Studies of the tumor microenvironment

Local and Systemic Effects Exerted by the Cross-talk Between Tumor and Stroma Cells in Pancreatic cancer

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“The only thing that comes to a sleeping man is dreams”
Lesane Parish Crooks

“Reach for the stars, so if you fall you land on a cloud”
Kanye Omari West

“With survival of the fittest, everyday is a challenge”
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List of papers

This thesis is based upon the following papers, which are referred to in the text in the following order:

Paper I

Paper II

Paper III

Paper IV
Abbreviations

AP1  Activator protein 1
APC  Antigen presenting cells
α-SMA  α-smooth muscle actin
BDCA  Blood dendritic cell antigen
bFGF  Basic fibroblast growth factor
BRCA2  Breast Cancer 2 susceptibility protein
BSA  Bovine serum albumin
CAFs  Cancer associated fibroblasts
COX-2  Cyclooxygenase type 2
CP  Chronic pancreatitis
CTLA4  Cytotoxic T-Lymphocyte Antigen 4
DCs  Dendritic cells
DCIR  Dendritic cell immunoreceptor
DC-LAMP  DC-lysosome associated membrane protein
ECM  Extracellular matrix
EGFR  Epithelial growth factor receptor
EP  Prostaglandin E receptor
FAMMM  Familial multiple mole melanoma syndrome
FCS  Fetal calf serum
FGF-2  Fibroblast growth factor 2
GM-CSF  Granulocyte macrophage colony stimulating factor
HGF  Hepatocyte growth factor
IFN  Interferon
IDO  Indoleamine 2,3-dioxygenase
iNOS  Inducible nitric oxide synthase
JAK  Janus kinase
K-RAS  Kirsten rat sarcoma viral oncogene homolog
MAPK  Mitogen activated protein kinases
MMPs  Matrix metalloproteinases
MDCs  Myeloid dendritic cells
MDSCs  Myeloid derived suppressor cells
NO  Nitric oxide
NFκB  Nuclear factor kappa-light-chain-enhancer of activated B cells
PanIN  Pancreatic intraepithelial neoplasia
PDAC  Pancreatic duct adenocarcinoma
PDCs  Plasmacytoid dendritic cells
PDGF  Platelet derived growth factor
PD1-L  Programmed death 1 ligand
PGE\textsubscript{j}  Prostaglandin E\textsubscript{j}
PIGF  Placenta growth factor
PI3K  Phosphatidylinositol 3-kinases
PSCs  Pancreatic stellate cells
Rho  Rho-associated kinase
STAT  Signal transducer and activator of transcription
TAMs  Tumor associated macrophages
TGF-β  Transforming growth factor beta
TH1  T helper 1
TH2  T helper 2
TIR  Toll/IL-1R
TLR  Toll-like receptor
TRAIL  TNF-related apoptosis inducing ligand
Tregs  T regulatory cells
TNF-α  Tumor necrosis factor- α
US  United States
VEGFA  Vascular endothelial growth factor A
VEGFR  Vascular endothelial growth factor receptor
5-Fu  5-fluorouracil
Abstract

Pancreatic cancer is one of the most lethal cancers and despite all research efforts the last 50 years, there is still no effective therapy for this terrible disease. Until quite recently most research in the field of pancreatic duct adenocarcinoma (PDAC) was focused on the tumor cells and mechanisms essential for their proliferation and survival. However, the tumor does not only consist of tumor cells, rather a combination of tumor cells and numerous stroma cell types creating the tumor microenvironment. The tumor cells have developed the ability to activate the surrounding cells to produce factors important for the progression of the tumor. Cancer associated fibroblasts (CAFs) are the major stroma component and as much as 70% of the total PDAC tumor mass consists of these cells. I have investigated the mechanisms involved in the cross-talk between tumor cells and CAFs and distinguished the local and systemic effects of this communication. Tumor derived IL-1α was identified as an important factor creating the inflammatory profile seen in CAFs. In PDAC patients, IL-1α was detected in 90% of the tumors and high expression was associated with poor clinical outcome. Moreover, the PDAC tumors had elevated expression levels of many inflammatory factors that were induced in CAFs by tumor derived IL-1α in vitro. Consequently, this high expression of inflammatory factors in CAFs will attract immune cells including tumor associated macrophages (TAMs), dendritic cells (DCs), and CD8+ T cells. This indicates an immune suppressive role of CAFs, protecting the tumor cells by acting as decoy targets for immune cells homing into the tumor. The inflammatory factors produced in the PDAC microenvironment did not only affect the infiltrating immune cells, but had also systemic effects that included decreased levels of blood myeloid and plasmacytoid DCs in PDAC patients. Furthermore, the DCs were partly activated and had a semi mature phenotype and impaired immunostimulatory function. Low levels of blood DCs were directly associated with poor patient prognosis and the same was seen for low expression of ICOSL by the DCs.

The findings presented in this thesis indicate an essential role for the cross-talk between tumor cells and stroma in the production of tumor promoting factors. Treatment of PDAC patients with drugs that target the IL-1α signaling pathway could prevent the communication between these cells, thus reduce the amount of inflammatory factors both locally and systemically. Altogether, our findings support the idea that neutralization of the IL-1α signaling molecule could be a promising therapy for pancreatic cancer.
Introduction

Epidemiology

Half a century of research has only resulted in minor advances in patient survival in pancreatic duct adenocarcinoma (PDAC), which still has one of the worst outcomes of all types of cancer. This is in contrast to several other cancers, like colon, breast, and prostate, that have had considerable improvement in prognosis over the last twenty years (Li, Xie et al. 2004). To emphasize the severity of this devastating disease, PDAC is only the 10th most frequent cancer in the western world, but with an overall 5-years survival of less than 5%, it is number four concerning cancer mortality (Jemal, Siegel et al. 2007). In Sweden, PDAC is a common gastrointestinal cancer with 1500 new cases annually (Cancerfondsrapporten 2010).

Historically, the incidence of PDAC in the US increased significantly among both males and females from the 1930s and throughout the 1960s. The incidence was stabilized among males during the 1970s and slightly declined during the 1980-90s, while the incidence in females increased throughout the 1970s and stabilized during the 1980-90s (Wingo, Cardinez et al. 2003). Males have throughout the last century been associated with a higher incidence of PDAC, but during the 90s a change in this trend was observed and a slightly higher incidence is now detected among females in both the US and in Sweden (Cancerfondsrapporten 2010). PDAC is rare among young people (<40 years) and it occurs primarily later in life with a peak incidence in the seventies and eighties (Yeo, Hruban et al. 2002).

The pancreatic gland

The pancreas is a retroperitoneal organ situated behind the stomach and the spleen (Figure 1). Its size varies from 12.5 to 15cm and its weight from 60 to 100g. The pancreas consists of three different sections i.e. head (neck), body, and tail, with both exocrine and endocrine functions. Acini cells in the exocrine pancreas secrete three categories of enzymes, each involved in digestion of different food contents, i.e. the proteolytic enzymes trypsinogen, chymotrypsinogen, and procarboxypeptiase, for protein digestion; pancreatic amylase for carbohydrate digestion; and lipase for digestion of fat. The enzyme cocktail secreted by the acini cells is, together with pancreatic juice produced by the duct cells, drained through the pancreatic duct and into the duodenum. The pancreatic juice, which is rich in sodium bicarbonate, neutralizes the highly acidic gastric content to protect the small intestine and
allow optimal function of the pancreatic enzymes (Guturu, Shah et al. 2009; Sherwood. 2009).

Figure 1. Pancreas location.
The pancreas is located behind the stomach and is connected to the small intestine through the Ampulla of vater, where the common bile duct and the main pancreatic duct are joined together to transport digestive enzymes and bile to the small intestine.

Pancreatic stellate cells (PSCs) are resident cells in the exocrine pancreas and are present in low numbers in the periacinar space where they encircle the base of the acinus. In normal healthy pancreas these cells are found in a quiescent state showing a “star” shaped morphology and are characterized by the presence of desmin, vitamin droplets, and glial fibrillary acidic proteins (Omary, Lugea et al. 2007). Upon activation, in response to injury or inflammation, the PSCs lose their vitamin droplets and adapt to a myofibroblast like phenotype also known as cancer associated fibroblasts (CAFs) in solid tumors. These cells serve as key players in the pathobiology of the major disorders of the exocrine pancreas, including chronic pancreatitis and pancreatic adenocarcinoma. (Omary, Lugea et al. 2007; Guturu, Shah et al. 2009).

The endocrine part of the pancreas consists of small clusters of cells, called endocrine islets or islets of Langerhans, and includes several different types of endocrine cells. The two most predominant cell types are the insulin and amylin producing β-cells and the glucagon producing α-cells, which constitute about 75% and 20% of the total endocrine
mass, respectively. These two hormones are essential for the physiological control of glucose homeostasis. The remaining part of the endocrine islets consist of somatostatin secreting δ-cells (4%) and an even smaller fraction of pancreatic polypeptide secreting cells (1%), F cells that produce adrenomedullin and ε-cells producing ghrelin. Moreover, the islets also contain other bioactive agents, including neuropeptides associated with the nerve terminals, such as neuropeptide Y, calcitonin gene-related peptide, and substance P, and agents like pancreastatin, a proteolytic cleavage product of chromogranin (Barreto, Carati et al.).

Pancreatic cancer
Pancreatic duct adenocarcinoma (PDAC) is the predominant tumor in the pancreas, constituting for 85-90% of all the pancreatic tumors, and is usually referred to as pancreatic cancer (Sohn, Yeo et al. 2000). Most of the duct adenocarcinomas arise in the head and neck (60%) of the gland while 15% are located to the body and only 5% in the tail. In 20% of the cases the tumor is located in the entire gland (Allen-Mersh 1982; Lillemoe, Yeo et al. 2000). The tumor metastasizes to a wide variety of tissues and organs, but the most common ones include regional lymph nodes, duodenum, liver, and peritoneum. Other less common metastatic sites are the brain, lungs, kidneys, and skeleton (Pneumaticos, Savidou et al.; Borad, Saadati et al. 2009). The metastatic spread to distant organs and tissues is responsible for about 90% of PDAC deaths (Keleg, Buchler et al. 2003).

Numerous of rather rare types of cancer are found in the pancreas. The most frequent ones are serous cystadenoma/carcinoma (0.8%), solid pseudopapillary tumor (1%), acinar cell carcinoma (1.2%), and pancreatic islet cell tumors (2%), with insulinoma as the most common endocrine tumor (Bardeesy, Morgan et al. 2002) (Abraham, Klimstra et al. 2002; Mulkeen, Yoo et al. 2006). Serous cystadenoma/carcinoma and solid pseudopapillary tumors have a low malignant prospective, while acinar cell carcinoma (mean survival of 19 months) and some of the endocrine tumors are more malignant (mean survival of 40-60 months) (Mulkeen, Yoo et al. 2006)

PDAC development and biology
The transformation of normal duct epithelial cells into invasive adenocarcinoma is believed to gradually develop through the formation of lesions of different morphological grades,
consequently initiating diverse changes in the morphology and functions of the cells. These precursor lesions of PDAC are classified as PanIN (Pancreatic Intraepithelial Neoplasia) and are graded from PanIN-1A (flat mucinous epithelium) to PanIN-3 (in situ carcinoma). PanIN-1A includes lesions of flat mucinous epithelium without any signs of cell abnormalities (atypia), whereas lesions with papillary architecture without atypia are categorized as PanIN-1B. Lesions with increased cell abnormalities and a prevalence of papillary architecture are categorized as either PanIN-2 (low to moderate grade of dysplasia) or PanIN-3 (high grade dysplasia), the latter is considered to be the stage immediately preceding stromal invasion. PDAC tumors are normally featured by a vast desmoplastic stromal reaction (growth of fibrous and/or connective tissue) similar to the morphology observed in chronic pancreatitis, including massive fibrosis and infiltration of immune cells (Figure 2). The fibrotic stroma enwraps the cancer cells (Korc 2007; Mahadevan and Von Hoff 2007) and may account for as much as 70% of the total tumor mass (Froeling, Marshall et al.). PanIN-1B lesions have been shown to obstruct the respective duct, decreasing the flow of pancreatic juice, thereby promoting apoptosis of acinar cells followed by their replacement by fibrosis (Detlefsen, Sipos et al. 2005). The establishment of a fibrotic stroma is probably an early and a very important event in the progression of PDAC.

![Figure 2. PDAC progression model. Cancer development from normal pancreatic ducts via PanIN lesions to invasive adenocarcinoma, and the creation of a functional microenvironment including a desmoplastic reaction (CAFs), immune cell infiltration and tumor angiogenesis.](image)

Mutations in the K-RAS oncogene are one of the earliest genetic abnormalities observed in PDAC and are present in 36% of PanIN-1A, 44% of PanIN-1B, and 87% of all PanIN lesions.
K-RAS mutations have been shown to be essential for spontaneous development of PanIN lesions and invasive adenocarcinomas in mice models. The development of PanIN lesions is also associated with the loss of three different tumor suppressor genes, CDKN2A/INK4A, TP53, and DPC4/SMAD4/MADH4. The CDKN2A/INK4A gene encodes the cell cycle checkpoint protein p16 and loss of p16 function is seen in 71% of PanIN-3 lesions and in 90% of PDAC tumors. The TP53 gene is inactivated in 50-75% of all PDAC tumors and as a consequence of this alteration, the cells are permitted to bypass the DNA damage checkpoints and apoptotic signals. Mutations in the TP53 tumor suppressor gene are usually found in PanIN-3 lesions and are most likely a late event in PDAC development. DPC4 (Deleted in Pancreatic cancer carcinoma 4) is commonly inactivated in PDAC and loss of this tumor suppressor gene results in decreased growth inhibition and uncontrolled proliferation. Loss of DPC4 expression is also a late genetic event in PDAC development and is only found in PanIN-3 lesions (31-41%) (Figure 2) (Hilgers, Rosty et al. 2002; Feldmann, Beaty et al. 2007). Another important genetic event is the loss of telomeric integrity within the epithelial duct cells. Telomeres are important sequences at the end of the chromosome arms that stabilize the chromosome during cell division. More than 90% of the lowest grade of PanIN lesions demonstrate marked shortening of telomeres and the genomic instability observed in PanINs is likely a consequence of this early genetic event and the chronic stress in the tumor microenvironment (Feldmann, Beaty et al. 2007).

