-Expression in gastrointestinalen Tumoren, insbesondere im Pankreaskarzinom und kolorektalen Karzinom, ist zum heutigen Stand noch unklar. Daher war es das Ziel dieser Arbeit, die Bedeutung von Foxp3 im Pankreaskarzinom und kolorektalen Karzinom weiter aufzuklären. Um seine prognostische Relevanz hinsichtlich der Tumorprogression sowie das Patienten-Überleben zu untersuchen, wurden Gen- und Proteinexpressionsanalysen in Tumorgeweben aus Patientenkohorten mit kolorektalem Karzinom durchgeführt. Die Ergebnisse aus den Tumorgeweben wurden mit klinikopathologischen Parametern und dem Gesamtüberleben der Patienten korreliert. Sowohl in den humanen Pankreaskarzinomzelllinien PANC1, PaCa DD 135, PaCa DD 159 und PaCa DD 185 als auch in den humanen Kolonkarzinomzelllinien SW480 und SW620 konnte tumorzellvermittelte Foxp3-Expression nachgewiesen werden. Zusätzlich wurden auch in den ex vivo Gewebeproben Foxp3-exprimierende Tumorzellen vorgefunden. Dabei nahm der Anteil an Foxp3-positiven Tumorzellen stadienabhängig von frühen zu fortgeschrittenen Tumorstadien (UICC I/II zu UICC III/IV) zu. Zudem waren Patienten mit einer starken Expression von Foxp3 im Vergleich zu Patienten mit niedrigem Foxp3-Expressionsprofil in den Tumorzellen von einer schlechten klinischen Prognose gekennzeichnet. Hohe bzw. niedrige Foxp3-Expressionen in tumorinfiltrierenden T-Zellen zeigten dagegen keinen signifikanten Einfluss auf das Gesamtüberleben der Patienten. In der Korrelationsanalyse ergab sich außerdem eine signifikante Verknüpfung von Foxp3-Expression mit der Expression der immunsuppressiven Zytokine IL-10 und TGF-β in den Tumorzellen. Diese Beobachtungen lassen vermuten, dass Foxp3-positive Tumorzellen durch die Sekretion von immunsuppressiven Zytokinen wie IL-10 und TGF-β im Tumormikromilieu die Aktivierung naiver T-Zellen inhibieren. Damit würden Antitumorimmunreaktionen unterdrückt und das Tumorwachstum begünstigt.

Chronische Entzündungsreaktionen sind wichtige epigenetische Faktoren in verschiedenen Tumorentitäten. Neuere Daten deuten darauf hin, dass Karzinogenese und Tumorprogression in Verbindung mit inflammationsinduzierter Aktivierung von Toll-like Rezeptoren (TLR) stehen. In dieser Arbeit wurde insbesondere der Einfluss der beiden Rezeptoren TLR7 und TLR8 auf die Tumorzellproliferation und Chemotherapieresistenz von gastrointestinalen Tumoren wie das kolorektale Karzinom und das Pankreaskarzinom untersucht. Mit Hilfe von Gen- und Proteinexpressionsanalysen wurde die tumorzellvermittelte Expression von TLR7 und TLR8 in vitro in verschiedenen humanen Kolon- als auch Pankreaskarzinomzelllinien nachgewiesen. Zusätzlich wurde verstärkte TLR7 und TLR8 Expression in Tumorgewebeproben aus Patienten mit Pankreaskarzinom als auch bei chronischer Pankreatitis vorgefunden, wobei die Expression in fortgeschrittenen Tumorstadien (UICC III) gegenüber früheren Stadien (UICC II) und chronischer Pankreatitis signifikant erhöht war. In vitro und in vivo Untersuchungen im xenogenen Tumormodell mit humanem Pankreaskarzinom zeigten für TLR7- und TLR8-exprimierende PANC1-Pankreaskarzinome signifikant gesteigerte Tumorproliferationen. Zusätzlich wurde durch die gezielte TLR7/8 Stimulation mit der Substanz R848 eine ausgeprägte Chemotherapieresistenz gegenüber 5-Fluorouracil (5-FU) induziert. Die Aktivierung von TLR7 und TLR8 führte darüber hinaus zu einer verstärkten Expression von NF-kB, COX-2, sowie den proinflammatorischen Zytokinen IL-1β, lL-8 und TNF-α. Diese Beobachtungen legen nahe, dass die TLR7/8 Signalgebung zu inflammatorischen, antiapoptotischen und proliferationsfördernden Prozessen im Tumormikromilieu beiträgt und unterstreichen die Bedeutung der Toll like Rezeptoren 7 und 8 als potentielle therapeutische Zielstrukturen in inflammationsassoziierten Tumorerkrankungen.

