Tumour-specific immune responses and tumour stroma analysis in a murine model for pancreatic adenocarcinoma

Von der Naturwissenschaftlichen Fakultät der
Gottfried Wilhelm Leibniz Universität Hannover
zur Erlangung des Grades

Doktor der Naturwissenschaften
Dr. rer. nat.
genehmigte Dissertation

von
M.Sc. Benjamin Vermeer
geboren am 21. August 1972
in Amstelveen, Die Niederlande

2007

1st Referee:	Prof. Dr. T. F. Greten
2nd Referee:	Prof. Dr. W. Müller
Date of defence:	July 19, 2007

I dedicate this thesis to my Oma and my parents

Kurzzusammenfassung

Das humane Pankreaskarzinom steht an vierter Stelle der Todesursachen bei Krebserkrankungen in den Vereinigten Staaten. In der hier vorgestellten Arbeit wurden tumorimmunologische Untersuchungen an EL-TGF-α x $Trp53^{-/-}$ transgenen Mäusen, welche spontan im Alter von drei Monaten duktale Pankreaskarzinome entwickelten, durchgeführt, mit dem Ziel neue immunologische Therapieansätze zu evaluieren und etablieren.

Zunächst konnte in dieser Arbeit gezeigt werden, dass Tiere mit Pankreaskarzinomen spontan zelluläre und humorale Tumor-spezifische Immunantworten entwickeln, die ein progressives Tumorwachstum jedoch nicht aufhalten können. In Paralleluntersuchungen wurden Pankreastumorzellen gleichen Ursprungs subkutan in Wildtypmäuse implantiert. Diese Tumore induzierten eine ausgeprägte Tumor-spezifische Immunantwort, die dazu führte, dass die Tumore abgestoßen wurden. Im Gegensatz dazu wuchsen subkutan applizierte Tumorzellen in Interferon (IFN)-γ-knockout-Mäusen aus, was darauf hindeutet, dass die Abstoßung des Tumors IFN-γ vermittelt ist. Obwohl gezeigt wurde, dass $CD4^+CD25^+$ regulatorische T-Zellen die Tumor-spezifische Immunantwort unterdrücken können, sind die Frequenzen von $CD4^+CD25^+$ regulatorische T-Zellen in sowohl spontanen als auch in subkutanen Tumoren gleich. Interessanterweise, wenn Tumor-spezifische T-Zellen transferriert werden in entweder EL-TGF-α x $Trp53^{-/-}$ transgene Mäuse mit spontanen Pankreastumoren oder in C57BL/6 Wildtyp Mäusen mit subkutanen Tumoren, dann zeigten die Tumor-infiltrierten T-Zellen unterschiedliche „homing“ und phänotypische Eigenschaften.

Weiterhin wurde die humorale Immunantwort sowohl in Mäusen mit spontanen Tumoren als auch bei Tieren mit subkutan applizierten Tumorzellen untersucht. Hierbei konnte gezeigt werden, dass spontane Pankreastumore hauptsächlich eine Immunoglobulin (Ig) G2b Antikörperantwort induzierten im Gegensatz zu Tieren mit subkutan applizierten Tumoren, die eine IgG1 Antikörperantwort aufzeigten.

In weiteren Untersuchungen konnte gezeigt werden, dass die systematische Gabe von Cyclophosphamid eine Tumor-spezifische T-Zell vermittelte Immunantwort verstärkt, die zu einem verzögerten Tumorwachstum führte. Erwartungsgemäß fand sich dieser Befund nicht in T-Zell defizienten Tieren, was die Wichtigkeit einer potentiellen Immuntherapie hervorhebt.

Schließlich wurde untersucht, welchen Einfluss eine lokale Entzündungsreaktion auf die Tumor-spezifische Immunantwort hat. EL-TGF-α x $Trp53^{-/-}$ transgene Mäuse mit spontanen Tumoren und chronischer Pankreatitis einerseits und C57BL/6 Wildtyp Mäusen mit subkutanen Tumoren und chronischer Pankreatitis andererseits zeigten beide tendenziell eine verminderte Tumor-spezifische Immunantwort gegen das murine Pankreaskarzinom.

Diese Ergebnisse der vorliegenden Arbeit machen deutlich, dass transplantierte subkutane Pankreastumormodelle nur bedingt zum Verständnis der Tumorimmunologie beitragen und spontane murine Pankreastumormodelle geeigneter sind für vorklinische Studien zur Entwicklung von neuen Therapieansätzen.

Schlagworte: Pankreaskarzinom, Tumor-spezifische Immunantwort, Tumor-Stroma

Abstract

Human pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States. In the present study, tumour immunological studies on EL-TGF-α x $Trp53^{-/-}$ transgenic mice, which develop spontaneous ductal pancreatic adenocarcinoma three months after birth, were performed with the aim to evaluate and establish new immunotherapeutic approaches.

At first, this study showed that animals with pancreatic cancer develop spontaneous cellular and humoral tumour-specific immune responses, which nevertheless were not able to inhibit progressive tumour growth. In a parallel study, pancreatic tumour cells derived from the spontaneous tumour were subcutaneously injected into wild type mice. These subcutaneous tumours induced distinct tumour-specific immune responses, which led to tumour rejection. In contrast, subcutaneously injected tumours grew progressively in interferon-γ knockout mice, which indicated that the rejection of the tumour is an interferon-γ mediated process. Although $CD4^{+}CD25^{+}$ T regulatory cells have been shown to suppress tumour-specific immune responses, the frequencies of $CD4^{+}CD25^{+}$ T regulatory cells were similar in both the spontaneous and the subcutaneous tumours. Interestingly, if tumour-specific T cells were transferred into either EL-TGF-α x $Trp53^{-/-}$ transgenic mice with spontaneous pancreatic tumours or into C57BL/6 wild type mice with subcutaneous tumours, then the tumour-infiltrating T cells demonstrated different homing and phenotypic properties.

Furthermore, the humoral immune response was investigated both in mice with spontaneous pancreatic tumours and in mice, which were subcutaneously injected with the tumour cells. Hereby it could be demonstrated that spontaneous pancreatic tumours mainly induced an immunoglobulin G2b antibody response, in contrast to mice with subcutaneously administered tumours, which induced an immunoglobulin G1 antibody response.

It could be shown in additional studies that the systematic administration of cyclophosphamide amplified the tumour-specific T cell mediated immune response, which led to a delay in tumour growth. As expected, this finding was not detected in T cell-deficient mice, which emphasized the importance of a potential immunotherapy against pancreatic cancer.

Finally, the influence of a local inflammatory reaction on tumour-specific immune responses was investigated. Thereby, EL-TGF-α x $Trp53^{-/-}$ transgenic mice with spontaneous pancreatic tumours and C57BL/6 wild type mice with subcutaneous pancreatic tumours, both suffering from chronic pancreatitis, revealed a decrease in the tumour-specific immune response against the murine pancreatic carcinoma.

The results of the current study clearly demonstrate that transplantable pancreatic tumour models are only a poor model to understand tumour-specific immune responses. Therefore, spontaneous tumour mouse models are more suitable for the study of the disease and should be used for preclinical testing of possible new therapeutic approaches.

Keywords: pancreatic adenocarcinoma, tumour-specific immune response, tumour stroma

1 Introduction

Paul Ehrlich was one of the first who noted that the immune system could inhibit a potential outgrowth of carcinomas (Ehrlich, 1909). In the midpoint of the twentieth century, scientists introduced his idea of immune control of neoplastic disease as cancer immunosurveillance (Burnet, 1957; Burnet, 1970). Thereby they suggested that tumours arise with a frequency that is similar to infection with pathogens and that the immune system constantly recognizes and eliminates these tumours based on their expression of antigens.

1.1 Tumour immunology

With the availability of inbred strains of mice, the idea that tumours were immunologically distinguishable from normal cells could be critically tested. The demonstration that mice could be immunized against syngeneic transplants of tumours induced by chemical carcinogens, viruses or other means established the existence of "tumour-specific antigens" and provided strong evidence for the immunosurveillance hypothesis (Klein, 1966; Old and Boyse, 1964; Darnell and Posner, 2003; Dunn et al., 2002). In addition, studies using gene-targeted mice that lack the *recombinase activating gene* (*RAG)-1* or *RAG-2* revealed that these immuno-deficient mice, which suffer a complete block in lymphocyte development at the gene-rearrangement stage and thus lack T, B and natural killer T (NKT) cells, have an enhanced susceptibility to chemically (e.g. methylcholanthrene (MCA)) induced and spontaneous tumours (Shankaran et al., 2001; Smyth et al., 2001a). Similar results were obtained in different mouse models reviewed and summarized by Dunn *et al.* (Dunn et al., 2002; Dunn et al., 2004b). The induction of tumour-specific immune responses is provoked by the adaptive immune system including interactions of antigen-presenting cells (APCs), cytotoxic cluster of differentiation (CD)8^+ T cells (CTLs), T_{Helper} (T_H) and plasma cells as well as the effector cells of the innate immune system, such as natural killer (NK) and NKT cells, which can recognize transformed cells and finally eliminate them. These observations demonstrate that components of the immune system have an influence on the tumour development in mice.

1.1.1 Adaptive and innate effector mechanisms in cancer immunity

Preclinical and clinical studies have demonstrated that activation of both $CD4^+$ and $CD8^+$ T cells is critical for generating the most potent anti-tumour immune responses (Marchand et al., 1995; Rosenberg et al., 1998; Jager et al., 1999). CTLs are able to lyse tumour cells directly upon recognition of peptide-major histocompatibility complex (MHC)-class I complexes expressed by the tumour. APCs can initiate antigen-specific T and B cell responses by capturing the antigens that are secreted or shed by tumour cells or released after cell lysis. As professional APCs, dendritic cells (DCs) are the most powerful stimulators of naïve T cells. Processing and presentation of tumour antigens by MHC class I and class II molecules on a single DC can enable priming and activation of both $CD4^+$ and $CD8^+$ T cells. APCs are able to present endocytosed tumour antigens not only to $CD4^+$, but also to $CD8^+$ T cells (cross priming) (Albert et

al., 1998; Huang et al., 1994). DCs and macrophages produce interleukin (IL)-12, which has been shown to possess potent anti-tumour activity in a wide variety of murine models of various histologies (Wolf et al., 1991; Brunda et al., 1993; Brunda, 1994; Zou et al., 1995; Robertson and Ritz, 1996; Nastala et al., 1994; Tannenbaum et al., 1996; Wigginton et al., 1996). As mentioned previously, $CD4^+$ T_H1 cells are essential for maintaining CTL effector functions. Costimulation occurs via $CD4^+$ T cell derived cytokines (e.g. T_H1 cytokines such as IL-2 and interferon (IFN)-γ or T_H2 cytokines such as IL-4) and also through the APC itself via CD40-CD40L or B7-CD28 interactions. In addition, memory $CD4^+$ T cells and $CD8^+$ T cells play a critical role in maintaining the protective immunity (Smyth et al., 2001b; Greten and Jaffee, 1999; Swain et al., 1991; Mackay, 1993).

The innate immune system consists of cells that protect the host in a non-specific manner. Amongst the cells of the innate immune system are natural NK cells, NKT cells, and macrophages. MHC class I-deficient tumour cells can spontaneously be killed by NK cells (Hoglund et al., 1988; Whiteside and Herberman, 1995). NK cells mediate cytotoxicity via perforin, produce a variety of cytokines, such as IFN-γ and are highly responsive to many cytokines, including IL-2, IL-12, IL-15 and IFNs.

NKT cells, a subset of T cells that express NK cell markers such as NK1.1, have been shown to exhibit the capacity to regulate both CTL and NK cell anti-tumour activity (Godfrey et al., 2000; Smyth et al., 2000). NKT cells have a restricted T cell receptor (TCR) repertoire and recognize the nonclassical MHC class I protein CD1d in the context of glycolipids. Several studies have demonstrated the importance of NKT cells in immune regulation and in the prevention of autoimmune diseases in mice, which supports the theory that NKT cells can act as suppressor cells (Hong et al., 2001; Sharif et al., 2001; Miyamoto et al., 2001). NKT cells express a variety of cell-death-inducing effector molecules and have been demonstrated to kill tumour target cells *in vitro*. The incidence of methylcholanthrene-induced tumour development is higher in NKT cell-deficient mice (Smyth et al., 2000). In addition, NKT cells rapidly secrete large amounts of both pro-inflammatory T_H1 cytokines such as IFN-γ, and anti-inflammatory T_H2 cytokines, such as IL-4 and IL-10, bridging the innate and adaptive immune system (Smyth et al., 2002).

1.1.2 Tumour antigens

Tumour rejection usually requires $CD4^+$ and $CD8^+$ T cells that recognize tumour-specific peptides on MHC class I and class II molecules, respectively. Upon activation, CTLs can directly kill tumour cells by cytotoxic molecules, such as perforin or granzyme B, and secrete different effector cytokines, e.g. IFN-γ and the tumour necrosis factor (TNF)-α. CTLs are known so far as the main effector cells in most tumours. However, their survival and activation is dependent on T_H cells (Bourgeois et al., 2002; Schoenberger et al., 1998).

Most of the murine and human tumour antigens known so far have been identified by screening tumour-derived cDNA libraries, using tumour-reactive T cell lines and clones from cancer patients (van der Bruggen et al., 1991; van der Bruggen et al., 2002; Rosenberg, 1996; Lurquin et al., 1989; De Plaen et al., 1988; Boon, 1993). Novel and previously defined tumour antigens have been identified by the serological analysis of recombinant cDNA expression libraries (SEREX) method, including two melanoma antigens (MAGE-1 and tyrosinase), both originally identified by cloning the epitopes

recognized by CTLs. The esophageal cancer-associated antigen NY-ESO-1, also detected by SEREX, was subsequently shown to be reactive with T cells (Chen et al., 1997; Greten and Jaffee, 1999; Rosenberg, 1999). So far, only a few $CD4^+$ T cell epitopes from tumour antigens have been identified, such as tyrosinase, MAGE-1 or NY-ESO (Topalian et al., 1994; Chaux et al., 2001; Jager et al., 2000; Zarour et al., 2000; Zeng et al., 2001; Zeng et al., 2000; Topalian et al., 1996; Zarour et al., 2002).

Additionally, potential target antigens have been defined by gene expression profiling searching for overexpressed gene products in neoplastic cells compared with normal tissues. Microarray-based studies of gene expression in a large number of tumour types have repeatedly revealed novel features of human cancer and have led to the identification of a very large number of potential tumour antigens (Mohr et al., 2002). Most of the tumour antigens identified fall into three categories: 1) Tumour-specific antigens, 2) Tumour-associated antigens (TAAs) that are present in tumour but silent in normal tissue, also called cancer testis antigens, and 3) Differentiation antigens expressed both in tumour and normal tissue. Examples of tumour-associated antigens that fall into each of these categories are provided in **Table 1.1**. It should be stressed that these categories are not mutually exclusive and tumour antigens may fall into more than one category. For example, the p53 tumour-suppressor gene product is frequently mutated in cancer cells, resulting in the accumulation of p53 protein in these cells in concert with reduced cell-cycle regulatory control by the tumour cells. Based on these parameters, p53 would then be simultaneously classified as both an overexpressed / accumulated tumour antigen and a mutated tumour antigen. In a similar fashion, tyrosinase represents a normal melanocytic protein that can be both overexpressed and altered in its posttranslational modification, leading to differential recognition of melanoma cells versus normal melanocytes by specific T lymphocytes.

Category	Example Antigen	Cancer Histology
Oncofetal	CEA	Colorectal carcinoma
	Immature laminin receptor	RCC
	TAG-72	Prostate carcinoma
Oncoviral	HPV E6, E7	Cervical carcinoma
Overexpressed/accumulated	BING-4	Melanoma
	Calcium-activated chloride channel 2	Lung carcinoma
	Cyclin-B_1	Multi
	9D7	RCC
	Ep-CAM	Breast carcinoma
	EphA3	Multi
	Her2/neu	Multi
	Telomerase	Multi
	Mesothelin	Ductal pancreatic carcinoma
	SAP-1	Colorectal carcinoma
	Survivin	Multi
Cancer-Testis	BAGE family	Multi
	CAGE family	Multi
	GAGE family	Multi
	MAGE family	Multi
	SAGE family	Multi
	XAGE family	Multi
	NY-ESO-1/LAGE-1	Multi
	PRAME	Multi
	SSX-2	Melanoma, Multi
Lineage Restricted	Melan-A/MART-1	Melanoma
	Gp100/pmel17	Melanoma
	Tyrosinase	Melanoma
	TRP-1/-2	Melanoma
	P.polypeptide	Melanoma
	MC1R	Melanoma
	Prostate-pecific antigen	Prostate
Mutated	β-catenin	Melanoma, Prostate, HCC
	BRCA1/2	Breast, ovarian carcinoma
	CDK4	Multi
	CML66	CML
	Fibronectin	Multi
	MART-2	Melanoma
	p53	Multi
	Ras	Multi
	TGF-βRII	Colorectal carcinoma
Posttranslationally altered	MUC1	Ductal carcinoma, RCC
Idiotypic	Ig, TCR	B, T leukemia, lymphoma, myeloma

BRCA = breast cancer antigen; CDK4 = cyclin-dependent kinase-4; CEA = carcino-embryonic antigen; CML66 = chronic myelogenous leukemia (antigen) 66; CT= cancer testis; HPV = human papilloma virus; Ep-CAM = epithelial cell adhesion molecule; Ig = immunoglobulin; MART-1/-2 = melanoma antigen recognized by T cells-1/-2; MC1R = melanocortin-1-receptor; SAP-1 = stomach cancer- associated protein tyrosine phosphatase-1; TAG-72 = tumor antigen-72; TCR = T cell receptor; TGF-βRII = transforming growth factor-β receptor II; TRP = tyrosinase-related protein.

Table 1.1 ***General categories and examples of tumour antigens*** *(adapted from Zarour et al., 2003).*

1.1.3 Tumour immunotherapy

While early localized cancer may effectively be treated by radical excision, metastatic cancer is fatal in most cases. Nevertheless, some patients show spontaneous regression of both primary tumours and metastasis, which is largely attributed to the adaptive

immune response, and the presence of tumour-infiltrating CTLs (Cho et al., 2003; Haanen et al., 2006; Kondratiev et al., 2004). The increased tumour incidence in immune suppressed individuals indicates that cancer, at least in part, can be controlled by the immune system (Adami et al., 2003). Therefore, efforts are being made to stimulate the patient's immune effector cells to recognize and destroy cancer cells.

1.1.3.1 Cancer vaccines

Several different active immunotherapies, such as vaccination with whole cells, proteins, peptides, nucleic acids encoding the respective antigens or combinations thereof, are currently under investigation (Otto et al., 2005; Slingluff, Jr. et al., 2004; Sondak and Sosman, 2003; Su et al., 2003; Trefzer et al., 2004). Although immune-based treatments are able to induce tumour responses in selected patients, a cancer vaccine that can reliably induce tumour destruction or improve the survival of patients is yet to be established. In this respect, it is important to note that the majority of current vaccination strategies are based on TAAs. This strategy is actually prone to several pitfalls since cancer cells may be subject to several immune evasion mechanisms (Ahmad et al., 2004; Pawelec, 2004; thor Straten et al., 1999).

1.1.3.2 Adoptive transfer

Adoptive T cell therapy generates large number of tumour-specific T cells that are transferred into cancer patients. In this situation, a variety of factors influence tumour rejection. The specific effector T cells should be easily expanded and their survival supported before they are transferred at a high number into patients (Dudley and Rosenberg, 2003; Lyman et al., 2004; Nguyen et al., 2002). Furthermore, T cells should have a high avidity against the tumour cells (Sadovnikova and Stauss, 1996). The tumour antigen should be expressed in large amounts and cannot be selected against (Spiotto et al., 2002). Finally, the tumour burden should be low, such that the T cells can efficiently infiltrate into the tumour tissue (Ganss et al., 2004; Garbi et al., 2004).

1.1.4 Tumour immune escape mechanisms and possible therapeutic approaches

Although tumour cells are derived from normal tissue cells and thus bear "self signals" in classical immunological terms, it is evident that the immune system is able to recognize tumour cells as a harassment for the body and consequently tries to eliminate these cells. By contrast, tumour cells acquire various characteristics which allow them to evade this immunological surveillance.

1.1.4.1 Alterations of the antigen processing machinery

The decrease of MHC class I molecules is a frequent mechanism used by tumours to escape from recognition and destruction by CTLs (**Figure 1.1**a) (Garrido et al., 1993; Hicklin et al., 1999). The presentation of TAA-derived peptides to CTLs can be lost by deletion, silencing or mutations of the TAA gene itself or defects in the antigen presentation pathway, such as loss or mutation of human leukocyte antigens (HLA) class I heavy chains, β2-microglobulin, or components of the protein processing machinery, e.g. the transporter for antigen presentation (TAP)1 and tapasin. It has been proposed that the major force contributing to the appearance of these MHC class I-deficient tumour clones is T lymphocyte immunoselection (Garcia-Lora et al., 2003; Jager et al., 1997). This hypothesis is further supported by animal studies of Garcia-Lora and co-workers, demonstrating that the MHC class I phenotype of a particular

tumour depends on the immune status of the host; in this manner, metastatic colonies derived from a mouse B9 fibrosarcoma clone displayed a MHC class I-negative phenotype in immunocompetent mice and a MHC class I-positive phenotype in immuno-deficient athymic nude mice (Garcia-Lora et al., 2001).

As described above, a low level or loss of MHC class I molecules may lead to less efficient rejection of a tumour by CTLs. Concomitantly, yet another tumour immunosurveillance is mediated by NK cells stimulated by the 'missing self' signals (Ljunggren and Karre, 1990). NK cells monitor MHC class I cell surface expression and eliminate those cells with downregulated HLA / H-2 class I molecules (Moretta et al., 1996). Thus, variants of the tumour that have low levels of MHC class I molecules, although less sensitive to cytotoxic T cells, become susceptible to NK cells. However, under physiological conditions, only few NK cells are found in tumours or they may lack them completely (Ravenswaay Claasen et al., 1992).

The ability of MHC class I-negative tumour cells to escape NK cell recognition may be attributed to the susceptibility to NK-mediated versus T cell-mediated anti-tumour mechanisms. In contrast to the commonly accepted paradigm that low expression of MHC class I favours escape from T cell-mediated immunity, several groups found in human uveal melanoma a correlation between HLA class I downregulation and favourable prognosis, resulting in a better survival of the patient (Ericsson et al., 2001; Jager et al., 2002).

A factor important for the final outcome of the MHC class I-negative tumour–host interaction may be the expression of non-classical MHC class Ib molecules, such as HLA-G and HLA-E. Expression of HLA-G and HLA-E has been described in several tumours of different origins, and this expression protects tumour cells from lysis by T and NK cells via ligation of the inhibitory receptors, such as ILT2 or CD94/NKG2A, respectively (**Figure 1.1**c) (Algarra et al., 2004; Wiendl et al., 2002; Malmberg, 2004; Wischhusen et al., 2005). Interestingly, IFN-γ was shown to protect targets from NK cells via the upregulation of HLA-E (Cerboni et al., 2001).

Additionally, tumour cells can evade an attack from NK cells through their expression of MHC class I chain-related glycoprotein A (MICA). NK cells express activating receptors, such as NKG2D, which react with the antigens MICA. These antigens are often upregulated as a consequence of neoplastic transformation (Pende et al., 2002). MICA may be proteolytically cleaved from the cell surface by matrix metalloproteinases (MMPs), expressed in the tumour microenvironment, and soluble MICA prevents activation of effector cells by binding to NKG2D (**Figure 1.1**c) (Salih et al., 2002).

1.1.4.2 Soluble immunosuppressive factors

Another mechanism by which tumours escape from the immune system, may be achieved by utilizing soluble immunosuppressive factors (**Figure 1.1**b). These have been found in mice with experimentally induced tumours and patients with cancer (Kiessling et al., 1999). Apparent unresponsiveness of the immune system in patients with cancer has been attributed to the secretion of immunosuppressive cytokines, such as IL-10 and the transforming growth factor (TGF)-β. TGF-β is often found at high levels in different malignancies and is associated with a poor prognosis as well as a lack of response to tumour immunotherapy (Gorsch et al., 1992). IL-10 is frequently detected in high amounts in ovarian cancer patients (Pisa et al., 1992). Secretion of

TGF-β and IL-10 by tumour cells themselves or stroma cells may inhibit the eradication of tumour cells by preventing T cell proliferation and differentiation or T cell stimulatory functions of APCs (**Figure 1.1**b) (Fontana et al., 1989; Gorelik and Flavell, 2001; Mocellin et al., 2004). IL-10 is described to regulate the differentiation, maturation and functional status of DCs, thus interfering with the induction of anti-tumour responses (De Smedt et al., 1997). Furthermore, IL-10 seems to suppress T cell-mediated immunity by downregulating the function of TAP, and the expression of MHC class I molecules on target cells (Kurte et al., 2004). In growing murine tumours, T cells condition tumour-infiltrating macrophages (TIMs) to produce IL-10, which in turn favours the tumour growth. In contrast, upon cyclophosphamide (Cy)-induced T cell inactivation, TIMs immediately start to produce IFN-γ and rejection of the tumour occurs (Ibe et al., 2001).

A functionally distinct subpopulation of T lymphocytes called $CD4^+$ T regulatory (T_{reg}) cells (also called $CD4^+$ suppressor T cells) also express IL-10 and TGF-β and are able to suppress the anti-tumour CTL and NK activities (Seo et al., 1999). T_{reg} cells, initially identified by Sakaguchi *et al.*, co-express CD4 and CD25 and have negative immune regulatory function (Sakaguchi et al., 1995). In particular, T_{reg} cells control key aspects of immunological tolerance to self-antigens and foreign antigens in humans and mice. Elimination of these cells, which comprise five to ten percent of $CD4^+$ T cells in humans, results in the development of autoimmune disease such as diabetes or hypothyroidism due to thyroiditis (Sakaguchi et al., 1995; Roncarolo et al., 2001). The concomitant transfer of $CD4^+CD25^+$ T cells abrogates the development of these autoimmune diseases. T_{reg} cells have been shown to infiltrate several types of human tumours, including lung, breast and pancreas carcinomas, thereby suppressing the function of tumour-reactive T cells (Liyanage et al., 2002; Woo et al., 2002). Blockage or elimination of these cells enhanced tumour immunosurveillance and efficacy of anti-tumour vaccines (Shimizu et al., 1999). Furthermore, the depletion of $CD4^+CD25^+$ cells in combination with the injection of an antibody capable of blocking cytotoxic T lymphocyte-associated antigen (CTLA)-4 increased reactivity to a known tumour-associated antigen, and resulted in tumour rejection (Sutmuller et al., 2001). In BALB/c mice, the depletion of $CD4^+CD25^+$ cells resulted in mammary tumour regression and enhanced susceptibility to thyroiditis (Wei et al., 2004).

1.1.4.3 Tumour counterattack

The mechanism termed tumour counterattack involves the upregulation of the death-inducing Fas ligand (FasL/CD95L) to subvert the immune system, and to suppress the anti-tumour defence. FasL has been reported to be expressed on many human tumour cells of various origins, including melanoma, colon, breast and esophagal carcinoma (Andreola et al., 2002; Okada et al., 2000; Reimer et al., 2000; Bennett et al., 1998). Expression of FasL in tumours implies that cancer cells are themselves resistant to Fas-induced cell death, which preserves them from undergoing apoptosis-mediated killing by cytotoxic T cells (Rivoltini et al., 1998). In parallel, the discovery of tumour-associated FasL raised the possibility that FasL could mediate immune privilege in human tumours by enabling the tumour cells to actively kill tumour-infiltrating lymphocytes (TILs) and thus suppress the anti-tumour response, a phenomenon called tumour counterattack (**Figure 1.1**c) (Igney et al., 2000). With regard to the tumour counterattack hypothesis, the *in vivo* results are very controversial. Various animal studies directly demonstrate a suppression of T cells *in vivo*, e.g. in allogeneic mice

injected with FasL-transfected colon carcinoma cells, allo-antibodies were absent, whereas allospecific T lymphocytes were abolished and T_H cell activity was reduced (Arai et al., 1997). By contrast, other researchers have shown that FasL expression on tumours inhibits the expansion of cytotoxic anti-tumour T cells *in vitro*, but induces the rejection or delayed tumour growth *in vivo* (Igney et al., 2005; Seino et al., 1997).

Another direct feature of cancer cells against T cell anti-tumour immune response is the induction of T cell anergy by T cell receptor stimulation in the absence of adequate co-activation, such as lack of CD28 or ICOS, or active negative signalling (Carreno and Collins, 2002; Carreno and Collins, 2003).

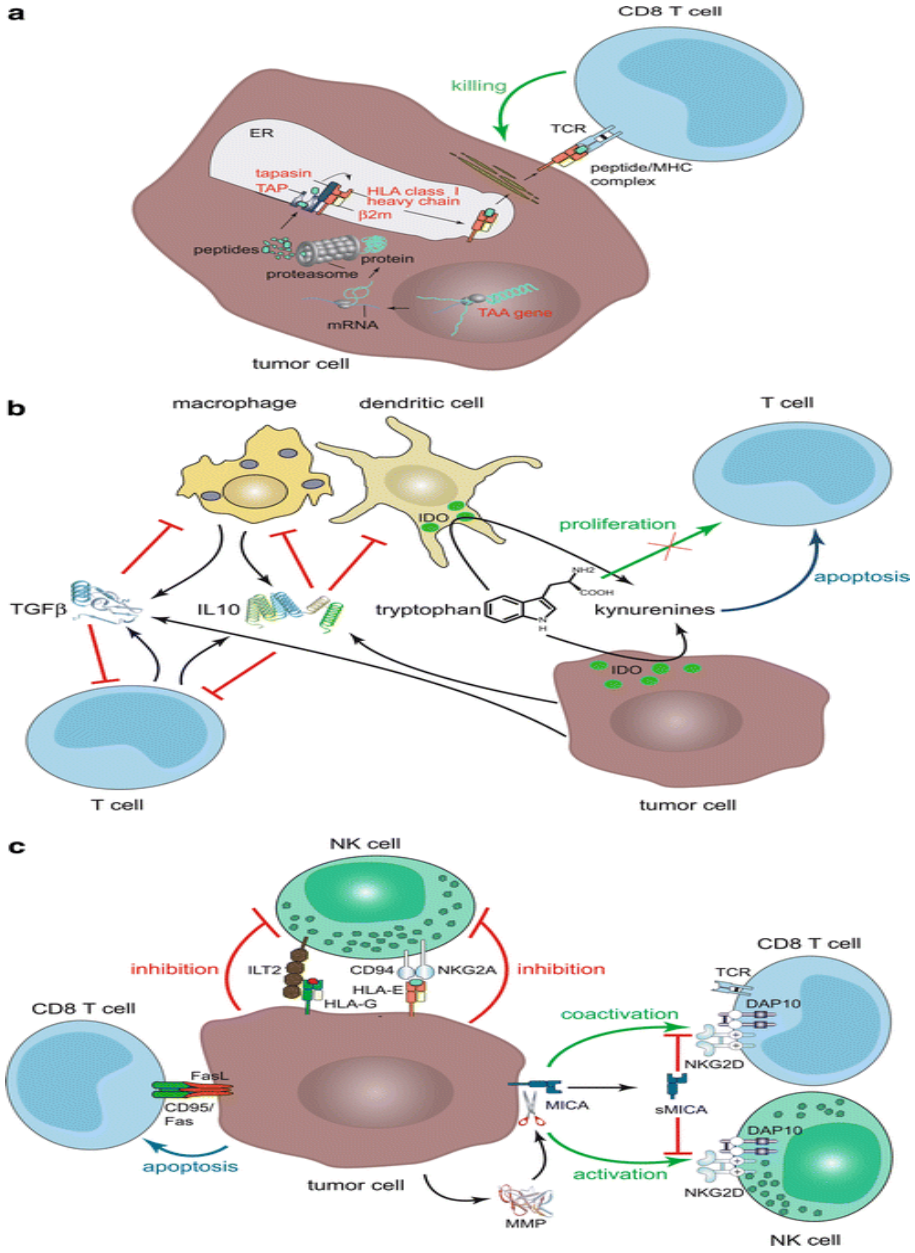

Figure 1.1 ***Tumour immune escape mechanisms.*** *(a) Alterations in expression of tumour-associated antigens (TAA) and MHC class I abrogate antigen presentation. Cellular components that can contribute to immune evasion by mutation or altered expression are highlighted in red. (b) Secretion of inhibitory molecules (IL-10, TGF-β) by tumour and stroma cells may reduce anti-tumoural activity of effector cells. IDO expression in tumour cells and/or TIMs or APC in the tumour draining lymph nodes may inhibit T cell proliferation. (c) Activation of cytotoxic effector cells may be prevented by enhanced negative and/or reduced activating signalling. (1) Expression of FasL by tumour cells may induce apoptosis of tumour-reactive T cells. (2) Upregulation of MHC class Ib antigens (HLA-E, HLA-G) inhibit cytotoxicity of NK and T cells by delivering inhibitory signals via CD94/NKG2A and ILT2, respectively. (3) Activation or coactivation of NKG2D expressing NK and T cells respectively may be prevented by loss of MIC expression or the cleavage of MICA by MMPs; soluble MICA binds to and thereby blocks activating effects of NKG2D on effector cells (adopted from Hofmeister et al., 2006).*

1.1.4.4 Tumour-derived exosomes and microvesicles

More than 25 years ago, Taylor and colleagues were the first to describe the ability of tumours to release or shed membrane vesicles (MV) or exosomes, expressing molecular markers, which are characteristic for tumour plasma membranes (Taylor et al., 1983). Numerous studies have described the isolation of exosomes from tumour cells *in vivo* and *in vitro*, and have demonstrated the presence of tumour-associated antigens, FasL, apoptosis-inducing ligand (APO2L) / tumour necrosis-factor apoptosis-inducing ligand (TRAIL) and class I MHC antigens, thus providing an explanation for their involvement in immune suppression. Recent studies shed new light on the role of FasL in immune evasion of tumours of varying origin, including colorectal cancer cells, ovarian tumours and oral cell carcinoma (Andreola et al., 2002; Andre et al., 2002; Martinez-Lorenzo et al., 2004; Wolfers et al., 2001; Huber et al., 2005; Taylor et al., 2003; Kim et al., 2005). Common to all of these studies is that the body fluids of the patients contained FasL-positive microvesicles, which induced apoptosis of activated T cells. This apoptosis-inducing pathway via the release of FasL-positive MVs may indeed play a significant role in eliminating the most effective component of the anti-tumour T cell response, and provides an explanation for the observed spontaneous T cell apoptosis in the peripheral circulation of patients with cancer (Kim et al., 2004).