Symptoms

The symptomatic course of PDAC is typically brief and progressive and the adenocarcinoma will usually remain silent until it extend and impose on other organs. When the adenocarcinoma erodes towards the rear wall of the abdomen, it affects the nerve fibers and causes pain (55.2%), and this is usually one of the first symptoms, but at this stage the cancer is unfortunately beyond cure. The majority of the patients are typically presented with a yellowish skin color (jaundice) (70.6%) as a result of obstruction of the intrapancreatic portion of the common bile duct, but it rarely draws attention to the invasive adenocarcinoma soon enough (el-Kamar, Grossbard et al. 2003; Hua, Liang et al. 2009). Involuntary weight loss is another frequent symptom for advanced PDAC, caused by decreased secretion of pancreatic enzymes as a consequence of hypercatabolism of pancreatic tissue, leading to malnutrition. The anorexia/cachexia syndrome is also involved in the weight loss and is mediated through the release of cytokines and other factors secreted by the tumor. The weight loss is predictive
of poor clinical outcome and greater morbidity and is further associated with weakness, fatigue, depression, and general poor quality of life (el-Kamar, Grossbard et al. 2003).

Treatment

The only treatment available that offers some hope for cure is radical surgery, but owning to late presentation of symptoms, only 10 to 15% of the patients are candidates for surgical resection (Onoue, Terada et al. 2004). Nevertheless, the aggressive nature and high recurrence rate of PDAC tumors has resulted in disappointing five year survival rates of 11% to 21% after resection (Sohn, Yeo et al. 2000; Diener, Heukaufer et al. 2008). Pancreaticoduodenectomy or Whipple resection (after Allen O. Whipple) (Figure 3) is the most common surgical procedure for resection of tumors in the head of the pancreas. The traditional Whipple resection is a resection of the entire pancreatic head, lower part of the stomach, distal bile duct, including the gallbladder, and duodenum (McGrath, Sloan et al. 1996).

Figure 3. Whipple resection.
Schematic figure of a traditional Whipple resection, involving the removal of the gallbladder, common bile duct, antrum of the stomach, the pancreatic head, duodenum and jejunum (marked in dark color).
For a majority of PDAC patients, surgery is not an option and chemotherapy remains the only treatment. Unfortunately, chemotherapy in advanced PDAC is primarily aimed at palliating symptoms and to ensure a better quality of life and do not change the poor prognosis (Squadroni and Fazio). Two chemotherapies are mainly used, i.e. gemcitabine and 5-fluorouracil (5-Fu). Gemcitabine was found superior to 5-Fu concerning clinical response (23.8% vs. 4.8%), median overall (5.6 vs. 4.4 months) and 1 year survival (18% vs. 2%) and has ever since its approval in 1997 been the standard first-line palliative treatment worldwide for patients with PDAC (Welch and Moore 2007). During the last decade various cytotoxic agents (cisplatin, oxaliplatin, 5-Fu, capecitabine, irinotecan, exetecan, or pemetrexed) have been tested in combinations with gemcitabine, but without benefits for the overall survival time (Stathis and Moore; Welch and Moore 2007). Targeted therapies focusing on multiple signaling pathways involved in the development and progression of PDAC have been tested inducing different inhibitors against RAS, matrix metalloproteinases (MMPs), VEGF, VEGFR, and EGFR. All trials, however, failed to improve the overall survival time and rate when compared with Gemcitabine alone (Stathis and Moore).

Risk Factors
The bad prognosis for individuals with PDAC has lead to focus on why some individuals are more likely than others to develop this type of cancer, and numerous risk factors have been identified. Tobacco smoking is the most important environmental factor and is thought to be involved in as much as 15-30% of all cases (Mulder, van Genugten et al. 1999; Mulder, Hoogenveen et al. 2002). The incidence of PDAC has risen dramatically in many countries as they have become more westernized in their way of living. In accordance, high intake of fat/cholesterol, meat, dairy products, as well as high intake of energy, fried foods, carbohydrates, salt, and general obesity (BMI ≥ 30 kg/m²) has in several independent studies been shown to be associated with development of PDAC (Michaud, Giovannucci et al. 2001). Patients with diabetes mellitus (type 2), have a twofold increased risk of developing PDAC and even the pre-diabetic state glucose intolerance and insulin resistance may play a role in the carcinogenesis (Michaud, Liu et al. 2002; Ghadirian, Lynch et al. 2003; Wang, Herrington et al. 2003). A high consumption of coffee or alcohol has shown no or negligible effects on PDAC development, while increased consumption of fresh fruits and vegetables, fiber, natural foods, and Vitamin C seems to have preventive effects (Ahlgren 1996; Talamini, Bassi et al. 1999; Ghadirian, Lynch et al. 2003).
In approximately 5-10% of all cases, various inherited genetic disorders could play a role (Lynch, Smyrk et al. 1996; Lynch, Brand et al. 2001). The genetic disorders predisposing PDAC include hereditary pancreatitis (30% higher risk), multiple endocrine adenomatosis type 1, glucagonoma syndrome, Lynch 2 variant, Gardner’s syndrome, early-onset familial breast cancer syndrome BRCA2 (Breast Cancer 2 susceptibility protein) germ line mutation, and familial multiple mole melanoma syndrome (Lynch, Smyrk et al. 1996; Yeo, Hruban et al. 2002; Ghadirian, Lynch et al. 2003). Children of parents diagnosed with PDAC have 1.68 fold increased risk for developing PDAC and the mean diagnostic age for this group is 10-15 years earlier (Hemminki and Li 2003).
The tumor microenvironment

Inflammation and carcinogenesis

Cancer originates from mutations that override critical pathways regulating tissue homeostasis, cell survival and cell death (de Visser, Eichten et al. 2006). Germline mutations are rare in cancers, whereas somatic mutations and environmental factors are linked to the vast majority of cancers. Numerous environmental risk factors and initiators of cancer are associated with chronic inflammatory conditions (Grivennikov, Greten et al.). Tumor development and progression has for a very long time been linked to inflammation, based on the hypothesis that some classes of irritants, such as pathogens, asbestos fibers, silica particles, cigarette smoking and even obesity, initiate inflammation and tissue injury, resulting in enhanced cell proliferation (Grivennikov, Greten et al.; Coussens and Werb 2002; Malfertheiner and Schutte 2006; Tan, Fattman et al. 2006). Increased proliferation per se is not enough to cause cancer as it is an important feature in normal homeostasis and wound healing. However, an environment rich in growth factors, agents promoting DNA damage, and proliferating cells, like in inflammation, could lead to cells with the ability to continue to proliferate, even after the inflammation is removed (Coussens and Werb 2002).

In the field of cancer research, much effort has been focused on tumor cell lines and the genetic abnormalities ensuing in their production of growth and anti apoptotic factors. However, a human tumor is not a homogenous self-sufficient mass of mutant cells. The tumor cells are highly heterogeneous with diverse differentiation grades. Furthermore, they are embedded in a non malignant stroma composed of numerous cell types, such as cancer associated fibroblasts (CAFs), epithelial cells, immune cells, and blood and lymph vessels, and extracellular matrix. The success of tumor cells seems to be dependent on their ability to control and shape the surrounding microenvironment to favor their own survival (de Visser, Eichten et al. 2006).

Cancer associated fibroblasts (CAFs)

A hallmark of chronic inflammatory tissues and adenocarcinomas, in particular chronic pancreatitis (CP) and PDAC, is the presence of an abundant fibrotic component, i.e. desmoplasia (Figure 4) (Korc 2007; Mahadevan and Von Hoff 2007).
Figure 4. The cancer associated fibroblasts in PDAC. Hematoxylin–phloxin–saffron staining of a tumor tissue section derived from PDAC patient 065. The staining show the massive desmoplastic reaction, i.e. cancer associated fibroblasts, surrounding the tumor nests. Photo Vegard Tjomsland

The activated fibroblasts found in PDAC tumors originate from PSCs. In normal healthy pancreas these cells are found in a quiescent state where they are characterized by the presence of desmin, vitamin droplets, and glial fibrillary acidic proteins. Upon activation, in response to injury or inflammation, the PSCs lose their vitamin droplets and adapt to a myofibroblast like phenotype (Guturu, Shah et al. 2009). Several major signaling pathways have been found to be involved in the regulation of PSCs differentiation, including mitogen activated protein kinases (MAPK), phosphatidylinositol 3-kinases (PI3K), Rho-associated kinase (Rho), Janus kinase (JAK)/signal transducer and activator of transcription (STAT), activator protein 1 (AP1), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and transforming growth factor β (TGF-β)/SMAD. (Omary, Lugea et al. 2007). In PDAC, tumor cells have the ability to activate PSCs by the release of cytokines (e.g. IL-1, IL-6, CXCL8, and tumor necrosis factor α (TNF-α)) and growth factors (e.g. platelet derived growth factor (PDGF) and TGF-β) (Mahadevan and Von Hoff 2007). When activated, PSCs have the ability to produce autocrine factors, such as PDGF, TGF-β, IL-1, IL-6, TNF-related apoptosis inducing ligand (TRAIL), and cyclooxygenase type 2 (COX-2), that perpetuate the activated phenotype (Omary, Lugea et al. 2007). The transformation into cancer associated fibroblasts (CAFs) is linked with several genetic and morphological changes, including expression of α-smooth muscle actin (α-SMA or ACTA2), vimentin, CXCL12, and podoplanin and secretion of large
amounts of extracellular proteins (Eyden 2008; Gonda, Varro et al. 2009). In PDAC, the CAFs outnumber the tumor cells and may account for 70% of the total tumor mass (Froeling, Marshall et al.) and new evidence points to an important role of the fibrosis in PDAC. The interlinked relationship between tumor cells and its stroma, has been shown to promote tumor growth, and metastasis by supporting vascularization, recruitment of inflammatory cells, and activation of fibroblasts (Liao, Luo et al. 2009).

Tumor infiltrating immune cells

Another stromal component important for creation and homeostasis of the inflammatory tumor microenvironment is the infiltrating immune cells. Infiltrating immune cells support tumor progression by the release of growth and survival factors, matrix remodeling factors, and reactive oxygen species (Erez, Truitt et al.). Tumor cells have the ability to produce numerous of chemotactic cytokines and chemokines that attract these leukocytes. During carcinogenesis even the surrounding stroma cells acquire this feature and increase the amount of chemoattractants produced (Coussens and Werb 2002). Among the cells in the stroma, CAFs are a major producer of several chemotactic cytokines, including CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, and CXCL12), CC chemokines (CCL2 and CCL20), and chemotactic growth factors (VEGF and PDGF) (Kogan-Sakin, Cohen et al. 2009; Tjomsland, Spångeus et al. 2010). The inflammatory microenvironment in cancer includes a diverse leukocyte population of neutrophils, dendritic cells (DCs), macrophages, eosinophils, mast cells, and T cells. These cells will also recruit additional immune cells to the tumor milieu through secretion of chemotactic factors.

Dendritic cells

DCs are professional antigen presenting cells (APCs), specially equipped for capture, processing, and presentation of antigens and subsequent activation of naïve T cells, central memory T cells, and B cells (Chehimi, Campbell et al. 2002; Vakkila, Thomson et al. 2004). DCs are named by their ability to stretch out very long motile arms, i.e. dendrites, and this ability gives them a very large contact surface that they can use to sense their surroundings. These cells are a heterogeneous population of bone marrow origin and are generally divided into three differentiation stages, precursors, immature, and mature DCs (O’Neill, Adams et al. 2004). Two principal populations of DCs exist in human blood and tissues, i.e. the myeloid
DCs (MDCs) derived from myeloid precursors and plasmacytoid DCs (PDCs) derived from lymphoid precursors developing within primary lymphoid tissues from CD34+ human stem cells (Blom, Ho et al. 2000). In blood, the MDCs and PDCs constitute less than 1% of the peripheral blood mononuclear cells (PBMCs) (Hashizume, Horibe et al. 2005; Steinman 2007). These two subtypes do not only differ in phenotype, but also in tissue distribution, cytokine production and growth requirements.

DCs have a big repertoire of surface receptors that are used for their different functions and the profile of this repertoire is affected by the microenvironment and the stimuli given to the DCs. MDCs and PDCs share several common features such as the expression of MHC class II molecules (HLA-DR), CD4, DCIR, PD1-L, B7H3, ICOSL, and lack of cell lineage specific markers for T cells (CD3), monocytes (CD14), B cells (CD19 and CD20), and NK cells (CD56). Different lectin binding receptors, e.g. DC-SIGN, MMR, DCIR, and DEC-205 are involved in the uptake and transport of antigens into special compartments in the DCs (O’Neill, Adams et al. 2004; Steinman 2007). The interaction with other immune cells such as T cells involves members of the B7 receptor family and some of these costimulatory molecules are of great importance for the activation of effector T cells and include CD80, CD86, and CD40, whereas others are considered important for suppressing immune responses, i.e. PD1-L and B7H3. DCs also express receptors guiding their distribution into different tissues and/or migration to sites of inflammation including CCR1, CCR2, CCR5 and CCR8. The expression of most

**MDCs**
- BDCA1
- BDCA3
- CD11c
- TLR1
- TLR2
- TLR3
- TLR5
- TLR6
- TLR8

**PDCs**
- BDCA2
- BDCA4
- CD45RA
- TLR7
- TLR9
- CD45RA
- CD123
- Type 1 interferons

Figure 5. Characteristic surface markers for immature MDCs and PDCs in peripheral blood.
chemokine receptors on circulating blood MDCs and PDCs are comparable, but the levels of CCR5, CCR7, and CXCR3 are higher on PDCs than on MDCs (Penna, Sozzani et al. 2001). DCs express an array of cytokine receptors including IL-6 receptor, IL-10 receptor, IL-18 receptor, IFN-γ receptor, Granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor (Ghirelli, Zollinger et al.; O’Neill, Adams et al. 2004). Some surface molecules distinguish the blood MDCs from the PDCs, and include CD11c, CD1c (blood DC antigen (BDCA1)), and CD141 (BDCA3), TLR3, and TLR5 for MDCs, and CD45RA, and CD123, TLR7, and TLR9 for PDCs (Figure 5) (Ju, Clark et al.; Cao and Liu 2007).

The MDCs are ubiquitously distributed within the body and are found in, e.g. skin, liver, lung, heart, intestine, pancreas, and lymphoid system. MDCs migrate from the bone marrow into the peripheral blood and out in peripheral tissues. Immature DCs in tissues are characterized by high endocytic activity and are constantly sampling their surroundings and have the role of sentinels that can sense danger, i.e. pathogens and tissue damage, by their different pattern recognition receptors and alert the immune system. This exposure leads to their reprogramming and migration to the lymphoid tissue where they can mount a specific immune response against the pathogen (Steinman 2007). The quality of the immune response depends on the initial activation and programming the DCs received at the site of it activation.