## VII. ABBREVIATIONS

| | |
|---|---|
| 5-FU | 5-fluorouracil |
| Akt | protein kinase B |
| AP | alkaline phosphatase |
| AP-1 | activator protein 1 |
| APC | antigene-presenting cell |
| Bcl | b cell lymphoma |
| BSA | bovine serum albumin |
| CD | cluster of differentiation |
| CDK | cyclin D-dependent kinase |
| Chron. pancr. | chronic pancreatitis |
| cIAP | baculoviral IAP repeat-containing protein |
| CK | cytokeratin |
| COX-2 | cyclooxygenase-2 |
| CRC | colorectal cancer |
| CTL | cytotoxic (T) lymphocyte |
| CTLA4 | cytotoxic T lymphocyte antigen 4 |
| Cy | cyanine |
| DAB | 3,39-diaminobenzidine |
| DAMP | damage-associated molecular pattern |
| DAPI | 49,6-diamidino-2-phenylindoldihydrochlorid |
| DC | dendritic cell |
| DMEM | dulbeccos modified eagles medium |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| DPBS | dulbecco's phosphate buffered saline |
| DTT | dithiothreitol |
| ECL | enhanced chemoluminescence |
| EDTA | ethylendiamintetraacetate |
| EGF | epidermal growth factor |
| EpCAM | epithelial cell adhesion molecule |
| ERK | extracellular-signal regulated kinase |
| FBS | fetal bovine serum |

| | |
|---|---|
| FITC | fluorescein isothiocyanate |
| Foxp3 | forkhead box protein 3 |
| FS | forward scatter |
| G418 | geneticin selective antibiotic |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GI | gastrointestinal |
| GUSB | beta-glucuronidase |
| HBV | hepatitis B virus |
| HCC | hepatocellular carcinoma |
| HCV | hepatitis C virus |
| HE | hematoxylin and eosin |
| HEPES | 2-(4-(2-hydroxyethyl)- 1-piperazinyl)-ethansulfonicacid |
| HES | hairy and enhancer of split |
| HEY | enhancer of split with YRPW motif |
| HIF | hypoxia-inducible factor 1-alpha |
| HPRT1 | hypoxanthine phosphoribosyltransferase 1 |
| HRP | horseradish peroxidase |
| IAP | inhibitor of apoptosis protein |
| IBD | inflammatory bowel disease |
| IFN | interferon |
| IgG | immunoglobulin G |
| IKK | IκB kinase |
| IL | interleukin |
| IL2RB | interleukin-2 receptor subunit beta |
| $LD_{50}$ | median lethal dose |
| LRR | ligand recognition region |
| mAb | monoclonal antibody |
| MAPK | mitogen-activated protein kinases |
| MMP | matrix metalloproteinase |
| MTS | 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2h-tetrazolium, inner salt |
| MyD88 | myeloid differentiation factor 88 |
| NaCl | sodium chloride |
| NaDOC | sodium deoxycholat |

| | |
|---|---|
| NaF | sodium fluoride |
| NFAT | nuclear factor of activated T cells |
| NF-κB | nuclear factor kappa B |
| NK cell | natural killer cell |
| NRP | neuropilin |
| NT | normal tissue |
| pAb | polyclonal antibody |
| PAMP | pathogen associated molecular pattern |
| PBMC | peripheral blood mononuclear cell |
| PE | phycoerythrin |
| Pen/strep | penicillin/streptomycin |
| PGE | prostaglandin |
| PMSF | phenylmethylsulfonyl fluoride |
| PRR | pattern recognition receptor |
| pTreg cell | peripherally derived Treg cell |
| R848 | resiquimod |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| RT-qPCR | real-time quantitative reverse transcription pcr |
| SDS | sodium dodecyl sulfate |
| SF | sodium fluoride |
| Smad3 | mothers against decapentaplegic homolog 3 |
| ssRNA | single stranded RNA |
| Stat5 | signal transducer and activator of transcription 5 |
| TAA | tumor-associated antigens |
| TCR | T cell receptor |
| TGF-β | transforming growth factor beta |
| $T_H$ cell | T helper cell |
| TICAM | toll-like receptor adaptor molecule |
| TIM | T cell Ig and mucin |
| TIR | toll/interleukin-1 receptor |
| TIRAP | TIR domain containing adaptor protein |
| TLR | toll-like receptor |
| TMR | transmembrane region |