1.1.5 Tumour microenvironment

In the early growth of tumours, cancer cells form a premalignant (neoplastic) lesion that is embedded in the microenvironment of a given tissue (usually epithelium), but separated from the surrounding tissue and contained within the boundary of a basement membrane (Hanahan and Weinberg, 2000). This is called carcinoma *in situ* (CIS). But cancers are not only autonomous masses of mutant cells. The basement membrane, innate and adaptive immune cells, epithelial cells, fibroblasts, cells that form the blood and lymphatic vasculature, specialized mesenchymal cell types that are unique to each tissue, capillaries and extracellular matrix surrounding the cancer cells constitute the tumour microenvironment (also called tumour stroma) (**Figure 1.2**) (Ronnov-Jessen et al., 1996; Schreiber H. and Rowley D.A., 1999).

The stroma cells can actively contribute to the tumourgenesis by secreting growth supporting factors, enzymes degrading the extracellular matrix, cytokines, or angiogenic factors. The microenvironment influences the stroma cells in such a way that they promote tumour progression by supporting recruitment of reactive stroma fibroblasts, secretion of proteolytic enzymes and peptide mediators, production of modified extracellular matrix, lymphoid and phagocytic infiltration, and allowing vasculo- and angiogenesis (Kalluri, 2003).

Often, substantial numbers of T cells and macrophages are found in the stroma of tumours. Nevertheless, the tumour grows progressively (Mantovani et al., 1992). A previous study revealed that between six and nine days after the injection of tumour cells into naïve mice, CTLs become activated but then were downregulated by the progressively growing tumour correlating with the appearance of T_{reg} cells in the tumour stroma (North and Bursuker, 1984).

Since development and progression of tumours is not only dependent on the cancer cells themselves but also on the stroma cells, the tumour stroma may also serve as a target for immune intervention. In contrast to cancer cells, tumour stroma cells are genetically

more stable, so that at least some immune evasion mechanisms of tumours do not apply to these cells. Nevertheless stroma cells differ from their normal counterparts by upregulation or induction of various antigens. Some of the tumour stroma-associated antigens are highly selective for the tumour microenvironment. It should be noted that some tumour stroma-associated antigens may be expressed in the neoplastic cells as well and that they are not confined to one histiotype; indeed, they may be expressed by a broad spectrum of solid tumours. Thus, therapies designed to target the tumour are not restricted to a selected tumour entity.

Stroma fibroblasts, endothelial cells and macrophages are promising cellular targets for stroma-directed cancer immunotherapy. Cancer-associated fibroblasts (CAFs) contribute to the development of cancer by secreting growth promoting factors, such as the TGF-β, matrix degrading enzymes like the matrix metalloproteinases (MMPs), and angiogenic factors like the vascular endothelial growth factor (VEGF) (Egeblad and Werb, 2002; Kalluri and Zeisberg, 2006).

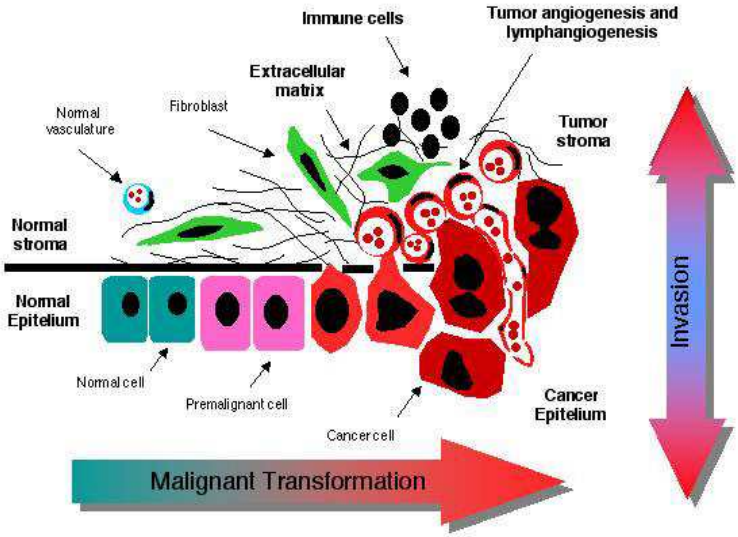

Figure 1.2 ***Tumour stroma.*** *Solid tumours are composed of malignant cells embedded in stroma consisting of non-malignant cells and extracellular matrix. A substantial number of immune cells, such as granulocytes, macrophages (TIMs) and lymphocytes (TILs) as well as non-bone-marrow-derived cells, such as endothelium (TECs) and other cells of the vasculature and fibroblasts (CAF), called stroma cells, can be part of the tumour stroma. The formation of new blood vessel and lymphatics (tumour angiogenesis and tumour lymphangiogenesis, respectively) is a characteristic feature of the tumour stroma and crucial for the tumour development (from the homepage of the "Centre Hospitalier Universitaire Vaudois", Lausanne, Switzerland).*

1.2 Pancreas and pancreatic cancer

In the upper abdomen, behind the stomach and the intestine, lies the pancreas, which is a small, spongy organ (**Figure 1.3**a). The pancreas is an endocrine as well as an exocrine gland, which has two main functions. It produces a fluid which contains enzymes that are needed to digest food and it secretes hormones which, among other things, help to maintain and regulate the body sugar levels (glucose metabolism).

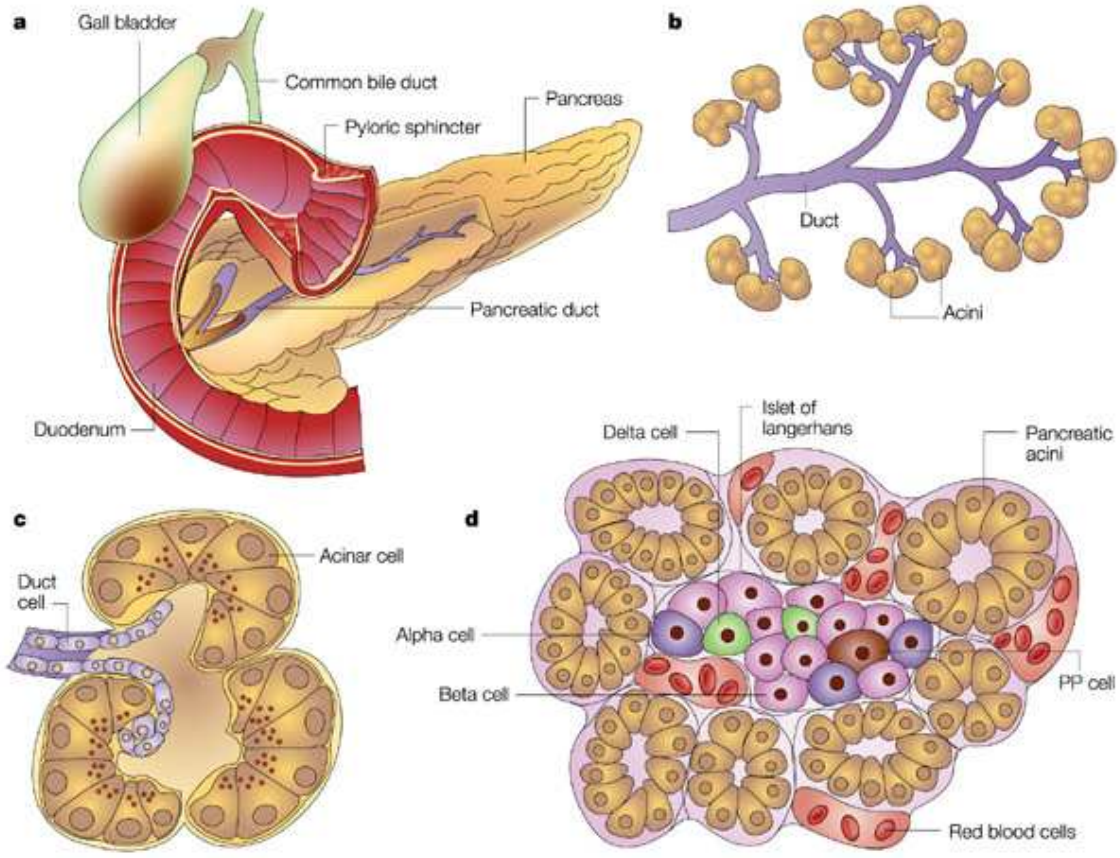

Figure 1.3 ***Anatomy of the pancreas.*** *(a) The pancreas lies behind the stomach and the intestine. Two major physiological processes are regulated by two separate functional units of the pancreas: digestion and glucose metabolism. (b and c) The exocrine pancreas consists of acinar and duct cells. The acinar cells that are organized in grape-like clusters produce digestive enzymes and constitute the bulk of the pancreatic tissue. (a) The ducts, which add mucous and bicarbonate to the enzyme mixture, form a network of increasing size, culminating in main and accessory pancreatic ducts that empty into the duodenum. (d) The endocrine pancreas, which consists of four specialized cell types (shown in different colours) organized into the Langerhans Islet and embedded within acinar (exocrine) tissue, secretes insulin and glucagon into the bloodstream (adapted from Bardeesy and DePinho, 2002).*

The pancreatic enzymes are produced in acinar cells, which form the exocrine gland (**Figure 1.3**b and c). Acinar cells release salts and enzymes into small tributaries which collect and transport this pancreatic fluid. These small ducts coalesce into the main pancreatic duct (**Figure 1.3**a and b), which eventually join with the bile duct and empty its combined digestive contents into the duodenum.

Inside of specialized groups of cells called the Islet of Langerhans (**Figure 1.3**d), the pancreas produces hormones, which are secreted directly into the blood stream. These hormones have numerous effects. The hormone insulin is produced by so-called beta cells and is mainly responsible for lowering the level of glucose in the blood (**Figure 1.3**d). Alpha cells of the Islet of Langerhans produce the hormone glucagon, which tends to increase the level of blood sugar (**Figure 1.3**d). Other hormones, as well as various peptides, are produced by the endocrine pancreas, e.g. somatostatin, a hormone which inhibits the secretion of insulin.

Cancer that starts in the pancreas is called pancreatic cancer. In up to 95% of the cases, pancreatic cancer arises from the exocrine portion of the organ. The least common exocrine cancer comes from the acinar cells. Most of the exocrine tumours (~90%) are from ductal cells, those which line the pancreatic ducts. These tumours are classified as carcinomas, a word that refers to tumours arising from a lining cell. The adenocarcinomas (cancerous glandular tumours) are by far the most common type of pancreatic cancer. Adenocarcinomas begin in the glandular ducts and are very aggressive neoplasms.

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States. This is mainly because pancreatic cancer is diagnosed late in the course of the disease, when the tumour has spread locally or metastasized, and curable resection is no longer possible. So far, surgical resection offers the only possibility for cure, yet fewer than 15% of patients are candidates for tumour resection at the time of diagnosis (Lillemoe et al., 2000). The lethality of this cancer is related to its rapid growth and propensity to invade adjacent organs and metastasize. The overall five-year survival rate in the United States for patients with pancreatic cancer is not more than five percent (Jemal et al., 2006). However, after a successful pancreaticoduodenectomy the five-year survival rate approaches 20% overall, and patients with a small tumour (<3cm), negative lymph nodes and negative resection margins have a 40% chance to survive five years (Yeo et al., 1995). Thus, early detection of pancreatic cancer, when resection is still an option, is crucial for a better patient outcome. In addition, novel strategies must be developed that can halt the progression of premalignant conditions to improve the prognosis of pancreatic cancer. Recent studies revealed that immunotherapy against cancer is an effective alternative to the classical cancer therapies, such as surgery, chemo- and radiotherapy (Jones et al., 2000; Rosenberg, 1999; Steinman and Dhodapkar, 2001). Cancer immunotherapy comprise peptide vaccinations, antibody therapies, vaccinations with DCs, adoptive transfers of CTLs, cytokine and tumour cell vaccinations.

1.2.1 Genes associated with the development of pancreatic cancer

The specific genes associated with the development of pancreatic cancer can be divided into three gene classes: oncogenes, tumour suppressor genes and DNA mismatch repair genes.

The pancreatic cells are non-dividing cells. Consequently, if these cells enter into the G1 phase and pass through the G1-S checkpoints, these events could point to an initial event during the tumourgenesis. The vast majority (~90%) of human ductal pancreatic adenocarcinomas have an activating point mutation in the *Kirsten-ras (*K-*ras)* proto-oncogene, which results in a hyperactive Ras protein (Almoguera et al., 1988). K-*ras* belongs to the Ras family and encodes a guanosine triphospate (GTP)-binding protein involved in signal transduction. Constitutive activation of K-*ras* occurs by point mutations, locking the protein in a GTP-bound state that leads to a transformation of the cell and a proliferative response.

The tumour suppressor gene *p53* is inactivated in about 70% of the pancreatic adenocarcinomas (Barton et al., 1991; Casey et al., 1993; DiGiuseppe et al., 1994a; Lundin et al., 1996; Rozenblum et al., 1997). In most cases, this gene is inactivated by loss of one allele accompanied by an intragenic mutation of the other allele. This results in the loss of important controls of the cell growth, such as arresting cell cycle and/or activating apoptosis (Kern et al., 1991; Kern et al., 1992). Genomic stress, inappropriate growth factor stimulation or expression of oncogenic Ras induces p53, which acts as a G1-S checkpoint by blocking the cell cycle if the DNA is damaged (Livingstone et al., 1992; Serrano et al., 1997). The p53 level is increased in damaged cells, which allows time to repair DNA during the cell cycle blockade. If the damage is severe, this p53 protein can cause apoptosis (Lowe et al., 1993). In 50% of the pancreatic carcinomas the tumour suppressor gene *deleted in pancreatic carcinoma-4* (*DPC-4*) is inactivated (Hahn et al., 1996; Rozenblum et al., 1997; Schutte et al., 1996). This gene has homology to the *small mother against decapentaplegic* (*SMAD*) gene family, which plays a role in the TGF-β receptor II signal transduction pathway (Derynck et al., 1996; Grau et al., 1997; Niehrs, 1996). The $p16^{\text{inhibitor of Cdk4 (INK4)a}}$ (multiple tumour suppressor (MTS) 1) protein is a cell cycle inhibitor molecule of the cycline-dependent kinase (Cdk) 4 that promotes progression of the cell division cycle (Kamb et al., 1994). Activated Ras of primary cells is able to induce the expression of the $p16^{INK4a}$ molecule, which is encoded by the INK4a locus. Approximately 95% of the pancreatic cancers reveal an inactivated form of $p16^{INK4a}$ (Hu et al., 1997; Huang et al., 1996a; Seymour et al., 1994; Serrano et al., 1996). Inactivation occurs in 15% of the cancers through methylation of its promotor region. The remaining cases show homozygous deletion (~40%) or deletions accompanied by intragenic mutations in the other allele (~40%) (Schutte et al., 1997). Other tumour suppressor genes which have been described, such as *breast cancer* (*BRCA*) *2*, *retinoblastoma* (*Rb*), *MAP kinase-kinase* (*MKK*) *4*, and *serine/threonine kinase* (*STK*) *11* are inactivated at a much lower frequency in pancreatic cancer than *p53*, *DPC 4* and *p16* (Giardiello et al., 1987; Goggins et al., 1996; Huang et al., 1996b; Ozcelik et al., 1997; Su et al., 1998).

The genetic alterations of genes are accompanied by pathomorphological changes of the pancreas. Thereby, pancreatic ducts and ductules adjacent to infiltrating cancers show hyperplasias and pancreatic intraepithelial neoplasias, which are the precursor lesions to pancreatic cancers (Brat et al., 1998; Cubilla and Fitzgerald, 1976; DiGiuseppe et al.,

1994b; Klimstra and Longnecker, 1994). Three human precursor lesions of pancreatic ductal adenocarcinoma are known: pancreatic intraepithelial neoplasm (PanIN), mucinous cystic neoplasm (MCN), and intraductal papillary mucinous neoplasm (IPMN) (**Figure 1.4**) (Brugge et al., 2004; Maitra et al., 2005). The PanIN can be graded in three stages (PanIN-1,2,3), with the earliest stage characterized by the appearance of a columnar, mucinous epithelium and with increasing architectural disorganization and nuclear atypical through stages two and three (**Figure 1.4**). Finally the PanIN-3 transform into frank pancreatic ductal adenocarcinoma with evidence of invasions beyond the basement membrane. MCNs are defined by large mucin-producing epithelial cystic lesions that harbour a distinctive ovarian-type stroma with a variable degree of epithelial dysplasia and focal regions of invasion. IPMNs resemble PanINs at the cellular level but grow into large cystic structures. MCNs and IPMNs are less common precursor lesions than PanIN. Various genetic events involved in the pancreatic adenocarcinoma progression are divided into those, which predominantly occur early (e.g. K-*ras* mutation, $p16^{INK4a}$ loss) or late (e.g. *p53* loss, *SMAD4/DPC* loss) in pancreatic ductal adenocarcinoma (Hezel et al., 2006).

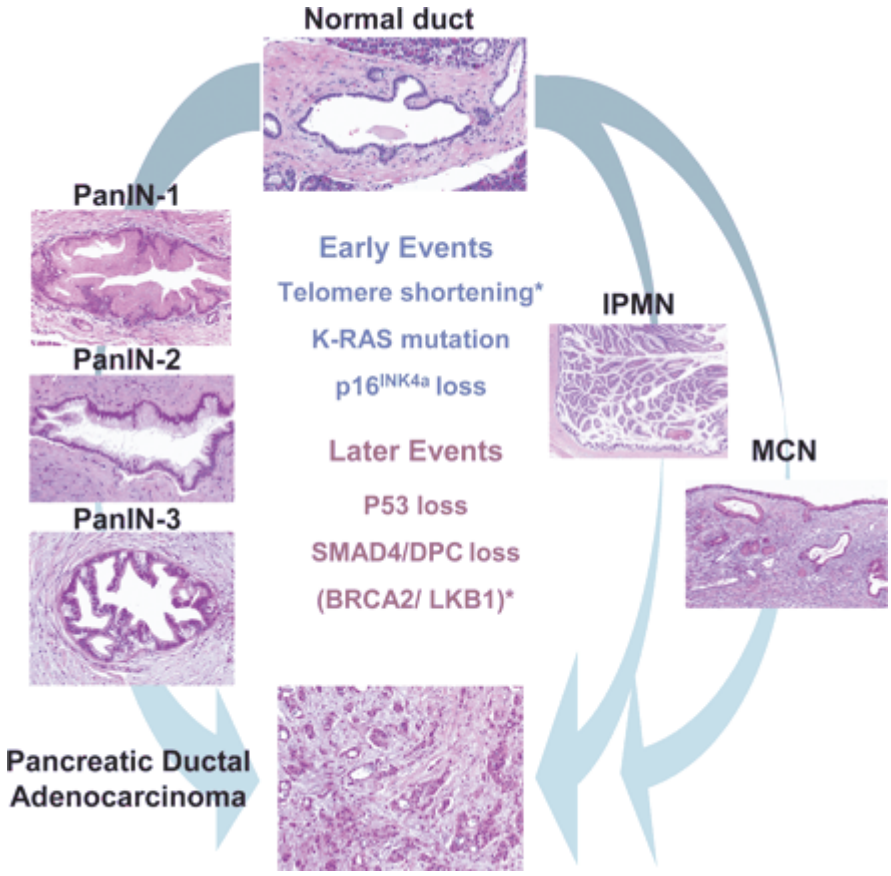

Figure 1.4 ***Genetic events and pancreatic premalignant lesions involved in the initiation and promotion of pancreatic adenocarcinoma.*** *The figure shows three known precursor lesions of the pancreatic ductal adenocarcinoma: PanIN, MCN, and IPMN. PanIN can be differentiated into three grades with an increasing atypical state of pancreatic epithelial neoplasia (left, PanIN-1, 2, 3). The potential progression of MCNs and IPMN to pancreatic ductal adenocarcinoma is displayed on the right side. Genetic alterations documented in the pancreatic ductal adenocarcinomas also occur in the PanIN, and to a lesser extent in MCNs and IPMNs, in an apparent temporal sequence. These genetic events can be divided in those that predominantly occur early or late in the pancreatic ductal adenocarcinoma progression. Asterisks indicate events that are not known to be common to all precursors (adapted from Hezel et al., 2006).*

1.3 Cancer and inflammation

The first reported link between cancer and inflammation was observed in the nineteenth century, when Virchow assumed that the origin of cancer was at sites of chronic inflammation. He suggested that some irritants, together with tissue injury and ensuing inflammation enhance cell proliferation (Balkwill and Mantovani, 2001). In recent years, the causal relationship between inflammation and cancer has been widely accepted (Balkwill and Mantovani, 2001; Balkwill et al., 2005; Coussens and Werb, 2002). Epidemiological studies revealed that chronic inflammation predisposes individuals to certain cancers, and conversely that non-steroidal anti-inflammatory agents protect against several tumours.

The inflammation and wound healing processes are intricate biological responses that initiate a multifactorial network of chemical signals and involve complex interactions between different cell types (**Figure 1.5**a). This involves the activation and directed migration of leukocytes, such as monocytes, neutrophils, eosinophils, and lymphocytes, from the venous system to the sites of damage. These cells regulate the expression of cytokines, which promote cell activation and proliferation, and chemokines, which induce chemotaxis and migration. The profile of cytokine / chemokine persisting at an inflammatory site is important in the development of chronic disease. The pro-inflammatory cytokine TNF-α controls inflammatory cell populations as well as mediates many of the other aspects of the inflammatory process. TGF-β1 is also important, both positively and negatively influencing the processes of inflammation and repair (Moustakas et al., 2002). The key concept is that normal inflammation together with wound healing is usually a self-limiting procedure. However, dysregulation of any of the converging factors can lead to abnormalities and ultimately to the development of cancer.

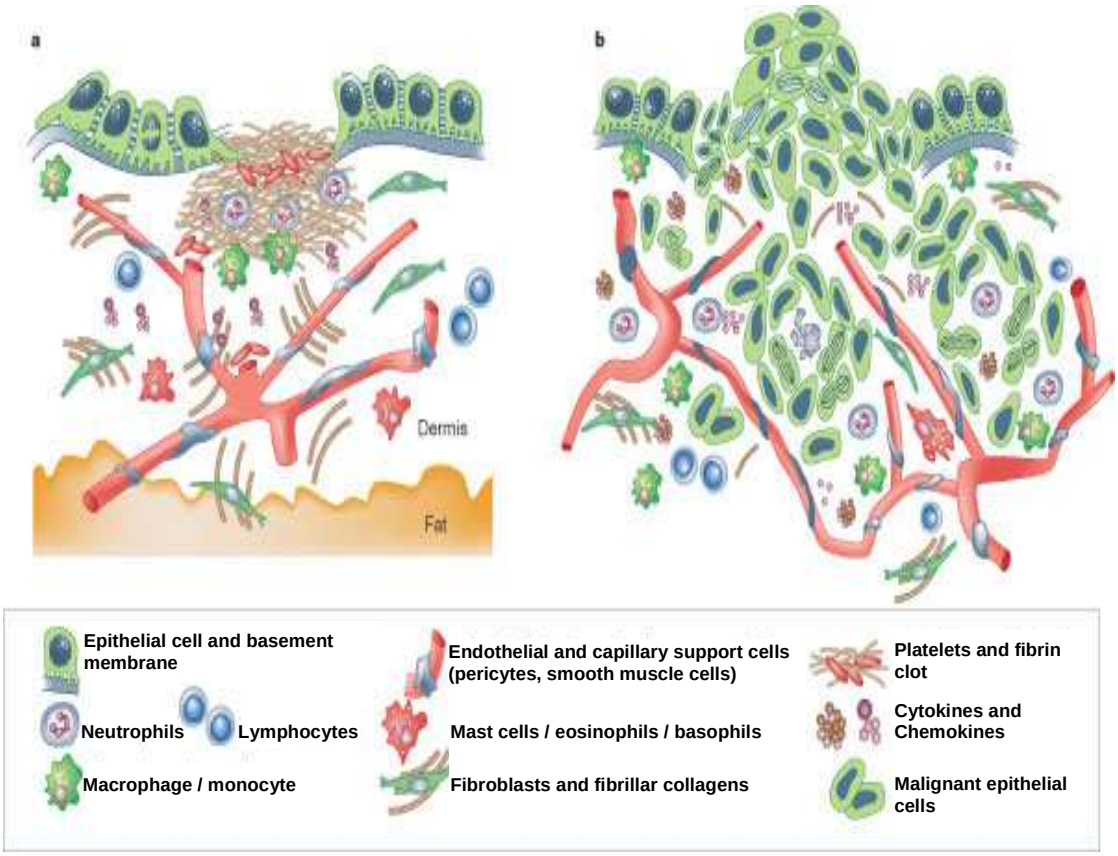

Figure 1.5 ***Comparison between wounded and tumour tissue.*** *(a) Wounded tissue with a highly organized and segregated architecture. Epithelial cells are separated from the vascularized stroma compartment. Due to wounding, platelets become activated and form a haemostatic plug, from which they release vasoactive mediators. Chemotactic factors initiate granulation, tissue formation, activation of fibroblasts, and induction and activation of proteolytic enzymes necessary for remodelling of the extracellular matrix. Granulocytes, monocytes, and fibroblasts are recruited, the venous network is restored, and re-epithelialization occurs across the wound. Epithelial and stroma cell types engage in a reciprocal signalling dialogue to facilitate healing. Once the wound is healed, the reciprocal signalling subsides. (b) Invasive carcinomas are less organized. Neoplasia-associated angiogenesis and lymph angiogenesis produce a chaotic vascular organization of blood vessels and lymphatics where neoplastic cells interact with mesenchymal, haematopoietic and lymphoid cell types and a remodelled extracellular matrix. Although the vascular network is not disrupted in the same way during neoplastic progression as it is during wounding, many reciprocal interactions occur in parallel. Neoplastic cells produce an array of cytokines and chemokines that are mitogenic and/or chemoattractants for granulocytes, mast cells, monocytes / macrophages, fibroblasts, and endothelial cells. In addition, activated fibroblasts and infiltrating inflammatory cells secrete proteolytic enzymes, cytokines, and chemokines, which are mitogenic for neoplastic cells, as well as endothelial cells involved in neoangiogenesis and lymph angiogenesis. These factors potentiate tumour growth, stimulate angiogenesis, induce fibroblast migration and maturation, and enable metastatic spread via engagement with either the venous or lymphatic networks (adapted from Coussens and Werb, 2002).*

Most premalignant and malignant tissues show signs of inflammation. This involves the movement of innate immune cells into the tissue, the presence of cytokines and chemokines, changes in the tissue structure (remodelling), and the formation of new blood vessels (angiogenesis) (**Figure 1.5**b). In the developing tumour stroma a diverse leukocyte population exists including DCs, macrophages, neutrophils, eosinophils, mast cells, and lymphocytes. These cells are capable of producing cytokines, cytotoxic mediators including reactive oxygen species, serine and cysteine proteases, MMPs and membrane perforating agents, and soluble mediators of cell killing, such as TNF-α and IFN-γ (**Figure 1.5**b) (Kuper et al., 2000; Wahl and Kleinman, 1998).

TIMs have a dual role in tumour growth. On the one hand they are able to kill neoplastic cells following activation by IL-2, IFN and IL-12 (Brigati et al., 2002; Tsung et al., 2002). On the other hand, TIMs release potent angiogenic and lymphangiogenic growth factors, cytokines and proteases, which promote tumour growth and metastasis (Schoppmann et al., 2002; Balkwill and Mantovani, 2001; Mantovani et al., 2002). TIMs and tumour cells can both express IL-10, which effectively inhibits the anti-tumour response by cytotoxic T cells. Increased expression of genes associated with macrophage infiltration, such as CD68, forms part of the molecular signatures that herald poor prognosis in certain cancers (Paik et al., 2004).

1.3.1 Pancreatic cancer and chronic pancreatitis

Chronic pancreatitis is a progressive and destructive inflammatory process, characterized by endocrine and exocrine pancreatic dysfunction that ends in total destruction of the pancreas and results in permanent, malabsorption of dietary nutrients, diabetes mellitus, and severe, unrelenting pain (Etemad and Whitcomb, 2001). The pathological hallmarks of chronic pancreatitis are glandular atrophy, ductal changes, and fibrosis (Stevens et al., 2004). The origin of chronic pancreatitis is mixed, with ~70% of the cases being attributed to alcohol abuse. The remaining cases are classified as idiopathic chronic pancreatitis, including tropical pancreatitis, which is a major cause of childhood chronic pancreatitis in tropical regions, or unusual causes including hereditary pancreatitis, cystic fibrosis, and chronic pancreatitis-associated metabolic and congenital factors. However, recent studies have revealed that subjects with chronic pancreatitis usually have multiple risk factors, including a number of underlying genetic susceptibility gene mutations (Etemad and Whitcomb, 2001). The process leading to chronic pancreatitis appears to require the interaction of environmental factors (e.g. alcohol and tobacco smoking), factors that lead to recurrent pancreatic injury (e.g. recurrent acute pancreatitis (RAP)), and/or an altered immune response leading to chronic inflammation and fibrosis (Whitcomb, 2004). The limited number of subjects with severe alcoholic chronic pancreatitis, who lived for an additional 20-30 years after development of chronic pancreatitis due to the complications of chronic pancreatitis or a destructive lifestyle, make it difficult to address the relationship between chronic pancreatitis and pancreatic cancer. Few epidemiological studies show first results, which assume a correlation between both diseases (Lowenfels et al., 1993; Whitcomb and Pogue-Geile, 2002). But so far, neither morphological nor molecular genetic studies have demonstrated without a doubt the existence of a progression from chronic pancreatitis to pancreatic cancer.