PDCs are located in lymphoid nodes, spleen, tonsils, and Peyer’s patches and have morphologic features similar to plasma cells (Ghirelli, Zollinger et al.; O’Neill, Adams et al. 2004; Cao and Liu 2007). PDCs produce a vast amount of type 1 IFNs when exposed to pathogens and were first known as interferon producing cells before it was clear that they belonged to the DC family. PDCs regulate inflammation and are an important link between innate and adaptive immunity through the production of type 1 IFNs (Colonna, Trinchieri et al. 2004). The primary locations of PDCs are blood and around high endothelial venules in T cell areas of lymphoid organs where they can induce tolerance through secretion of IL-4 and IL-10 or T helper 1 (TH1) responses depending on their initial stimuli and activation (Ghirelli, Zollinger et al.; Colonna, Trinchieri et al. 2004; Shurin, Shurin et al. 2006). The role of PDCs has been studied in antiviral immunity, but PDCs are also involved in induction of tumor immunity and peripheral tolerance (Pan, Ozao et al. 2008). Interestingly, PDCs can express large amount of IDO and in the lymphoid organs this encourage T cell death (Herbeuval and Shearer 2007).

The balance between GM-CSF and IL-3, the only known cytokines that promote the survival and differentiation of PDCs, has been shown to regulate PDCs ability to promote TH1 or T helper 2 (TH2) responses. PDCs activated with GM-CSF produced more IFN-γ and
less IL-4 and IL-10 compared to PDCs activated by IL-3, indicating an important role for GM-CSF in the modulation of a TH1 response (Ghirelli, Zollinger et al.). PDCs migrate from the bone marrow to the peripheral blood, but in contrast to MDCs, they relocate directly from the blood into secondary lymphoid tissue without the need to encounter any antigen (Liu 2005; Cao and Liu 2007).

Studies of mouse pancreas have found DCs in the Langerhans islets, whereas very little is known about the MDCs and PDCs location in the normal human pancreas (Calderon, Suri et al. 2008).

DCs in the tumor microenvironment

DCs are believed to be among the first cells migrating to the tumor site where they can identify and eliminate tumor cells on the basis of their expression of tumor specific antigens or molecules induced by cellular stress (Shurin, Shurin et al. 2006). This recruitment is propagated through the release of cytokines, such as VEGF, β-defensin, CXCL12, HGF, and CXCL8, by the tumor and stroma cells (Murdoch, Muthana et al. 2008). Infiltrating DCs are found in different tumors and several lines of evidence have suggested that these cells play a role in anti tumor immune responses. For instance, several studies indicate that high levels of infiltrating DCs are associated with better clinical outcome in a variety of human cancers (Shurin, Shurin et al. 2006; Talmadge, Donkor et al. 2007). Unfortunately, the inflammatory nature of the tumor microenvironment will influence the infiltrating leukocytes, e.g. DCs, by turning them into cells with the ability to suppress immune responses instead of activating them. The production of both chemotactic and immunosuppressive chemokines by the tumor and stroma cells is a terrible combination, giving rise to tumor infiltrating suppressor cells that will contribute to the survival and progression of the tumor. As a consequence, the tumor specific T cells that should destroy the tumor are incapacitated.

One mechanism involved in sequestering of DCs in the tumor is the production of CXCL8 by tumor and stroma cells (Feijoo, Alfaro et al. 2005) seeing that the DCs express receptors, e.g. CXCR1 and CXCR2, that pull them towards CXCL8. The maturation process induces downregulation of tissue retaining receptors on the DCs including CXCR1, CXCR2, CCR1, CCR2, CCR4, CCR5, and CCR6 and upregulation of CCR7 that allows migration to the lymphatics. Migration to lymphoid tissues by CCR7 positive DC is driven by CCL19 and CCL20, which are expressed at high levels in the T cell area of lymph nodes by interstitial DCs and stromal cells (Talmadge, Donkor et al. 2007). DC maturation enhances the expression
of MHC I and II molecules and costimulatory molecules including B7-1 (CD80) and B7-2 (CD86), cytokine secretion, and ability to prime naïve T cell responses (Figure 6). In addition, mature DCs express CD83 and CD208 (DC-LAMP: DC lysosome associated membrane protein), (Ladanyi, Kiss et al. 2007). DC-LAMP expression is induced in the later stages of DC maturation and its definite function has yet to be established (Elliott, Scolyer et al. 2007). CD83, a transmembrane-bound glycoprotein and member of the IgG superfamily, is the best known cell surface marker for mature human DCs. CD83 is detected inside monocytes, macrophages, and immature DCs, but only mature DCs and some activated T and B cells show stable surface expression (Prechtel, Turza et al. 2007). The exact role for CD83 in the activation of T cells has not been quite established, but evidence points to enhancing effects on the T cell stimulatory capacity of mature DCs (Prechtel, Turza et al. 2007).

![Mature DCs](image)

Figure 6. Phenotypic features of mature DCs.

DCs in healthy tissues have a phenotype favoring tissue surveillance and maintenance of peripheral tolerance (Talmadge, Donkor et al. 2007), whereas tumor infiltrating DCs have an altered phenotype with features characteristic of both mature and immature DCs, i.e. semi mature DCs (Fainaru, Almog et al.). For instance, colon cancer patients with high infiltration of CD208+ DCs in their tumors had poor prognosis (Melief 2008), whereas the presence of CD83+ and/or CD208+ DCs are associated with better clinical outcome compared to immature DCs in melanoma, breast, and colorectal cancer (Movassagh, Spatz et al. 2004; Talmadge, Donkor et al. 2007). Moreover, DCs with a more immature phenotype promote tumor angiogenesis and tumor growth, while their mature counterparts do not (Fainaru,
Almog et al.). Furthermore, the DCs themselves, i.e. PDCs, located in ovarian cancer induced angiogenesis through production of TNF-α and CXCL8, thus contributing to the progression of the tumor (Yigit, Massuger et al.).

These observations demonstrate an important role, not only for the amount of infiltrating DCs, but also for the maturation state and location of the DCs inside the tumor (Talmadge, Donkor et al. 2007). This manipulation of the DC activation status is probably a consequence of tumor/stroma derived factors, restricting the development of fully functional mature DCs (Lechner, Liebertz et al.; O’Neill, Adams et al. 2004; Murdoch, Muthana et al. 2008). DCs found in tumors are not fully matured cells instead they have a semi mature phenotypic profile (Belkaid and Oldenhove 2008). The tumor microenvironment can via expression of the COX-2 metabolite prostaglandin E$_2$ (PGE$_2$) induce indoleamine 2,3-dioxygenase (IDO) expression in some of the DCs. IDO positive DCs have the capacity to suppress the immune system by the induction of regulatory T cells (Tregs), thereby inhibiting specific tumor cell immune responses (Munn and Mellor 2007; Belkaid and Oldenhove 2008; Katz, Muller et al. 2008). Moreover, the IDO immune suppressor mechanism is also used by Tregs, as these cells can trigger high IDO expression in DCs through the cross linking of CTLA4 to CD80 and CD86. New evidence also points to a closely coupled positive feedback system in which Tregs induce IDO and IDO drives the differentiation of new Tregs (Curti, Trabanelli et al.; Munn and Mellor 2007). These synergistic tolerogenic mechanisms enhance the suppressor function in Tregs and inhibit the cytotoxic T cell killing of tumor cells. (Munn and Mellor 2007).

Tumor associated macrophages

Macrophages are differentiated from the mononuclear phagocytic lineage and express CD14, CD68, CD163, CD16, CD312, and CD115, all markers for this lineage (Qian and Pollard). These highly flexible, multifunctional cells are characterized by their ability to engulf microbes, apoptotic and necrotic cells, secrete a broad array of immune modulatory cytokines and adapt their phenotype to the microenvironment they reside within (Murdoch, Muthana et al. 2008). Most commonly activated macrophages are classified as M1 or M2 cells. The M1 macrophages are involved in the response of TH1 cells to pathogens and are characterized by high capacity to present antigen, production of proinflammatory cytokines (IL-1, IL-12, TNF-α, IFN-γ), generation of reactive oxygen intermediates, nitric oxide (NO), and the ability to kill pathogens and cells. By contrast, the M2 macrophages express an immunosuppressive
phenotype, initializing TH2 type responses through production of IL-10, ensuing humoral immunity, and the promotion of angiogenesis, tissue remodeling and repair (Qian and Pollard; Murdoch, Muthana et al. 2008; Porta, Rimoldi et al. 2009). The recruitment of monocytes to the tumor is mainly driven by CCL2, a chemokine produced principally by CAFs (Murdoch, Muthana et al. 2008; Tjomsland, Spångeus et al. 2010), while differentiation and growth of macrophages are regulated by several growth factors, including CSF-1, GM-CSF, and IL-3. Tumor infiltrating macrophages, i.e. tumor associated macrophages (TAMs) have generally a M2 skewed phenotype. TAMs are believed to support tumor progression by production of a wide array of growth factors and cytokines important in lymphogenesis and angiogenesis. TAMs also exhibit important immune suppressive features in tumors, through the production of IL-10, effectively diminishing the functionality of tumor specific cytotoxic T cells (Coussens and Werb 2002). High levels of TAMs are observed in most malignant tumors and are associated with poor prognosis.

**Tumor angiogenesis**

Blood vessels are developed through two different mechanisms, the formation by differentiation of endothelial cell precursors during embryogenesis and formation by angiogenesis where new blood vessels are created by budding or splitting of pre-existing vessels (Li and Eriksson 2001). Most blood vessels remain quiescent during adulthood, but retain the ability to expand rapidly in response to physiological stimulus, such as hypoxia for blood vessels and inflammation for lymph vessels. This process is regulated by the balance between angiogenic stimulators and inhibitors, and when skewed it results in an angiogenic switch. Reactivation of angiogenesis is a crucial event in wound healing and tissue repair, but also in other conditions like malignant and inflammatory disorders (Carmeliet 2005).

As often in the case of malignancy, the steps involved in the progression are frequently paralleled by normal physiological events. The similarities between wound healing and tumors are obvious, and angiogenesis is not an exception. The process of wound healing is very complex and it involves a cross-talk between epithelial cells and the stromal microenvironment, through numerous paracrine, autocrine, and mechanical factors (Condon 2005; Ishii, Imamura et al. 2009). The initial steps of repairing injured tissue include activation of aggregating thrombocytes that attract numerous immune cells and myofibroblasts. Macrophages activated at the site of injury produce growth factors, such as TGF-β, VEGF, PDGF, and FGF-2. These factors do not only stimulate angiogenesis, but also activate
local fibroblasts to proliferate and produce components of the extracellular matrix (ECM), including type 1 and 3 collagen and fibronectin (Condon 2005). Once the wound healing process is completed, the majority of the fibroblasts are removed by apoptosis (Rasanen and Vaheri). Of note, angiogenesis is essential for the growth of solid tumors beyond 1-2 mm³ (Bhowmick, Neilson et al. 2004; Dineen, Lynn et al. 2008). The cancer cells could promote angiogenesis directly by secreting VEGF, bFGF, CXCL8, and placenta growth factor (PIGF), and also indirectly by taking over the role of the thrombocytes, attracting immune cells, and by the transformation of stellate cells to highly proliferating CAFs. TAMs produce and express numerous of proangiogenic and angiogenesis modulating factors, such as VEGF, bFGF, HGF, VEGFR1, tissue factor 3, MMP7, MMP9, MMP12, IL-1β, CXCL8, and COX-2 (Murdoch, Muthana et al. 2008).

CAFs role in tumor angiogenesis

CAFs seem to play an essential role in the tumor vascularization, supporting the creation of new blood vessels both directly and indirectly by producing ECM components, chemokines (CXCL8 and CXCL12), MMPs (MMP1-3, 7, 9, and 13-14), VEGF, and COX-2 (Rasanen and Vaheri). The phenotype of CAFs is well adjusted for supporting angiogenesis, by high expression of proangiogenic ELR+ CXC chemokines and no expression of angiostatic ELR negative CXC chemokines, such as CXCL4, CXCL9 and CXCL10 (Coussens and Werb 2002; Tjomsland, Spångeus et al. 2010). Moreover, CAFs are the key partners of TAMs in the tumor microenvironment and by over-expressing chemokines, such as CCL2, they recruit these cells into the tumor stroma and the infiltration has been found to correlate with the levels of CCL2 and disease stage in several adenocarcinomas (Ksiazkiewicz, Gottfried et al.; Rodrigues-Lisoni, Peitl et al.).

Tumor associated factors

Inflammation is an essential event in the development and progression of tumors and is suggested to be the seventh hallmark of cancer (Colotta, Allavena et al. 2009). The inflammatory environment is created by the release of proinflammatory cytokines, defined as “alarm cytokines” present early in the carcinogenesis, such as TNF-α, IL-1α, and IL-1β. These cytokines initiate inflammatory responses and are secreted by infiltrating leukocytes
and malignant cells. Besides being inflammatory initiators, these cytokines also induce expression of other proinflammatory genes, such as COX-2, inducible nitric oxide synthase (iNOS), chemokines, cytokines, and MMPs (Apte and Voronov 2008)

Interleukin 1 (IL-1)

The IL-1 family includes eleven different ligands that share some amino acid sequence homology. The most extensively studied family members are IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1RA), but during the last years the effects exerted by IL-18 and IL-33 have also been well characterized. The release of IL-18 induces IFN-γ expression in IL-12 primed naïve T cells and promotes the differentiation of TH1 cells, while IL-33 promotes responses mediated by TH2 cells by binding to the IL-1 receptor protein. The last six members of the IL-1 family have not yet been fully elucidated and if they function as agonists or antagonists is rather unclear. The IL-1 agonists, IL-1α and IL-1β, are exceptionally potent inducers of inflammation, serving as multifunctional cytokines, primarily affecting inflammatory and immune responses, hematopoiesis, and regulation of other homeostatic functions of the body (Apte and Voronov 2002). The two agonists and IL-1RA are all products of different genes, located close to each other in the human chromosome 2q14 region. IL-1α and IL-1β only share 22% of the amino acid sequences, while IL-1RA has 18% and 26% homology with IL-α and IL-1β, respectively, they all bind to the same receptors (Burger, Dayer et al. 2006).