| | |
|---|---|
| TNF-α | tumor necrosis factor alpha |
| Treg cell | regulatory T cell |
| tTreg cell | thymus-derived Treg cell |
| TXA2 | thromboxane A2 |
| UICC | union internationale contre le cancer |
| VEGF | vascular endothelial growth factor |

## VIII. FIGURES PAGE

## IX. TABLES PAGE

## X. REFERENCES


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**EIDESSTATTLICHE ERKLÄRUNG**

Hiermit erkläre ich an Eides statt, die Dissertation: „Immunity, Inflammation and Cancer: The role of Foxp3, TLR7 and TLR8 in gastrointestinal cancer“, eigenständig, d. h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, den 13.11.2015

## LEBENSLAUF

***Publikationen:***

2013:

Kim M, **Grimmig T**, Grimm M, et al. Expression of Foxp3 in colorectal cancer but not in Treg cells correlates with disease progression in patients with colorectal cancer. PLoS One. 2013;8(1):e53630.

Pelz JO, Vetterlein M, **Grimmig T**, et al. Hyperthermic Intraperitoneal Chemotherapy in Patients with Peritoneal Carcinomatosis: Role of Heat Shock Proteins and Dissecting Effects of Hyperthermia. Ann Surg Oncol. 2013;20(4):1105-13.

**Grimmig T**, Kim M, Germer CT, et al. The role of FOXP3 in disease progression in colorectal cancer patients. Oncoimmunology. 2013;2(6):e24521.

2015:

**Grimmig T**, Matthes N, Hoeland K, et al. TLR7 and TLR8 expression increases tumor cell proliferation and promotes chemoresistance in human pancreatic cancer. Int J Oncol. 2015;47:857-66.

***Kongressbeiträge (Erstautorin):***

2011:

**Chirurgische Forschungstage**, Dresden; Vortrag „Resistance to nutrient stress - characteristic for a minor tumor cell population in colon and pancreatic cancer responsible for further tumor growth?"

2012:

**Deutscher Krebskongress**, Berlin; Poster „Cancer initiating cells in colon and pancreatic cancer are resistant to nutrient stress - a characteristic for tumor growth?"

**Chirurgische Forschungstage**, Regensburg; Vortrag „Expression of Foxp3 in colorectal cancer but not in Treg cells correlates with disease progression in patients with colorectal cancer"

2013:

**International Meeting on Molucular-Based Treatment of GI Cancer**, Göttingen; Poster „The role of Foxp3 in disease progression in patients with colorectal cancer" und Poster „Prognostic relevance of circulating chemoresistant tumor cells in the blood of patients with colorectal cancer" (Posterpreis Travel Grant)

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**Deutscher Krebskongress**, Berlin; Poster „Chronic inflammation-mediated tumor cell proliferation and chemoresistance is driving tumor progression in pancreatic cancer”

**Kongress der Deutschen Gesellschaft für Chirurgie**, Berlin; Vortrag „Chronische Inflammation im Pankreaskarzinom: Die Bedeutung intrazellulärer Signalwege für die Tumorprogression“ und Vortrag „The role of ABCB5 expression and circulating tumor cells for tumor-specific outcome analysis in colorectal cancer“

**AACR Annual Meeting**, San Diego, USA; Poster „The potential of PD-1/PD-L1 signaling inhibition outlined from clinical analysis of colorectal cancer”

**Chirurgische Forschungstage**, Hannover; Vortrag: „Clinical significance and therapeutic potential of the PD-1/PD-L pathway in human colorectal cancer”

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**AACR Annual Meeting**, Philadelphia, USA; Poster „Hyperthermia and Chemotherapy mediated effects on tumor cell proliferation and increase of Heat Shock Protein (HSP) expression profiles in human colon cancer cells”

**Jahrestagung der Vereinigung der Bayerischen Chirurgen**, Dachau; Vortrag: „HIPEC bei isolierter Peritonealkarzinose: Effekte der Hyperthermie auf den Zelltod“

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