1.4 Mouse models for cancer

Animal models are essential to study the feasibility and toxicity of cancer therapies before designing a clinical trial (Jaffee et al., 1995). To date, most of the published studies are based on transplantable tumour cell lines, which were cultured *in vitro* and subcutaneously injected into experimental animals. These subcutaneous tumours are of only restricted use as a model for tumour patients. In contrast to the patient tumours, these models are in most cases not induced organ-specifically. Therefore, the tumour stroma develops differentially. Additionally, these subcutaneous injected tumour cells have a different growth kinetic in the mouse than the carcinoma in the patient. Another problem and the key to developing an effective anti-tumour response (immunotherapy) is to understand why the immune system is unable to detect transformed cells and is subsequently tolerant of tumour growth and metastasis (Smyth et al., 2001b).

To date, general tumour progressor models in animals have been rarely described. Tissue-specific expression of the simian virus 40 (SV40) T antigen develops a carcinoma in the particular tissue, whereas the overexpression of the Her-2/neu in the breast leads to a mammary carcinoma (Chailley-Heu et al., 2001; Gingrich et al., 1996; Hanahan, 1985; Harris et al., 1999). Other transgenic mouse models overexpress TAA, such as the carcinoembryonic antigen (CEA), the prostate-specific antigen (PSA), the mucin (MUC) 1 glycoprotein or the friend murine leukaemia virus (FMuLV) envelope protein, which were developed to study the T cell tolerance against possible tumour antigens (Clarke et al., 1998; Rowse et al., 1998; Wei et al., 1997).

For many human tumours, successful mouse modelling has been facilitated either by the availability of appropriate cell type-specific promotor elements for transgene targeting and conditional gene deletion, by an epithelium accessible and susceptible to chemical carcinogenesis, or by the identification of heritable tumour predisposition following chemical mutagenesis (Bolt et al., 2000; Hutchinson and Muller, 2000; Moser et al., 1992; Saran et al., 2000; Su et al., 1992). Significant rates of spontaneous or viral-mediated tumourgenesis have also provided effective mouse models for other tissues (Hook et al., 2000; Malkinson, 2001). In contrast to the successful murine modelling of most common human tumours, the generation of appropriate mouse models for pancreatic cancer has turned out to be complicated. Combined with this problem is the real sense of urgency, since pancreatic cancer is one of the most lethal of all human malignancies.

1.4.1 Mouse models for human pancreatic cancer

The mouse pancreas was one of the very first organs in which tissue-specific transgene expression was accomplished, and among the first tissues in which transgenic tumour induction was achieved (Ornitz et al., 1985; Quaife et al., 1987; Swift et al., 1984; Ornitz et al., 1985; Quaife et al., 1987; Swift et al., 1984). These mouse models were generated by using the tissue-specific promotor / enhancer elements in the rat elastase I locus. These elements predominantly targeted pancreatic acinar cells and produced acinar cell neoplasms when coupled to either H-*ras* (proto-oncogene, belonging to the Ras family) or SV40 T-antigen. On the other hand, the expression of a *c-myc* transgene driven by the same promotor produced mixed acinar/ductal neoplasms (Sandgren et al., 1991). However, the generation of these mouse models of the exocrine pancreatic

tumour do not reflect the classic pancreatic ductal adenocarcinoma, the predominant form of human pancreatic cancer.

In the decade of the 1990s two important insights, that are related to the pancreatic tumourgenesis were discovered. First, the genetic basis of pancreatic ductal adenocarcinoma was revealed, with activation of K-*ras* and inactivation of the $p16^{INK4a}$, *p53*, and *SMAD4* tumour suppressor genes identified as characteristic features of invasive pancreatic cancer (Hruban et al., 2001b). Second, consensus was reached regarding the role of PanIN as a direct non-invasive neoplastic precursor to human pancreatic cancer (Hruban et al., 1999; Hruban et al., 2001a). Due to the knowledge gained, regarding the genetic background, the PanIN precursors, and the identification of transcription factors and signalling pathways regulating normal pancreatic development, much progress was made in mouse models for pancreatic ductal adenocarcinoma. Recently, researchers developed a transgenic mouse model with a constitutive activation of a mutant K-*ras* allele and a deletion of a conditional *Ink4a/alternative reading frame (Arf)* tumour suppressor allele, whereas activated K-ras serves to initiate PanIN lesions and the INK4A/ARF tumour suppressor function to constrain the malignant conversion of these PanIN lesions into a lethal ductal adenocarcinoma of the pancreas (Aguirre et al., 2003). These mice succumbed to invasive and metastatic pancreatic cancer within eleven weeks.

Transgenic mice overexpressing the TGF-α under the control of the pancreas-specific rat elastase promotor (EL-TGF-α-human growth hormone (hGH)), develop tubular structures and fibrosis in the pancreas (Sandgren et al., 1990). Thereby, TGF-α is able to promote the progression throughout G1, but not the S phase, in premalignant lesions (Wagner et al., 2001). Acinar cells of the pancreas of these mice transdifferentiate into ductal cells representing premalignant lesions, accompanied by an induction of the epidermal growth factor receptor (EGFR) expression in the resulting metaplastic ducts that proliferate due to an autocrine loop (Wagner et al., 1998). Malignant pancreatic tumours emerge in about 30% of the EL-TGF-α-hGH transgenic mice. This murine tumour progression model for the human pancreatic adenocarcinoma is similar to the human disease, regarding the genetic alterations, the cellular differentiation, and the growth characteristics. Crossbreeding the EL-TGF-α-hGH mice with Trp53 (p53)-deficient mice accelerated the pancreatic tumour formation and increased their incidence (**Figure 1.6**) (Wagner et al., 2001). Heterozygous loss of *p53* in EL-TGF-α-hGH mice (EL-TGF-α x $Trp53^{+/-}$) resulted in an even more increased incidence of pancreatic tumours (77%) and a decreased tumour-free survival of about 220 days. 100% of the EL-TGF-α x $Trp53^{-/-}$ transgenic mice developed pancreatic adenocarcinoma within 120 days after birth. Thereby, some tumours form ductal structures in dense connective tissue, others show frequent mitotic figures surrounded by sparse fibrosis. In addition, metastasis to the liver and lung, local invasive growth with duodenal obstruction, and malignant ascites are also observed in EL-TGF-α x $Trp53^{-/-}$ mice (Wagner et al., 2001). The tumour spectrum of Trp53-deficient mice is dominated by lymphomas and sarcomas (Jacks et al., 1994). This tumour spectrum is shifted to ductal pancreatic cancer in crossbred mice. Only about three percent of these mice bear both a large epithelial pancreatic tumour and an extrapancreatic sarcoma. Pancreatic tumour cells of this murine model bear strong similarities in genetic changes with the human pancreatic tumour cells. In addition to the loss of the *p53* wild-type allele, inactivation of the tumour suppressor protein $p16^{INK4a}$ and the low frequency of

the loss of heterozygosity of the *SMAD4/DPC4* locus occur in this murine and in most human pancreatic tumour cells (Hahn et al., 1996; Serrano et al., 1996; Wagner et al., 2001). Thus, EL-TGF-α x Trp53-deficient mice develop spontaneous pancreatic tumours with pathomorphologic features and genetic alterations close to the human disease.

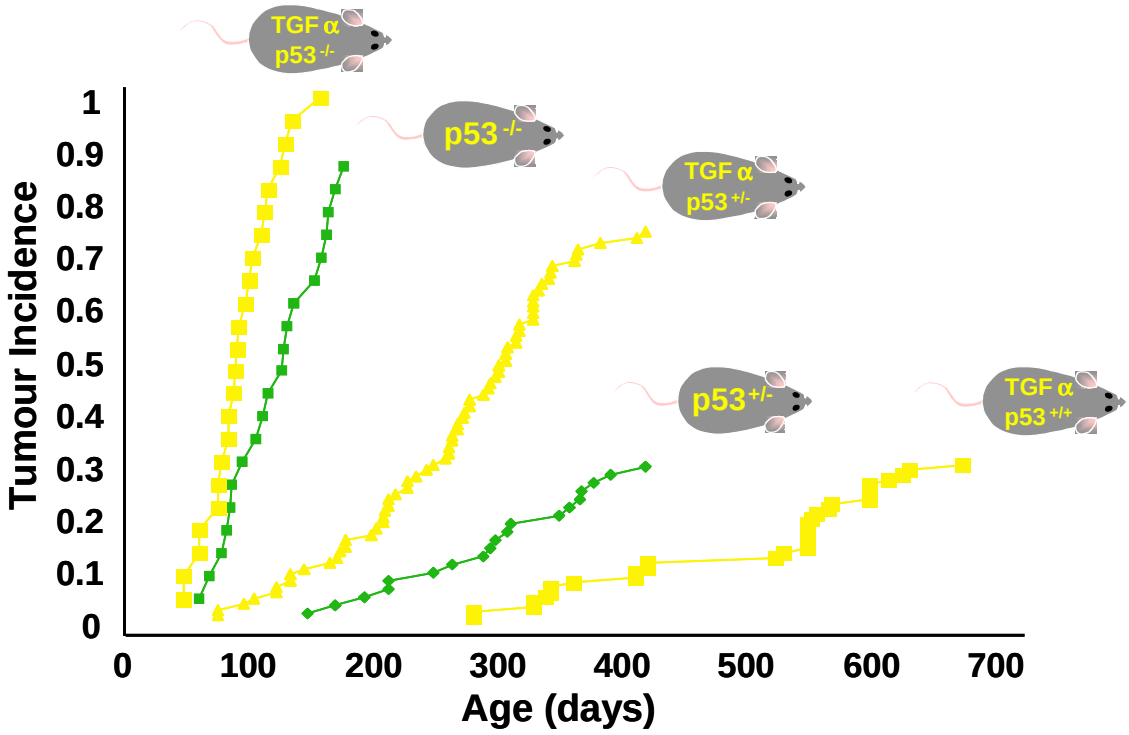

Figure 1.6 ***Comulative tumour incidence of EL-TGF-α x Trp53$^{-/-}$, Trp53$^{-/-}$, EL-TGF-α x Trp53$^{+/-}$, Trp53$^{+/-}$, and EL-TGF-α x Trp53$^{+/+}$*** *(adapted from Wagner et al., 2001).*

2 Materials and Methods

2.1 Materials

2.1.1 Mice

The p53-deficient mice and the transforming growth factor (TGF)-α transgenic mice, which express a TGF-α-human growth hormone (hGH) fusion gene under the control of the elastase (EL) promotor (EL-TGF-α-hGH transgenic mice, line no. 2261-3), have been previously described (Jacks et al., 1994; Sandgren et al., 1990). TGF-α transgenic mice were kindly provided by Dr. Roland Schmid, TU München, Germany. EL-TGF-α x $Trp53^{-/-}$ mice were obtained by crossing a TGF-α transgenic mouse with a $Trp53^{-/-}$ mouse purchased from Jackson Laboratories (Bar Harbor, ME, USA). IFN-γ-deficient mice (B6.129S7-$Ifn\gamma_{tm1Ts}$) were kindly provided by Dr. Siegfried Weiss, GBF, Braunschweig, Germany. TGF-α transgenic, p53-deficient, IFN-γ-deficient and nu/nu mice were bred in the animal facilities of the Medizinische Hochschule Hannover, Germany. Mice were backcrossed for a minimum of ten generations on C57BL/6 background. Enhanced green fluorescent protein ($EGFP^{+}$) transgenic mice were kindly provided by Dr. Michael Ott, Medizinische Hochschule Hannover, Germany. C57BL/6 mice were obtained from Charles River (Sulzfeld, Germany). NOD SCID beige mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were kept under specific pathogen-free conditions, and all experiments were conducted according to the German animal protection law (animal experiment number T 01 / 449).

2.1.2 Chemicals and enzymes

2-Mercaptoethanol	Merck
4´, 6-Diamidin-2´-phenylindol (DAPI)	Sigma
7-Amino-actinomycin (7-AAD)	BD PharMingen
0.9% Sodium chloride	DeltaSelect
Acetone	Roth
Agarose	Invitrogen
Ammonium sulphate	Merck
Bromphenolblue	Invitrogen
Bovine serum albumin (BSA)	Serva
Caerulein	Sigma
Ciprofloxacin	Bayer
Collagenase I	Sigma Aldrich
Cyclophosphamide	Baxter Oncology
Cytofix / cytoperm	BD Biosciences
Dimethylsulfoxid (DMSO)	Merck

Dispase II	Roche Diagnostics
dNTP-mix (10 mmol/l)	Invitrogen
DNA Marker: 1 kb DNA marker	New England Biolabs
100 bp Marker	New England Biolabs
p-Xylene-bis (N-pyridinium bromide) (D.P.X.)	Polyscience
DMEM (+4500mg/L Glucose, + GlutaMAX I)	Gibco
EDTA	Sigma
Eosin	Merck
Ethanol	Merck
Ethidiumbromid	Sigma Aldrich
FACSflow	BD Biosciences
FACSrinse	BD Biosciences
Fast Red	Sigma Aldrich
Foetal calf serum (FCS) (Lot Nr. 0357 H)	Biochrom
Bicoll separation solution (Ficoll)	Biochrom
Glycerin	Sigma Aldrich
Haematoxylin	Merck
Hemalaun	Merck
Liquemin N 25000 (L-Heparin)	Roche
HEPES-buffer	Biochrom
Hot Star Taq polymerase	Qiagen
Isopropanol	Merck
L-glutamine	Biochrom
Methanol	Roth
$MgCl_2$ (25 mmol/l)	Qiagen
Mitomycin C	Medac
Mounting medium	DakoCytomation
Non-essential amino acids (100 x)	Biochrom
Paraformaldehyde	Sigma
Penicillin/Streptomycin (10000 U / 10000 µg/ml)	Biochrom
PharM Lyse™ lysing solution (10 x)	BD Biosciences
Phosphate buffered saline (PBS) w/o Ca^{2+} Mg^{2+}	Gibco
Propidiumiodid	Calbiochem
Proteinase K	Roche

RPMI-1640	Gibco
SDS	Merck
Sodium azide (NaN_3)	Merck
Sodium pyruvate	Biochrom
Staphylococcal Enterotoxin B (SEB)	Sigma
Tris-acetate EDTA (TAE) buffer (10 x)	Invitrogen
Tetramisole hydrochloride (Levamisole)	Sigma
Tissue Tek (O.C.T.)	Sakura
Tris	Roth
Trypan blue (0.4%)	Sigma
Trypsin / EDTA solution (0.25% / 0.02%)	Biochrom
Tween® 20	Calbiochem
Water (sterile)	DeltaSelect and B. Braun
Xylene	Roth

2.1.3 Cell culture media, buffers, and solutions

Cell culture media:

Culture media for T cell generation	Roswell Park Memorial Institute (RPMI)-1640
	10% FCS
	100 U/ml Penicillin
	100 µg/ml Streptomycin
	2 mmol/l L-glutamine
	1% Non-essential amino acids
	1 mmol/l Sodium pyruvate
	50 µmol/l 2-Mercaptoethanol
Culture Media for tumour cell lines	Dulbecco's Modified Eagle's Medium (DMEM)
	10% FCS
	100 U/ml Penicillin
	100 µg/ml Streptomycin
	2 mmol/l L-glutamine
	1% Non-essential amino acids
	1 mmol/l Sodium pyruvate
	100 mmol/l HEPES

Buffers and solutions for cell preparation:	
Wash buffer for cultured cells	0.9% Sodium chloride (NaCl)
Lysis solution for erythrocytes	1 x PharM Lyse™ lysing solution in water (H_2O)
Collagenase/dispase digestion for TILs isolation	200 U/ml Collagenase type I 0.7 U/ml Dispase II
Buffers for cell analysis by FACS:	
FACS buffer	Phosphate buffered saline (PBS) 1% (w/v) Bovine serum albumin (BSA) 0.5% Sodium azide (NaN_3)
Fixation and permeabilization buffer	1 x Cytofix/cytoperm
Permeabilization / wash buffer	BD 10 x Perm / wash™
Buffers and solutions for histology:	
Tris-buffered saline (TBS) buffer	10 mmol/l Tris/HCl pH 7.5 150 mmol/l NaCl
TBS Tween	10 mmol/l Tris/HCl pH 7.5 150 mmol/l NaCl 0.05% (v/v) Tween 20
Blocking buffer	3% BSA in TBS
Wash buffer	PBS
Tissue fixation	4% Paraformaldehyde (PFA) in PBS, pH 7
Tissue fixation on sections	Methanol/Aceton (1:1) or 4% PFA in PBS, pH 7

Other buffers:

Proteinase K digestion buffer	0.5 mg/ml Proteinase K 0.1% Sodiumdodecylsulfate (SDS) 0.1 mol/l NaCl 50 mmol/l Tris/HCl pH 8.0 10 mmol/l Ethylendiamintetra acetic acid (EDTA)
10 x PCR buffer	Qiagen
$MgCl_2$ (25 mmol/l)	Qiagen
Buffer for running agarose gel electrophoresis	1 x TAE buffer
5 x Sample buffer	50 ml Glycerin 2 ml Tris 0.5 mol/l pH 7.5 Two knife tips bromephenolblue Ad 100 ml H_2O

2.1.4 Antibodies for FACS, immunohistochemistry and immunofluorescence analysis

The listed monoclonal antibodies (mAbs) were used for the detection of surface and intracellular markers. The antibodies were conjugated with fluorescein isothiocyanat (FITC), R-phycoerythrin (PE), allophycocyanin (APC), peridinin chlorophyll protein (PerCP), or biotin and obtained from BD PharMingen (Heidelberg, Germany) or eBioscience (Heidelberg, Germany). Biotinylated (bio) antibodies were revealed by PE-streptavidin-, APC-streptavidin conjugates (BD PharMingen, Heidelberg, Germany), or cyanine (Cy) 3-streptavidin conjugate (Caltag, Hamburg, Germany). Propidiumiodid (Calbiochem, Läufelfingen, Switzerland) or 7-AAD (BD PharMingen, Heidelberg, Germany) were used for staining of dead cells. Flow cytometry was carried out using a FACSCalibur. Analysis of the acquisition was done by CellQuest (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) or FlowJo (Tree Star, Inc., Ashland, OR, USA) software. Antibodies used in the experiments were titrated before use.

Antibodies for FACS analysis:

Anti-mouse CD3-APC	clone 145-2C11	BD PharMingen
Anti-mouse CD4-bio	clone GK1.5	BD PharMingen
Anti-mouse CD4-PE	clone GK1.5	BD PharMingen
Anti-mouse CD4-FITC	clone GK1.5 and H129.19	BD PharMingen
Anti-mouse CD8-bio	clone 53-6.7	BD PharMingen

Anti-mouse CD8-FITC	clone 53-6.7	BD PharMingen
Anti-mouse CD8-PE	clone 53-6.7	BD PharMingen
Anti-mouse CD11b	clone M1/70	BD PharMingen
Anti-mouse CD25-APC	clone 7D4	BD PharMingen
Anti-mouse CD62L-PE	clone MEL-14	BD PharMingen
Anti-mouse CD40L-PE	clone MR1	BD PharMingen
Anti-mouse / rat Foxp3-PE	clone FJK-16s	eBioscience
Anti-mouse IFN-γ-PE	clone XMG1.2	BD PharMingen
Anti-mouse I-A/I-E-PE	clone M5/114.15.2	BD PharMingen
Rat IgG_1-FITC (isotype control)	clone R3-34	BD PharMingen
Rat IgG_1-PE (isotype control)	clone R3-34	BD PharMingen
Rat IgG_{2b}-FITC (isotype control)	clone A95-1	BD PharMingen
Rat IgG_{2b}-PE (isotype control)	clone A95-1	BD PharMingen
Rat IgG_{2a}-PE (isotype control)	clone eBR2a	eBioscience
Goat anti-mouse IgG-FITC		Southern Biotech
Streptavidine-APC		BD PharMingen
Streptavidine-PE		BD PharMingen

Antibodies for immunohistochemistry and immunofluorescence analysis:

Rat anti-mouse CD4	clone RM4-5	BD PharMingen
Rat anti-mouse CD8	clone 53-6.7	BD PharMingen
Rat anti-mouse CD11b	clone M1/70	BD PharMingen
Goat anti-rat IgG-Cy3		Jackson (Dianova)
Streptavidine-Cy3		Caltag

2.1.5 Cytokines, consumed materials and kits

Cytokine used for cell culture:

Proleukin IL-2	Chiron

Consumed materials:

Cannulas and syringes	B. Braun
Cap lock reaction tubes (0.5, 1.5, and 2 ml)	Sarstedt
Cap lock sterile disposable PP RNase-free tubes (1.5 ml)	Eppendorf
Cover slides	Menzel-Glaser

Cryogenic vial (2 ml)	Nalgene Nunc
FACS tubes	Micronic
Filter cap tissue culture flasks (25 cm^2, 75 cm^2, and 175 cm^2)	Greiner
Gloves	Kimberly Clark
Multiwell tissue culture plates (6-, 24-, and 96-well), sterile	Falcon, Greiner and Nalgene Nunc
Nylon mesh (70 µm)	Heidland
Pasteur pipettes (22.5 mm)	Brand
Petri dishes	Greiner and Corning / Costar
Pipette tips (10-, 200-, and 1000 µl)	Biozym and Sarstedt
Quartz cuvette (UVette)	Eppendorf
Serological pipettes	Sarstedt
Sterile filter (0.22 µm and 0.45 µm), 50 ml	Millipore
SuperFrost Plus microscope slides	Menzel-Glaser
Tissue culture plates (60 mm and 100 mm), sterile	Nalgene Nunc
Tissue culture tubes (15 ml and 50 ml), sterile	Greiner

Kits:

Cytometric Bead Array (CBA)	BD PharMingen
IFN-γ Capture staining kit	Miltenyi
BD Cytofix / cytoperm IFN-γ intracellular staining kit	BD Biosciences
PE anti-mouse/rat Foxp3 staining set	eBioscience
Real time-PCR	BIO-RAD
RNeasy isolation kit	Qiagen

2.1.6 Devices

-80°C refrigerator		GFL
Autoclave Typ GE406		Getinge
Balance (440-33)		Kern
Calliper rule		Roth
Cell culture incubator Heracell		Heraeus
Cell-freezing container Nalgene Cryo 1°C “Mr. Frosty”		Nalgene Nunc
Centrifuges:	Z200 M/H	Hermale
	Megafuge 1.0 R	Heraeus

Cryotom	Microm
FACSCalibur	Becton Dickinson
Fluorescence microscope (Diaphot 300)	Nikon
Freezer and refrigerator	Liebherr
Gamma-irradiator (Gammacell 2000)	Molsgaard
Gel Doc 2000	Bio-Rad
Gel electrophoresis chamber	Biozym
Heating block	Kleinfeld
Lab-pH-meter	ionoLab
Liquid nitrogen tank	Locator
Light microscope BX40	Olympus
Microtom	Reichert-Jung
Microwave	Siemens
Neubauer chamber	Brand
Pipetboy (accu-jet)	Brand
Pipettes (2, 10, 20, 200, and 1000 µl)	Gilson
Power supplier (Power Pac 300)	Bio-Rad
Spectrophotometer	Pharmacia Biotech
Sterile bench	Gelaire
Thermocycler: Mastercycler	Eppendorf
Thermocycler	Biometra
Thermomagnetic stirrer	IKA RCTbasic
Vortex	Heidolph
Water bath	GFL

2.2 Methods

2.2.1 Methods for mice experiments

2.2.1.1 Tumour transplantation

To analyse the *in vivo* growth of the generated cell lines, that were derived from EL-TGF-α x $Trp53^{-/-}$ mice, 1 x 10^7 mPAC cells were injected subcutaneously (s.c.) in 200 µl sterile PBS in the hind flank of the mice. Tumour development was monitored every second day by measuring the tumour with the calliper rule. For ethical reasons, animals were sacrificed when tumours reached a diameter greater than 15 mm.

2.2.1.2 Adoptive Transfer

Enhanced green fluorescent protein positive ($EGFP^+$) transgenic mice were injected s.c. into the left hind flank with 1 x 10^7 regressor tumour cells. At day 14, splenocytes and draining lymph node cells from injected $EGFP^{+/-}$ mice were isolated and depleted of erythrocytes. Cells were washed with 0.9% NaCl solution and resuspended in RPMI medium at final concentration of 4 x 10^6 cells/ml.

To induce apoptosis, tumour regressor cells were treated with 50 ng/ml mitomycin C (Medac, Hamburg, Germany) for one hour at 37°C / 5% CO_2. Treated cells were washed three times and resuspended in RPMI complete medium to a final concentration of 1 x 10^5 cells/ml.

1 x 10^5 treated tumour regressor cells and 4 x 10^6 isolated $EGFP^+$ splenocytes/draining lymph node cells were incubated for five days at 37°C / 5% CO_2 per well of a 24 well plate. After 24 hours, 20 U of IL-2 was added to each well. Cells were harvested, washed and resuspended in PBS. Twelve week old EL-TGF-α x $Trp53^{-/-}$ transgenic and s.c. regressor or progressor tumour-bearing (about five millimetres) wild type (C57BL/6) mice were injected intravenously (i.v.) with 2 x 10^7 cells into the tail vein. Mice were scarified for the analysis of tumour-infiltrating lymphocytes (TILs) twelve hours after transfer. Spontaneous pancreatic adenocarcinoma (PAC) from twelve week old EL-TGF-α x $Trp53^{-/-}$ transgenic mice and s.c. regressor or progressor tumours from murine pancreatic adenocarcinoma (mPAC) cell line injected wt mice were isolated. After mechanical dissociation, tumours were digested for 40 minutes at 37°C / 5% CO_2 in 0.64 mg/ml collagenase type I (Sigma, Taufkrichen, Germany) and 1.44 mg/ml dispase II (Roche Diagnostics, Mannheim, Germany) in RPMI medium. 0.5 mol/l EDTA (pH 7.2) was added for five minutes to stop the enzymatic reaction of collagenase. Digested tumours were passed over a 70 µm nylon mesh (Heidland, Gütersloh, Germany) and washed twice with 0.9% NaCl solution. Samples were washed once in RPMI medium and resuspended in 0.9% NaCl solution.

$EGFP^+$ TILs were stained with APC-conjugated anti-mouse CD3 (PharMingen, Heidelberg, Germany) and PE-conjugated anti-mouse CD62L (Caltag, Hamburg, Germany) with PE-conjugated isotype control. Samples were analyzed by FACS.

The total number of $EGFP^+$ / $mCD3^+$ TILs per milligram tumour weight was determined by the following equation: events of $EGFP^+$ / $mCD3^+$ cells divided by tumour weight used per sample. $CD62L^+$ cells of all $EGFP^+$ / $mCD3^+$ cells were calculated as a percentage.

2.2.1.3 Induction of chronic pancreatitis
C57BL/6 wild type or EL-TGF-α x Trp53$^{-/-}$ mice were prebled before induction of chronic pancreatitis. Twelve hours before injection of caerulein, food and drink were removed from the mice cages. After twelve hours of diet, five intraperitoneal (i.p.) injections of 50 µg/kg body weight (BW) caerulein (Sigma, Taufkirchen, Germany) (dissolved in PBS) were performed at hourly interval into each mouse. This procedure was repeated twice per week for a period of five weeks.

2.2.1.4 Cyclophosphamide treatment
Ten days after the s.c. injection of mPAC progressor tumour cells into C57BL/6 wild type, NOD SCID beige, or nude mice, 200 mg/kg BW cyclophosphamide (Baxter Oncology, Halle/Westfalen, Germany) (diluted in PBS) were injected i.p. into the tumour bearing mice. Tumour size was measured each second day with a calliper rule.

2.2.2 Methods of the cell biology

2.2.2.1 General cell culture
Sterile procedures and using sterile materials were required. Generally, adherent and suspension cells were grown in a 37°C incubator containing 5% CO_2. The saturated cultures were split every three days. Cell density was maintained between 50 and 90% confluence. To split and passage adherent cells, the cells were gently rinsed with 0.9% sodium chloride and trypsinized until the cells were easily detached. Trypsinization was quenched with medium prior to subculturing in fresh medium. If not mentioned otherwise, all centrifugation steps were performed at 250 g for ten minutes.

2.2.2.2 mPAC tumour cell lines generation and other tumour cell lines
Tumour cell lines were generated from pancreatic tumours of EL-TGF-α x Trp53$^{-/-}$ mice by fine needle aspiration or homogenization. Fibroblast overgrowth was controlled by differential trypsinization. Established primary cultures were passaged by trypsinization, when each culture formed an 80% to 100% monolayer. Established murine mPAC cell lines derived from EL-TGF-α x Trp53$^{-/-}$ mice were used for further *in vitro* and *in vivo* characterization and analysis. The main *in vivo* experiments of this study were performed with mPAC regressor and the *in vivo* passaged (ivp) variant mPAC progressor cell line.

EL-4 is a murine lymphoma cell line, established from a lymphoma induced in a C57BL/6 mouse by 9, 10-dimethyl-1, 2-benzanthracene (Gorer, 1950). RMA is a mouse T cell lymphoma of C57BL/6 origin (Karre et al., 1986). All cell lines were grown in DMEM complete medium (10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mmol/l L-glutamine, 1% non-essential amino acids and 1 mmol/l sodium pyruvate under standard culture conditions (37°C / 5% CO_2). All cell lines were tested for mycoplasma contamination using nested PCR. Two consecutive PCR runs were performed to increase the sensitivity of the PCR, employing a nested set of general, mycoplasma specific primers (Wirth et al., 1994; Hopert et al., 1993). As few as 1-2 mycoplasma genome copies are detectable using this nested PCR. Some cells were subjected to two weeks treatment with 40 µg/ml ciprofloxacin (Ciprobay 200; Bayer, Leverkusen, Germany) (Uphoff et al., 2002).

2.2.2.3 Cell count
Suspension and trypsinized viable cell counts were performed using a Neubauer chamber (Brand, Wertheim, Germany) with at least 200 cells being counted per sample. Cell viability was assessed using trypan blue exclusion (Sigma-Aldrich Chemie GmbH, Taufkrichen, Germany).

2.2.2.4 Cell freezing
To freeze suspension and trypsinized cells, the cells were centrifuged and the supernatant was removed. Immediately, one millilitre of 4°C cold freezing solution (90% heat-inactivated FCS, 10% DMSO) was added to 5 x 10^6 cells and the cells were transferred into two millilitre cryogenic vial. The vial was placed into ´Mr. Frosty` freezing box (Nalgene Nunc, International Hereford, U.K.) containing isopropanol and kept for at least 12 hours at -80°C. The vial was kept in a liquid nitrogen tank.

2.2.2.5 Cell thawing
To thaw the frozen cells, one vial was removed from liquid nitrogen and thawed rapidly at 37°C. Immediately, one millilitre medium was added to the vial and the cells were gently transferred to a 15 ml sterile conical screw cap tube. To allow for osmotic equilibrium, ten millilitre medium was gently added and the tube was mixed by inverting. After the cells were centrifuged and the supernatant removed, the cells were resuspended in the appropriate volume and cultured at 37°C / 5% CO_2.

2.2.2.6 Preparation of single cell suspensions
Spleens of mice were isolated and put into a sterile 50 ml sterile conical screw cap tube with five millilitre of sterile DMEM complete medium. Single cell suspensions were obtained by flushing spleens with DMEM complete medium followed by red blood cell lysis using 1 x PharM Lysis™ lysing solution (BD Biosciences, Heidelberg, Germany) or by disaggregation of mesenteric and inguinal lymph nodes by using the back of a syringe's plunger. Cells were passed through a mesh with 70 µm pore size and were washed with DMEM complete medium. Mice blood obtained from the tail artery or by cardiac puncture was collected in PBS containing heparin (50 U/ml), followed by cell separation on ficoll gradients. Single cell suspension of tumours were prepared as described previously 2.2.1.2

2.2.2.7 Flow cytometry and depletions
Flow cytometry was performed using a FACSCalibur and CellQuest (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) or FlowJo (Tree Star, Inc., Ashland, OR, USA) software. The manufacture's standard protocol was used for FACS acquisition and analysis.