Both IL-1α and IL-1β are synthesized as 31 kDa precursor peptides and further processed by either calpain proteases (IL-1α) or caspase-1 (IL-1β) to generate 17kDa mature IL-1α and IL-1β. The most obvious difference between IL-1α and IL-1β is that IL-1α is biological active both as precursor and as mature peptide, while pro-IL-1β is inactive and need to be processed to induce cellular responses. The cleavage of pro-IL-1β to the mature form also includes cell secretion of active IL-1β. Consequently, IL-1β is inactive intracellularly, while IL-1α has the ability to act through nuclear translocation exerting intracellular activities. Whereas IL-1β exert its functions as a secreted cytokine, IL-1α predominantly, but not only, act as an active membrane form (23kDa) derived from myristoylation of pro-IL-1α and is anchored to the membrane through a mannose-like receptor exerting its function by stimulating cells by direct contact (Burger, Dayer et al. 2006; Nazarenko, Marhaba et al. 2008). This confines the direct effects of IL-1α to its nearby surroundings, while IL-1β has the ability to induce inflammatory responses throughout the body. As a consequence, the production of IL-1β is more tightly regulated at several levels compared to IL-1α, including
gene transcription, mRNA turnover, translation, and the conversion of the inactive pro-IL-1β to the mature biological active form (Burger, Dayer et al. 2006; Apte and Voronov 2008). This is observed in fibroblasts simultaneously stimulated with recombinant TNF and IL-1, which gave increased expression of IL-1β mRNA and pro-IL-1β, but no secretion of IL-1β. Although, monocytes incubated at the same conditions produced high levels of soluble IL-1β, indicating differences in the way IL-1β is processed and produced by various cell types (Elias, Reynolds et al. 1989).

IL-1α, IL-1β, and IL-1RA have the ability to bind three different receptors, IL-1R1, IL-1R2, and IL-1RAP (IL-1R accessory protein). The IL-1 receptors are characterized by immunoglobulin like extracellular domains and except for IL-1R2, a cytoplasmic region of a conserved sequence called Toll/IL-1R (TIR) domain (Lee, Wang et al.; Apte, Dotan et al. 2006). IL-1 ligation to IL-1R1 induces recruitment of IL-1RAP followed by downstream signaling and activation NFκB. In tumors, activation of NFκB transcription factors are associated with tumor cell survival, while NFκB activation induces expression of proinflammatory cytokines in immune cells (Apte and Voronov 2008). IL-1RA binds to IL-1R1 with the highest affinity of all the ligands, but does not induce any intracellular response. The off rate for IL-1RA is slow and the binding to cell surface IL-1R1 is almost irreversible, thus it functions as an optimal inhibitor of the IL-1 agonists. IL-1R2 has a short cytoplasmic domain and is unable to transduce any intracellular signaling, and thus functions as a decoy receptor by binding IL-1β with higher affinity than IL-1α and IL-RA (Apte, Dotan et al. 2006; Burger, Dayer et al. 2006).

IL-1 and cancer
IL-1α and IL-1β is defined as proinflammatory cytokines predominantly produced by mononuclear cells, initiating immune responses, causing inflammation, and induction of proinflammatory genes. Furthermore, IL-1 is proposed to be involved in the earliest stages of carcinogenesis by stimulating phagocytes and fibroblasts to produce mutagenic reactive oxygen intermediates and can also stimulate proliferation of the pre-malignant cells. In the tumor arena, IL-1 is produced by the malignant cells in addition to stromal cells and infiltrating leukocytes in response to factors secreted by tumor cells or as part of the inflammatory response to the tumor (Apte and Voronov 2008). Moreover, high IL-1β concentrations within the tumor microenvironment in cancers such as melanomas, colon, lung, and head and neck cancers are associated with a more aggressive tumor phenotype (Kock, Schwarz et al. 1989; Chen, Colon et al. 1998; Gemma, Takenaka et al. 2001). IL-1α is expressed by the tumor cells
in several different malignant cell types, including breast, gastric, pancreatic, prostate, head and neck, liver, lung, cervix, and biliary duct (Chen, Malhotra et al. 1999; Tomimatsu, Ichikura et al. 2001; Singer, Hudelist et al. 2006; Rhim, Kim et al. 2008; Kogan-Sakin, Cohen et al. 2009; Melisi, Niu et al. 2009). In PDAC mouse models, liver metastasis has only been observed in cell lines expressing high levels of IL-1α. Moreover, exogenously added IL-1α favors the metastatic and invasive behavior of PDAC cells in vitro (Melisi, Niu et al. 2009). Tumor derived IL-1α has been shown to induce the overexpression of prometastatic factors, such as CXCL8 and IL-6 in both breast cancer cells and in stromal fibroblasts (Nozaki, Sledge et al. 2000). IL-1α was more pronounced in differentiated tumors and showed a significant correlation with liver metastasis in gastric tumors (Tomimatsu, Ichikura et al. 2001). A prostate cancer study revealed an IL-1 dependent upregulation of CXCL1, CXCL2, CXCL3, and CXCL8 in stromal cells incubated in condition medium from immortalized prostate epithelial cells (Kogan-Sakin, Cohen et al. 2009).

Tumor cells seem to have the ability to use IL-1 as an autocrine and also paracrine factor, promoting a tumor beneficial environment consisting of growth, angiogenic, anti-apoptotic, and immunosuppressive factors (Wolf, Chen et al. 2001; Voronov, Shouval et al. 2003; Niu, Li et al. 2004; Rhim, Kim et al. 2008; Kogan-Sakin, Cohen et al. 2009; Melisi, Niu et al. 2009; Tjomsland, Spångeus et al. 2010).

Interleukin 6 (IL-6)

IL-6 is a cytokine originally identified as a B cell differentiation factor that induced the final maturation of B cells into antibody producing plasma cells (Kishimoto, Akira et al. 1995). However it is now known that the cytokine affects a variety of biological functions, including acute phase reaction, cell growth, differentiation, survival, migration during immune responses, hematopoiesis, and inflammation (Ohtani, Ishihara et al. 2000; Park, Nakagawa et al. 2004). The members of the IL-6 family have all a 4-helical bundle structure and subunit in their respective receptor complexes; known as signal transducer gp130. Besides IL-6, this family includes ciliary neurotrophic factor, IL-11, leukemia inhibitory factor, oncostatin M, cardiotrophin 1, and cardiotrophin like cytokine (Febbraio 2007). The responses of IL-6 are transmitted through a glycoprotein complex consisting of one membrane bound binding receptor (IL-6Rα) and the signal transducer gp130. IL-6 can also bind to a soluble form of the IL-6Rα (sIL-6Rα) and this creates a complex, which bind and activates membrane bound gp130. The universal expression of gp130 in tissue provides IL-6 trans-signaling with the
ability to activate cells that do not express the IL-6Rα (McLoughlin, Jenkins et al. 2005). IL-6 signaling activates Janus kinases ensuing recruitment of signal transducing molecules such as STAT3 that translocates to the nucleus (Ohtani, Ishihara et al. 2000). STAT3 homodimers modulate the expression of proinflammatory genes (including IL-6 itself), crucial for the acute phase response and cancer promoting inflammatory conditions. STAT3 signaling is highly interconnected with NFκB signaling through its activation by IL-1 ligation, leading to release of several inflammatory factors important for STAT3 activation, including IL-6. STAT3 and NFκB are both consistently activated in tumors, transducing intracellular signals from extracellular stimuli leading to upregulation of genes involved in proliferation, survival, angiogenesis, migration, and inflammatory factors known to promote cancer. IL-6, produced either by fibroblasts or bone marrow derived myeloid cells, has the ability to activate STAT3 in both inflammatory cells and epithelial cells. This activation promotes carcinogenesis by upregulation of genes involved in cell proliferation and survival. The STAT3 induced release of inflammatory factors and ligation to their respective receptors also activates STAT3, thus creating a feed forward loop between tumor cells and immune cells in the tumor microenvironment (Yu, Pardoll et al. 2009). As a consequence, the persistent STAT3 activation mediates T cell infiltration in acute inflammation, alteration of DC differentiation by downregulation of costimulatory molecules, Treg expansion in tumors, TH17 cell development, and immune suppressive and tumor promoting effects by TAMs and myeloid derived suppressor cells (Park, Nakagawa et al. 2004; McLoughlin, Jenkins et al. 2005; Yu, Pardoll et al. 2009).

Chemokines
The chemokine superfamily includes about 50 low chemotactic cytokines and 20 different receptors, which are involved in several biological processes, such as immune cell chemotaxis, embryogenesis, angiogenesis, hematopoiesis, atherosclerosis, tumor progression, and HIV infection (Balestrieri, Balestrieri et al. 2008; Vandercappellen, Van Damme et al. 2008). The sequence homology among the chemokines is highly variable especially between different subfamilies. The chemokines act either as homeostatic or inflammatory cytokines and are classified based on their structural differences and functionality (Balestrieri, Balestrieri et al. 2008). Homeostatic chemokines are constitutively expressed in the body and plays a pivotal role in the development and maintenance of the hematopoiesis and the immune system, while the inflammatory chemokines are induced as a result of inflammatory stimuli.
Their expression is regulated by proinflammatory cytokines released into the inflammatory environment (Vandercappellen, Van Damme et al. 2008). The chemokines can further be divided into four subgroups, CXC, CC, CX3C, and C chemokine ligands, according to the number and the spacing of the first two conserved cysteine residues in the amino terminal part of the protein (Balestrieri, Balestrieri et al. 2008).

CXC chemokines

The CXC chemokine family includes 16 ligands and a total of 8 receptors and several of these chemokine can bind multiple receptors. The family is characterized by four highly conserved cysteine amino acid residues separated by a single non-cysteine residue, representing the letter X (cysteine - non cysteine - cysteine) (Balestrieri, Balestrieri et al. 2008). The CXC chemokine structure consists of a disordered N-terminus dictating the receptor specificity. The characteristic of the N-terminus also subdivides the CXC chemokines into two categories depending on the presence or absence of three amino acid residues, glutamine - leucine - arginine, the so called “ELR motif” (Strieter, Burdick et al. 2006). This motif is critical for the functional activity of the chemokine. CXC family members that contain the ELR motif (ELR+) are potent promoters of angiogenesis, while the members that lack the motif (ELR-) are angiostatic (Strieter, Belperio et al. 2004). The ELR+ angiogenic CXC chemokine members include CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8. The receptors for the ELR+ CXC chemokines are CXCR1 and CXCR2, but only CXCL6 and CXCL8 have the ability to bind to CXCR1 (Strieter, Burdick et al. 2006). These receptors are membrane bound G protein-coupled receptors that possess 78% amino acid level homology with each other. CXCR2 is expressed by endothelial cells and bind all ELR+ chemokines with high affinity and is found to be the putative receptor for ELR+ CXC chemokine induced angiogenesis (Addison, Daniel et al. 2000). Endothelial cells respond to the angiogenic chemokines by rapid accumulation of stress fiber, chemotaxis and enhanced proliferation resulting in the formation of neovascularization (Strieter, Belperio et al. 2004; Balestrieri, Balestrieri et al. 2008). Binding of CXC ELR+ chemokines to respective receptors on neutrophils, results in recruitment into inflamed tissue and these cells could also have an impact on angiogenesis through secretion of VEGF, which induces secretion of CXCL8 from endothelial cells that help formation and maintenance of CXCL8 dependent capillary like structures (Strieter, Burdick et al. 2006). In addition, ELR+ chemokines do not only attract the tumor infiltrating immune cells, but exert direct effects on tumor cells, contributing to tumor cell transformation, migration and
growth. CXCL8, previously known as IL-8, was the first described angiogenic chemokine and in addition to attract neutrophils, CXCL8 is the only member of the ELR+ family that also attract basophils, T cells, DCs, and monocytes, and the expression levels of CXCL8 correlate with bad prognosis in different solid tumors (Eck, Schmausser et al. 2003; Gordon, Li et al. 2005; Balestrieri, Balestrieri et al. 2008; Vandercappellen, Van Damme et al. 2008; Bendrik and Dabrosin 2009). CXCL8 is secreted by many different cell types, including tumor cells, after exposure to proinflammatory cytokines and mediators, such as TNF-α, COX-2, and IL-1 (Pold, Zhu et al. 2004; Strieter, Burdick et al. 2006; Vandercappellen, Van Damme et al. 2008). In prostate cancer, stroma cells express CXCL1, CXCL2, CXCL3, and CXCL8 due to the IL-1 produced by the epithelial cells and this facilitates inflammation, cancer development, and progression (Kogan-Sakin, Cohen et al. 2009). Moreover, pancreatic cancer cell lines expressing high levels of IL-1α enhanced the expression of all ELR+ chemokines, apart from CXCL7, in CAFs. Exposure to IL-1RA almost abolished the expression of these chemokines, demonstrating a fundamental role of IL-1α in the regulation of angiogenic CXC chemokines in pancreatic cancer (Tjomsland, Spångeus et al. 2010). The ELR+ CXC chemokines are essential mediators of angiogenesis during tumorigenesis and their expression levels correlate with tumor vascularity depending on the origin of the tumor, including CXCL8 and CXCL5 in non small cell lung cancer (NSCLC) and CXCL1, CXCL2, and CXCL3 in melanoma (Strieter, Belperio et al. 2004; Strieter, Burdick et al. 2005).

The remaining members of the CXC chemokine members are ELR- and inhibit endothelial cell proliferation, chemotaxis, hematopoiesis, and activation of TH1 cells, natural killer cells (NK), macrophages, and DCs. These CXC chemokines have angiostatic properties, thus inhibiting tumor progression and include the angiostatic CXCL4, CXCL4L1, CXCL9, CXCL10, CXCL11, CXCL13, CXCL14, CXCL16, and CXCL17. Of note, CXCL12 has demonstrated angiogenic activity by binding to its receptor CXCR4 and several studies show that CXCR4 promotes tumor progression by direct or indirect mechanisms. Moreover, CXCR4 is also essential in tumor cell migration to distant organs expressing CXCL12 (Balestrieri, Balestrieri et al. 2008). The common expression of CXCR4 on tumor cells and the expression of CXCL12 in numerous tissues, including liver, lung, lymph nodes, adrenal glands, and bone marrow, suggests direct homing of metastatic tumor cells to these organs. Evidence for this mechanism is higher levels of CXCR4 positive tumor cells in metastasis found in these organs compared to the primary tumors in vivo (Kulbe, Levinson et al. 2004). Of note, high expression of CXCL12 in the primary tumor can retain the tumor cells and function as an anti-metastatic mechanism (Ooi and Dunstan 2009).
CC Chemokines

The CC family of chemokines is characterized by two consecutive highly conserved cysteine residues and is the largest subgroup including 28 different cytokines and 10 receptors (Raman, Baugher et al. 2007; Richmond, Yang et al. 2009). Several members of the CC chemokine family are associated with the malignant process, including proliferation, angiogenesis, metastasis, and chemotaxis of leukocytes. CCL2, earlier known as monocyte chemoattractant protein, is found expressed by both tumor cells and none neoplastic cells, e.g. CAFs, and is upregulated in multiple cancers (Mishra, Banerjee et al.; Zhang, Patel et al.). The level of CCL2 expression in ovarian cancer correlates with the infiltration of macrophages and lymphocytes (Kakinuma and Hwang 2006). Moreover, the expression level of CCL2 has been shown to correlate with clinical stage and grade in patients with bladder cancer (Loberg, Ying et al. 2007). Nevertheless, high levels of CCL2 and subsequent infiltration of macrophages in pancreatic cancer are associated with good prognosis (Monti, Leone et al. 2003). CCL2 binds with high affinity to the G protein coupled receptor CCR2 and can regulate the recruitment of monocytes, memory T cells, NK cells and macrophages. Moreover, CCL2 has recently been shown to play a key role in development of chronic inflammation by promoting tumorigenesis and metastasis (Zhang, Patel et al.; Marra 2005). CCL2 can act as a potent proangiogenic factor, by binding to CCR2 expressed on endothelial cells, promoting creation of new blood vessels through endothelial cell migration.