2.2.2.8 IFN-γ capture assay
To detect mPAC-specific IFN-γ secretion, the "Cytokine secretion assay" for murine cells (Miltenyi Biotec, Bergisch Gladbach, Germany) was used, according to the manufacturer's recommendations. Briefly, 5 x 10^6 pooled mesenteric and inguinal lymph nodes and spleen cells of EL-TGF-α x $Trp53^{-/-}$ transgenic mice or wild type mice immunized with irradiated mPAC or an irrelevant tumour cell line were restimulated over night (max. 12-16 hours) with 1 x 10^5 mitomycin C treated mPACs in 48 well culture dishes. After washing, an IFN-γ-specific catch reagent was attached to the cell surface of all leukocytes. The cells were labelled for five minutes on ice and then

incubated for 45 minutes at 37°C to allow cytokine secretion. During incubation, the tube was turned every five minutes to prevent cell clumping. The secreted IFN-γ binds to the IFN-γ catch reagent on the positive cells. Cross-staining was avoided by keeping the cell density at 1 x 10^5 cells/ml. IFN-γ secreting cells were subsequently labelled with a second IFN-γ-specific antibody, the IFN-γ detection antibody conjugated to PE. The cells were counterstained with monoclonal antibody against CD8 (FITC labelled CD8a, Ly-2, 53-6.7) and the frequency of IFN-γ secreting $CD8^+$ T cells was determined by FACS analysis.

2.2.2.9 Apoptosis induction by mitomycin C treatment

Harvested 2 x 10^6 mPAC 6B regressor or progressor cells were diluted in 9.5 millilitre complete DMEM medium and transferred into a new sterile 15 ml sterile conical screw cap tube. 0.5 millilitre of mitomycin C solution (1 mg/ml in complete DMEM medium) was added and mixed. Tumour cells were incubated for one hour at 37 °C (leave lid slightly open). After incubation, cells were washed three times in complete DMEM medium.

2.2.2.10 Intracellular cytokine staining

Intracellular staining for IFN-γ was performed using the Cytofix/cytoperm kit (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) according to manufacturer's instructions. Briefly, pooled mesenteric and inguinal lymph nodes and spleen cells were restimulated as described for IFN-γ capture assay; in the last four hours of incubation, GolgiPlug (BrefeldinA) was added to the culture. Cells were first stained for surface expression of CD8 (FITC labelled CD8a, Ly-2 (53-6.7)) or CD4 (FITC labelled CD4, L3T4, (GK1.5 and H129.19)) and with 7-AAD for exclusion of dead cells. Then cells were fixed and permeabilized with the cytofix / cytoperm buffer. Intracellular IFN-γ was detected using an anti-IFN-γ-PE antibody (XMG1.2) (BD PharMingen, Heidelberg, Germany). The frequency of IFN-γ positive CD8 or CD4 T cells was determined by FACS analysis.

To identify T_{reg} cells, pooled draining lymph node and spleen cells or tumour-infiltrating cells were stained with anti-CD4, anti-CD25, and anti-Foxp3. Intracellular stain of Foxp3 was performed using the PE anti-mouse/rat Foxp3 staining set (eBioscience, Heidelberg, Germany). Briefly, 1 x 10^6 cells were stained for surface expression of CD4 (FITC labelled CD4, L3T4, (GK1.5 and H129.19) and CD25 (7D4) following the surface standard protocol. Cells were washed with FACS-buffer, then fixated and permeabilized for 14 hours at 4°C. Cells were washed twice with permeabilization buffer and then blocked with rat serum at 4°C for 15 minutes. Without washing, PE conjugated anti-Foxp3 antibody or isotype control diluted in permeabilization buffer were added to cells and incubated at 4°C for 30 minutes in the dark. Cells were washed with permeabilization buffer and resuspended in FACS buffer. Acquisition and analysis of T_{reg} cells were performed by FACSCalibur and FlowJo, respectively.

2.2.2.11 Serology

Blood from mice and rats were harvested by heart puncture or tail bleeding. Isolated blood was kept in a 1.5 ml cap lock reaction tube for twelve hours at 4°C. Blood was centrifuged at 7000 g in a table centrifuge at 4°C for five minutes. The serum (the supernatant) was harvested and kept at -20°C. To determine mPAC-specific serum

immunoglobulin (Ig)G titer, mPAC regressor and mPAC progressor tumour cell lines were stained using serum (1:50 dilution) obtained from mPAC bearing mice as primary antibody. After washing, a FITC-conjugated goat anti-mouse pan IgG secondary antibody (Southern Biotec, Birmingham, AL, USA) was used to detect bound serum IgG. mPAC specific IgGs were analysed by FACS.

2.2.2.12 Cytometric bead array (CBA)

Cytokine release in tumours was detected by using the mouse inflammatory CBA kit (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) that can simultaneously and quantitatively measure up to six different cytokines (IL-6, IL-10, IL-12, IFN-γ, monocyte chemoattractant protein-1 (MCP-1), and tumour necrosis factor-α (TNF-α) or IL-2, IL-4, IL-5, IFN-γ, and TNF-α) in a single sample. Different CBA beads are stabily labelled with a fluorescent dye, whose emission is at ~650 nm (FL 3). Each different group of beads is labelled with a discrete level of fluorescent dye so that it can be distinguished by its mean fluorescent intensity upon FACS analysis. The beads have been covalently coupled with capture antibodies specific for the different cytokines. CBA was performed following the manufacturer's recommendations. In brief, single-cell suspensions were generated from freshly isolated tumours and incubated for twelve hours at 37°C / 5% CO_2. Supernatants were harvested and cytokine content was determined as follows. The CBA capture beads were mixed with PE-coupled detection antibodies (specific for the defined cytokine) and standards, controls, or test samples, to form sandwich complexes. The mixture was incubated for two hours at room temperature with supernatant. Unbound detector antibody-PE reagent was removed by a single washing step before data acquisition was performed by flow cytometry using a FACSCalibur. Acquired data were analysed using the Becton Dickinson Cytometric Bead Array software.

2.2.3 Histological methods

2.2.3.1 Haematoxylin & Eosin (H&E) staining

Isolated tumours were immersion-fixed in four percent buffered formalin for 16 hours. Fixed tumours were dehydrated and then embedded in paraffin. Sections (6 µm) were stained with haematoxylin for three minutes, washed, and stained with 0.5% eosin for an additional three minutes. After an additional washing step with tap water, the slides were dehydrated in 70%, 96%, 100% ethanol, and in xylene before they were embedded in p-Xylene-bis (N-pyridinium bromide) (D.P.X.) (Polyscience, Eppelheim, Germany).

2.2.3.2 Immunohistochemistry staining of TILs

Freshly excised tumour tissues were embedded in O.C.T. medium (Tissue-Tek; Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands), immediately snap-frozen in liquid nitrogen, and stored at -80°C.

For immunohistochemistry, sections (5 µm) of frozen tissue were fixated for ten minutes with methanol/acetone (1:1) at -20°C. Sections were stained with purified anti-mouse CD4 (RM4-5; BD PharMingen, Heidelberg, Germany) or anti-mouse CD8a (53-6.7; BD PharMingen, Heidelberg, Germany) for twelve hours at 4°C. Reaction was visualized by the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method with anti-rat antibody (DakoCytomation, Copenhagen, Denmark) and monoclonal rat APAAP (DakoCytomation, Copenhagen, Denmark), each applied for 30 minutes and 15

minutes, respectively. Colour reaction was generated with 1 mg/ml fast red (Sigma-Aldrich, Taufkrichen, Germany) in APAAP substrate solution, stopped by PBS, and counterstained with hemalaun. Sections were mounted in mounting medium (DakoCytomation, Copenhagen, Denmark) and analysed by microscopy.

2.2.3.3 Immunofluorescence stain of TILs

For the detection of specific TILs by immunofluorescence stain, frozen sections (5 µm) were fixed with acetone at -20°C for ten minutes, followed by rehydration in PBS, pH 7.4. Probes were stained with rat anti-mouse CD4-, CD8-, or CD11c (BD PharMingen, Heidelberg, Germany) followed by application of the secondary Cy3-conjugated goat anti-rat IgG antibody (Jackson ImmunoResearch/Dianova, Hamburg, Germany). The nuclei were stained with 4´,6-diamidin-2´-phenylindol (DAPI) (Sigma, Taufkrichen, Germany). Stained tissue was mounted in mounting medium (DakoCytomation, Copenhagen, Denmark). Autofluorescence was excluded by parallel examination of the red (617 nm, Cy3) and green emission (528 nm, Cy2).

2.2.4 Molecular biological methods

2.2.4.1 PCR screening

The genotype of mice used in this study was determined by genomic polymerase chain reaction (PCR) of tail biopsies. PCRs were performed using Hot Star Taq (Qiagen, Hilden, Germany) and were initiated at 95°C for 15 minutes. The status of p53 was assessed by a multiplex PCR using the following primers: 5`p53 (5`-CCCGAGTATCTGGAAGACAG-3`) and 3`p53 (5`-ATAGGTCGGCGGTTCAT-3`), 5`neo (5`-CTTGGGTGGAGAGGCTATTC-3`) and 3`neo (5`-AAGTGAGATGACAGGAGATC-3`). A 600 base pairs (bp) fragment of wild type p53 and a 280 bp fragment of neomycin were amplified by PCR as follows: 94°C for 30 seconds, 58°C for one minute, 72°C for one minute. PCR was carried out for 30 cycles. The presence of TGF-α transgene was determined using hGH gene specific primers: 5`hGH (5`-GGCTTTTTGACAACGCTATG-3`) and 3`hGH (5`-TAGGAGGTC ATAGACGTTGC-3`) (600 bp). The PCR was performed for 40 cycles according to the following program: 94°C for 30 seconds, 58°C for one minute, 72°C for one minute. IFN-$\gamma^{-/-}$ mice were identified using multiplex PCR with 3 different primers: G-IFN725 (5`-TCAGCGCAGGGGCGCCCGGTTCTTTT-3`) and G-IFN482 (5`-AGAAGTAAGTGGAAGGGCCCAGAAG-3`) for wt IFN-γ (260 bp) and G-IFN484 (5`-AGGGAAACTGGGAGAGGAGAAATAT-3`) and G-IFN484 for neo-disrupted IFN-γ (1 Kb). Conditions were: 94°C for 30 seconds, 58°C for one minute and 72°C for one minute; 35 cycles.

2.2.4.2 Preparation of RNA and reverse transcription (RT) PCR

Total cellular RNA was isolated from 1 x 10^7 mPAC or control cell lines, using RNeasy Kit (Qiagen, Hilden, Germany) according to standard protocols. Residual chromosomal DNA was digested with DNAse I (Live technologies, Karlsruhe, Germany). First strand cDNA was synthesized from total RNA (2 µg) in a 20 µl reaction using Oligo(dT) primer and the enzyme Superscript™II (Live Technologies, Karlsruhe, Germany) according to manufacturer's recommendations. 1 µl of the primer was mixed with 2 µg total RNA, and sterile double distilled water was added to a total volume of 12 µl. The mixture was incubated at 70°C for ten minutes and quickly chilled on ice. After a

centrifugation step, 4 µl of 5 x first strand buffer, 2 µl of 0.1 mol/l dithiothreitol (DTT) and 1 µl of 10 mmol/l dNTP-mix were added. The contents were gently mixed and incubated at 42°C for two minutes, 1 µl Superscript^{TM}II was added. The reaction was continued for 50 minutes at 42°C followed by inactivation of reverse transcriptase. PCR was initiated at 95°C for 15 minutes (Hot Star Taq). The synthesized cDNA was analyzed using primers specific for Cytokeratin 18: 5`CK18 (5`-TGGTACTCTCTTCAATCTGCTG-3`) and 3`CK18 (5`-CTCTGGATTGACTGTGGAAGTG-3`) and Cytokeratin 19: 5`CK19 (5`-CATGGTTCTTCTTCAGGTAGGC-3`) and 3`CK19 (5`-GCTGCAGATGACTTCAGAACC`-3) (174 bp). The PCR was performed for 30 cycles according to the following program: 94°C for 15 seconds, 54°C for 30 seconds, 72°C for 30 seconds. A 158 bp fragment of TGF-α was amplified using the following primers: 5`TGF-α (5`-GTGGTGTCTCACTTCAACAAG-3`) and 3`TGF-α (5`-TGCCAGGAGATCTGCATGCTC-3`). Conditions were: 94°C for 30 seconds, 60°C for one minute, 72°C for one minute; 30 cycles, and for human Growth Hormone: 5`hGH (5`-CCGACACCCTCCAACAGGGA-3`) and 3`hGH (5`-CCTTGTCCATGTCCTTCCTG-3`) the PCR conditions were: 94°C for 30 seconds, 58°C for one minute, 72°C for one minute; 35-40 cycles (Raccurt et al., 2002).

2.2.4.3 Real time PCR

RNA expression normalized by the house-keeping gene GAPDH was measured by real time PCR with the iCycler System (BIO-RAD, München, Germany) using the iCycler iQ Real Time Detection System Software (Version 2.1), as described previously (Jochheim et al., 2004). Primer sequences and TaqMan probes for real time PCR were kindly provided by Dr. Florian Greten, Technical University of München**,** Germany, or designed, using the primer design software Primer 3 of the Whitehead Institute for Biomedical Research, Boston, and purchased from suppliers (Eurogenetic, Belgium).

2.2.4.4 Agarose gel electrophoresis

To visualize the isolated total RNA and the DNA fragments (mouse and tumour cell genotyping), agarose gel electrophoresis was used. 1 or 2 g agarose (Serva, Heidelberg, Germany) in 100 ml 1 x TAE buffer was boiled in a microwave. Before pouring the gel on electrophoresis plate, 2.5 µl ethidium bromide (10 mg/ml in distilled H_2O) was added. Samples with (1/10 volume of RNA sample or 3/4 volume of DNA sample) 5 x sample buffer were loaded on the soaked plates in the gel electrophoresis chamber that was filled with 1 x TAE buffer. Electrophoresis was done at ~100 V until the samples reached the appropriate distance. The samples were visualized by Gel Doc 2000 (Bio-Rad, München, Germany) and analysed by molecular analysis software (Bio-Rad, München, Germany).

2.2.4.5 Quantification of RNA concentration by spectrophotometer analysis

To determine the concentration and purity of the isolated total RNA obtained from RNeasy isolation kit method, the absorption of diluted solution was measured by UV spectrophotometer at 260 nm and 280 nm. The dilution was performed to achieve an absorption between 0.1 and 1.0 OD. The used equations are:

RNA concentration (µg/µl) = absorbance at 260 nm x dilution factor x 40 x 1000

Purity = absorbance at 260 nm/absorbance at 280 nm

3 Results

3.1 Subcutaneous and spontaneous pancreatic tumours

3.1.1 Expression of cytokines in spontaneous and subcutaneous pancreatic tumours

The innate and adoptive immunity both play an important role in the immune-surveillance and specific anti-tumour immune responses. The local tumour microenvironment is crucial for the ability of the tumour to grow and to metastasize. Many studies have shown the importance of tumour-stroma interactions as well as the infiltrating immune cells.

Several tumour cell lines from spontaneous pancreatic tumours of twelve week old EL-TGF-α x $Trp53^{-/-}$ mice were generated by *in vitro* culturing. Fibroblasts and non adherent cells were selected out by short trypsination. After three months, the cultured tumour cells were expanded. Upon injecting these cell lines subcutaneously into immune competent mice, solid tumours developed but after five days the tumours started to regress until they fully disappeared by day ten. These tumour cell lines were called murine pancreatic adenocarcinoma regressor (mPAC regressor).

In order to study the cytokine environment of pancreatic tumours, five day old subcutaneous mPAC regressor tumours from C57BL/6 wild type (wt) mice injected with mPAC regressor cells and spontaneous tumours from twelve week old EL-TGF-α x $Trp53^{-/-}$ mice were isolated. Cell suspensions were prepared therefrom and cultured overnight. The cytokines in the supernatant of the cultures were measured by applying the cytometric bead array (CBA) assay (**Figure 3.1**).

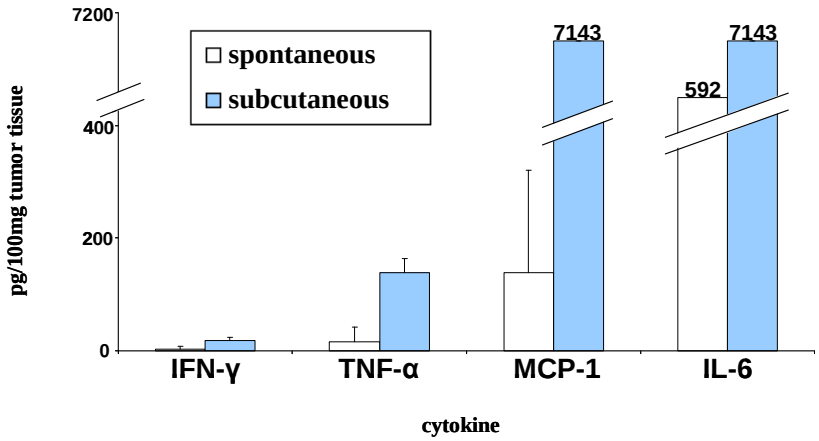

Figure 3.1 ***Cytometric bead array (CBA) analysis for the detection of the cytokine profiles of spontaneous pancreatic adenocarcinoma and subcutaneous regressor tumours.*** *Spontaneous (white column) and subcutaneous (blue column) tumours were explanted and cut into small pieces. Tumour pieces were incubated in complete medium for twelve hours at 37°C / 5% CO_2. IFN-γ, TNF-α, MCP-1, and IL-6 secretion was analysed in cell supernatants by CBA.*

Tumour explants of subcutaneous injected tumours secrete significant amounts of IFN-γ (18 pg/100 mg tissue), tumour necrosis factor-α (TNF-α; 138 pg/ 100 mg), macrophage chemotactic protein-1 (MCP-1; >7143 pg/ 100 mg tissue), and interleukin-6 (IL-6; >7,143 pg/ 100 mg tissue). In contrast, single cell suspensions of spontaneous tumours secrete significantly less IFN-γ (3 pg/ 100 mg), TNF-α (15 pg/ 100 mg tissue), MCP-1 (183 pg/ 100 mg tissue), and IL-6 (591 pg/ 100 mg tissue).

In addition, the RNA from spontaneous and subcutaneous tumours from different mice was isolated and the relative mRNA expression for the cytokines as measured by CBA was determined by applying the quantitative real time polymerase chain reaction (real time PCR) method. A representative of two independent experiments is shown in **Figure 3.2**.

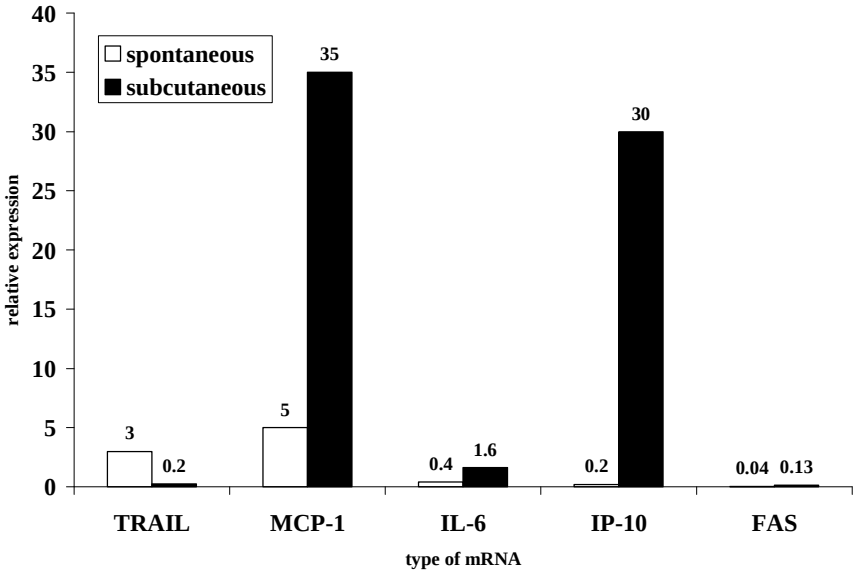

Figure 3.2 ***Real time PCR to analyse the relative expression of mRNA in cells of the spontaneous and subcutaneous tumour environment.*** *mRNA was isolated from subcutaneous (black columns) and spontaneous tumour (white column) by using the RNA isolation kit. SYBR-Green real time PCR was used to quantitatively analyse the relative mRNA expression of the tumour environment.*

The high expression of MCP-1 and IL-6 in subcutaneous murine pancreatic adenocarcinoma (mPAC) regressor tumours in comparison to spontaneous tumours could be confirmed by real time PCR. In addition, a significantly higher expression of interferon-γ inducing protein (IP-10), mainly secreted by activated T cells, was determined in the supernatant of the subcutaneous regressor tumour cell culture. The FAS receptor protein, which plays a known role in the nonimmune surveillance against tumour development and is responsible for the programmed cell death (apoptosis), was not expressed in cells of the subcutaneous and spontaneous tumour stroma. Like T cells and monocytes, tumour cells are also able to produce TNF-related apoptosis-inducing ligand (TRAIL). TRAIL, which induces apoptosis of activated T cells and tumour cells, is significantly less expressed in subcutaneous than in spontaneous tumour tissue.

3.1.2 Histological analysis of the microenvironment of the spontaneous and subcutaneous pancreatic tumour

Different types of cells are part of the tumour stroma, which can influence tumourgenesis. The tumour environment consists of many types of cells among them immune cells, which can also influence tumour initiation and progression. To analyse the immune cells that are attracted by the tumour, immunofluorescence and immunochemistry staining were used to detect these cells in the spontaneous and subcutaneous tumours. Tumours were isolated from mice bearing the subcutaneous regressor tumour or the spontaneous tumour (**Figure 3.3**). Sections of frozen tissue were fixed and stained with rat anti-mouse CD4 (**Figure 3.3**) or CD8 (**Figure 3.4**) followed by application of secondary cyanin (Cy)3-conjugated goat anti-rat IgG. The nuclei were counterstained with 4´,6-diamidin-2´-phenylindol (DAPI, **Figure 3.3** and **Figure 3.4**).

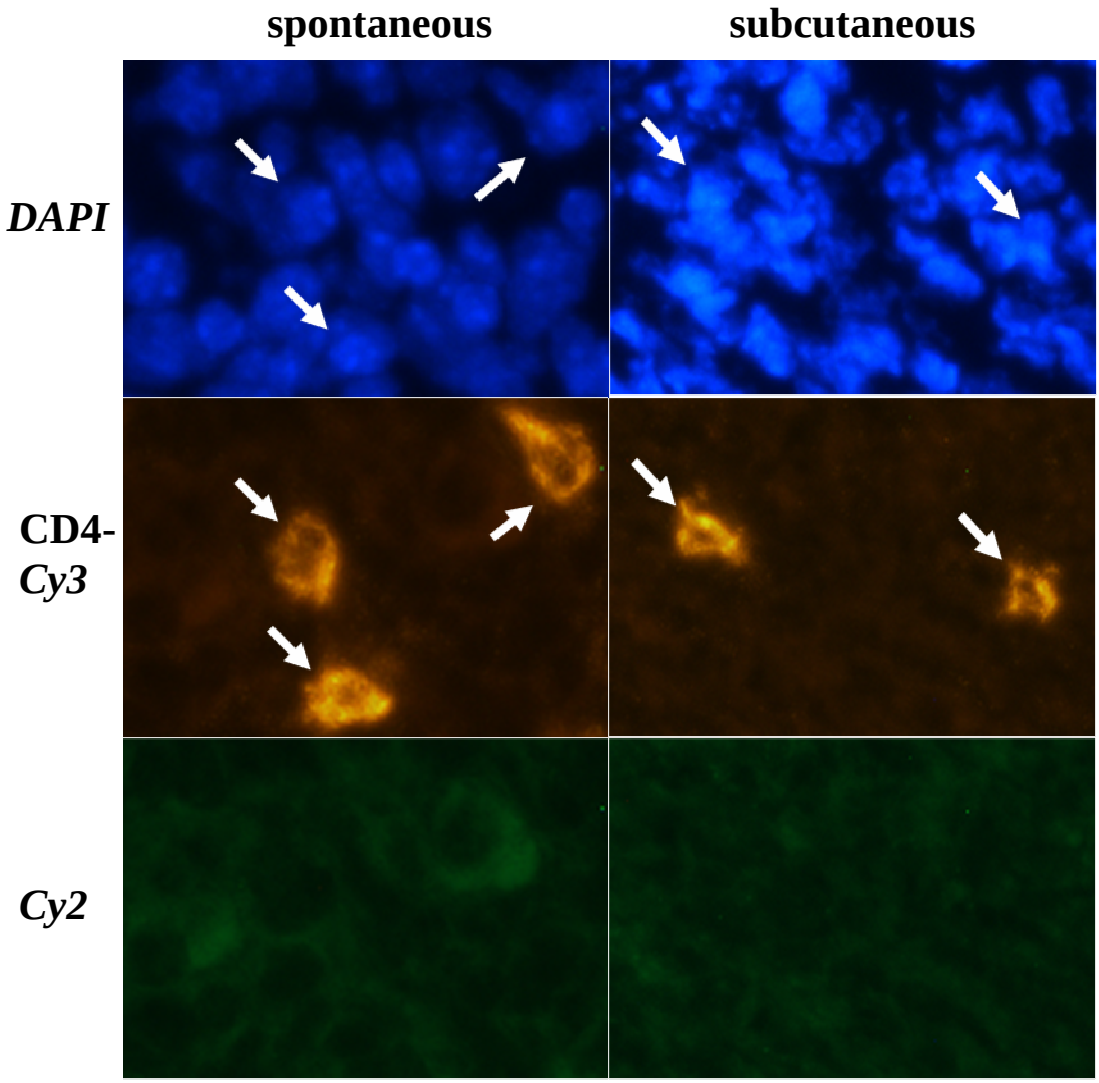

Figure 3.3 ***Immunofluorescence staining of CD4 expressing cells in spontaneous and subcutaneous tumours.*** *Spontaneous (left) and subcutaneous (right) pancreatic tumours were isolated form twelve week old EL-TGF-α x Trp53$^{-/-}$ or mPAC regressor cell injected C57BL/6 mice, respectively. Tumours were snap frozen in liquid nitrogen. Frozen sections were fixed and stained for the nuclei (DAPI, blue, top) and CD4 (CD4-Cy3, orange, middle). Cy2 (bottom) was used to check any background staining. White arrows indicate the CD4^{+} cells. Representative sections are shown.*

Spontaneous as well subcutaneous tumours were infiltrated by CD4 cells. The white arrows mark the CD4 positive cells. No unspecific binding is indicated by the bottom pictures, controlling the Cy2 channel.

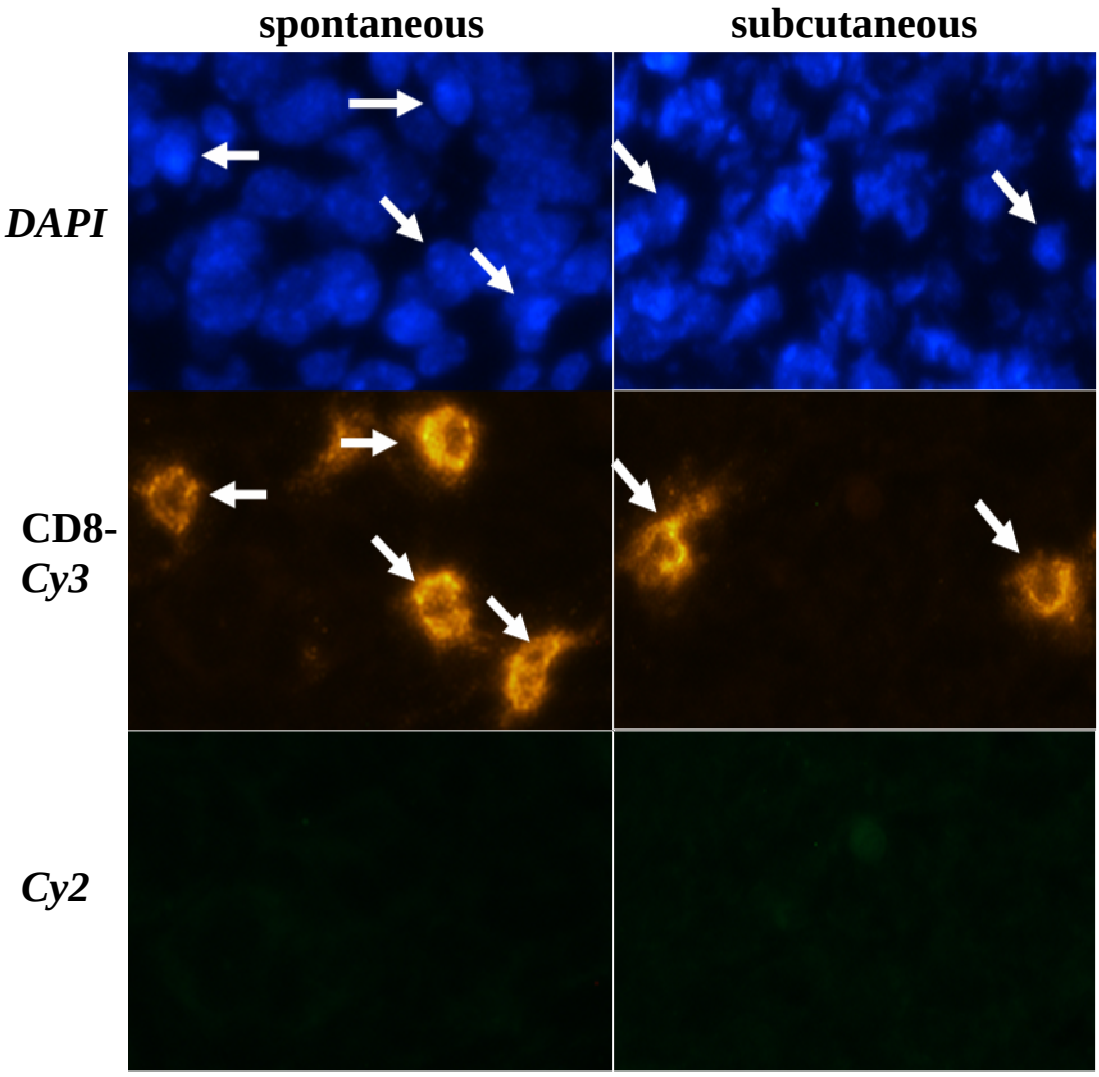

Figure 3.4 ***Immunofluorescence staining of CD8 expressing cells in spontaneous and subcutaneous tumours****. Spontaneous (left) and subcutaneous (right) pancreatic tumours were isolated form twelve week old EL-TGF-α x Trp53$^{-/-}$ or mPAC regressor cell injected C57BL/6 mice, respectively. Tumours were snap frozen in liquid nitrogen. Frozen sections were fixed and stained for the nuclei (DAPI, blue, top) and CD8 (CD8-Cy3, orange, middle). Cy2 (bottom) was used to check any background staining. White arrows indicate the CD8^{+} cells. Representative sections are shown.*

Like CD4 cells, CD8 cells are found in the subcutaneous and spontaneous tumours. The histology results indicate that CD4^{+} and CD8^{+} cells are involved in the tumour microenvironment. But their functionality and their relevance must still be investigated.

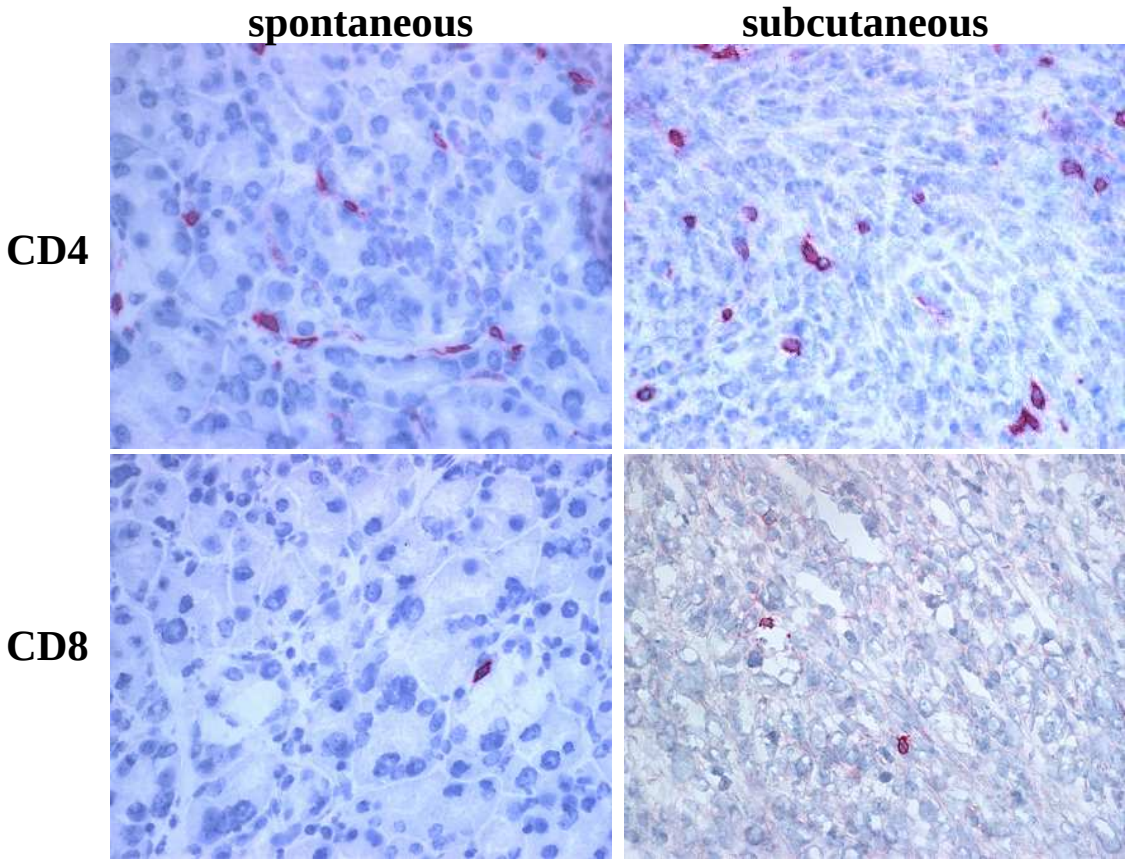

Figure 3.5 ***Immunohistochemistry staining of tumour-infiltrating CD4$^+$ and CD8$^+$ cells.*** *Sections from spontaneous pancreatic tumours (left) and of seven day old subcutaneous regressor tumours (right) were isolated and snap frozen. Frozen sections were fixed and stained with anti-CD4 (top) or anti-CD8 (bottom) antibodies followed by incubation with rabbit anti-rat immunoglobulin. Positive cells were visualized using the APAAP system and fast red. Tissue was counterstained with hemalaun. Representative sections are shown.*

Immunohistochemistry staining confirms the infiltration of CD4$^+$ and CD8$^+$ cells into both types of tumours (**Figure 3.5**). Isolated spontaneous tumours from twelve week old EL-TGF-α x Trp53$^{-/-}$ mice and subcutaneous regressor tumours from C57BL/6 mice were stained with anti-CD4 or anti-CD8 antibodies. More CD4$^+$ (red stained) infiltrating cells occurred in both tumours compared to CD8$^+$ cells. In subcutaneous tumours more CD4$^+$ and CD8$^+$ positive cells were detected than in spontaneous tumours. Three independent counts of three different tumours each resulted in a higher number of CD4 cells in both tumours compared to the CD8 infiltrating cells (**Figure 3.6**). Interestingly, there are more infiltrating CD8 cells in subcutaneous tumours.

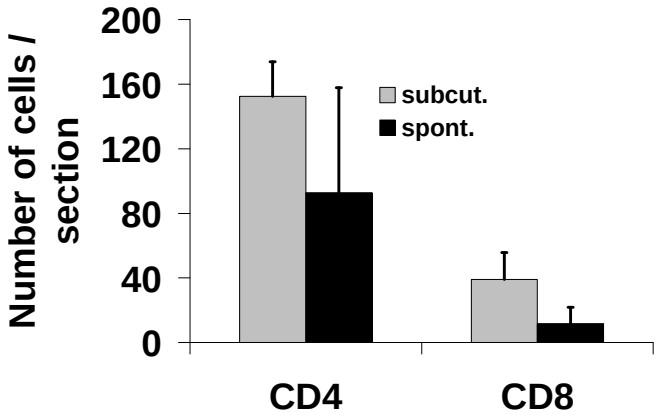

Figure 3.6 ***Histological analysis of tumour-infiltrating CD4 or CD8 T cells in subcutaneous and spontaneous pancreatic tumours.*** *Subcutaneous (grey columns) or spontaneous (black columns) pancreatic tumours were explanted and snap frozen in liquid nitrogen. Sections of frozen tissue were fixed and stained with anti-CD4 (left two columns) or anti-CD8 (right two columns) antibodies. Positive cells were visualized using the APAAP system and fast red staining. $CD4^+$ or $CD8^+$ cells were counted by three independent persons and the average including the standard deviation (SD) calculated.*

To confirm and quantify the results of infiltrating CD4 and CD8 cells, FACS analysis of tumour-infiltrating T cells from different mice with subcutaneous or spontaneous tumours was performed (**Figure 3.7**). Spontaneous tumours from twelve week old EL-TGF-α x $Trp53^{-/-}$ mice and subcutaneous regressor tumours from C57BL/6 mice were explanted and single cell suspensions were made. Cells were stained with anti-CD3, anti-CD4 and anti-CD8 antibodies. Tumour-infiltrating T cells were analysed by FACS. Theses data showed more $CD8^+$ T cells in rejected subcutaneous tumours than in spontaneous tumours (**Figure 3.7**). The difference seen for $CD4^+$ T cells was less profound.

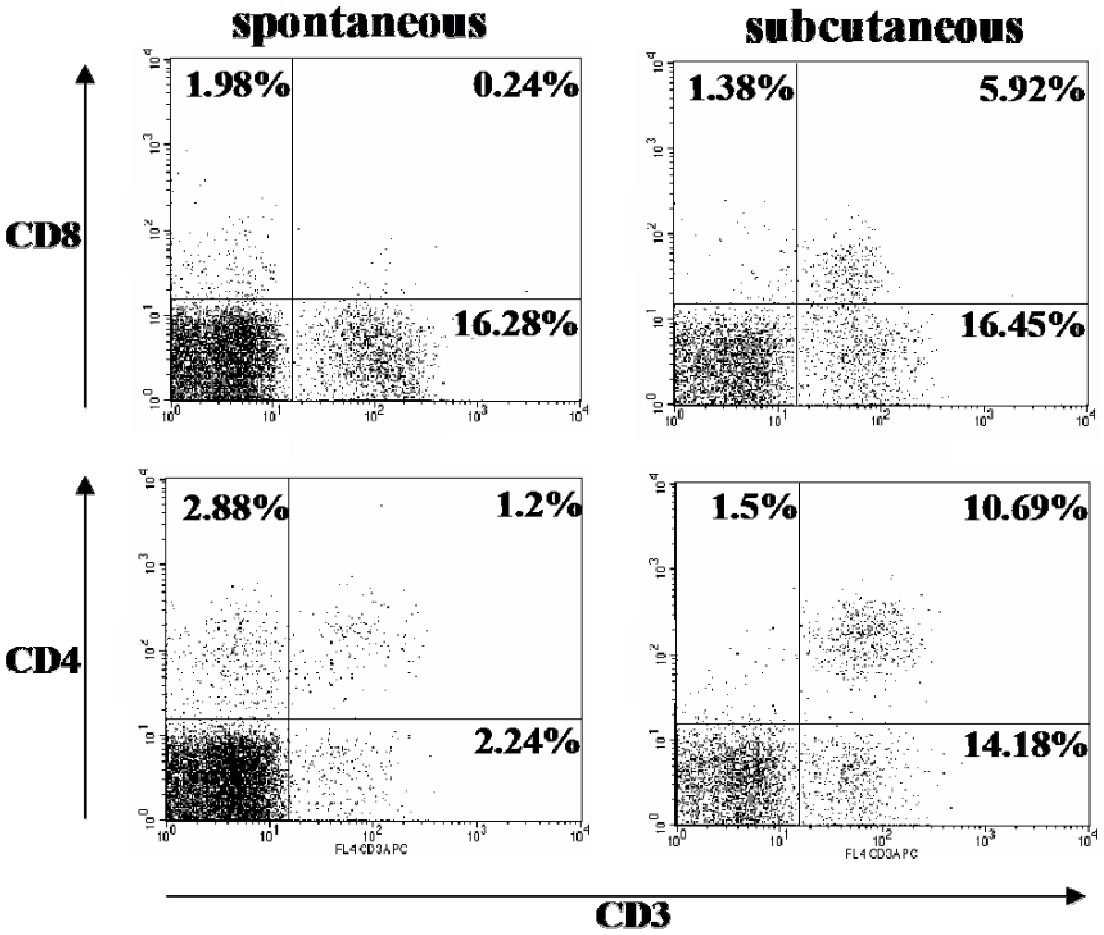

Figure 3.7 ***FACS analysis of tumour-infiltrating CD4$^+$ and CD8$^+$ T cells.*** *Spontaneous tumour of an EL-TGF-α x Trp53$^{-/-}$ mouse (left) and a subcutaneous regressor tumour (right) of an mPAC regressor cell injected C57BL/6 mouse were isolated. Tumours were digested and single cell suspensions were stained with anti-CD3, anti-CD4 and anti-CD8 antibodies. Tumour-infiltrating T cells were analysed by FACS. A representative FACS of tumour-infiltrating T cells is shown.*

Statistical analysis of three independent experiments revealed tumour-infiltrating CD4$^+$ cells in both types of tumours with no significant differences in the number of cells per milligram tumour tissue (**Figure 3.8**). In contrast, significantly more CD8$^+$ cells are found in subcutaneous tumours than in spontaneous tumours.

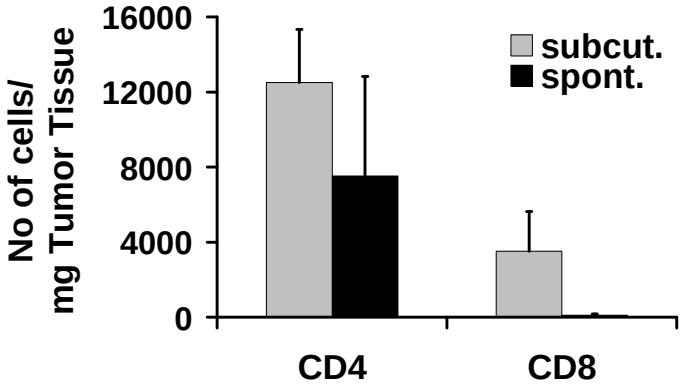

Figure 3.8 ***FACS analysis of tumour-infiltrating CD4$^+$ or CD8$^+$ T cells in subcutaneous and spontaneous pancreatic tumours.*** *Spontaneous tumours of EL-TGF-α x Trp53$^{-/-}$ mice (black columns) and subcutaneous regressor tumours (grey columns) of mPAC regressor cell injected C57BL/6 mice were isolated. Tumours were digested and single cell suspensions were stained with anti-CD3, anti-CD4 (left columns) and anti-CD8 (right columns) antibodies. Tumour-infiltrating T cells were analysed by FACS. At least three independent experiments are combined. The average of all experiments is shown, including the calculated SD.*

3.1.3 Activation- and migration-status of tumour-specific CTLs in spontaneous or subcutaneous tumour

Since no T cell receptor transgenic T cells are available for this tumour model, tumour-specific cytotoxic T lymphocytes (CTLs) were generated by using the mPAC regressor tumour cell line. The tumour cells were subcutaneously injected into transgenic enhanced green fluorescent protein (EGFP) mice (Okabe et al., 1997)**.** Splenocytes and draining lymph node cells were isolated from these mice on day 14 and restimulated with irradiated mPAC regressor cells for five days. Generated CTLs were harvested and i.v. injected into spontaneous or subcutaneous tumour bearing mice. The specificity of CTLs for both tumours was analysed by looking for EGFP$^+$ tumour-infiltrating T cells. Four, twelve, and 24 hours after adoptive transfer, tumours were analysed for EGFP$^+$ tumour-infiltrating T cells. Approximately twice more transferred EGFP$^+$ cells were found in the tumours twelve hours after transfer, compared to four and 24 hours after transfer (data not shown). Specific CD3$^+$ anti-tumour T cells were found in the spleen of spontaneous and subcutaneous tumour bearing mice (**Figure 3.9**). Compared to the spontaneous tumour of the EL-TGF-α x Trp53$^{-/-}$ mice, more specific adoptively transferred T cells migrate into the subcutaneous tumour. Combined data collected from three independent experiments reveal a six-fold higher number of transferred infiltrating T cells in subcutaneous tumours than in spontaneous tumours (**Figure 3.10**).

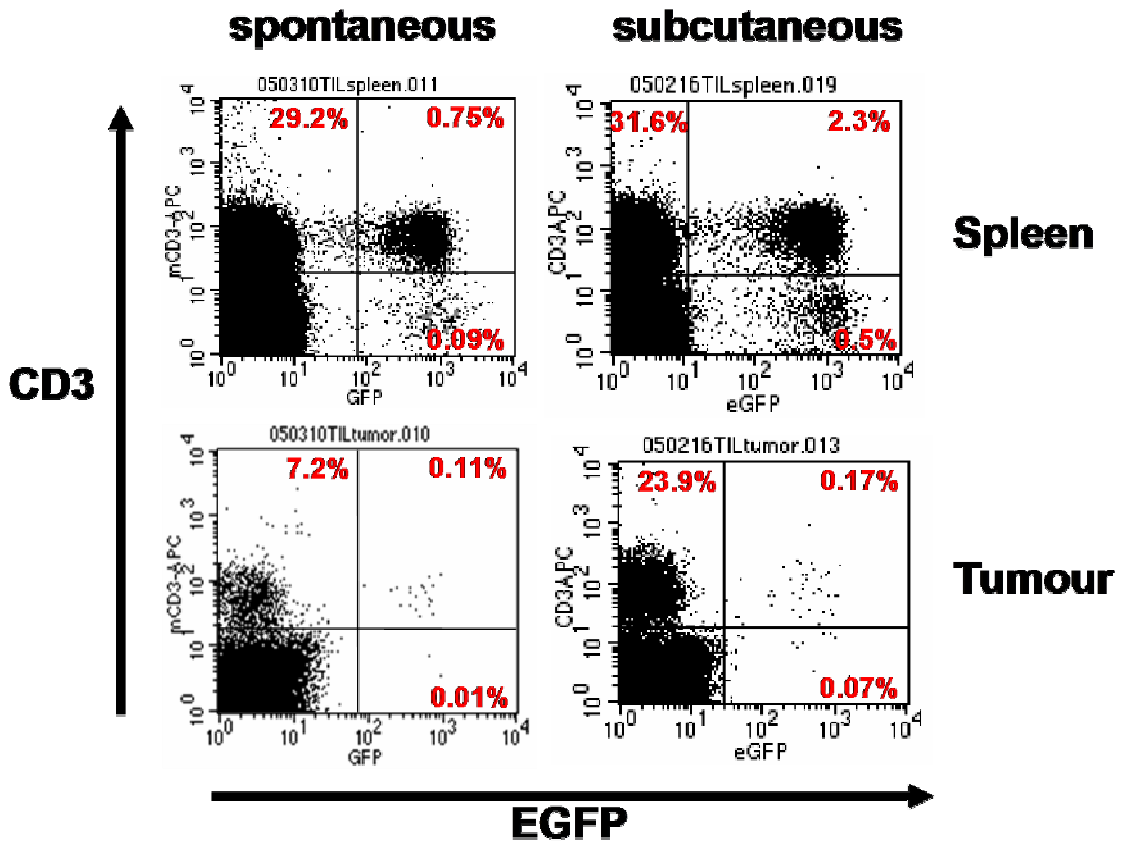

Figure 3.9 ***FACS analysis of tumour-infiltrating T cells in subcutaneous and spontaneous pancreatic tumours after adoptive T cell transfer.*** *Tumour-specific CTLs were generated from mPAC regressor vaccinated EGFP transgenic mouse and i.v. injected into twelve week old EL-TGF-α x Trp53$^{-/-}$ mouse with spontaneous tumours (left) and wild type (wt) mouse with subcutaneous tumour (right). Tumours and splenocytes/draining lymph node cells were isolated twelve hours after adoptive T cell transfer. Tumour-infiltrating T cells and splenocytes/draining lymph node cells were stained for CD3^{+} cells and analysed by FACS. A representative FACS is shown of specific EGFP^{+} CTLs reaching the spleen (top) and the tumour (bottom) after transfer.*

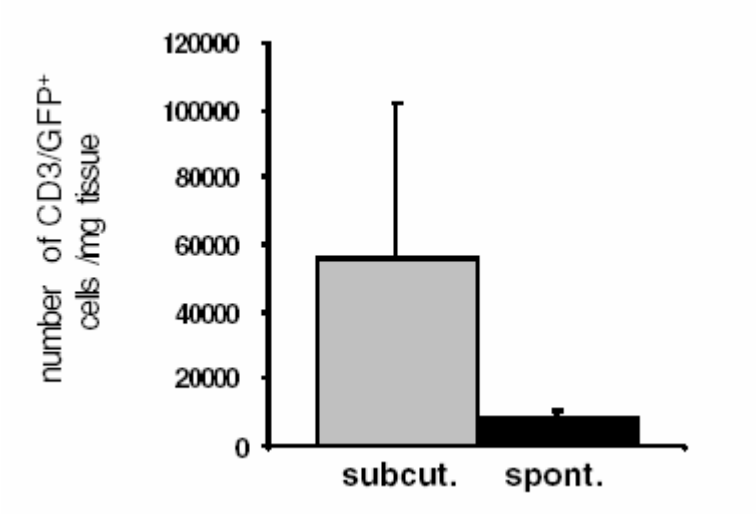

Figure 3.10 ***Analysis of tumour-specific infiltrating T cells after adoptive T cell transfer.*** *Proliferating mPAC regressor tumour cells were subcutaneously injected into transgenic EGFP mice. Splenocytes and draining lymph node cells were isolated from these mice on day 14 and restimulated with irradiated mPAC regressor cells for five days. Generated CTLs were harvested and i.v. injected into spontaneous (black column) or subcutaneous tumour (grey column) bearing mice. The specificity of CTLs for both tumours was analysed by looking for $EGFP^+$ tumour-infiltrating T cells twelve hours after adoptive transfer. Tumour-infiltrating T cells were stained for $CD3^+$ cells and analysed by FACS. Combined data of three independent experiments are analysed. Absolute numbers of adoptively transferred $CD3^+/CD8^+$ T cells are shown.*

More transferred tumour-specific T cells are found in the subcutaneous tumour than in the spontaneous pancreatic tumours. Although the stimulator cells, the mPAC regressor tumour cells, have the same origin as the spontaneous tumour itself (**Figure 3.19**), the T cell homing was much more significant in the subcutaneous than in the spontaneous tumour model. This significant difference in T cell homing could be an explanation for the stronger immune response and the regression in the subcutaneous tumour model.

In order to clarify the phenotype of the tumour-infiltrating cells, analyses of specific functional intracellular and surface markers of these T cells were performed. First, the percentage of activated $CD4^+$ T cells was determined by their expression of CD25 (IL-2 receptor). Tumour-infiltrating immune cells of spontaneous tumour and subcutaneous regressor tumour bearing mice were isolated and stained with anti-CD4 and anti-CD25. Statistical analysis of at least three independent experiments is shown in **Figure 3.11**. In summary, 9% of the $CD4^+$ tumour-infiltrating cells of the subcutaneous regressor tumour express CD25 on the cell surface, in contrast to 1% $CD25^+$ cells in spontaneous tumours (**Figure 3.11**).

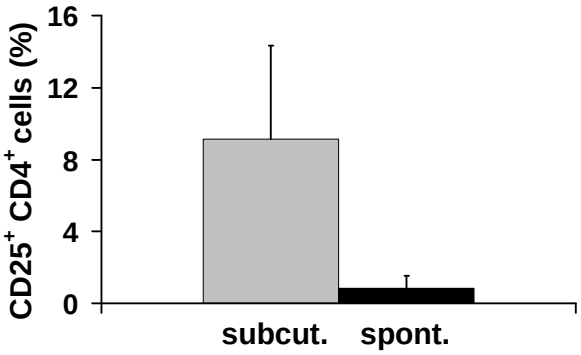

Figure 3.11 ***FACS analysis of tumour-infiltrating CD4$^+$CD25$^+$ cells.*** *Subcutaneous regressor tumours from C57BL/6 mPAC regressor cell injected mice and spontaneous tumours from twelve week old EL-TGF-α x Trp53 mice were explanted. Tumour-infiltrating cells were isolated and stained with anti-CD4 and anti-CD25. Cells were analysed by FACS. Data from three independent experiments were combined by taking the average and calculating the SD.*

The natural T regulatory (T_{reg}) cell is a specific T cell subset, which plays an important role in maintaining immunological self-tolerance, and controlling effector T cells directly or indirectly through antigen presenting cells (APC). This T cell subset also expresses the receptors CD4 and CD25, but can be distinguished from other activated T cells by their specific expression of the transcription factor fork head box p3 (Foxp3). Compared to the splenocytes and draining lymph node cells of naïve C57BL/6 wt mice, there is also a high percentage of T_{reg} cells of all the activated CD4$^+$ cells in twelve week old EL-TGF-α x Trp53$^{-/-}$ spontaneous and five day old subcutaneous tumour bearing mice (**Figure 3.12**).

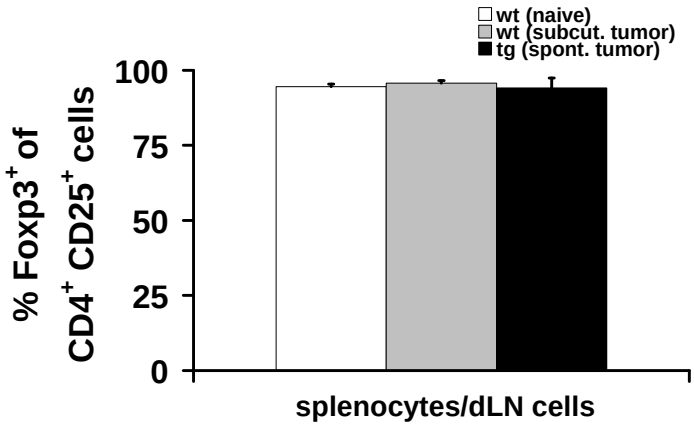

Figure 3.12 ***FACS analysis for T_{reg} cells in the spleen and draining lymph node of subcutaneous or spontaneous pancreatic tumour bearing mice.*** *Isolated splenocytes and draining lymph node cells from spontaneous (black column) and regressor tumour bearing mice (grey column) were stained with anti-CD4 and anti-CD25 antibodies. The Foxp3 expression was investigated by intracellular staining with anti-Foxp3. As control, splenocytes and draining lymph node cells from naïve C57BL/6 mice were stained with anti-CD4, anti-CD25 and anti-Foxp3 antibodies (white column). The average of all experiments is shown, including the calculated SD.*

Even in both tumours, most of the activated tumour-infiltrating $CD4^+$ T cell subsets are characterized by a regulatory phenotype (**Figure 3.13**). Although on average there is a difference in the percentage of T_{reg} cells in mice with spontaneous pancreatic tumours, compared to the subcutaneous regressor tumour bearing mice; this difference is not significant.

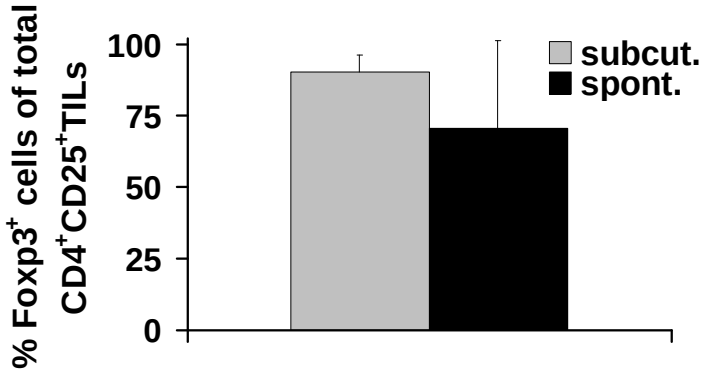

Figure 3.13 ***FACS analysis of T_{reg} cells in subcutaneous and spontaneous pancreatic tumours.*** *Tumour-infiltrating cells were isolated from subcutaneous (grey column) and spontaneous pancreatic tumours (black column). T_{reg} cells were detected by $CD4^+CD25^+$ and $Foxp3^+$ surface and intracellular staining, respectively, and analysed by FACS. At least three independent experiments with different tumour bearing mice were combined and charted. The average of all experiments is shown, including the calculated SD.*

To find out if these features especially occur in the subcutaneous and spontaneous pancreatic tumours, tumours from different origins were analysed for the percentage of T_{reg} cells in their TIL population (**Figure 3.14**). The subcutaneous tumours of the murine thymoma cell line EL-4 as well the murine lymphoma cell line RMA isolated from C57BL/6 wt mice showed a similar percentage of T_{reg} cell infiltration into their tumours. Therefore, it is rather a common feature that T_{reg} cells are widely distributed in tumours.

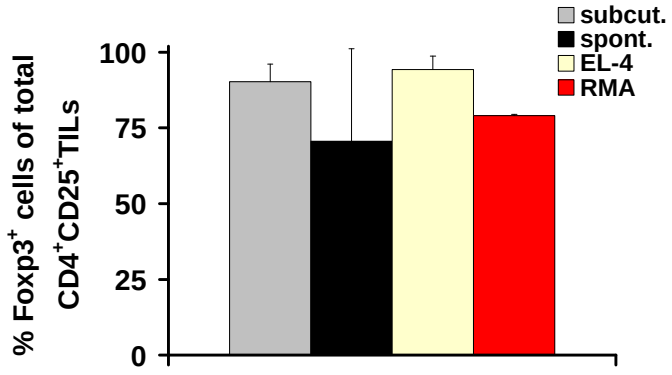

Figure 3.14 ***FACS analysis of T_{reg} cells in different tumours.*** *Tumour-infiltrating cells from subcutaneous regressor (grey column), spontaneous (black column), thymoma EL-4 (yellow column) and lymphoma RMA tumour (red column) were isolated and stained for $CD4^+$, $CD25^+$ and intracellular for $Foxp3^+$. Cells were analysed by FACS. Three mice from each tumour were independently analysed and combined later for evaluation. The average of all experiments is shown, including the calculated SD.*

The leukocyte receptor CD62L is, among other molecules, required for lymphocyte homing to peripheral lymph nodes and contributes to neutrophil migration at inflammatory sites. Subcutaneous and spontaneous pancreatic tumour bearing mice were i.v. injected with 2 x 10^7 tumour-specific generated CTLs. Twelve hours after transfer, mice were killed, splenocytes and draining lymph node cells (pooled and referred to as spleen in **Figure 3.15**), and tumour-infiltrating cells were also isolated. Cells were stained for CD3 and CD62L and analysed by FACS (**Figure 3.15**). Only transferred ($EGFP^+$) cells were analysed for the T cell receptor CD3 and the homing receptor CD62L. In all tumour bearing mice, most of the detected transferred $CD3^+EGFP^+$ cells in the spleen and the draining lymph nodes express CD62L. More cells of the splenocytes and draining lymph node cells (pooled and referred to as spleen) from the subcutaneous tumour bearing mice express CD62L compared to the spontaneous tumour bearing EL-TGF-α x $Trp53^{-/-}$ transgenic mice (**Figure 3.15**). Interestingly, the tumour-infiltrating $EGFP^+$ cells of the subcutaneous tumour have a significantly lesser expression level of CD62L. This is not the case for the spontaneous tumour.

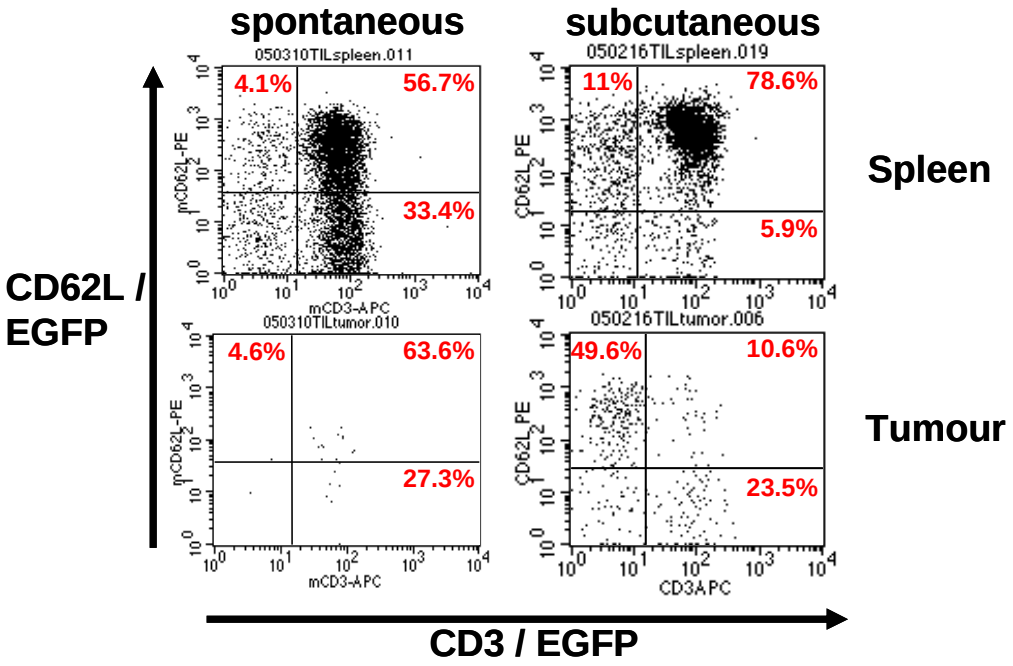

Figure 3.15 ***FACS analysis of splenocytes/draining lymph node cells and tumour-infiltrating lymphocytes on the expression of the homing factor CD62L after adoptive transfer.*** *Mice bearing subcutaneous or spontaneous pancreatic tumour were i.v. injected with tumour-specific EGFP$^+$ CTLs. Twelve hours after transfer, splenocytes and draining lymph node cells (top), as well as tumour-infiltrating cells (bottom) of subcutaneous (right) and spontaneous (left) pancreatic tumour bearing mice (top) were isolated. Splenocytes and draining lymph node cells were pooled and referred to as spleen. Cells were stained for CD3 and CD62L. Gated on EGFP$^+$ cells, cells were analysed for CD3 and CD62L expression by FACS. A representative FACS is shown.*

Statistical analysis of at least three mice per group revealed that less than half of the CD3$^+$EGFP$^+$ cells are CD62L$^+$ cells in the subcutaneous pancreatic tumour (**Figure 3.16**). Compared to the CD3$^+$EGFP$^+$ cells of the spontaneous pancreatic tumour, about 75% are positive for CD62L. Compared to the corresponding spleen, about 90% of the CD3$^+$EGFP$^+$ cells from subcutaneous regressor tumour also express CD62L. No significant loss of the homing receptor CD62L occurs between the splenocytes and the tumour-infiltrating cells of the spontaneous tumour bearing mice.