However, angiogenesis induced by CCL2 is also associated with recruitment of monocytes from the bloodstream into the tissue. In the tissue, CCL2 will direct the differentiation of monocytes into M2 macrophages (TAMs) (Zhang, Patel et al.; Raman, Baugher et al. 2007; Richmond, Yang et al. 2009). In addition, TAMs produce CCL2, thus contributing to further recruitment of macrophages and CCL2 induced massive tumor angiogenesis (Raman, Baugher et al. 2007). The binding of CCL2 to CCR2 on prostate cancer cells ensues in enhanced expression of VEGFA, demonstrating another CCL2 mediated angiogenesis mechanism. In mice, administration of CCL2 neutralizing antibodies significantly reduced the tumor growth and decreased microvascular density of the tumor (Zhang, Patel et al.). Moreover, neutralization of CCL2 prevented the formation of lung metastasis in a mouse model for breast cancer, suggesting a role for CCL2 in the metastasis in breast cancer (Raman, Baugher et al. 2007). These promising results obtained in mice have resulted in a phase I clinical trial investigating the effects of the CCL2 antibody, CNTO 888, on human solid tumors. Preliminary results show no dose-limiting toxicity in the patients (Garber 2009).

Besides CCL2, several other members of this family have been shown to modulate angiogenesis, including CCL1, CCL11, CCL15, CCL16, and CCL23, while CCL21 has been...
confirmed as angiostatic by binding to CXCR3 (Zhang, Patel et al.). The major contribution of CCL2 in recruiting and activation of inflammatory cells may be its ability to drive the immune system towards a more TH2 mediated response, which normally mediates humoral immunity and suppresses anti tumor activity (Coussens and Werb 2002; Kakinuma and Hwang 2006; Raman, Baugher et al. 2007). In contrast, the presence of CCL5 is associated with infiltration of CD8+ T cells thereby acting as an anti tumor agent in non small lung cancer (Kakinuma and Hwang 2006; Raman, Baugher et al. 2007). Moreover, CCL2 has also been found to induce migration of Tregs in vitro, suggesting a possible role for CCL2 dependent recruitment of natural Tregs to the site of inflammation (Zhang, Patel et al.; Huang, Lei et al. 2007).

Several chemokines can attract DCs into tumors, including CCL5, CCL20, CXCL8 and CXCL12 (Raman, Baugher et al. 2007). CCL20 is expressed in colon, pancreas, prostate, lung, cervix, skin, and lymphatic tissues where it exerts important homeostatic functions. The expression of CCL20 is augmented by proinflammatory cytokines, including TNF-α and IL-1, and it is expressed by tumor and stroma cells in several solid tumors, such as pancreatic, renal, breast, colorectal, and papillary thyroid cancer (Williams 2006; Raman, Baugher et al. 2007; Ghadjar, Rubie et al. 2009). Tumor cell expression of CCR6, receptor for CCL20, is associated with liver metastasis in colorectal cancer patients, due to the production of CCL20 in the liver (Ghadjar, Rubie et al. 2009). The chemokine receptor CCR7, expressed by naïve T cells and responsible for the migration of mature DCs from the site of inflammation to the lymphatic tissue, has been associated with lymph node metastasis in melanoma, esophageal cell carcinoma, head and neck, breast, gastric, and non small lung cancer (Kulbe, Levinson et al. 2004; Raman, Baugher et al. 2007). Tumor cells expressing CCR7 have a migration resembling the chemokine directed lymphocyte migration by responding to the CCR7 ligands, CCL19 and CCL21, produced within the secondary lymphoid organs (Kulbe, Levinson et al. 2004; Kakinuma and Hwang 2006).

Cyclooxygenase 2 (COX-2)
Cyclooxygenase enzymes catalyze the conversion of arachidonic acid into prostaglandin H$_2$, the precursor of several bioactive molecules, including prostaglandins, prostacyclin, and thromboxane. Two cyclooxygenase isoforms are identified and include the cyclooxygenase-1 (COX-1) and the inducible COX-2. COX-1 is constitutively expressed in several tissues and is essential in maintaining various homeostatic conditions, such as protection of mucosal integrity, platelet function, and maintenance of in renal blood flow, glomerular filtration, and ovulation (Ramalingam and Belani 2004). COX-2 is an inducible immediate early gene
upregulated in association with inflammation by proinflammatory cytokines, such as TNF-α and IL-1 (Liu, Reinmuth et al. 2003; Itatsu, Sasaki et al. 2009). The COX-2 metabolite PGE₂ binds with high affinity to G-protein-coupled prostaglandin E receptors EP1, EP2, EP3, and EP4. These receptors are classified according to their different signaling pathways and the biological effect depends on the specific receptor subtype expressed at the cell surface (Alvarez-Soria, Largo et al. 2007). Activation of the EP1 receptor leads to an increase in intracellular calcium, while EP2 and EP4 receptors induce adenylate cyclase activity resulting in an increase in cyclic adenosine monophosphate (cAMP) (Tober, Thomas-Ahner et al. 2007). The adenylate cyclase family regulates the production of cAMP, a prototypical second messenger that has impact on every aspect of a cell's life cycle, from differentiation to its death (Willoughby and Cooper 2007). EP2 receptor mediates the mitogenic effect of PGE₂ in esophageal squamous cell via activation of the Erk/AP-1 pathway (Yu, Wu et al. 2008). The EP3 receptor is alternatively spliced, yielding three variants, EP3a, EP3b, and EP3g. Signaling through EP3a and EP3b has been shown to inhibit the activation of adenylate cyclase, whereas EP3g signaling has been shown to have both stimulatory and inhibitory effects on adenylate cyclase. Moreover, all the EP3 isoforms can also induce increases in intracellular calcium (Tober, Thomas-Ahner et al. 2007).

Elevated levels of COX-2 and its metabolite PGE₂ are associated with variety of cell proliferative events, including differentiation, apoptosis, metastasis, and angiogenesis in human tumor carcinogenesis (Schlosser, Schlosser et al. 2002; Chu, Lloyd et al. 2003; Furukawa, Nishikawa et al. 2003; El-Rayes, Zalupski et al. 2005). PGE₂ exhibits potent immunosuppressive effects, orchestrating an imbalance between TH1 and TH2 cytokines and has shown to be a key modulator of DC function, altering cytokine production as well as MHC class II molecules (Yang, Yamagata et al. 2003). Tumor derived PGE₂ also exerts indirect effects on tumor development by induction and accumulation of different types of immune suppressor cells, such as expansion of natural Tregs and induction of IL-10 producing regulatory T cells (Bergmann, Strauss et al. 2007). The expression of COX-2 enzyme has in several types of cancer been associated with poor prognosis. For instance, COX-2 is overexpressed in breast cancer tumors, and high expression is associated with poorer prognosis (Basu, Pathangey et al. 2005). Moreover, subjects who used nonsteroidal anti-inflammatory drugs on regular basis showed decreased incidence of breast, colorectal, esophageal, and lung cancer (Basu, Pathangey et al. 2004; Ramalingam and Belani 2004). However, therapy with NSAIDs is associated with serious side effects such as gastrointestinal bleeding and ulcer formation. Subsequently, this resulted in selective inhibitors, focusing only on COX-2. The selective inhibitors retained the anti-inflammatory effect of the non selective NSAIDs and reduced
Vascular endothelial growth factor (VEGF)

The VEGF family includes VEGFA, VEGFB, VEGFC, and VEGFD, which share structural features but display different biological activities. The fifth member of the family, PIGF belongs to the VEGF family based on its binding to VEGFR-1 and appears to be a functional homolog of VEGFB (Li and Eriksson 2001). The members of the VEGF family are expressed in many tissues by a number of different cells, including macrophages, DCs, tumor cells, and fibroblasts, and their expression is elevated during development, tissue remodeling, wound healing, and carcinogenesis (Nam, Park et al.; Dineen, Lynn et al. 2008; Tjomsland, Spångeus et al. 2010).

VEGF upregulation is regulated by pathological conditions such as hypoxia, hypoglycemia, growth, and inflammatory factors, including PDGF, TNF-α, COX-2, IL-1, and IL-6 (Nam, Park et al.). VEGFA is the most potent angiogenic factor and exerts its cellular functions by binding with high affinity to tyrosine kinase receptors, VEGFR-1, and VEGFR-2 (Guo, Xu et al. 2001). Activation of VEGFR-2 by VEGFA is directly associated with angiogenesis by inducing vascular permeability and endothelial cell proliferation (Roland, Dineen et al. 2009). For this reason angiogenesis has become a critical target for cancer therapy and inhibition of VEGFR-2 activity using monoclonal antibodies has resulted in significant reduced tumor burden, microvessel density, macrophage infiltration, and reduced number of metastatic events in preclinical pancreatic and breast cancer models (Dineen, Lynn et al. 2008; Roland, Dineen et al. 2009). Even though binding of VEGFA mediates its angiogenic potential though activation of VEGFR-2, it binds VEGFR-1 with much higher affinity, yet inducing a weaker signal compared to the VEGFR-2 ligation. The binding of VEGFA to the VEGFR-1 modulates angiogenesis through the sequestering of VEGFA, thus preventing over activation of the angiogenic receptor. Nevertheless, activation of VEGFR-1 by its ligands, VEGFA, VEGFB, and PIGF, are associated with tumor cell survival, progression, negative regulation of VEGFR-2, recruitment and activation of bone marrow progenitors, inflammatory cells, smooth muscle cells, and DCs (Fischer, Mazzone et al. 2008; Roland, Dineen et al. 2009). PIGF indirectly stimulates angiogenesis by recruiting and activating various cell types that upregulate VEGFA and other angiogenic factors, including fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), CXCL8, CXCL12, granulocyte colony-stimulating factor (G-CSF), and MMP9. Moreover, the binding of PIGF to VEGFR-1 also leads to intermolecular crosstalk between VEGFR-1 and VEGFR-2, which amplifies VEGFR-2 signaling and consequently...
enhances the responses to VEGFA. VEGFB is believed to have a restricted role in angiogenesis, seeing that mice lacking VEGFB do not display vascular defects. Nevertheless, VEGFB has been detected in several solid tumors and the expression correlates with microvascular density in oral squamous cell carcinoma (Fischer, Mazzone et al. 2008).

Metastasis to the regional lymphatic tissue is a frequent event in all malignant tumors. The tumor utilizes the lymphatic vasculature as a route of dissemination through lymphangiogenesis, the creation of lymphatic vessels from pre-existing lymphatic vessels (Whitehurst, Flister et al. 2007). Two members of the VEGF family, VEGFC and VEGFD, specifically activate VEGFR-3, which is exclusively expressed on lymphatic epithelium. VEGFC can bind both VEGFR-2 and VEGFR-3 and is frequently increased in tumors, whereas VEGFD is highly expressed in normal tissue and often down regulated in tumors. Moreover, VEGFC expression correlates with increased lymphatic vessel invasion, metastasis, and poor survival in many cancers (Mattila, Ruohola et al. 2001; Whitehurst, Flister et al. 2007; Muders, Zhang et al. 2009). VEGFC promotes lymphangiogenesis by increasing endothelial cell proliferation, migration and sprout formation. VEGFC and CCR7 expressed on tumor cells can also act synergistically to promote lymphatic invasion, by VEGFC promoted lymphatic secretion of CCL21, which in turn drives CCR7-dependent tumor migration toward the lymphatic tissue (Issa, Le et al. 2009). More recently, VEGFC has also shown to be important for tumor progression independently of lymphangiogenesis, by stimulating tumor cell proliferation, protection from cell stress induced cell death, and with autocrine functions resulting in tumor cell invasion of lung, breast and gastric cancers (Muders, Zhang et al. 2009). The expression of VEGFC is not regulated by hypoxia, but instead by cytokines and growth factors, including PDGF, EGF, TGF-β and IL-1 (Enholm, Paavonen et al. 1997; Mattila, Ruohola et al. 2001; Watari, Nakao et al. 2008).
Aims

PDAC tumors are characterized by their massive fibrotic component, i.e. CAFs and these cells can exceed the tumor cells in quantity. The high amount and the location of CAFs in close connection to tumor cells indicate that there is a cross-talk between PDAC cells and CAFs. We aimed to investigate if and how these cells interact with each other and elucidate the effects the cross-talk had on the tumor microenvironment and systemically.

Specific aims

Paper I: To elucidate the effect cross-talk between tumor cells and CAFs exerts on their functionality and the mechanisms involved in this cross-talk.

Paper II: To determine the impact of IL-1α on human PDAC tumors and evaluate the amount and location of different immune cells in the tumors.

Paper III: To evaluate the systemic impact of PDAC tumors on the levels and viability of MDCs and PDCs in the peripheral blood of PDAC patients.

Paper IV: To investigate the systemic effects PDAC tumors exert on peripheral blood MDCs and PDCs regarding phenotype and functionality and the involvement of inflammatory factors in this process.
Material and Methods

Blood samples from patients and controls
Twenty ml heparinized peripheral whole blood samples were obtained from controls, at one occasion, and from patients at two time points, one week prior surgical removal of the tumor (Whipple resection) and 8-12 weeks after the surgery. The age matched controls were recruited randomly from department of Transfusion Medicine at Linköping University Hospital (Linköping, Sweden) and from the senior division of Linköping orienteering club.

Density gradient separation of peripheral blood mononuclear cells
Peripheral blood mononuclear cells (PBMCs) were isolated from heparin treated whole blood by Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. The plasma layer was collected after the density centrifugation, aliquoted and stored at -70°C until analysis. The cellular interface containing the PBMCs was harvested and washed twice in PBS (PAA Laboratories GmbH, Germany). The PBMCs were re-suspended in freezing media (fetal bovine serum containing 8% DMSO (Sigma-Aldrich, Schnelldorf, Germany)) and cryo preserved.

Propagation of PDAC and CAF cell lines
Primary PDAC or CAF cell lines were propagated from pancreatic tumor tissue biopsies obtained from patients. The tumor samples were cut into small pieces and incubated in HBSS buffer (Invitrogen) supplemented with 0.3M CaCl₂ and 1mg/ml Collagenase II (Invitrogen) under gentle agitation at 37°C for 1h. The samples were further processed through a syringe and a 100µm filter followed by centrifugation. The cell pellet was re-suspended in RPMI 1640 (Fisher Scientific, Pittsburgh, PA), supplemented with 20% FCS (Invitrogen), 2mM HEPES (Invitrogen), 30µg/ml Gentamycin (Invitrogen), and 1% Fungizone (Invitrogen) and cultured in tissue flasks. The cells were detached using Trypsin-EDTA (Invitrogen) when they reached confluence and labeled with CD326 microbeads and positively selected according to manufacturer’s description (Miltenyi Biotec). The primary PDAC cell lines were cultured for five passages, harvested, and cryo preserved. CD326 negative cells, i.e. CAFs, were cultured for two passages, harvested, and cryo preserved. The primary PDAC cell lines: PC013, PC065, and PC077, and the commercial PDAC cell line BXPC-3 (LGC Standards) in addition to three PDAC derived CAF cell lines, CAF039, CAF055, and CAF073 were used in this study.
Immunohistochemistry (IHC)

Formalin fixed paraffin embedded samples were cut in 5 µm sections. The sections were then re-hydrated and antigen retrieval was performed in a microwave oven for 15 min (350W) using citrate buffer (pH 6.0). Endogenous peroxidase was eliminated by 10 minutes incubation in H2O2, and non specific binding was avoided by incubating with Background Sniper (Biocare Medical) or 1% Bovine serum albumin for 10 min. The samples were immunostained with primary antibodies incubated overnight at room temperature. The next day, the sections were incubated with alkaline phosphatase conjugated anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch) for one hour or by using LSAB2 System-HRP kit (K0675, Dako) containing biotinylated link and streptavidin conjugated HRP according to manufacturer protocol. Alkaline phosphatase was detected by Vulcan fast red chromogen 2 solution (Biocare Medical) according to the manufacture's protocol. HRP was detected by development in Tris-buffer containing diaminobenzidine tetrahydrochloride (DAB) (Saveen-Werner AB) and 10µl of 30% H2O2 (Figure 8). Counter-staining was performed with methyl green solution (0.1M sodium Acetate buffer, pH 4.2) containing 1% Methyl-green (Sigma Aldrich) or hematoxylin. Images representative for the patients and the controls were processed using Quantimet 500MC image processing analysis systems linked to a Leica DM LB microscope (Leica Microsystems) supported by Leica QWin software version 3 (Leica Microsystems).

Figure 7.
Principle of Immunohistochemistry
The antigen is recognized by a primary antibody and detected by a secondary antibody conjugated with biotin. A complex consisting of avidin, biotin and HRP binds to the biotin conjugation on the secondary antibody and a brown signal is detected when developing the sample in diaminobenzidine (DAB).
Quantification with Real-time PCR
Total RNA was prepared from the samples using RNA Easy Mini kit (Qiagen) and cDNA was synthesized with SuperScript III Reverse Transcriptase First-Strand cDNA Synthesis kit according to the manufacturer’s protocol (Invitrogen). Quantitative PCR was performed with Fast SYBR Green Master Mix (Version 09/2007; Applied Biosystems, Foster City, CA) on 7900 Fast Real-Time PCR system with 7900 System SDS 2.3 Software (Applied Biosystems) according to the manufacturer’s protocol. The results were analyzed using the ΔΔCt method (Livak and Schmittgen 2001) and presented as either normalized data or as relative gene expression.

Figure 8. qRT-PCR
A) The primers and the TaqMan probe bind to the single stranded DNA and the polymerase starts making a complementary strand and on its way it cleaves the probe. When the reporter is disconnected from the quencher it will emit light when exposed to a light source or a laser beam. The emitted light can be measured and quantitative results obtained by comparing the intensity of the emitted light between endogenous controls and factors of interest.
B) SYBR Green is a cyanine dye that emits green light when exposed to blue light (488nm), but only when binding to double stranded DNA. Quantitative results are obtained when comparing the intensity of the emitted light between endogenous controls and factors of interest.
Tissue samples from patients and controls

Tumor tissue samples from a total of 30 patients with PDAC and normal pancreatic tissue obtained from 10 individuals who had died of hypothermia, were used in this study. The patients had been recruited from patients undergoing pancreatic Whipple resection at Linköping University Hospital. The final diagnosis was histologically confirmed by two pathologists, independently investigating the samples. All samples were coded to protect the identities of the subjects participating in this study. The study protocol and patient consent documents were approved by the Regional Ethics committee in Linköping, Sweden (Dnr. M38-06). The PDACs were staged according to the 1997 International Union against Cancer classification (TNM=Tumor, Node, Metastasis).

Flow cytometry acquisition and analysis

Figure 9. Fluorescence activated cell sorting (FACS). Cells marked with antibodies conjugated with fluorochromes are directed into a hydrodynamically focused single stream. When the cells pass through the laser beam the fluorochromes become excited and emitted light is transferred to detectors that convert the signal into electrical signals that can be processed by computers.

PBMCs were suspended in PBS supplemented with 0.2% BSA (FACS wash) and labeled with lineage cocktail, HLA DR, CD11c, and CD123 mAbs to detect MDCs and PDCs. The antibody incubation was carried out at 4°C for 60 min. After the incubation unbound antibodies were
removed by spinning down the samples and replacing the supernatant with new FACS wash. This procedure was repeated 2 times. Four or eight color flow cytometry was performed using FACS Calibur and FACS ARIA flow cytometer (Figure 9) (Becton Dickinson, San Jose, CA). The acquired data was analyzed using the FLOW-JO software, v7.0 (Tree Star Inc, Ashland, OR).

Statistics
The statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). A p-value of <0.05 was considered statistically significant and error bars throughout indicate standard error of the mean (SEM). Non-parametric data was analyzed using the Wilcoxon matched pairs test followed by Mann-Whitney test and Paired t-test was used for normalized data. Survival curves were analyzed by the Kaplan-Meier survival method, and statistical significance was determined using Log-rank (Mantel-Cox) test and a p value <0.05 was considered statistically significant.
Result and Discussion

Paper I

Most research in the field of pancreatic cancer has focused on the malignant cells and the identification of mechanisms essential for their survival. However, a tumor does not solely consist of malignant cells so investigations focusing only on the tumor cells do not mimic the genuine conditions of solid tumors existing in humans. Tumors are believed to develop in a darwinistic setting, and one essential part is the ability of the malignant cells to create a tumor friendly microenvironment with stroma cells that can produce factors necessary for tumor progression. This is primarily achieved by recruitment of inflammatory cells and the creation of a massive fibrotic component that enwraps the tumor cells. At the tumor site, the tumor cells instruct the stroma to release factors favoring tumor progression. The microenvironment created during the process of wound healing is optimized for cell growth and angiogenesis and much evidence points to that tumors have developed special features to create and exploit this physical condition to its advantage. In normal settings, the mechanism of wound healing will be switched off when tissue reconstruction is accomplished, but in tumors, the malignant cells will continue to drive the “wound healing process” by providing the non-malignant stroma cells with activation signals. PDAC is strongly associated with a dense desmoplastic reaction that may account for as much as 70% of the total tumor mass. The close contact between the tumor cells and the fibrous stroma, makes CAFs a natural target for tumor cell influence. It is of great value to understand the mechanisms behind the cross-talk between tumor cells and CAFs in PDAC, seenig that this could lead to development of drugs eliminating CAF derived factors. In this study have we focused on the inflammatory environment created by the cross-talk between PDAC cells and CAFs.

Principal findings

The gene expression levels were compared between primary tumor (PC013) and CAF (CAF039) cell lines after 5 days of coculture and we found that the CAFs were highly affected by the cross-talk with the tumor cells. The gene profiles for cocultured CAFs showed a vast upregulation of genes associated with inflammation. KEGG pathway analysis indicated an important role of IL-1 in the upregulation of the inflammatory factors expressed by CAFs. Both PDAC and CAF cell lines expressed IL-1β mRNA, but IL-1α was only detected in the tumor cells. To confirm the protein expression of IL-1α and IL-1β, both cell lysate and supernatants were analyzed by ELISA. Although both CAF039 and PC013 expressed high...
levels of IL-1β mRNA, neither of the cell lines expressed IL-1β as protein. This eliminated IL-1β and confirmed IL-1α as the inducer of the inflammatory genes. To verify our findings, additional tumor cell lines and CAF cell lines were analyzed for the expression of IL-1β and IL-1α. The results were in accordance to our previous data and none of the cell lines expressed IL-1β as protein. IL-1α protein was detected in 3 out of 4 tumor cell lines and none of the CAF cell lines tested. The different tumor cell lines showed great diversity in the expression of IL-1α, fluctuating from very high to undetectable expression. To study the involvement of IL-1α in the upregulation of inflammatory genes in CAFs they were cocultured with IL-1α positive or negative tumor cell lines. The results revealed increased expression of IL-1α, IL-1β, IL-6, CCL20, VEGF, CXCL8, and COX-2 in CAFs cocultured with IL-1α expressing PDAC cell lines. Moreover, the increase correlated with the expression levels of IL-1α (Figure 10).

The IL-1α negative cell line even reduced the CAFs’ expression of these factors. The data was confirmed by analyzing the protein levels of IL-6 and CXCL8, from single and cocultured PDAC and CAF cell lines, which indicated a strong link between the PDAC IL-1α levels and the protein production of IL-6 and CXCL8 by CAFs. The PDAC cell line with the highest IL-1α expression did also induce the expression of other ELR+ CXC chemokines besides CXCL8, including CXCL1, CXCL2, CXCL3, CXCL5 and CXCL6. A similar effect was not found in CAFs cocultured with tumor cell lines expressing moderate to low levels of IL-1α, which decreased the levels of CXCL1 and CXCL2 in CAFs, while the remaining chemokines were unchanged. The IL-1α negative cell line had negative effects on the expression of almost all ELR+ CXC chemokines by CAFs.

The IL-1α activity was blocked, using its natural antagonist IL-1RA, to confirm the involvement of IL-1 in the upregulation of the inflammatory gene profile in CAFs. Single cultured CAFs significantly downregulated the expression of IL-6, CCL20, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8 when blocking IL-1 signaling. The neutralization of IL-1 significantly reduced the expression of IL-1α, IL-1β, IL-6, CCL20, COX-2, CCL2, CXCL1, CXCL3, CXCL5, CXCL6, and CXCL8 in CAFs cocultured with tumor cells. Moreover, IL-1RA treatment of single and cocultured tumor cell lines showed decreased levels of the ELR+ CXC chemokines, but little or no impact on the expression of the other inflammatory factors. Finally, the protein levels of IL-6 and CXCL8 were slightly reduced in the supernatants from single cultured PDAC cells and significantly reduced in the supernatants from single cultured CAF. Supernatants from the cocultures of tumor and CAF cell lines showed the biggest decrease in the protein levels of IL-6 and CXCL8.

PDAC patient tissues were immunostained with specific antibodies to confirm the presence and location of IL-1α, IL-1RA, IL-1R1, and CXCL8 in the tumors. IL-1α and IL-1RA
were exclusively found located inside the tumor cells and the expression detected in the patient seems to be identical to what was found in the primary cell line obtained from the patient. The major expression of the active IL-1 receptor was detected in the fibrotic stroma, while the receptor was expressed to a less extent among the tumor cells. CXCL8 expression was primarily detected in IL-1α expressing tumors and generally located in the fibrotic parts of the tumors. Moreover, PDAC tissue samples expressed superior mRNA levels of IL-1α, IL-1R1, IL-1Ra, IL-6, CXCL8, VEGFA, CCL2 and CCL20 compared to healthy pancreatic tissue.

Figure 10. Cross-talk between PDAC cells and CAFs
Tumor derived IL-1α binds to IL-1R1 on CAFs and induces the expression of inflammatory genes, such as IL-6, COX-2, VEGFA, and ELR-CXC chemokines.

Conclusion
At an early stage in the carcinogenesis tumor cells have to be able to create an inflammatory microenvironment. This inflammatory milieu supports the malignant cells directly by inducing tumor cell proliferation and survival or indirectly by promoting angiogenesis, migration, and immune suppression, thus preventing elimination of the tumor by the immune system. Our findings demonstrate that CAFs exceed tumor cells in the expression of inflammatory genes and that the tumor cells have the ability to further increase the expression of those genes. CAFs are major components in the PDAC tumors and the ability to activate these non-malignant cells is probably an essential event in the development and progression of the tumor. In PDAC tumors, IL-1α is an important factor used by the malignant cells to induce the production of inflammatory factors in non-malignant cells, such as CAFs and probably
also immune cells expressing IL-1R1. CAFs expressed high levels of IL-1R1, but coculture decreased the levels of this receptor and this could be a result of a negative feedback loop caused by high receptor ligation.

The inflammatory genes, including IL-6, CCL20, CCL2, VEGFA, COX-2, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8, expressed by CAFs when cocultured with IL-1α positive tumor cell lines are strongly associated with tumor angiogenesis and by using IL-1α to activate CAFs, the tumor cells create an environment optimal for angiogenic development. Angiogenesis is crucial for the progression of the tumor, as tumors can not grow beyond 2 mm³ without a functional blood supply. Besides promoting angiogenesis these factors probably also promote metastasis of tumor cells. The high levels of chemokines produced by CAFs create a chemokine gradient attracting different immune cells into the tumor. It is most likely that the same chemokine gradient retains the immune cells in the tumor microenvironment, while factors such as COX-2 and IL-6 impair the functionality of these cells. As a consequence of these events, the immune cells become a part of the tumor stroma, thereby contributing to the progression of the tumor by producing cytokines, chemokines, and growth factors important for angiogenesis and tumor cell survival. Furthermore, these factors will attract even more immune cells into the tumor and most likely contribute to the activation of CAFs by releasing IL-1.

By neutralizing IL-1α, the tumor cells lost much of their capacity to induce inflammatory genes in CAFs, but some of the genes were only somewhat influenced by the neutralization, pointing to the involvement of other mechanisms. IL-1 induces the expression of proinflammatory genes by activating the transcription factor NFκB, which indirectly activates STAT3, by the release of cytokines belonging to the IL-6 family.