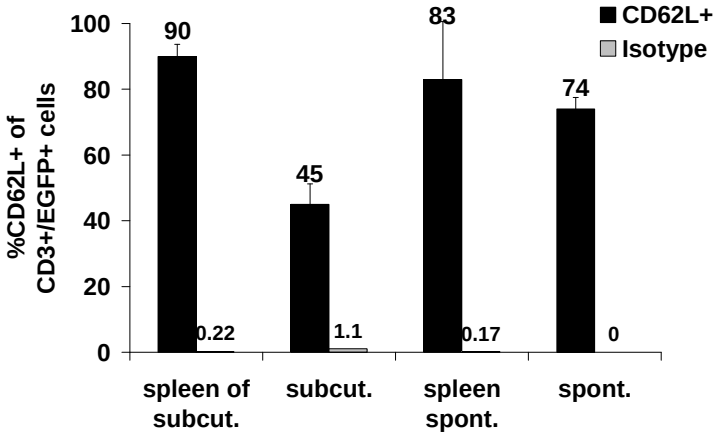

Figure 3.16 ***FACS analysis of CD62L$^+$ splenocytes (first and third black columns) or tumour-infiltrating T lymphocytes (second or fourth black column) after adoptive transfer of tumour-specific EGFP$^+$ CTLs.*** *Isolation of splenocytes and tumour-infiltrating cells of subcutaneous (first four left columns) and spontaneous (four right columns) pancreatic tumour bearing mice, after adoptive transfer of EGFP$^+$ CTLs. Phenotype of EGFP$^+$ T cells of spleen and tumour-infiltrating EGFP$^+$ T cells with regard to surface expression of the homing marker CD62L. Grey columns are the corresponding isotype controls. Three independent experiments are combined in this statistic. The average of all experiments is shown, including the calculated SD.*

3.1.4 Analysis of tumour-specific humoral immune responses in mice with subcutaneous and spontaneous pancreatic tumours

The principal antibody isotype in the blood and extracellular fluid is IgG. IgG is responsible for many functional activities in the humoral immune response, such as pathogen opsonization (IgG1) or neutralization (IgG2), and activation of the complement system (IgG3). It was previously shown that subcutaneous injection of the mPAC regressor tumour cell line into the T and B cell-deficient RAG$^{-/-}$ mouse had an accelerated tumour growth compared to the T cell-deficient (nude) mice. In order to examine the role of B cells and their antibody production in the development of pancreatic tumours, sera were isolated from EL-TGF-α x Trp53$^{-/-}$ and mPAC regressor injected immune competent C57BL/6 wt mice. The sera of the twelve week old EL-TGF-α x Trp53$^{-/-}$ transgenic mice and C57BL/6 wt mice 21 days after subcutaneous mPAC regressor injection were used as primary antibody to stain mPAC cells (**Figure 3.17**). The conjugated goat anti-mouse panIgG secondary antibody was used to detect the mPAC cells bound to the tumour-specific serum IgG. Similar to the wt mice injected with mPAC cells (right blue line), the spontaneous tumour bearing transgenic mice develop a tumour-specific IgG antibody response (left blue line). A slightly stronger antibody immune response was observed in the C57BL/6 wt mice vaccinated with the mPAC regressor cell line. A control tumour cell line did not show any specific staining (data not shown).

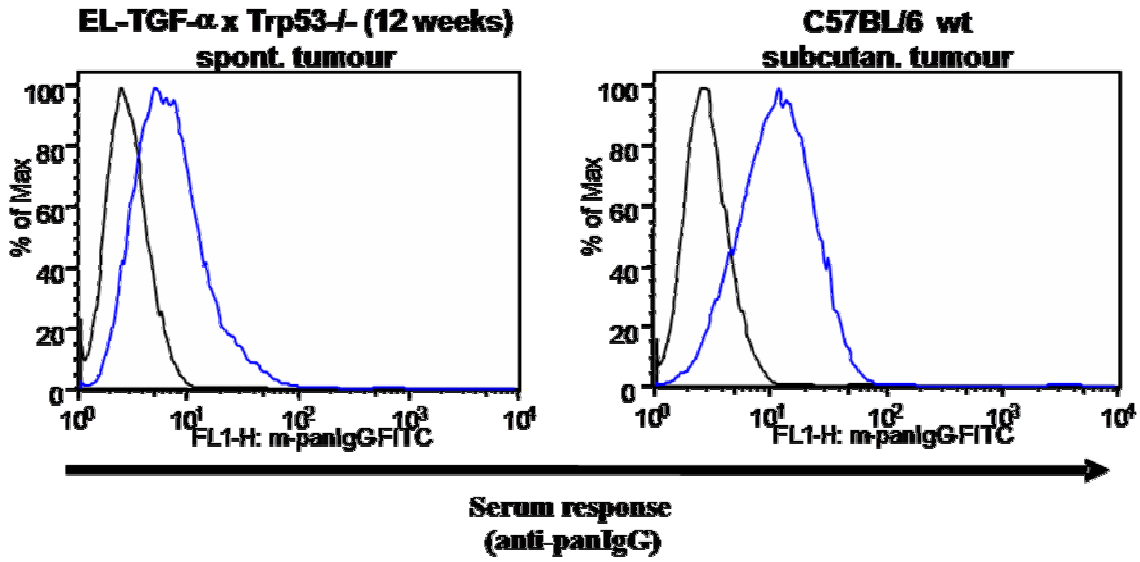

Figure 3.17 ***FACS analysis of tumour-specific antibody response.*** *Sera from twelve week old EL-TGF-α x Trp53$^{-/-}$ spontaneous tumour bearing mice and mPAC-vaccinated C57BL/6 wt mice were isolated. Pancreatic tumour cells were incubated with serum (diluted 1:50) of the described mice. Tumour-specific antibodies were detected by using FITC-labelled panIgG. Antibody response (blue line) was observed in spontaneous (left) and subcutaneous (right) tumour bearing mice. Serum from naive C57BL/6 wt was used as control (black line). A representative FACS analysis of stained pancreatic tumour cells by sera from subcutaneous and spontaneous pancreatic tumour bearing mice is shown.*

The tumour-specific antibody was titrated by using different dilutions of the serum. The higher the dilution, the weaker was the staining/detection of the tumour-specific antibody (data not shown). This confirms a tumour-specific antibody response in spontaneous and subcutaneous tumour bearing mice. To determine the type of humoral immune response, which could be responsible for the subcutaneous pancreatic tumour regression, the IgG subtype of the tumour-specific antibody was examined (**Figure 3.18**). Anti-IgG1, anti-IgG2a, or anti-IgG2b was used as secondary antibodies.

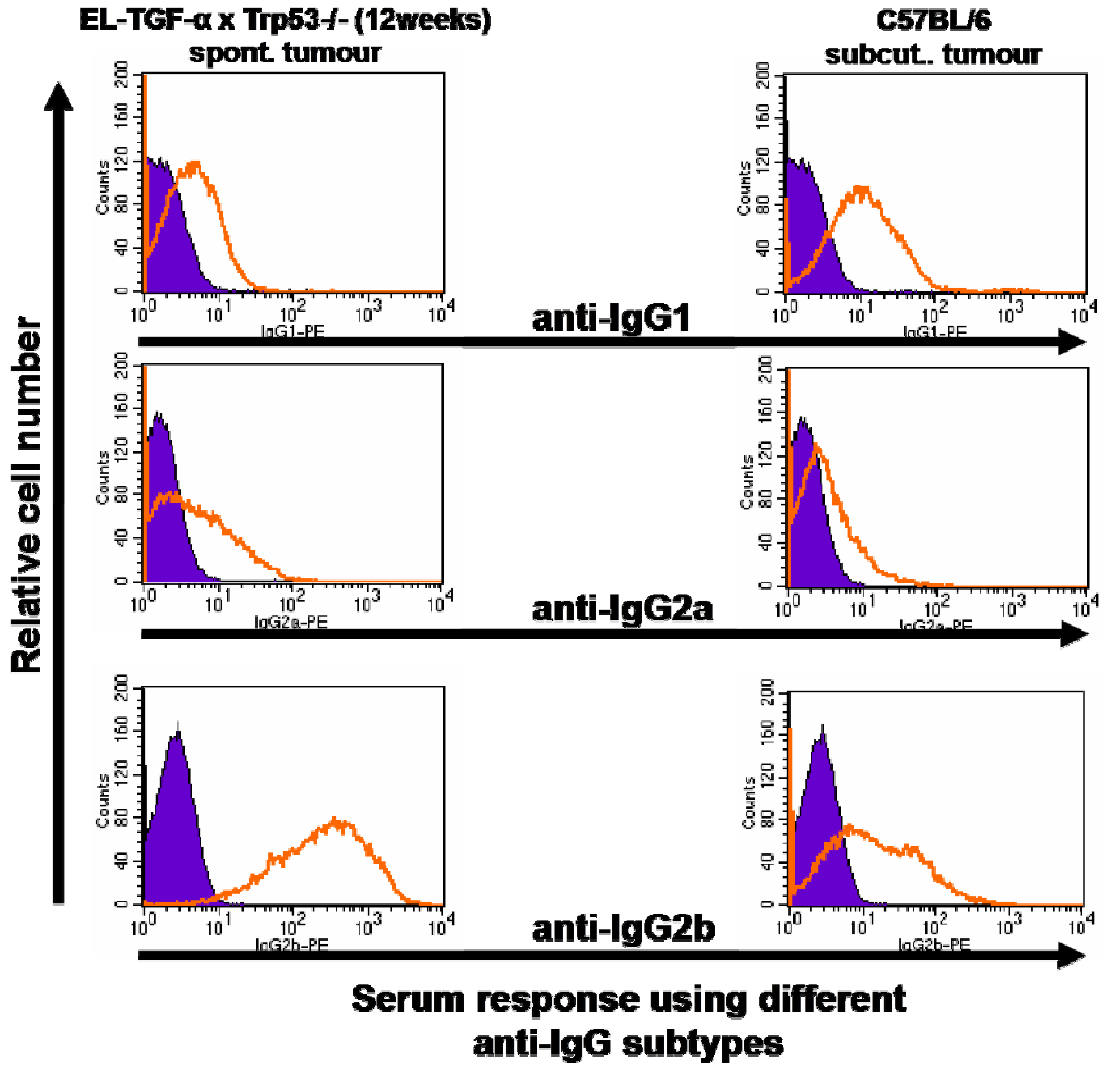

Figure 3.18 ***FACS analysis of anti-IgG subtype determination.*** *Sera from twelve week old EL-TGF-α x Trp53$^{-/-}$ spontaneous tumour bearing mouse (left row) and C57BL/6 wt subcutaneous tumour bearing mouse (right row) were isolated. Pancreatic tumour cells were stained with these sera. Tumour-specific antibodies were detected using different IgG subtypes antibodies, anti-IgG1 (top), anti-IgG2a (middle) and anti-IgG2b (bottom). A representative FACS analysis of stained pancreatic tumour cells by sera from subcutaneous and spontaneous pancreatic tumour bearing mice is shown.*

To specify the humoral immune response in twelve week old transgenic EL-TGF-α x Trp53$^{-/-}$ spontaneous tumour bearing mice and in mPAC vaccinated C57BL/6 mice, different subtypes of the isotype IgG were tested. Spontaneous tumour bearing mice show a strong IgG2b antibody response, which indicates a T_H1 response and neutralization function of the tumour-specific antibody. However, mPAC regressor vaccinated C57BL/6 mice develop antibodies in the IgG1 isotype form. This indicates mainly a T_H2 immune response. In both types of tumour bearing mice, antibodies with the IgG2a isotype form also recognize mPAC cells, although to a lesser extent.

3.2 Pancreatic tumour cell lines with the same origin behave differently *in vivo*

As mentioned previously (3.1.1), mPAC regressor cell lines were generated from spontaneous pancreatic tumours of twelve week old EL-TGF-α x Trp53$^{-/-}$ mice. Although after ten days, all of the induced mPAC regressor tumours were rejected in immune competent C57BL/6 mice, in about 20% of the cases, the tumours grew again after 90 days, but this time progressively. From these subcutaneous progressively growing tumours cell lines were generated again. These pancreatic tumour cell lines grew progressively after subcutaneous injection into immune competent C57BL/6 mice and were called murine pancreatic adenocarcinoma progressor (mPAC progressor). Both types of tumour cell lines (mPAC regressor and mPAC progressor) express the transgenic markers for the spontaneously developing tumour from the EL-TGF-α x Trp53$^{-/-}$ mouse (**Figure 3.19**). In addition, western blot analysis showed an expression of the epithelial tumour marker cytokeratin 8/18 in both tumour cell lines (data not shown).

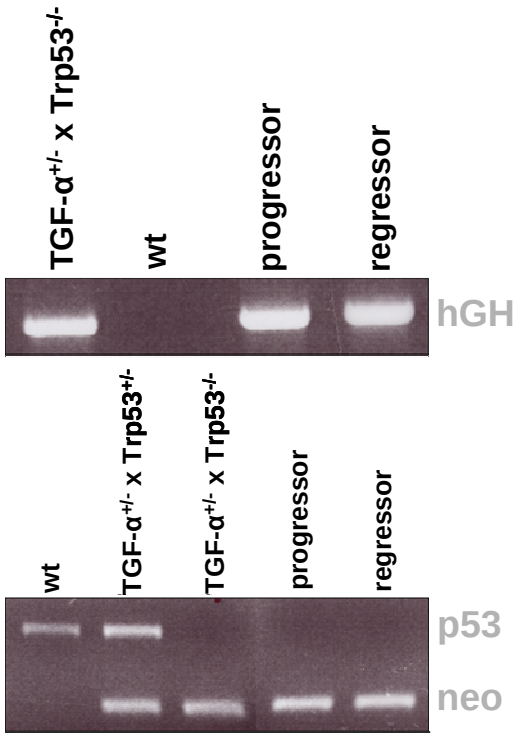

Figure 3.19 ***DNA analysis of mPAC progressor and regressor tumour cell line for transgenic markers by PCR.*** *DNA was isolated from mPAC progressor and regressor tumour cell line. The gene of the transgenic human growth hormone (hGH) was detected in both cell lines using specific primer. The neo construct but not that caused by the insertion of neo disrupted p53 gene was detected in the tumour cell lines. The tumour cell lines show the same genotype as EL-TGF-α x Trp53$^{-/-}$ transgenic mice. As control, C57BL/6 wt and EL-TGF-α x Trp53$^{+/-}$ were used to detect the wt p53 gene. The hGH was not detected as expected in C57BL/6 wt mice (top "-").*

3.2.1 Expression of cytokines in regressive and progressive subcutaneous pancreatic tumours

A cytokine profile of the solid mPAC regressor and progressor tumour was generated to discover the differences between these two tumours regarding their *in vivo* growth kinetics. C57BL/6 immune competent mice were injected with the mPAC regressor or progressor tumour cell line. Five day old regressor and ten day old progressor tumours were explanted and cut into small pieces. The tumour pieces were incubated *in vitro* for twelve hours at 37°C / 5% CO_2 in complete medium. Culture supernatants from mPAC regressor and progressor derived tumours were harvested and analysed for several cytokines using the CBA (**Figure 3.20**).

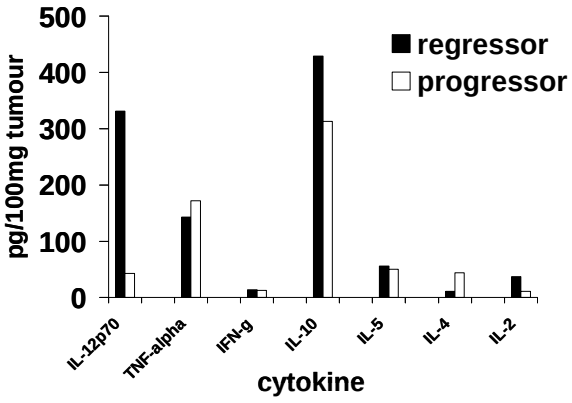

Figure 3.20 ***CBA analysis of in vitro regressor and progressor tumour culture.*** *200 mg of explanted regressor and progressor tumour were cut into pieces and cultured for twelve hours at 37°C / 5% CO_2 in complete medium. Supernatants were harvested and the expression of the cytokines IL-12p70, TNF-α, IFN-γ, IL-10, IL-5, IL-4, and IL-2 was analysed by cytometric bead array. Graph indicates the average of two independent experiments.*

Significant differences in the levels of IL-12p70 subunit between the *ex vivo* regressor and progressor tumour cultures were seen. The IL-12 cytokine is mainly produced by macrophages and dendritic cells and activates NK cells. In addition, IL-12 induces CD4 T cell differentiation into $T_{Helper}1$ (T_H1) cells. In contrast, IL-4 is more detected in the supernatant of the progressor tumour. It activates B cells and induces the differentiation into T_H2 cells. Minor differences could be detected in the expression of IL-2. IL-2 and IL-10 are expressed slightly higher in the regressor tumour culture. The T cell growth factor IL-2 is responsible for the proliferation of T cells and is produced by the same type of cells. Macrophages and T cells both produce IL-10, which acts as a potent suppressor of macrophage functions.

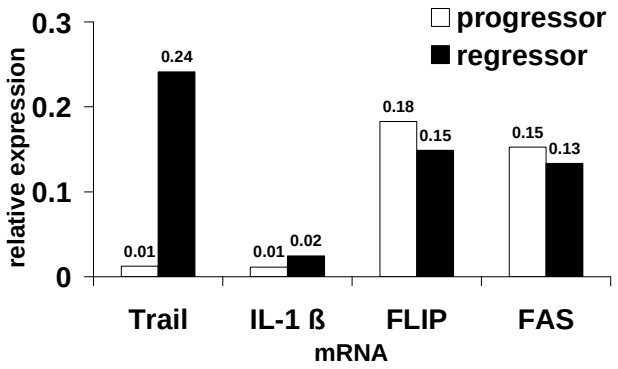

Figure 3.21 ***Real time PCR analysis to detect the relative expression of mRNA in cells of the mPAC progressor and regressor tumour environment.*** *mRNA from explanted mPAC progressor (white columns) and regressor (black columns) tumours were isolated and the relative expression of the indicated mRNA was determined by quantitative SYBR-Green real time PCR. TRAIL and IL-1β are expressed higher in the mPAC regressor tumour. In contrast, FLIP and FAS mRNA could be detected slightly more in the mPAC progressor tumour environment.*

For the detection of RNA expression of specific cytokines in the mPAC progressor or regressor tumour environment, C57BL/6 wt mice were injected with the mPAC regressor or progressor tumour cell line. Five millimetres of the mPAC regressor and progressor tumours were explanted and their RNA isolated. Quantitative expressions of the TRAIL, IL-1β, FLICE (caspase-8) inhibitory protein (FLIP), and FAS mRNA were detected by SYBR-Green real time PCR (**Figure 3.21**). There was an induction of TRAIL mRNA in the mPAC regressor tumour that was 20-fold greater than the mPAC progressor tumour. Twice as much IL-1β mRNA was produced in the mPAC regressor tumour. In contrast, slightly higher expression of the members of the apoptosis pathway FLIP and FAS was seen in the mPAC progressor tumour. The mRNA expressions of IP-10 and MCP-1a are six and two fold higher in mPAC regressor than in progressor tumour, respectively (**Figure 3.22**). mPAC regressor tumour stroma produced three or two fold higher amounts of TGF-β2 or vascular endothelial growth factor (VEGF) mRNA, respectively, than the mPAC progressor tumour stroma (**Figure 3.23**).

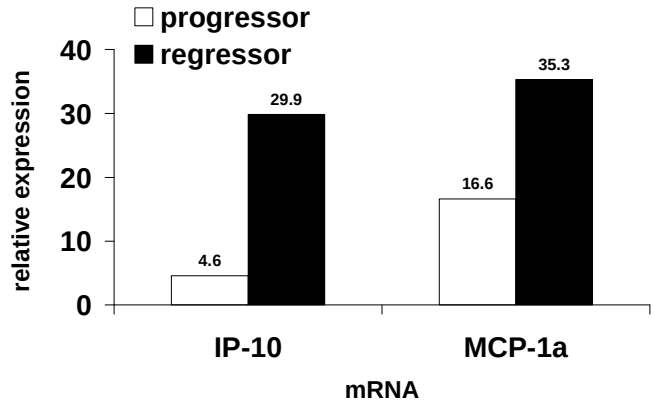

Figure 3.22 ***Real time PCR analysis to detect the relative expression of IP-10 and MCP-1a mRNA in cells of the mPAC progressor and regressor tumour environment.*** *mRNA from explanted mPAC progressor (white columns) and regressor (black columns) tumours were isolated and the relative expression of the indicated mRNA was determined by quantitative SYBR-Green real time PCR. IP-10 and MCP-1a are more strongly expressed in the mPAC regressor tumour than in the progressor tumour.*

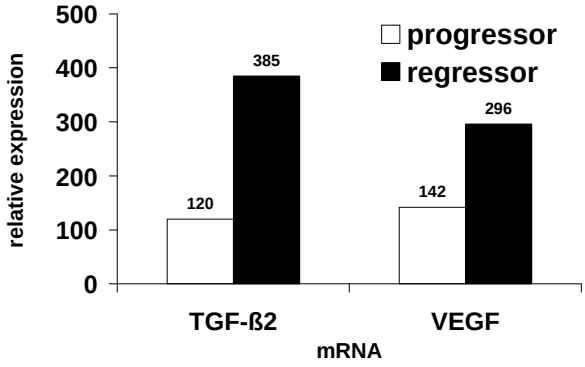

Figure 3.23 ***Real time PCR analysis to detect the relative expression of TGF-β2 and VEGF mRNA in cells of the mPAC regressor and progressor tumour environment.*** *mRNA from explanted mPAC progressor (white columns) and regressor (black columns) tumours were isolated and the relative expression of the indicated mRNA was determined by quantitative SYBR-Green real time PCR. TGF-ß2 and VEGF were more strongly expressed in the mPAC regressor tumour than in the progressor tumour.*

Studying the tumour-infiltrating cells in the mPAC regressor and the progressor tumours could help to understand the growth kinetics of these tumours. Subcutaneous regressor and progressor tumours from injected immune competent C57BL/6 wt mice were isolated and snap frozen. Sections were fixed and used for detection of $CD4^+$ or $CD8^+$ cells in the tumours by staining with anti-CD4 and anti-CD8 antibodies. Cells were visualized by using the APAAP-Fast Red system (**Figure 3.24**). The mPAC regressor and progressor tumour show no difference in the distribution of $CD4^+$ cells. In contrast, the mPAC regressor tumour seems to be more infiltrated by $CD8^+$ cells than

the progressor tumour. FACS analysis revealed significantly higher $CD4^+$ and $CD8^+$ infiltration in the mPAC regressor compared to the progressor tumour (**Figure 3.25**). No significant differences in the number of $CD4^+$ and $CD8^+$ cells were seen in the splenocytes and draining lymph node cells of the mPAC regressor and progressor tumour bearing mice (**Figure 3.26**).

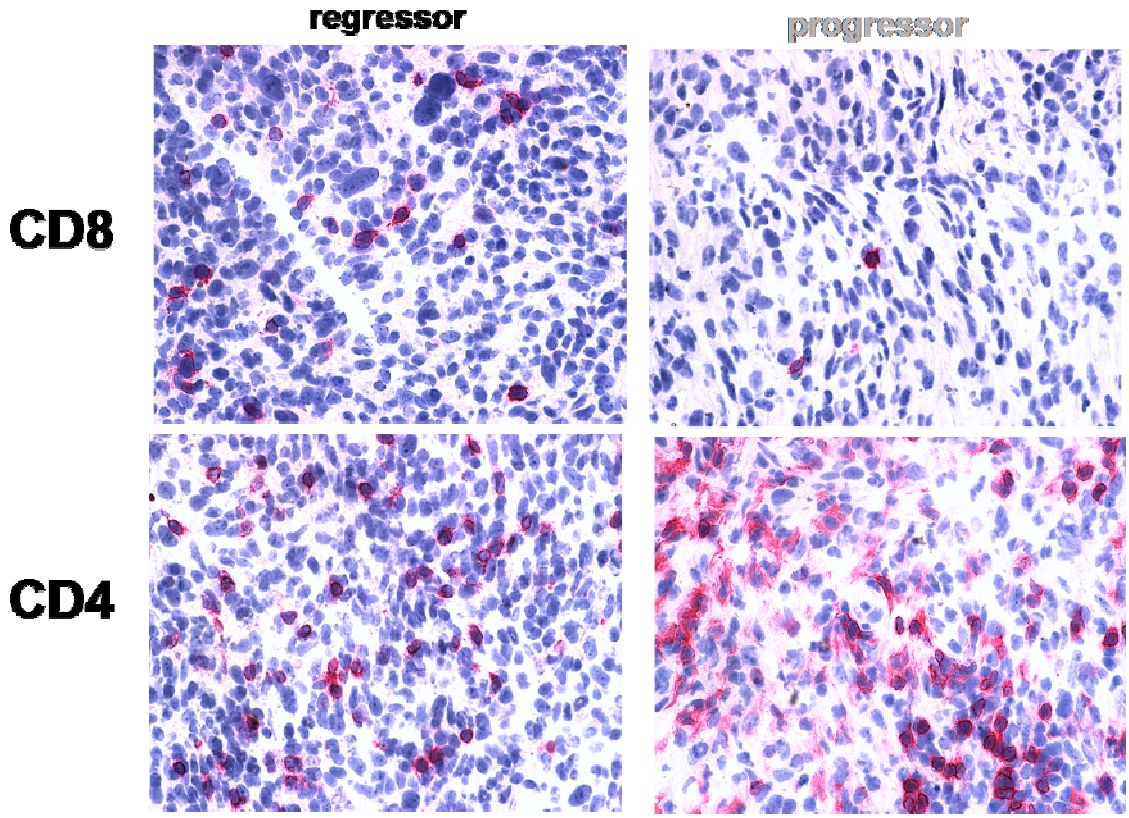

Figure 3.24 ***Immunohistochemistry staining of mPAC regressor and progressor tumour-infiltrating T cells.*** *Subcutaneous mPAC regressor (left) or progressor (right) tumours were explanted and snap frozen in liquid nitrogen. Sections of frozen tissue were fixed and stained with anti-CD4 (bottom) or anti-CD8 (top) antibodies. Positive cells were visualized in red using the APAAP system. In both tumours, $CD4^+$ and $CD8^+$ infiltrating cells were detected, but $CD8^+$ cells to a lesser extent in the progressor tumour, compared to the regressor tumour.*

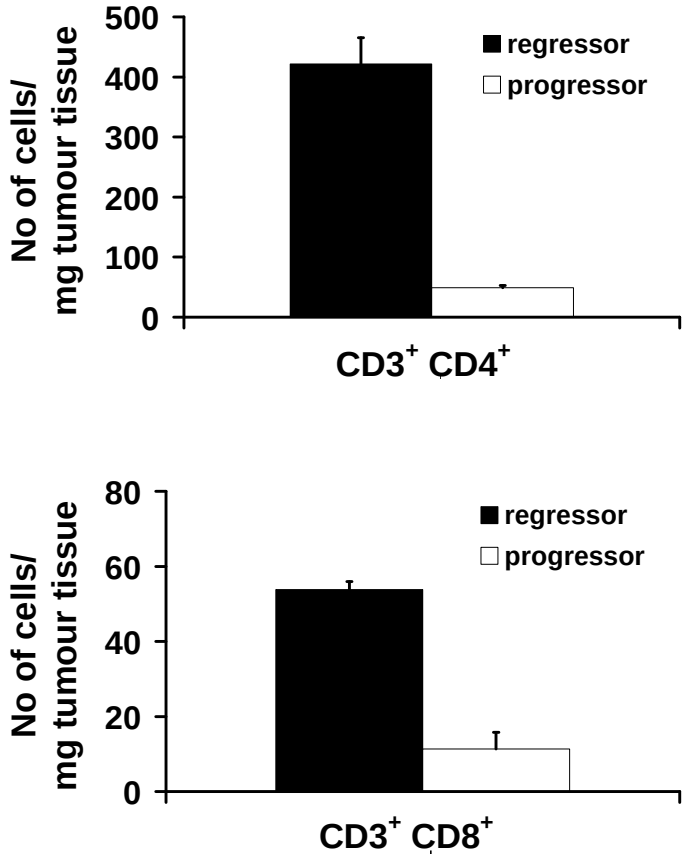

Figure 3.25 ***FACS analysis of tumour-infiltrating CD4$^+$ and CD8$^+$ cells in mPAC regressor and progressor subcutaneous tumours.*** *Subcutaneous regressor and progressor tumours from C57BL/6 wt were isolated. Tumour-infiltrating cells were isolated from both tumours, stained with anti CD3 / anti-CD4 (top graph) and anti CD3 / anti-CD8 (bottom graph) antibodies and analysed by FACS. Both CD4$^+$ and CD8$^+$ lymphocytes are significantly higher infiltrated in regressor (black column) than in progressor (white column) tumours. At least three independent experiments with different tumour bearing mice were combined and charted. The average of all experiments is shown, including the calculated SD.*

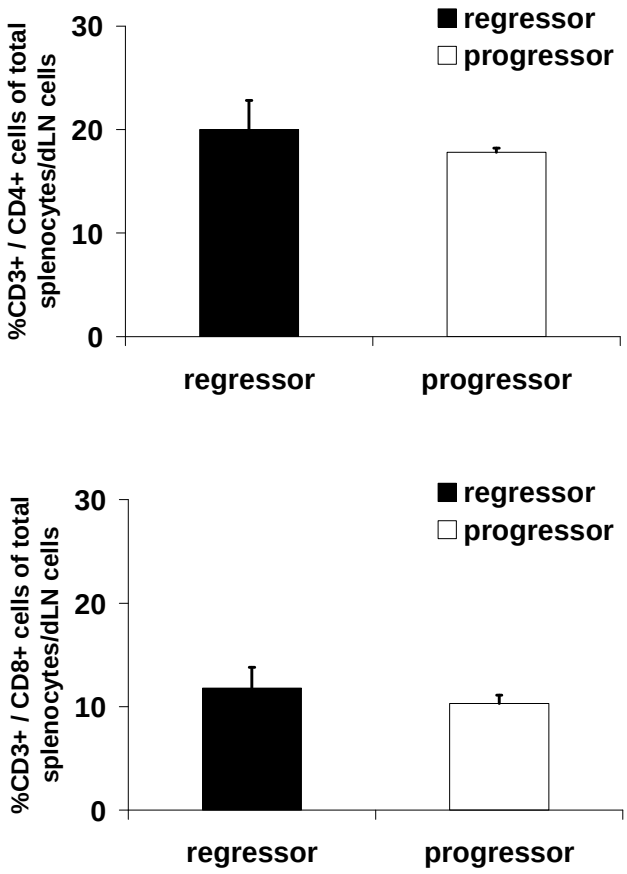

Figure 3.26 ***FACS analysis of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in splenocytes and draining lymph node cells of mPAC regressor and progressor subcutaneous tumour bearing mice.*** *Splenocytes and draining lymph node cells from regressor and progressor tumour bearing C57BL/6 wt mice were isolated. Cells were double stained with anti-CD3 / anti-CD4 (top graph) or anti-CD3 / anti-CD8 (bottom graph) antibodies and analysed by FACS. Both $CD4^+$ and $CD8^+$ cells are similarly distributed in splenocytes and draining lymph node cells of regressor (black column) and progressor (white column) tumour bearing mice. At least three independent experiments with different tumour bearing mice were combined and charted. The average of all experiments is shown, including the calculated SD.*

Myeloid and natural killer (NK) cells are also able to infiltrate tumours and influence the tumourgenesis. Therefore, tumour-infiltrating cells were stained with anti-CD11b and analysed in mPAC regressor and progressor tumour sections (**Figure 3.27**) and in three independent FACS experiments (**Figure 3.28**). The histology results indicate a higher infiltration of $CD11b^+$ cells in the centre (bottom left) and the surrounding (top left) tumour tissue of the regressor tumour compared to the progressor tumour. The FACS analysis confirms these results and indicates a significant difference between the two tumours (**Figure 3.28**).