When first activated, the STAT3 pathway induces production of inflammatory factors that have the potential to activate STAT3, thus creating a positive feedback loop. Both NFκB and STAT3 are observed constitutively activated in a number of human tumor cell lines and primary tumors. The activation of both pathways could explain why factors known to be induced by either of these pathways, e.g. IL-6, COX-2, and VEGFA were only partially reduced after IL-1 neutralization, while factors only associated to NFκB, such as ELR+ CXC chemokines were almost eliminated. Blocking of both these pathways should have the potential to drastically decrease the inflammation caused by the cross-talk between tumor and stroma cells.
Paper II

Background

Solid tumors are characterized by an inflammatory microenvironment consisting of malignant and stroma cells, such as CAFs and different types of leukocytes. In paper I we showed that tumor cells use IL-1α to enhance the expression of inflammatory genes in CAFs. The inflammatory gene profile was associated with factors important for angiogenesis, metastasis, but also attraction and differentiation of leukocytes. Leukocytes such as macrophages, DCs, T cells, neutrophils, eosinophils, and mast cells migrate towards the chemokine gradient established by the cross-talk between malignant cells and the tumor stroma. The infiltration of leukocytes has the potential to eliminate tumor cells, but when failing the immune cells will be under the control of the tumor and a component in the tumor microenvironment.

The tumor infiltrating immune cells support the progression of the tumor and attract new leukocytes to the tumor by releasing cytokines, cytotoxic mediators, and MMPs. The quantity of TAMs correlates with the expression of CCL2 and is a prognostic factor in many cancers. Moreover, DCs are retained in the tumor by CXCL8 and have phenotypic and functional abnormalities. The prognostic value of infiltrating DCs fluctuates; which could be a result of phenotypic diversity among DCs in different solid tumors. CAFs, a major component of the PDAC tumors, were shown in paper 1 to be the main contributor of inflammatory factors, thus important in leukocyte trafficking. The high expression of chemoattractants by stroma cells could affect the location of immune cells inside the tumor, i.e function as decoy targets protecting the malignant cells from immune cell confrontations. Characterization of the amount and location of different immune cells inside the tumor and the inflammatory factors involved in leukocyte migration and differentiation could give valuable information of the involvement of immune cells in development of PDAC.

In this paper we wanted to characterize the expression, location, and phenotypical differentiation of immune cells in the tumor environment, and further link these findings to the interactions between malignant cells and stroma cells observed in paper I.

Principal findings

IL-1α was in paper I found to be exclusively expressed by tumor cells both in vitro and in vivo, and the expression of this highly potent proinflammatory cytokine was responsible for the upregulation of inflammatory genes in CAFs. In this paper we investigated the gene and protein expression of IL-1α in 30 PDAC tumors and 10 healthy controls. The controls were all negative, while 90% of the tumors were positive. To evaluate if the expression levels
of IL-1α had any impact on the clinical outcome, the patients were divided into two groups with 15 patients in each, based their expression levels of IL-1α mRNA. The group of patients expressing low gene levels of IL-1α showed significantly better prognosis than the patient group with high levels of IL-1α. This was further confirmed by semi-quantification of the protein expression of IL-1α in PDAC tumors, and patients expressing low/negative levels of IL-1α had significantly better prognosis than patients with moderate/high levels of IL-1α. In paper 1, IL-1α was found to induce the expression of several inflammatory factors and significant higher expression levels of COX-2, CXCL8, CCL2, and CCL20 were detected in the PDAC tumors compared to normal pancreas tissue. The expression levels of each of these factors were correlated to the levels of IL-1α and there was also a strong correlation between CXCL8 and of IL-1α expression in the tumors. The chemokines, CXCL8, CCL2, and CCL20 were primarily detected in the tumor stroma. COX-2 was expressed by both stroma and tumor cells, with the highest expression in stroma. The increased levels of chemotactic chemokines in the tumors should have the potential to bring immune cells into the tumor. CCL2 is essential for macrophage migration into tissue and significantly increased mRNA levels of the macrophage marker CD163 were found in the tumors. Moreover, PDAC tumors also had higher levels of CD163 positive macrophages compared to health controls and the cells were located to the fibrotic stroma surrounding the tumor nests. The PDAC tissues had significantly higher mRNA levels of markers associated with MDC, CD1a and CD1c, and the PDCs marker CD303, compared to normal pancreas tissues. DCs, detected by the marker S100, were located in the Langerhans islets in normal pancreas, while in PDAC the DCs were located in the fibrotic stroma encapsulating the tumor nests. Moreover, gene levels of CD83 and CD208, markers associated with DC maturation, were upregulated in PDAC tissues, while the expression of CD209, associated to immature DCs, was downregulated. CD83 positive DCs were located in tumor stroma and their levels were significantly higher in PDAC tissues compared to the normal pancreas. Furthermore, infiltrating cytotoxic T cells (CD8+) were located almost exclusively in the PDAC tissue and positioned in the fibrotic tissue, close to the tumor nests.

Conclusion
The success of the tumor depends on the malignant cells ability to create a well balanced microenvironment supporting the growth and protecting the tumor from elimination by
the immune system. In paper I we identified IL-1α as a main factor used by the tumor cells to induce proinflammatory genes in CAFs. The importance of IL-1α in the tumor setting was further confirmed in the present study by prolonged survival for patients expressing low or no levels of IL-1α. Moreover, the induction of expression of CXCL8 by CAFs in paper 1 was IL-1α dependent and we show a strong correlation between the expression of IL-1α and CXCL8 in the PDAC tumor tissue. The tumor cells seem to use IL-1α in the regulation of CXCL8 in the microenvironment, but the levels of CXCL8 per se did not correlate to patient survival. Probably due to that IL-1α has the capability to induce expression of several inflammatory genes, each with ability to affect the progression of the tumor, and collectively these factors has a negative impact on the patient's clinical outcome. Moreover, the tumor expression of COX-2 did not follow the IL-1α expression, pointing to that other factors regulate this gene. The finding corresponds to what was seen in paper I, where IL-1RA only in part decreased the levels of COX-2. Nevertheless, COX-2 was upregulated in the tumor microenvironment and the stroma contributed significantly to the expression. The COX-2 metabolite PGE₂ is a key modulator of DC functionality and exhibits potent immunosuppressive effects. In paper 4, PGE₂ induced the expression of CD83, CD86, and CCR7, while not affecting the expression of CD40 and ICOSL, thus promoting a semi mature phenotype, with decreased T cell stimulatory capacity. The high levels of COX-2 observed in the tumor tissue could affect the DC activation levels and induce partly matured cells, i.e. CD83+ cells in the PDAC tumors. Cocultured CAFs were found (paper I) to be the main producer of the chemokines, CXCL8, CCL2, and CCL20. CCL2 is strongly associated with infiltration of macrophages and CXCL8 and CCL20 attract DCs to the tumor site. No correlation was seen in infiltration of immune cells and levels of chemokines and this is probably due to that several different chemokines could attract the same type of cells. The immune cells were all located inside the fibrotic stroma, indicating new important roles for CAFs in the tumor microenvironment, by functioning as a decoy target that sequesters the immune cells. As a consequence they miss their target cells and the tumor cells can avoid elimination by the immune cells. CXCL8 has been observed to sequester DCs in the tumor microenvironment, thus hindering the mounting of tumor specific immune response by blocking the migration of DCs to lymphoid tissue. This was supported by our findings of elevated levels of DCs with increased expression of CD83 and CD208 in addition to decreased levels of CD209, pointing to the retention of activated DCs in the PDAC tumors. High levels of infiltrating CD208 positive DCs in colon cancer were associated with a poor clinical outcome. Furthermore, immune cell retention in the tumor also supports the progression of the tumor. Macrophages are important in the angiogenic process by providing the tumor with VEGFA, VEGFC, and FGF. Furthermore, the
TAMs could also produce IL-1, important for tumor creation and sustenance of the tumor microenvironment. The amount of TAMs found in the tumor microenvironment has also been shown to correlate with tumor progression and patient survival in several different cancers.

These findings point to a crucial role for IL-1α in the regulation of the tumor microenvironment in PDAC patients. The chemotactic gradient created by the interaction between tumor cells and stroma cells attracted immune cells and retained these cells in the fibrotic stroma, indicating an important immune suppressing function for the tumor stroma. Eliminating IL-1 signaling in the tumor could prevent angiogenesis, tumor cell migration and retention of DCs and other immune cells in the tumor tissue.

**Paper III**

**Background**

DCs are APCs crucial for the activation of an adaptive immune response against pathogens and also tumor cells. The DCs are divided into two distinct subgroups based on the origin of their progenitors, MDC from the myeloid lineage and the PDCs from the lymphoid lineage. These two subgroups of DCs are ubiquitously distributed within the body and constitute about 1% of the total amount of the peripheral blood mononuclear cells. The findings in paper I and II demonstrate how the tumor cells shape their local environment, by creating an inflammatory microenvironment that supports the tumor through growth stimulation, angiogenesis, metastasis and immune suppression. The microenvironment was found to release a broad array of inflammatory factors that could be distributed through the bloodstream to the entire body. Impaired function and reduced numbers of blood DCs have been reported in several types of solid and blood cancers, suggesting a systemic effect exerted by the tumor. Moreover, similar effects on blood DC numbers and phenotype have been observed in individuals with chronic infections and autoimmune diseases, which support the involvement of inflammatory factors in the reduction and impairment of blood DCs.

The tumor cells are responsible for the chronic inflammatory conditions established in the tumor, by the release of proinflammatory factors such as IL-1α, thus inducing the expression of inflammatory genes in the surrounding stroma. By resecting the tumor, the source of inflammation should be removed and the DCs should be able to retain their normal phenotype and numbers.

In this paper we wanted to investigate if the PDAC tumors exerted systemic effects on
peripheral blood MDCs and PDCs and compare the results to other types of cancer and inflammatory conditions in the pancreas.

Principal findings:
PDAC patients had significantly decreased levels of peripheral blood MDCs and PDCs presurgery, while biliary duct adenocarcinoma (BDAC), ampullary carcinoma, and endocrine carcinoma showed no significant decrease. Of note, patients with chronic pancreatitis had similar levels of MDCs as the PDAC patients, but did not show any significant reduction in the levels of PDCs. Removal of the tumor only slightly increase the levels of MDCs and PDCs in the blood of PDAC patients. Chronic pancreatitis patients had slightly less DCs compared to pre surgery. The surgery affected the levels of circulating DCs in ampullary carcinoma and endocrine carcinoma, which resulted in significantly decreased levels of both MDCs and PDCs post surgery. Besides MDCs and PDCs the DC compartment also includes a population of cells referred to as non-DCs (linage-HLA-DR⁺CD11c CD123⁻). The total amount of non-DCs were found comparable between the controls and the different patient groups. On the other hand, in the DC compartment the frequency of the non-DCs significantly increased in PDAC and chronic pancreatitis both pre and post surgery. The increased ratio of non DCs is directly related to that neither tumor nor chronic inflammation reduced the levels of non-DCs. Spontaneous apoptosis was detected by Annexin V and PDAC, BDAC, ampullary carcinoma, and chronic pancreatitis showed increased levels of apoptotic MDCs and PDCs pre surgery. The removal of the tumor further increased the amount of apoptotic DCs. These findings indicated a role for apoptosis in the reduction of peripheral blood MDCs and PDCs in PDAC patients.

Inflammatory factors released from the tumor and/or stroma cells have the potential to affect the DCs. We investigated the plasma for a large selection of inflammatory factors, PGE₂ and CXCL8 were the only factors significantly increased in PDAC patients. The level of COX-2 metabolite PGE₂ was significantly enhanced in PDAC patients, pre and post surgery, while chronic pancreatitis had enhanced but not significantly increased levels of PGE₂. CXCL8 was increased in PDAC, endocrine carcinoma, and chronic pancreatitis patient plasma pre surgery and the levels were significantly decreased in PDAC patients post surgery, but still higher than the controls.

The amount of peripheral blood DCs (pre surgery) was higher in PDAC patients surviving more than two years after surgery compared to patients surviving less than one year. The levels of DCs in this group were similar to levels in ampullary carcinoma and endocrine...
carcinoma patients, which both are associated with a much higher 5 years survival rate than PDAC (60% vs. 3-5%). The PDAC patient group with low MDC and PDC levels had a one year survival rate of 53%, compared to 83% for the group of patients with high MDC and PDC levels.

Conclusion
DCs are recognized as the main initiators of the adaptive immune response and play an essential role in tumor surveillance. Findings from several types of solid cancers indicate that anti-tumor immunity may be related to the amount and/or function of DCs. In this paper, we found decreased levels of both MDCs and PDCs in PDAC patients both pre and post surgery. The reduced levels of the DC subsets were also seen in patients with chronic pancreatitis, indicating an important role for inflammatory factors in the decrease of these cells. In paper I we demonstrated how the tumor cells could use IL-1α to induce upregulation of inflammatory genes in CAFs, thus creating an inflammatory microenvironment. The inflammatory factors produced by cells in the tumor microenvironment, released/leaking into the blood flow have the ability to affect cells in the entire body. We found increased levels of both PGE₂ and CXCL8 in the plasma from PDAC patients and the same factors were found expressed in PDAC tumors in paper II. Furthermore, CXCL8 was (paper I and II) found to be IL-1 dependent and by resecting the tumor, the levels dropped significantly. Surgery is also associated with increased inflammation as a result of the wound healing. This could be one explanation behind the reduced levels of DCs post surgery observed among patients with less severe cancer in the pancreas and chronic pancreatitis. These observations are supported by increased MDC and PDC apoptosis post surgery, which indicates a role for inflammatory factors in the apoptosis of the DC subsets. Moreover, PDAC and chronic pancreatitis tissues have a dense desmoplastic reaction creating an environment that should have stronger inflammation than the other types of cancer in pancreas. This could explain why PDAC and chronic pancreatitis patients have less blood DCs compared to the other patient groups.

IL-1α was (paper II) found to be a prognostic marker for PDAC and the factor sustaining the inflammatory environment in the tumor, providing inflammatory factors systemically that reduce the amount of DCs in the bloodstream. High levels of blood DCs seem to benefit the PDAC patients and by reducing the chronic inflammation the DC compartment could be restored, but studies in breast cancer show that the DCs need one year to recover (Chehimi, Azzoni et al. 2007; Pinzon-Charry, Ho et al. 2007). For the majority of the PDAC patients this
time frame is too long, as the cancer gives the patients a shorter life span than it takes for the immune system to recover.

Paper IV

Background

DCs are equipped for initiating a specific adaptive immune response against pathogens and tumor cells. This process is strictly regulated and the functionality of the DCs depend on their ability to differentiate into a fully mature DC after encountering an antigen. The transformation into a mature DC includes several important events, such as upregulation of positive costimulatory molecules, e.g. CD80, CD86, decrease in expression levels of negative costimulatory molecules, e.g. ICOSL, and B7H3, endocytic and antigen capturing receptors, e.g. DCIR, migration towards lymphatic tissue as a result of upregulation of CCR7 and downregulation of tissue retaining chemokine receptors, e.g. CCR1, CCR2, CCR5, and CCR8. This will shift the balance on the DC surface from molecules that can suppress T cell responses to molecules that can activate effector T cell responses and this is a very fine tuned process.

Partially mature DCs, i.e. semi mature DCs exist in HIV-1 and hepatitis C infected individuals and these changes in the DC phenotype give rise to impaired immune functions. In paper 3 we provide evidence for a systemic effect exerted by the tumor resulting in decreased levels of both MDCs and PDCs in the peripheral blood. In this paper we wanted to expand these findings by examining the phenotype and functionality of circulating MDCs and PDCs obtained from PDAC patients.

Principal findings

MDCs and PDCs from peripheral blood obtained from PDAC patients were analyzed for their expression of surface receptors known to be affected by the DC activation status pre (1 week) and post (8-12 weeks) surgical removal of the tumor. Significantly increased levels of CD83, CD40, DCIR, PDL-1, B7H3, CCR6, and CCR7 were detected in both MDCs and PDCs from PDAC patients compared to healthy controls. ICOSL was significantly decreased on both MDCs and PDCs pre surgery, but only on MDCs post surgery. Furthermore, only PDCs expressed significantly decreased levels of CCR2, both pre and post surgery. When the same surface markers were tested on MDCs and PDCs obtained from chronic pancreatitis patients we detected a similar phenotype as seen for the PDAC patients. These DCs expressed less
CD40 and PDL-1 and higher levels of ICOSL compared to the profile expressed by MDCs and PDCs from PDAC patients. These data indicate that PDAC patients have a DC compartment that is more affected than in chronic pancreatitis. Moreover, MDCs and PDCs obtained from PDAC patients had significantly reduced immunostimulatory capacity compared to healthy controls. Of note, low levels of ICOSL on both MDCs and PDCs and low levels of CCR2 on PDCs alone, were associated with poor clinical outcome.

We isolated MDCs and PDCs from healthy donors and incubated these cells in medium containing 25% plasma from a single or from several PDAC patients to investigate the involvement of blood related factors in the semi mature DC phenotype found in PDAC patients. These result showed that DCs cultured in plasma from single PDAC patients obtained a phenotype with several similarities to the MDC and PDC phenotype seen in PDAC patients ex vivo. CXCL8 and PGE$_2$, inflammatory factors upregulated in PDAC plasma (paper 3) were assessed for their effect on MDCs and PDCs derived from healthy individuals. PGE$_2$ enhanced the MDCs and PDCs expression of CD83, CD86, and CCR7, and declined the expression of B7H3 on MDCs. The expression of CD40, ICOSL, and PDL-1 by DCs was highly affected by the TLR ligation, whereas PGE$_2$ had lesser effects on the cells, indicating a role for PGE$_2$ in the induction of the semi mature MDCs and PDCs found in PDAC patients.

Conclusion

The data presented in this paper demonstrate the presence of MDCs and PDCs expressing a phenotype that is neither immature nor mature and we defined this phenotypic profile as semi mature (Figure 11). The correlation between poor patient survival and the low expression of ICOSL and CCR2 is probably due to that aggressive tumors have a higher capacity to exert systemic effects on the DCs compared to less aggressive PDAC tumors. These results indicate an essential role for the tumor microenvironment in the DC transformation into a semi mature DCs. Surgical resection of the tumor did not lead to recovered DC phenotypic profile and this is in accordance with the findings in paper III, where the removal of the tumor only slightly impacted on the levels of MDCs and PDCs. The blood DCs in PDAC patients could retain a semi mature phenotype associated with impaired immunostimulatory capacity for up to one year after the tumor removal, as it has been shown for breast cancer. Only a minority of the PDAC patients will be tumor free long enough to have a chance to recover their immune cell functionality. One explanation for this slow recovery could be that inflammatory factors such as PGE$_2$ still affect the DCs after the removal of the tumor. In paper III, we detected significantly increased levels of PGE$_2$ post surgery and this observation supports the
hypothesis. Inhibition of inflammatory factors, including PGE\textsubscript{2} and IL-1, could speed up the recovery of the immune function, ensuing in DCs capable to eliminate formations of new tumors.

Figure 11. Semi mature DCs in the blood of PDAC patients
The DCs obtained from the PDAC patients expressed levels of cell surface markers that was neither characteristic for immature nor mature DCs, but rather somewhere in between these two states of differentiation.
Conclusion

All solid tumors are associated with the presence of an inflammatory microenvironment consisting of both malignant and none-malignant cells. The ability of tumor cells to create and maintain a well balanced microenvironment is an essential event in the tumor evolution and the mechanisms behind this cross-talk between tumor and stroma cells are potential therapeutic targets in the future.

Our findings indicate that PDAC cells use IL-1α to induce a proinflammatory gene profile in CAFs, including several genes strongly associated with tumor angiogenesis, metastasis, migration, and immune suppression. Of note, the tumor levels of IL-1α also should also be considered as a prognostic marker in PDAC. The continuous production of IL-1α by the tumor cells followed by the activation of CAFs via the IL-1 receptor, creates a state of chronic inflammation that will go on and on as long as there are tumor cells present. By neutralizing IL-1α, the tumor cells lost much of their capacity to induce inflammatory genes in CAFs, but some of the genes were only partially influenced by the neutralization, pointing to the involvement of other mechanisms. The STAT3 pathway could be involved, as factors associated with NFκB activation are known to activate this pathway. Blocking of both these pathways should have the potential to drastically reduce the inflammation caused by the cross-talk between tumor and stroma cells.

The fibrotic stroma, i.e. CAFs, were the main producers of chemokines and also the predominant location of immune cells in the tumors. The tumor cells tactically disposition their environment; by initiating other cells to produce chemotactic chemokines, the immune cells will infiltrate these sites and leave the tumor cell areas alone. The infiltration of immune cells will further enhance the inflammation in the tumor by contributing with factors necessary for the progression of the tumor.

Our findings demonstrate that the inflammation induced in the tumor by the interaction between tumor and stroma cells is not only limited to the microenvironment, but expands to the entire body, affecting both DC subtypes in the peripheral blood. The amount of MDCs and PDCs in blood was decreased and they had a semi mature phenotype with impaired immunostimulatory activity. These effects were not reversed 3 months after the removal of the tumor and as a consequence, the PDAC patients probably have a DC compartment less effective in activating an immune response against the formation of secondary tumors. This could be one event responsible for the high tumor recurrence rate seen in PDAC patients.

To summarize, these findings show how tumor cells utilize stroma cells to create an inflammatory microenvironment, which also exerts systemic collectively effects by impairing
the levels, phenotype and functionality of DCs in the peripheral blood.
Future Challenges

The darwinistic evolution of tumors has resulted in the development of several mechanisms important for the progression of the tumor and it is a well established fact that patients are not cured by blocking only one pathway. Our studies indicate an essential role for IL-1α in the creation and maintenance of an inflammatory microenvironment in PDAC by inducing the expression of inflammatory genes in CAFs. Neutralization of IL-1α revealed several genes to be IL-1 dependent for expression, but factors such as COX-2, IL-6, and VEGFA were only partly decreased, pointing to the involvement of other mechanisms. STAT3 is found activated in most solid tumors and NFκB has been shown to indirectly activate STAT3, by the release of cytokines included in the IL-6 family. When first activated, the STAT3 pathway will produce inflammatory factors that have the potential to reactivate STAT3, thus creating a positive feedback loop. Moreover, both these pathways are observed constitutively activated in tumor cell lines and primary tumors. Blocking both IL-1 and STAT3 pathways could decrease the levels of inflammatory factors produced by CAFs even further and prevent the progression of the tumor by reducing the levels of factors associated with angiogenesis, growth, metastasis, and immune suppression.

CXCR4, a chemokine receptor binding the ELR- CXC chemokine CXCL12, is associated with tumor metastasis and was almost depleted in CAFs when cocultured with tumor cells. Coculture without direct cell contact using inserts, did not downregulate CXCL12, pointing to direct contact dependent mechanisms. As a consequence, low expression of CXCL12 in the tumor could promote metastasis by changing the chemokine gradient, so CXCR4 positive tumor cells in the tumor could migrate towards CXCL12 released from other parts of the body, for instance the liver or bone marrow. This could be an important mechanism in the formation of distant metastasis in PDAC and we want to evaluate the effect coculture of tumor cells and CAFs have on CXCL12/CXCR4 dependent migration of tumor cells.

The tumor microenvironment in PDAC contains several different types of none malignant cells and the success of the tumor depends on the ability of malignant cells to exploit these stroma cells to the benefit of the tumor. We have until recently been focusing on CAFs, but the tumors also include Langerhans islets and these endocrine cell clusters that produce inflammatory factors, including COX-2. We want to elucidate the mechanism behind the upregulation of COX-2 in tumor associated Langerhans islets and study the impact these endocrine islets have on the progression of the tumors.

DCs have been found to be sequestered in side the tumor by molecules such as CXCL8, preventing their migration into lymphatic tissue where they could elicit tumor specific
immune responses. We have shown systemic effects exerted by the PDAC tumors on the amount, phenotype, and functionality of DCs in the peripheral blood. Similar studies performed on tumor associated DCs could give important information about why the DCs fail to initiate an effective immune response against the tumor.
Populärvetenskaplig sammanfattning

Cancer i bukspottskörteln (pankreas cancer) är en av våra dödligaste cancerformer där endast en av 20 patienter förväntas överleva fem år. Trots intensiv forskning under de senaste 50 åren finns det fortfarande ingen effektiv behandling för denna sjukdom. Fram tills nyligen har forskningen nästan utslutande fokuserat på samspelet mellan tumörcellerna och de mekanismer de använder för att överleva och föröka sig. Detta kan i sig vara logiskt med tanke på att det just är tumörcellerna, direkt eller indirekt, som till slut dödar individen. Å andra sidan består cancervävnaden inte bara av tumörceller och genom att renodlat fokusera på samspelet mellan tumörcellerna riskerar man att missa viktiga signaler/interaktionsfaktorer som finns i verkligheten och som påverkar tillväxten. Tumörceller tros växa fram på ett Darwinistiskt sätt, dvs de tumörceller som har egenskaper att utnyttja omgivningen effektivast för överlevnad och tillväxt kommer att fortleva och föra sina egenskaper vidare. En sådan egenskap är förmågan att manipulera normala celler i omgivningen till att börja producera olika signalsubstanser och tillväxtfaktorer som kan gynna tumören, dvs tumören skapar en miljö som gynnar sin egen tillväxt. Den av tumören lokalt skapade miljön påminner mycket om den som ses lokalt vid sårläkning, dvs en miljö som är optimerad för celltillväxt och där nya blodkärl tillväxer. Av denna anledning kallas tumörer ibland för "sår som aldrig läker". Vid sårläkning stängs mekanismerna av då såret läkts, men vid cancer fortsätter signalerna. Bindvävsceller (fibroblaster) är en celltyp som tumörer ofta utnyttjar för att producera viktiga faktorer för tumöröverlevnad och tillväxt. Fibroblasterna förändras av tumörcellernas påverkan och blir sk cancerassocierade fibroblaster vilka i fallet med pankreas cancer kan utgöra så mycket som 70 % av tumörmassan. Då skapandet av denna lokala tumörmiljö, med interaktioner och utnyttjande av normala celler, tros vara avgörande för tumörens fortsatta utveckling är det sammantaget av stor vikt att vinna förståelse för de mekanismerna tumörcellerna använder i detta syfte för att kunna skapa framtida behandlingsstrategier för att stänga av dessa mekanismer. I denna avhandling har vi kunnat visa att tumörceller producerar en viktig signalmolekyl kallad IL-1α vilken påverkar fibroblasten genom att förmå dem att tillverka ett flertal faktorer som är viktiga för tillväxt av blodkärl (vilket är avgörande för syre och näringstillförsel till tumörcellerna), tillväxtfaktorer som stimulerar tumörcellöverväxning, immunmodulerande faktorer (som gör att tumören kan undgå kroppens effektiva immunförsvar) samt faktorer som möjliggör spridning av tumören till andra delar av kroppen. Genom att slå ut den här signalen har vi kunnat visa att tumörcellerna inte längre kan påverka fibroblasterna lika effektivt och således minskar ett flertal av de faktorer som gynnar tumörtillväxten. För att bekräfta upptäckten undersökte vi
pankreasvävnad från patienter med pankreascancer som opererats här i Linköping. Vi fann att 90% av patienterna hade IL-1α-producerande tumörceller och att produktionsnivån var kopplad till överlevnad, dvs de patienter med en tumör som inte producerade IL-1α eller som endast hade låga nivåer hade en längre överlevnad jämfört med de patienterna som hade en måttlig till hög produktion.

Vidare har vi studerat hur pankreas cancer påverkar dendritiska celler som finns i blodet. Dendritiska celler är viktiga försvarsceller som tar upp t.ex. virus, bakterier och tumörceller för att sedan presentera dem för och aktivera andra försvarsceller som är skapade för att specifikt och effektivt förgöra speciella faror som kroppen utsätts för. Våra resultat visar att tumörcellerna inte bara har en lokal påverkan på cellerna i sin närmiljö utan även påverkar celler i andra delar av kroppen i detta fall genom att minska antalet och påverka funktionen av dendritiska celler i blodet. Dessa förändringar kvarstod även efter att patienterna hade opererats, dvs efter att tumören hade avlägsnats. Antalet dendritiska celler i blodet var kopplat till patientöverlevnad, där patienter med högst antal kvarvarande dendritiska celler hade en längre medelöverlevnad.


Resultaten i avhandlingen visar på en viktig roll för kommunikationen mellan tumörceller och omgivande celler för att producera faktorer betydelsefulla för effektiv tumörtillväxt. Behandling med läkemedel mot IL-1α skulle kunna försvara kommunikationen mellan cellerna i mikromiljön och eliminera fibroblaster som försörjare av tumörcellerna. Detta skulle även kunna resultera i minskade nivåer av de faktorer som påverkar dendritiska celler negativt avseende antal och funktion hos patienter med pankreas cancer. Sammantaget har studierna visat på IL-1α som en lovande kandidat att utforska vidare för framtida potentiella läkemedel.
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