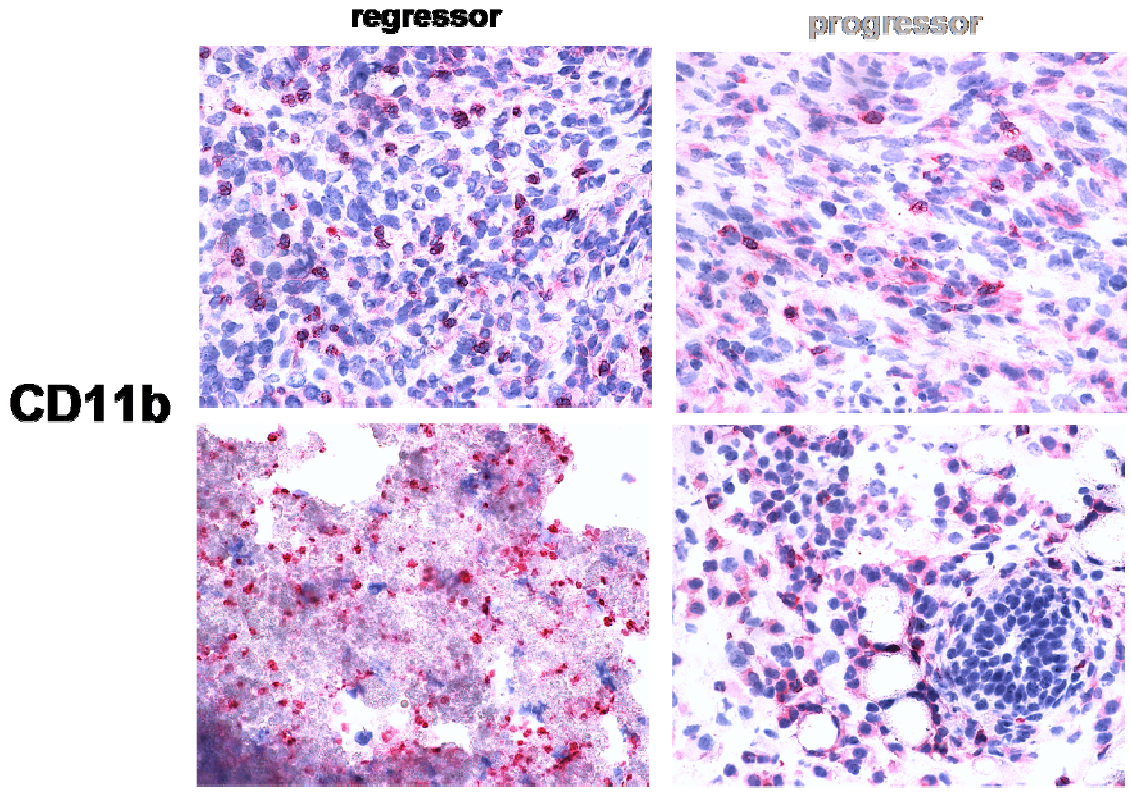

Figure 3.27 ***Immunohistochemistry staining of regressor and progressor tumour-infiltrating T cells.*** *Subcutaneous regressor (left panels) or progressor (right panels) tumours were explanted and snap frozen in liquid nitrogen. Sections of frozen tissue were fixed and stained with anti-CD11b (all panels) antibodies. Positive cells were visualized in red using the APAAP system. In both tumours CD11b$^+$ infiltrating cells were detected, but to a lesser extent in the progressor tumour. Top panels show the tumour tissue between the tumour centre and the outside of the tumour; the bottom panels show the tumour centre.*

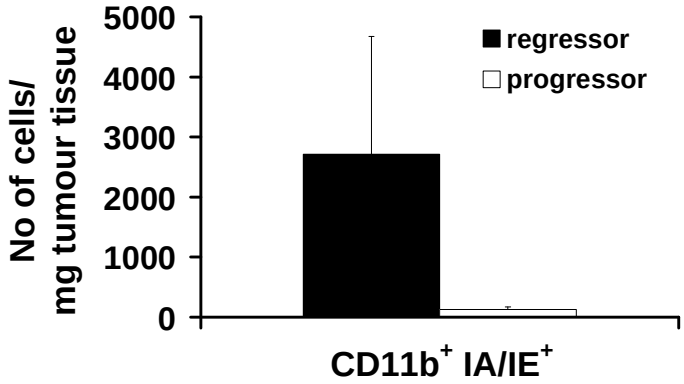

Figure 3.28 ***FACS analysis of tumour-infiltrating CD11b$^+$ and IA/IE$^+$ cells in mPAC regressor and progressor subcutaneous tumours.*** *Tumours from mice injected with either mPAC regressor or progressor cells were isolated. Tumour-infiltrating cells were isolated from both tumours, double stained with anti-CD11b and IA/IE antibodies and analysed by FACS. The regressor (black column) tumour shows a significantly higher infiltration of CD11b$^+$IA/IE$^+$ cells than the progressor (white column) tumour. At least three independent experiments with different tumour bearing mice were combined and charted. The average of all experiments is shown, including the calculated SD.*

Analysis of the splenocytes and draining lymph node cells of the mPAC regressor and progressor tumour bearing mice revealed a slightly increased number of macrophages (CD11b$^+$IA/IE$^+$) in mPAC regressor, compared to progressor tumour bearing mice (**Figure 3.29**).

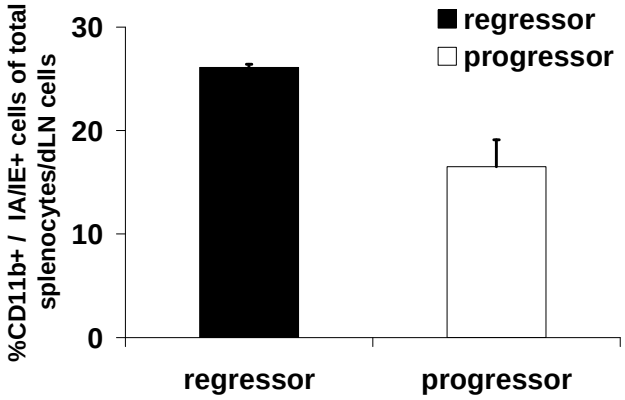

Figure 3.29 ***FACS analysis of CD11b$^+$IA/IE$^+$ cells in splenocytes and draining lymph node cells of regressor and progressor tumour bearing mice.*** *Splenocytes and draining lymph node cells from regressor and progressor tumour bearing C57BL/6 mice were isolated and stained for CD11b$^+$IA/IE$^+$. A significantly increased number of CD11b$^+$IA/IE$^+$ could be detected in splenocytes and draining lymph node cells of the regressor tumour bearing mice (black column). At least three independent experiments with different tumour bearing mice were combined and charted. The average of all experiments is shown, including the calculated SD.*

In order to test the effector function of the mPAC regressor or progressor tumour-infiltrating CD4 lymphocytes, isolated infiltrating cells were stained with anti-CD4 and anti-CD40 ligand (CD40L). The member of the membrane bound tumour necrosis factor (TNF)-family, CD40L, is particularly important for the $CD4^+$ T cell immune response. Induced CD40L on T cells ($CD4^+$) activates B cells and macrophages. There was no significant difference in CD40L expression of $CD4^+$ cells isolated from the mPAC regressor and progressor tumour (**Figure 3.30**).

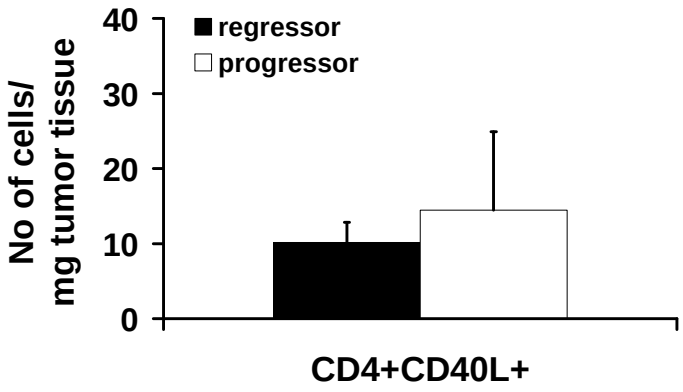

Figure 3.30 ***FACS analysis for the detection of CD40L expression on $CD4^+$ tumour-infiltrating cells.*** *Subcutaneous tumours were explanted from mPAC regressor (black column) or progressor (white column) tumour cell injected C57BL/6 wt mice. Tumour-infiltrating cells were isolated and stained for CD4 and CD40L. Three different tumours from mPAC regressor and progressor tumour bearing mice were analysed and the average charted, including the SD.*

To discover, if generated tumour-specific CTLs could target the progressor tumour *in vivo*, tumour-specific $EGFP^+$ CTLs from mPAC regressor-vaccinated $EGFP^+$ transgenic mice were transferred i.v. into subcutaneous progressor and regressor tumour bearing mice. Twelve hours later, the tumours were explanted, their tumour-infiltrating cells isolated, and stained for CD3, CD4, and CD8 (**Figure 3.31**). Combining FACS data from three different mice per group indicated a lesser infiltration of $EGFP^+CD8^+$ cells in progressor tumours than in regressor tumours. No significant differences between the mPAC regressor and progressor tumours could be detected, regarding the number of transferred $CD4^+$ cells found in the tumours.

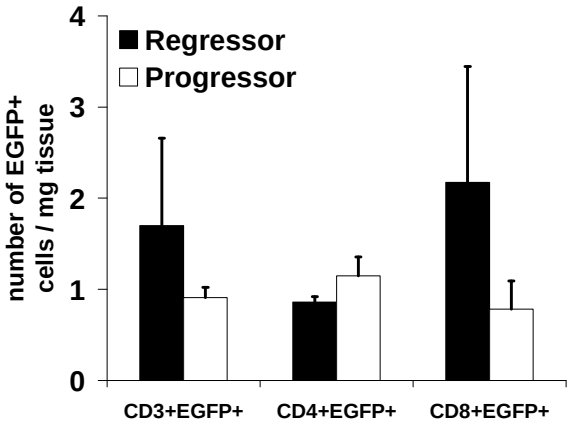

Figure 3.31 ***FACS analysis of tumour-specific EGFP$^+$ CTLs in mPAC regressor and progressor tumours after adoptive transfer.*** *Tumour-specific CTLs were generated from mPAC regressor-vaccinated EGFP transgenic mice and i.v. injected into C57BL/6 wt mice bearing subcutaneous progressor (white columns) or regressor tumour (black columns). Tumours were explanted twelve hours after adoptive T cell transfer and tumour-infiltrating T cells analysed by FACS. The average of three different mice is shown, including the calculated SD.*

3.2.2 Functional analysis of splenocytes from regressor and progressor tumour bearing mice

Anti-tumour cellular immune response is associated in most cancers with a cytotoxic CD8$^+$ T cell effector function. The effector cytokine in this process is IFN-γ, which can be produced by CTLs, inflammatory T_H1 lymphocytes and NK cells. To analyse tumour-specific immune responses in mPAC regressor and progressor tumour bearing mice, the IFN-γ response of T cells was examined (**Figure 3.32**). C57BL/6 wt mice were injected with irradiated regressor, progressor or the irrelevant tumour cell line RMA. Two weeks after vaccination, splenocytes and draining lymph node cells were isolated and restimulated overnight with irradiated regressor or progressor tumour cells. The secreted IFN-γ was detected by intracellular staining with anti-IFN-γ antibodies. Cells were counterstained with anti-CD8 and anti-CD4 antibodies. FACS analysis revealed that 0.26% CD8$^+$ cells of the regressor vaccinated wt mice secrete IFN-γ after restimulation with the regressor. No IFN-γ secreting CD8$^+$ cells were detected in progressor vaccinated mice after restimulation with the progressor (0%). 0.07% of CD8$^+$ splenocytes/draining lymph node cells of the progressor vaccinated mice secreted IFN-γ after restimulation. Immunization with irradiated regressor cells and progressor restimulation resulted in 0.04% of secreting CD8$^+$ cells. Splenocytes/draining lymph node cells from RMA vaccinated mice showed no IFN-γ secreting CD8$^+$ cells after restimulation with irradiated regressor or progressor tumour cells (data not shown).

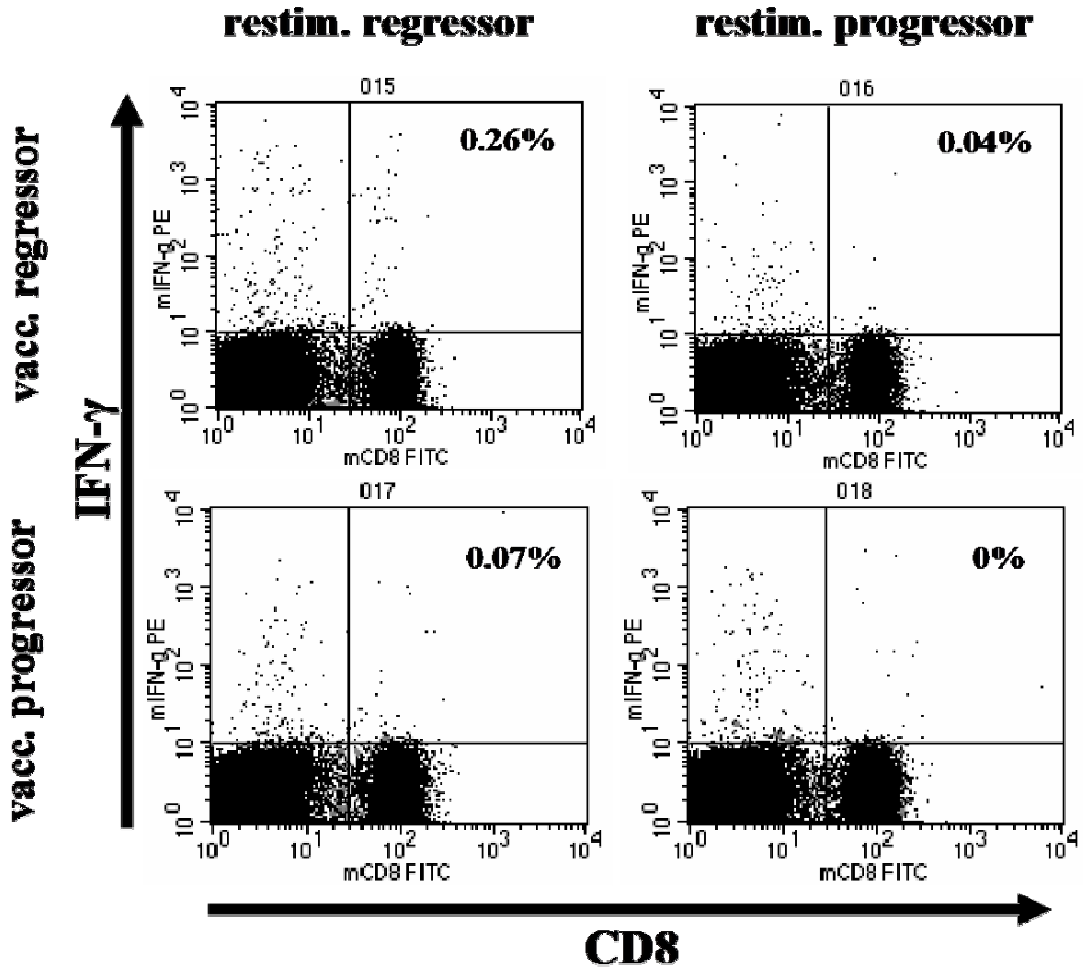

Figure 3.32 ***FACS analysis of tumour-specific IFN-γ response in mPAC regressor and progressor vaccinated C57BL/6 wt mice.*** *Pooled splenocytes and lymph node cells were isolated from regressor (top) or progressor (bottom) vaccinated mice. Cells were restimulated with irradiated regressor (left) or progressor (right) tumour cells. Intracellular staining of IFN-γ secreting CD8$^+$ cells was performed to detect tumour-specific IFN-γ response. FACS data shows a representative result from at least five independent experiments.*

A similar experimental set up was used to detect tumour-specific IFN-γ responses in CD4$^+$ cells of mPAC regressor and progressor tumour bearing C57BL/6 wt mice (**Figure 3.33**). But instead of using intracellular staining, IFN-γ$^+$CD4$^+$ cells were detected by the more sensitive capture IFN-γ secreting assay. Here, the secreted IFN-γ was captured on the IFN-γ secreting cells and subsequently labelled with a second IFN-γ specific antibody. Cells were counterstained with anti-CD4 antibodies. Naïve mice were used as control for background IFN-γ secretion. The regressor restimulated CD4$^+$ splenocytes of progressor vaccinated mice showed the highest number of IFN-γ producing CD4$^+$ cells (**Figure 3.33**, 0.9%). Fewer CD4$^+$ cells from regressor vaccinated mice secreted IFN-γ when restimulated with irradiated regressor tumour cells (0.6%). Progressor restimulation of CD4$^+$ cells from regressor and progressor vaccinated mice showed no significant differences in the number of IFN-γ secreting CD4$^+$ cells (**Figure 3.33** 0.4% and 0.6%, respectively). In contrast, only 0.2% of the naïve CD4$^+$ cells

produce IFN-γ without and after stimulation with irradiated regressor or progressor tumour cells (**Figure 3.33**).

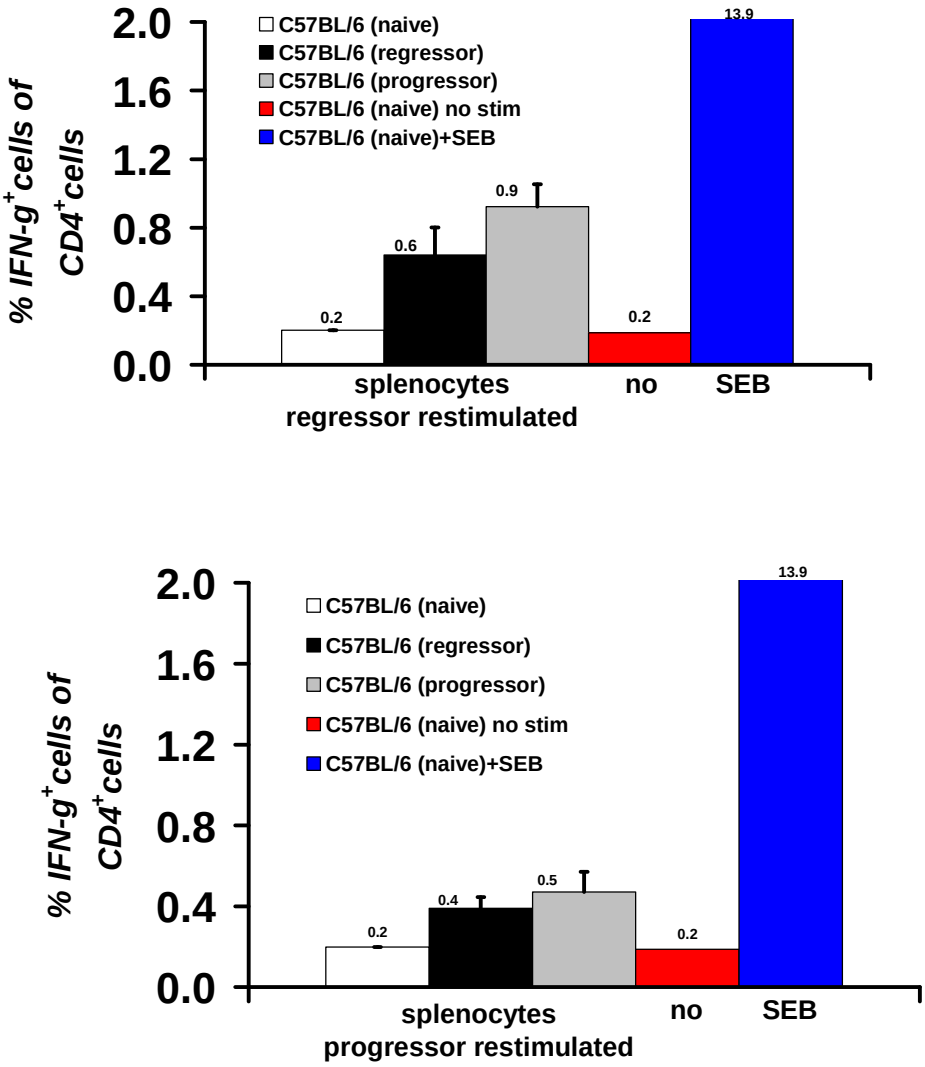

Figure 3.33 ***Combined FACS analysis for tumour-specific IFN-γ response of CD4+ cells from regressor and progressor vaccinated mice.*** *Splenocytes were isolated from regressor (grey column) and progressor (black column) vaccinated, or naive (white and red) mice. Cells were restimulated with irradiated regressor (top graph), progressor (bottom graph) tumour cells, without any restimulation (red column) or with staphylococcus enterotoxin B (SEB) (blue column) as positive control. Capture IFN-γ secretion assay was performed and cells were counterstained with anti-CD4 antibodies to detect the tumour-specific IFN-γ response of CD4+ cells. The average of three different mice is shown, including the calculated SD.*

In order to confirm the importance of the IFN-γ effector function of $CD4^+$ and $CD8^+$ T cells regarding the regressor tumour rejection, regressor tumour cells were subcutaneously injected into C57BL/6-IFN-γ knock out mice (**Figure 3.34**). The tumour growth was monitored and revealed a progressively regressor growth starting on day 55. IFN-γ seems to be an important factor in the final tumour rejection of the regressor injected tumour mice. Subcutaneously injected mPAC regressor tumour cells into C57BL/6 wt mice showed tumour growth in the first ten days, followed by a rejection of the tumour. The tumour did not develop again after rejection.

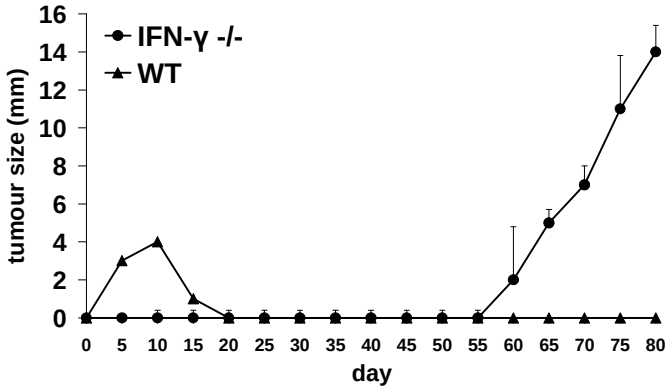

Figure 3.34 ***Analysis of tumour growth after subcutaneous injection of mPAC regressor tumour cells into IFN-$\gamma^{-/-}$ mice.*** *Three C57BL/6 wt and C57BL/6-IFN-$\gamma^{-/-}$ mice were subcutaneously injected with mPAC regressor tumour cells. Tumour growth was monitored and the average of the three mice is charted including the SD.*

Comparable tumour growth kinetics, as in IFN-γ knockout (IFN-$\gamma^{-/-}$) mice, was shown when mPAC regressor tumour cells were subcutaneously injected into B cell knockout mice ($IgH^{-/-}$). Together with the *in vivo* studies on T cell knockout (C57BL/6 nu/nu), or T cell and B cell knockout ($RAG^{-/-}$) mice, these results showed the importance of the effector cytokine IFN-γ and the tumour-specific antibodies in the cellular and humoral immune response against the subcutaneous mPAC regressor tumour development.

The progressor tumour cell line has the same origin, thus the same specific markers, as the mPAC regressor cell line (**Figure 3.19**). But in contrast to the mPAC regressor tumour, the mPAC progressor tumour cannot initiate a tumour-specific humoral immune response (data not shown). To discover if the induced antibody response of the regressor tumour cell line has any influence on the growth of the progressor tumour, immune competent C57BL/6 wt mice were first injected with regressor tumour cells. Because the antibody response was the highest on day 21, the progressor tumour cells were injected on this day into the same mice. The tumour growth was monitored (**Figure 3.35**). Progressor injected C57BL/6 wt mice were used as positive growth control. This experiment showed that the humoral immune response developed in the regressor vaccinated C57BL/6 wt mice had no significant influence on the progressor tumour growth. Even serum from regressor vaccinated C57BL/6 wt mice transferred into regressor tumour cells injected C57BL/6 nu/nu mice showed no delay in regressor tumour development (data not shown). *In vivo* cross experiments revealed that the preliminary injection of regressor into C57BL/6 wt mice had no effect on the progressor

growth (data not shown). Although progressor restimulated $CD4^+$ cells from progressor injected C57BL/6 wt mice secreted a similar amount of IFN-γ (**Figure 3.33**) in response to regressor injected mice, this cellular immune response had no inhibitory effect on the progressor tumour growth.

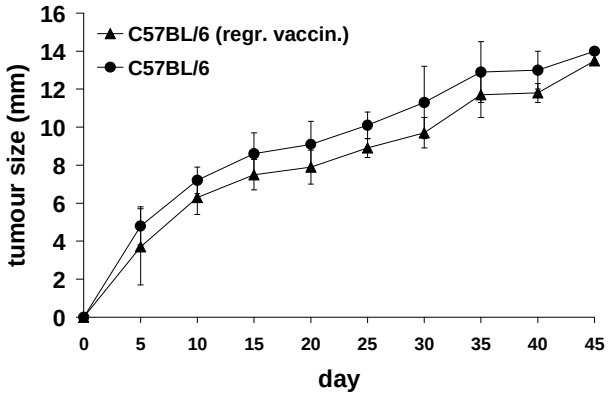

Figure 3.35 ***Tumour kinetics of progressor tumour cell injected C57BL/6 wt mice after regressor vaccination.*** *C57BL/6 wt mice were subcutaneously vaccinated with regressor tumour cells and three weeks later injected with progressor tumour cells. C57BL/6 wt mice injected with progressor tumour cells were used as positive control. Tumour growth was monitored and the average of three mice is charted, including the SD.*

3.3 Chronic pancreatitis and tumour-specific immune responses

3.3.1 Influence of chronic pancreatitis on the tumour-specific immune responses and tumour growth of mice with premalignant lesions or spontaneous tumours

To study the effect of inflammation in the pancreas by inducing pancreatitis, mice were injected intraperitoneal (i.p.) for five weeks, twice a week for five hours with caerulein 50µg/kg bodyweight (BW). The injection was performed in hourly intervals. Twelve hours before and during the injection the mice received no food. Twenty four hours after the last injection, blood was isolated from these mice and the sera were analysed for the amylase concentration. Humans and mice also suffering from pancreatitis have an increased plasma level of pancreatic amylase due to damage of the cells that produce amylase. The amylase level was measured using biochemical techniques. In **Figure 3.36** a representative analysis is shown to confirm pancreatitis in C57BL/6 wt mice. C57BL/6 mouse, which was injected with caerulein for five weeks to induce chronic pancreatitis, developed a five fold higher amylase concentration in blood, compared to a C57BL/6 wt mouse.

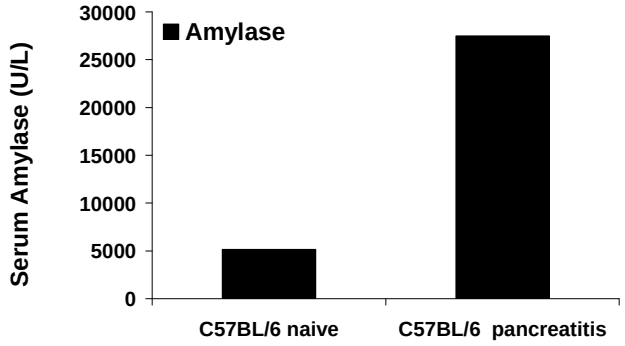

Figure 3.36 ***Amylase serum level (Units/Litre) in C57BL/6 naïve and C57BL/6 caerulein injected mouse.*** *C57BL/6 mouse was injected i.p. for five weeks twice a week for five hours in an hourly interval with 50µg/kg (BW) caerulein. A blood sample was taken 24 hours after the first injection and the serum level of amylase was detected by biochemical analysis (right column). As control, serum from C57BL/6 naïve mouse was analysed (left column).*

3.3.2 Influence of chronic pancreatitis on the tumour-specific immune responses in regressor tumour bearing mice

The aim of the following study was to examine the influence of chronic pancreatitis on the tumour-specific immune responses in regressor tumour bearing mice. Therefore, chronic pancreatitis was induced into C57BL/6 mice, as described above. After three weeks subcutaneously regressor tumour cells were injected into these mice. Two weeks later, splenocytes and draining lymph node cells were isolated and restimulated overnight with irradiated regressor tumour cells. The IFN-γ response of $CD8^+$ cells were detected by capture IFN-γ secreting assay and analysed by FACS (**Figure 3.37**). As control, splenocytes from naïve mice, from regressor tumour cell injected mice without chronic pancreatitis, and from mice suffering only from chronic pancreatitis were analysed. This analysis indicates that mice suffering from chronic pancreatitis developed a weaker IFN-γ immune response of $CD8^+$ cells against mPAC regressor tumours than mice without pancreatitis. Comparable results were found for the tumour-specific immune response of IFN-γ secreting of $CD4^+$ cells (**Figure 3.37**).

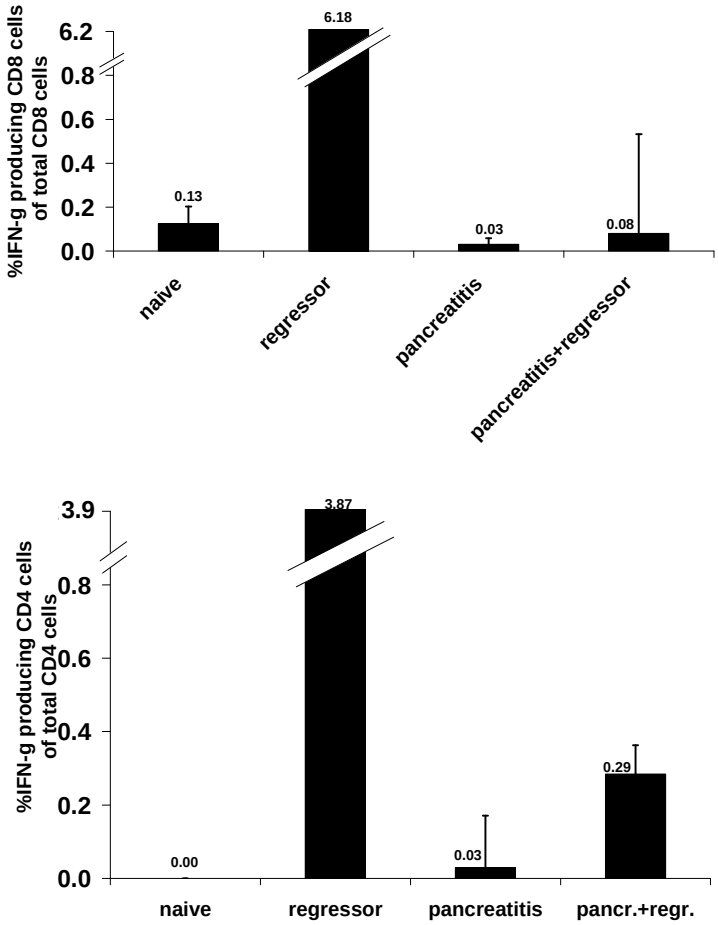

Figure 3.37 ***FACS analysis of IFN-γ secreting CD8$^+$ (top) or CD4$^+$ cells (bottom) from regressor tumour cell injected mice suffering from chronic pancreatitis.*** *mPAC regressor tumour cells were subcutaneously injected into C57BL/6 mice suffering from chronic pancreatitis (right column) and C57BL/6 wt mice (second left column). As controls, C57BL/6 mice suffering from chronic pancreatitis but without regressor tumour cell injection (second right column) and C57BL/6 naïve mice (left column) were used. Splenocytes and draining lymph node cells were isolated and (re)stimulated with irradiated regressor tumour cells. Capture IFN-γ secretion assay was performed and cells counterstained with anti-CD8 or anti-CD4 antibodies to detect the tumour-specific IFN-γ response of CD8$^+$ and CD4$^+$ cells. The average of three different mice is shown, including the calculated SD.*

3.3.3 Specificity of the pancreatic tumour-specific immune reduction in mice suffering from chronic pancreatitis

In order to confirm the specific influence of chronic pancreatitis on the tumour-specific immune response induced by the subcutaneous pancreatic regressor tumour cells, an irrelevant tumour was tested. Irradiated melanoma B78-OVA or pancreatic regressor tumour cells were subcutaneously injected into C57BL/6 wt or C57BL/6 mice suffering from chronic pancreatitis (**Figure 3.38**). Ten or 14 days later, splenocytes and draining lymph node cells were isolated and restimulated with OVA-peptide or irradiated regressor tumour cells. Capture IFN-γ detection assay was performed to analyse the tumour-specific response of specific CTLs. The tumour-specific immune response induced by the melanoma B78-OVA tumour was not reduced in mice suffering from

chronic pancreatitis. Chronic pancreatitis had no significant influence on the weak tumour-specific immune response of twelve week old EL-TGF-α x Trp53$^{-/-}$ spontaneous tumour bearing mice (data not shown). The same is true for mice with premalignant lesions in the pancreas (data not shown). Regressor tumour bearing C57BL/6 mice suffering from chronic pancreatitis developed a weaker IFN-γ immune response of CD8^{+} cells against mPAC regressor tumours than mice without pancreatitis. However, this difference is not significant.

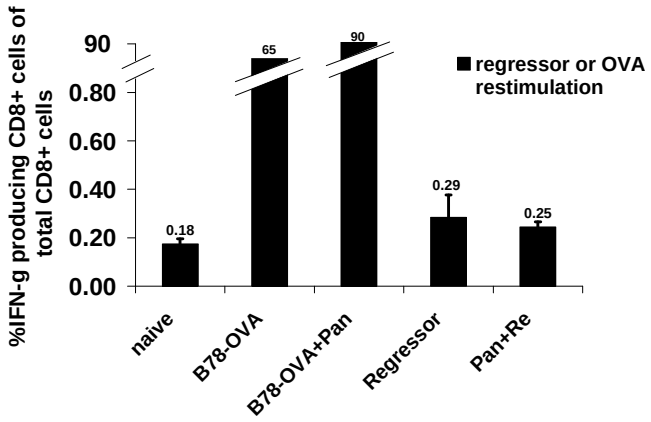

Figure 3.38 ***FACS analysis of IFN-γ secreting CD8^{+} cells from regressor or irrelevant tumour cell injected mice suffering from chronic pancreatitis.*** *mPAC regressor tumour cells or irrelevant B78-OVA tumour cells were subcutaneously injected into C57BL/6 mice suffering from chronic pancreatitis (right column, third left column, respectively) and C57BL/6 wt mice (second right, second left column, respectively). Splenocytes and draining lymph node cells were isolated and restimulated with irradiated mPAC regressor tumour cells or OVA peptide. As controls, splenocytes and draining lymph node cells from C57BL/6 naïve mice (left column) were used. Capture IFN-γ secretion assay was performed and cells counterstained with anti-CD8 antibodies to detect the tumour-specific IFN-γ response of CD8^{+}. The average of three different mice is shown, including the calculated SD.*

3.3.4 Tumour growth kinetics of subcutaneous regressor and spontaneous tumour bearing mice suffering from chronic pancreatitis

In order to view the effect of chronic pancreatitis and inflammation on the regressor and spontaneous tumour growth, subcutaneous as well spontaneous pancreatic tumours were examined in this model. First C57BL/6 mice suffering from chronic inflammation were monitored for tumour growth after the subcutaneous injection of regressor tumour cells. In one out of five mice, the regressor tumour developed to a progressively growing tumour, which started to grow on day 60 (data not shown).

Because there is variability in the tumour size of twelve week old EL-TGF-α x Trp53$^{-/-}$ transgenic mice, no conclusion can be drawn regarding the influence of chronic pancreatitis on the growth of spontaneous pancreatic tumours. However, the induction of chronic pancreatitis in four week old EL-TGF-α x Trp53$^{-/-}$ transgenic mice revealed after twelve weeks of age, a clear difference in the tumour histology, compared to spontaneous tumours of EL-TGF-α x Trp53$^{-/-}$ transgenic mice without suffering from

chronic pancreatitis (**Figure 3.39**). Cells in the spontaneous tumour of EL-TGF-α x Trp53$^{-/-}$ transgenic mice suffering from chronic pancreatitis were much more undifferentiated. The original pancreatic tissue structure was completely lost, compared to the spontaneous tumour of the twelve week old EL-TGF-α x Trp53$^{-/-}$ transgenic mice, without suffering from chronic pancreatitis.

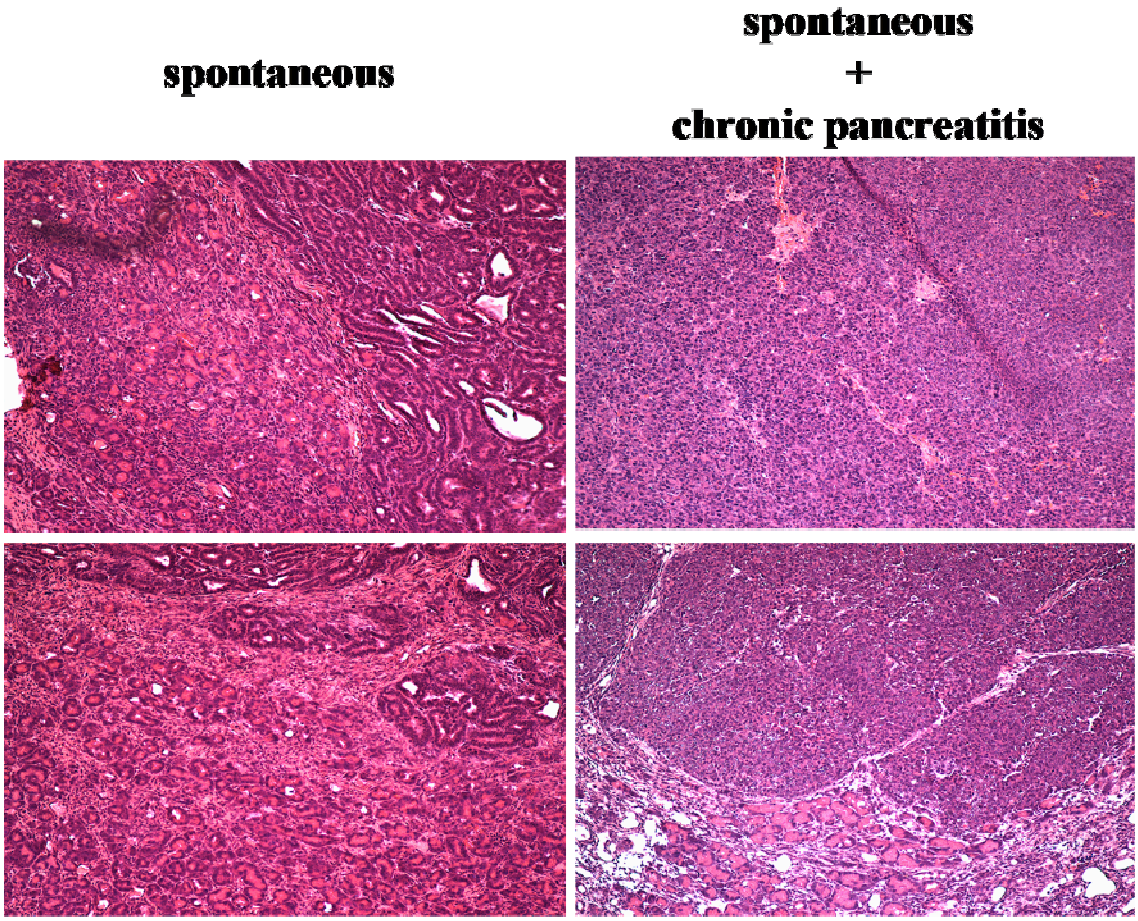

Figure 3.39 ***Histological analysis of spontaneous pancreatic tumours of EL-TGF-α x Trp53$^{-/-}$ transgenic mice with (right) and without chronic pancreatitis (left) by haematoxylin & eosin (H&E)-staining.*** *Four week old EL-TGF-α x Trp53$^{-/-}$ transgenic mouse was induced with chronic pancreatitis by caerulein injection over eight weeks (right). After twelve weeks, tumour was isolated and fixed. Sections of six µm were stained with H&E. As control, tumour from twelve week old EL-TGF-α x Trp53$^{-/-}$ transgenic mouse without chronic pancreatitis was isolated and analysed by H&E staining (left).*

3.4 Influence of tumour stroma on the growth of subcutaneous pancreatic tumours

3.4.1 Regressor and progressor tumour growth in different tissues

In order to analyse, if the tumour development of pancreatic tumour cell lines is dependent on the tumour tissue and their developing stroma, regressor and progressor mPAC cells were injected i.v., i.p. and into the liver. mPAC regressor tumour cells did not grow in the lung after i.v. injection or in the abdominal area after i.p. injection and also not in the liver (**Table 3.1**). In contrast, the progressor tumour cell line developed a solid tumour in the lung (**Figure 3.42**, red arrow) and in the liver (**Table 3.1**; **Figure 3.40** and **Figure 3.41**), but not in the abdominal area.

	Regressor tumour cells	Progressor tumour cells
s.c.	-	+
i.p.	-	-
i.v.	-	+
into liver	-	+

Table 3.1 ***Growth of regressor or progressor mPAC tumour cell lines in different murine tissues.*** *Regressor or progressor tumour cells were injected s.c., i.p., i.v., or into the liver in C57BL/6 wt mice. The growth was monitored and two weeks later the concerned organs were analysed.*

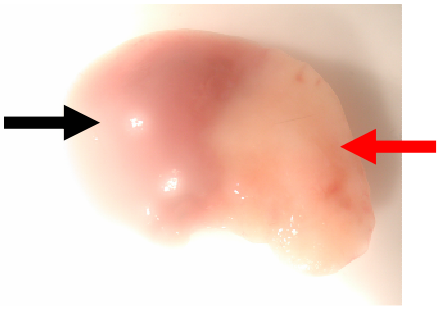

Figure 3.40 ***Progressor tumour cells injected into the liver of a C57BL/6 wt mouse.*** *C57BL/6 wt mouse was injected with 1 x 10^6 progressor tumour cells into the liver. After 14 days the mouse was killed and the liver analysed. The white, right part (red arrow) of the liver indicates the progressor tumour in the liver. The left part (black arrow) was unaffected.*

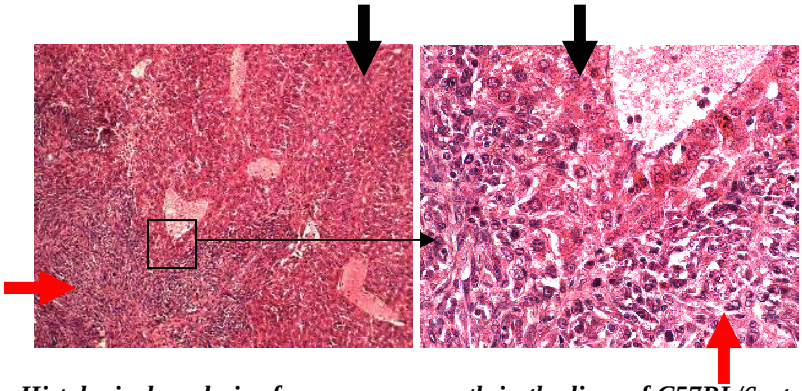

Figure 3.41 ***Histological analysis of progressor growth in the liver of C57BL/6 wt mice.*** *Progressor tumour cells were injected into the liver of a C57BL/6 wt mouse. After 14 days the liver was isolated, fixed, embedded, and sectioned. Sections were H&E stained. Left picture shows a 100x and the right picture a 400x magnification. The black arrows indicate the healthy liver tissue and the red arrows the progressor cell invaded tissue.*

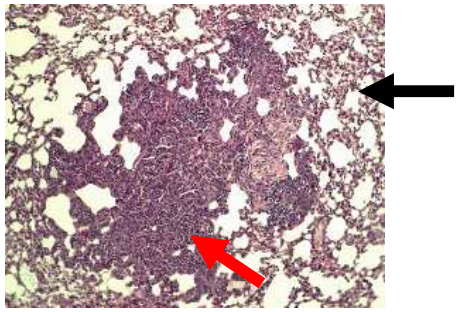

Figure 3.42 ***Histological analysis of progressor growth in the lung of C57BL/6 wt mice.*** *Progressor tumour cells were injected into the tail vein of a C57BL/6 wt mouse. After 14 days the lung was isolated, fixed, embedded in paraffin, and sectioned. Section was H&E stained. Picture shows a 100x magnification of the lung. Black arrow indicates the normal lung tissue. Red arrow indicates the invaded progressor tumour cells into the lung tissue.*

3.4.2 Growth kinetics of regressor or spontaneous tumours in C57BL/6 wt mice

55 days after injection of mPAC regressor tumour cells into T cell-deficient (NOD SCID) mice, the tumour started to grow. In order to analyse, if the developed regressor tumour grew in the T cell-deficient mice due to possible *in vivo* changes of the regressor tumour cells (selection; mutation (genotype or phenotype)), the tumour was isolated, cut into pieces and single cell suspension was made. Cells were subcutaneously injected into C57BL/6 wt mice and their kinetic tumour growth was analysed (**Figure 3.43**). In comparison, spontaneous tumour from twelve week old EL-TGF-α x $Trp53^{-/-}$ mouse was isolated and single cell suspension was made. These cells were subcutaneously injected into C57BL/6 wt mice and the tumour growth monitored. Single tumour cells derived from regressor tumour of T cell-deficient mice started to grow six days after subcutaneous injection into immune competent C57BL/6 wt mice. The tumour grew progressively. In contrast, single tumour cells isolated from spontaneous tumour and directly injected into C57BL/6 mice did not show any growth.

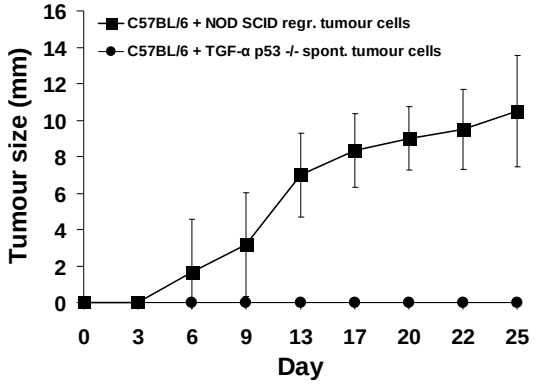

Figure 3.43 ***Tumour growth in C57BL/6 wt mice after transplanting regressor tumour cells isolated from T cell-deficient (NOD SCID) mice or spontaneous tumour cells isolated from EL-TGF-α x Trp53$^{-/-}$ mice.*** *Regressor tumour cells were injected into NOD-SCID mice. After solid tumour growth, the tumour was isolated, cut into small pieces and single cell suspension was made. Single cells were subcutaneously injected into immune competent C57BL/6 wt mice and tumour growth was monitored (square). In comparison, spontaneous tumour was isolated from twelve week old EL-TGF-α x Trp53$^{-/-}$ mouse, cut into small pieces and single cell suspension was made. Single cells were subcutaneously injected into immune competent C57BL/6 wt mice and tumour growth was monitored (circle). The average of three different mice is shown, including the calculated SD.*

3.4.3 Growth kinetics of transplanted tumour pieces in C57BL/6 wt mice

Tumour pieces already harbour a specific tumour stroma, including connective tissue, blood vessels, fibroblasts, tumour cells and infiltrating immune cells. In order to examine if pancreatic tumours with the same origin but with different tumour stroma have a comparable growth kinetic, progressor tumour pieces derived from mPAC progressor cell injected C57BL/6 wt mice, regressor tumour pieces derived from mPAC regressor injected NOD-SCID mice, or spontaneous tumour pieces derived from twelve week old EL-TGF-α x Trp53$^{-/-}$ mice were subcutaneously injected into immune competent C57BL/6 wt mice (**Figure 3.44**). The tumour growth was monitored. The progressor tumour pieces grew progressively. In contrast to the spontaneous tumour pieces, which started to grow but the tumour was rejected after seven days and did not reoccur. Interestingly, regressor tumour pieces from the regressor tumour of the T cell-deficient mice grew in the immune competent C57BL/6 wt mice. Both, regressor single cells and pieces derived from T cell-deficient mice, developed a progressive growing regressor tumour in C57BL/6 wt mice. In contrast, the originally mPAC regressor cell line was rejected in C57BL/6 wt mice.

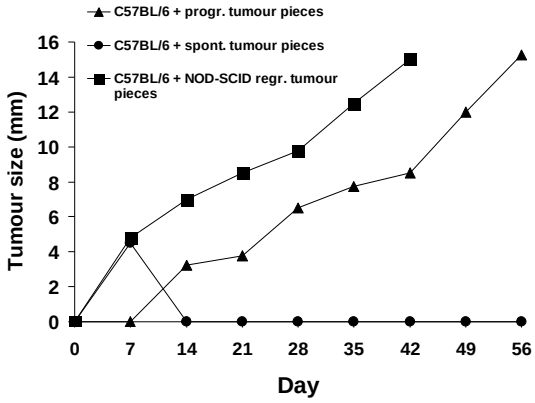

Figure 3.44 ***Tumour growth in C57BL/6 wt mice, after transplanting different pancreatic tumour pieces into C57BL/6 wt mice.*** *mPAC regressor cells were subcutaneously injected into NOD-SCID mouse. After solid tumour growth, the tumour was isolated and cut into small pieces. Pieces were subcutaneously injected into immune competent C57BL/6 wt mice and tumour growth was monitored (square). In comparison, progressor tumour derived from mPAC progressor cells injected C57BL/6 wt mice and spontaneous tumour isolated from twelve week old EL-TGF-α x Trp53$^{-/-}$ mouse were cut into small pieces and subcutaneously injected into immune competent C57BL/6 wt mice (triangle and circle, respectively). Tumour growth was monitored (triangle and circle, respectively). The average of three different mice is shown, including the calculated SD.*

3.4.4 Influence of cyclophosphamide on the *in vitro* and *in vivo* tumour growth of pancreatic tumours

Cyclophosphamide is known to influence the tumour stroma not only by functioning as a cytostatic drug to inhibit the tumour proliferation but also by disturbing the stroma cell interaction. These processes are dose-dependent. In order to inspect the influence of cyclophosphamide on the pancreatic tumours, mPAC progressor tumour cells were subcutaneously injected into C57BL/6 mice. Ten days later, different concentrations of cyclophosphamide (15-200 mg/kg BW) were injected i.p. into progressor tumour bearing mice. The tumour growth was monitored (**Figure 3.45**).

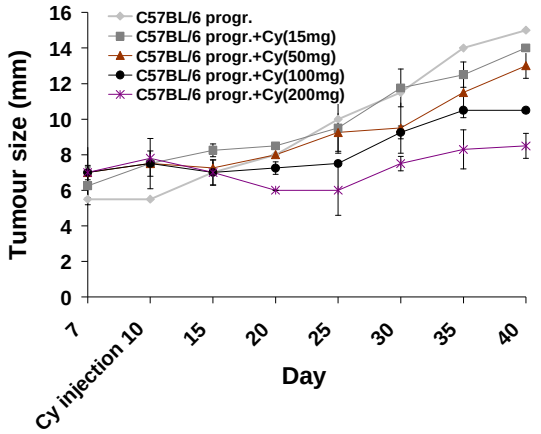

Figure 3.45 ***Progressor tumour growth, after injection of different doses of cyclophosphamide. C57BL/6 mice were subcutaneously injected with mPAC progressor cells.*** *After ten days, C57BL/6 progressor tumour bearing mice were injected i.p. with 15 mg/kg BW- (square), 50 mg/kg BW- (triangle), 100 mg/kg BW (circle), and 200 mg/kg BW- (star) cyclophosphamide. As control, C57BL/6 wt mice were subcutaneously injected with mPAC progressor cells but were not treated with cyclophosphamide (rhombus). The tumour kinetics as of day seven is charted. The average of three different mice is shown, including the calculated SD.*

The titration revealed the best inhibition of the progressor tumour growth by using 200 mg/kg BW of cyclophosphamide. The tumour was not rejected, but showed a strong delay in growth. *In vitro* treatment of progressor cells should clarify, if cyclophosphamide has a direct function as inhibitor on progressor tumour cell proliferation. Therefore, progressor cells and also regressor cells were cultured in complete medium including cyclophosphamide (**Figure 3.46**). As control, both tumour cell lines were cultured without cyclophosphamide. As seen in **Figure 3.46**, cyclophosphamide had no direct influence on the progressor and regressor tumour cell proliferation.

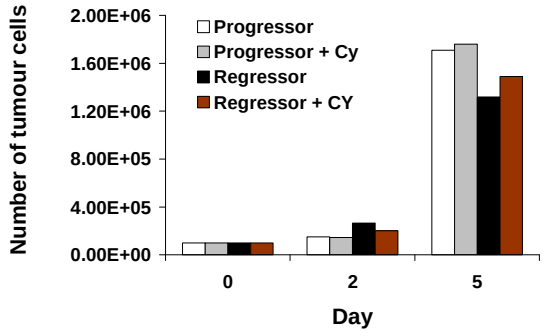

Figure 3.46 ***mPAC regressor and progressor cell growth in vitro, after treatment with cyclophosphamide.*** *mPAC regressor and progressor tumour cells were cultured in vitro with complete medium including 0.2 mg/ml cyclophosphamide (grey and brown column, respectively). As control, tumour cell lines were cultured without cyclophosphamide (progressor: white column; regressor: black column).*

3.4.5 Influence of cyclophosphamide on immune cells in the pancreatic tumour model

Because cyclophosphamide had no influence on the proliferating pancreatic tumour cell itself, the influence of cyclophosphamide on T cells of the tumour stroma was investigated. NOD SCID mice were injected with mPAC progressor tumour cells. After ten days, the mice were injected with cyclophosphamide and the tumour growth was monitored (**Figure 3.47**). As control, immune competent C57BL/6 progressor tumour bearing mice were used with or without treatment of cyclophosphamide. Cyclophosphamide had no influence on the progressor tumour growth in T cell-deficient mice. This result revealed that the delay in the growth of the mPAC progressor tumour is T cell dependent.

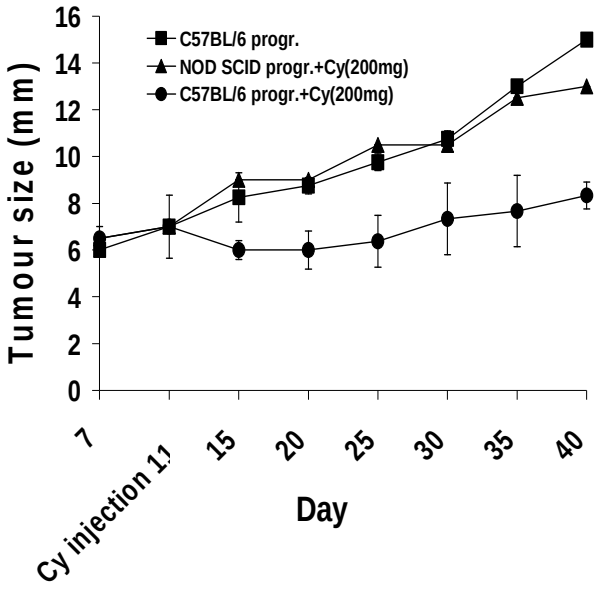

Figure 3.47 ***Influence of cyclophosphamide on T cells of the tumour stroma.*** *T cell-deficient NOD SCID mice were injected s.c. with mPAC progressor tumour cells. After ten days, mice were injected i.p. with 200 mg/kg BW cyclophosphamide and tumour growth was monitored (triangle). As control, C57BL/6 progressor tumour bearing mice were treated with (circle) or without cyclophosphamide (square). The average of three different mice is shown, including the calculated SD.*

4 Discussion

Human pancreatic cancer remains one of the most lethal of all human malignancies. About 90% of human pancreatic cancer originates from the ductal part of the exocrine pancreas. However, most of the pancreatic cancer mouse models generated to date, do not represent the ductal phenotype, but instead produce acinar, mixed acinar-ductal, or cystic carcinomas (Bardeesy and DePinho, 2002; Glasner et al., 1992; Grippo et al., 2003; Mukherjee et al., 2000; Ornitz et al., 1987; Quaife et al., 1987; Sandgren et al., 1991; Tevethia et al., 2006). In this study, transgenic mice expressing TGF-α in the pancreas were crossed with p53 knockout ($Trp53^{-/-}$) mice, resulting in the development of spontaneous ductal pancreatic adenocarcinomas. This EL-TGF-α x $Trp53^{-/-}$ transgenic mouse is the first described murine model for developing ductal adenocarcinoma of the pancreas that recapitulates the cellular differentiation, growth characteristics, and genetic alterations of the human disease (Wagner et al., 1998; Wagner et al., 2001). The close relation to the human pancreatic adenocarcinoma (PAC) has shown that the EL-TGF-α x $Trp53^{-/-}$ transgenic mouse is an informative animal model for ductal pancreatic cancer and it should, therefore, be useful for tumour immunological and tumour stroma studies.

For the first time this study investigates humoral and cell mediated tumour-specific immune responses in a genetic mouse model for pancreatic cancer. The immunological characterizations of the spontaneous tumour bearing EL-TGF-α x $Trp53^{-/-}$ transgenic mice and the comparison with a transplantable pancreatic tumour model in immune competent wild type mice, which were studied here, should provide a basis for evaluating immunotherapeutic approaches against autochthones tumours.

In addition to the tumour cell itself an ideal target for immunotherapeutic approaches against cancer could be the tumour stroma, which often favours the tumour growth (Lamagna et al., 2006). Therefore, the role of the tumour stroma in the development of pancreatic cancer was also explored by the specific manipulation of the stroma cells and the influence of chronic pancreatitis. The present study showed that a chronic inflammation in the pancreas inhibits the tumour-specific immune responses against pancreatic cancer. In addition, specific manipulation of the stroma cells influenced the progressive tumour growth *in vivo*.

The results of this study will help to improve current, immune-based cancer therapies for the prevention and treatment of pancreatic cancer.

4.1 Pancreatic adenocarcinoma is highly immunogenic and causes spontaneous tumour-specific immune responses

To date, only transplantable tumours have been tested for preclinical, immune based therapies (Greten and Jaffee, 1999). An important factor affecting the efficiency of immune-based therapies might be the host-tumour interactions, which are neglected in these models (Dunn et al., 2004a). Therefore, an important advantage for the evaluation of potential new immunotherapies is the use of spontaneous tumour models (Ostrand-Rosenberg, 2004).

Several cancer vaccines have already been tested in patients with pancreatic cancer (Jaffee et al., 2002). Despite this, only limited preclinical data exist, which support an immunotherapeutic approach for the treatment of pancreatic cancer. For the development of pancreatic cancer vaccines to date, mouse models have been used, which are SV40 T antigen-dependent (Ostrand-Rosenberg, 2004). Whereas the SV40 T antigen provides an opportunity to use SV40 T antigen-specific T cells as well as analysing antigen-specific immune responses, these mice either develop endocrine tumours or acinar adenocarcinomas, which differ significantly from human ductal adenocarcinomas (Ganss et al., 2002; Hanahan, 1985; Kern et al., 2001; Mukherjee et al., 2000). Therefore, the EL-TGF-α x $Trp53^{-/-}$ transgenic mouse model for human ductal adenocarcinomas is more helpful to identify new therapeutic options for pancreatic cancer.

4.1.1 Comparative analysis of subcutaneous and spontaneous murine pancreatic adenocarcinoma

Thus far, most transgenic mouse models that were used in tumour immunology are based on overexpression of a defined human antigen, such as HER-2/neu, CEA, or MUC-1, which facilitates the analysis of antigen-specific immune responses. Because the immunodominant tumour antigen in the EL-TGF-α x $Trp53^{-/-}$ tumour model is unknown, initial tumour cell lines from this spontaneous tumour were generated, which were used in the analysis of tumour-specific cellular and humoral immune responses. All established murine pancreatic adenocarcinoma (mPAC) cell lines grew progressively *in vitro*, but showed a regressive phenotype after subcutaneous injection into a syngenic immune competent C57BL/6 wt mice (Garbe et al., 2006). Thereby, the injected mPAC tumour cells form a solid tumour and grow until they are rejected starting on day six and fully disappear by day twelve (these cells have been called mPAC regressor tumour cells) (Garbe et al., 2006). In contrast, injection of mPAC regressor tumour cells into immune incompetent mice lacking T cells (nude mice) or T and B cells ($RAG^{-/-}$ mice) resulted in a slow but progressive growth (Garbe et al., 2006). This result indicated a T and B cell-dependent rejection of the subcutaneous mPAC regressor tumour. To rule out that the regression of the mPAC regressor tumour is due to partial histoincompatibility, mPAC regressor tumour cells were injected subcutaneously into the F1 generation of EL-TGF-α x $Trp53^{-/-}$ mice. In these mice, mPAC regressor tumour cells exhibited the same kinetics of initial growth and subsequent regression as observed in C57BL/6 wt mice (data not shown). For that reason, major and minor histocompatibility differences can be excluded as the cause for regression. The regression of transplanted syngenic tumours has already been described for several tumour cell lines isolated from spontaneous tumours. In most of these

studies the regression was T cell dependent, because the tumours grew progressively in nu/nu mice (Gross, 1943; Kripke, 1974; Martin et al., 1983; Old et al., 1962; Urban et al., 1982).

In order to identify the ongoing cellular and humoral anti-tumour immune responses in the spontaneous and subcutaneous mPAC tumour model, cytokines and chemokines in the pancreatic tumour environment, which could play an important function in the anti-tumour immune responses, were analysed by performing the cytometric bead array (CBA) and real time PCR assays (3.1.1). CBA revealed high IFN-γ, TNF-α, MCP-1, and IL-6 expression in the subcutaneous mPAC regressor tumour of the immune competent C57BL/6 wt mouse, as compared to the progressively growing spontaneous mPAC tumour of the EL-TGF-α x $Trp53^{-/-}$ mouse. As shown in most transplantable tumour models, IFN-γ is positively associated with rejection of the subcutaneous mPAC regressor tumour. The progressively growing spontaneous mPAC tumour showed a significantly lower IFN-γ inducible protein (IP)-10 (CXCL10) expression and hardly any IFN-γ expression. Activated T cells, NK cells, B cells, macrophages and DCs can produce IFN-γ (Ibe et al., 2001; Harris et al., 2000; Munder et al., 1998; Rottenberg et al., 2002). In most of the tumours, infiltrating T cells, NK cells and macrophages are known to be the major effector cells in the anti-tumour immune response.

The chemotactic chemokine monocyte chemoattractant protein-1 (MCP-1) is highly expressed in the subcutaneous mPAC regressor tumour and to a lesser extent in the spontaneous mPAC tumour. This indicates that tumour-infiltrating macrophages (TIMs) could be part of the stroma cells and could play a role in the cell-mediated anti-tumour immune responses. On the other hand, fibroblasts can also produce MCP-1 and are known to be part of the tumour stroma. However, the expression of MCP-1 revealed that the stroma cells could stimulate the activation and migration of monocytes. IFN-γ and MCP-1 could play an essential role in the anti-tumour immune response against the subcutaneous mPAC regressor tumour. To date, IFN-γ is known to be the major effector cytokine in nearly all described tumour models (Blankenstein and Qin, 2003; Qin and Blankenstein, 2000; Qin et al., 2003; van Elsas et al., 2001; Winter et al., 1999; Wu et al., 2004). Cytokines expressed by T_H2 cells, such as IL-4 and IL-5, were expressed at low levels in both tumours (data not shown).

NK and NKT effector cells can express IFN-γ and are known to eliminate early malformed cells and MHC class I-deficient tumour cells (Hoglund et al., 1988; Whiteside and Herberman, 1995). CTLs are the major effector cells in the anti-tumour immune response of many tumour models. The successful killing of tumour cells by CTLs is dependent on CD4 T_H1 cells, which are known to be essential in the maintenance of CTL effector functions (Bourgeois et al., 2002; Schoenberger et al., 1998). Both T cell subtypes can secrete IFN-γ in an anti-tumour immune response. The pro-inflammatory cytokine TNF-α and the chemokine MCP-1 can also be produced by cells from the innate and adaptive immune system. TNF-α can function as an effector cytokine in the anti-tumour immune response and can be expressed by macrophages, NK and NKT cells as well as T cells. The chemokine MCP-1 targets T cells, monocytes and basophile cells.

IL-6 is mainly produced by T cells, macrophages, and endothelial cells and stimulates the T and B cell growth and differentiation. Additional studies reveal that IL-6 enhances immunoglobulin secretion and cytolytic ability of NK cells, and is important for the

development and maintenance of CTLs (Galandrini et al., 1991). Since CTLs are the main effector cells in the anti-tumour immune response of many tumour models, the high expression of IL-6 in the subcutaneous mPAC regressor tumour indicates that CTLs have an important function in the regression of the subcutaneous mPAC regressor tumour. Interestingly, studies on human pancreas tumour cells have shown, that tumour-derived IL-6 enhances the humoral immune response and has an anti-metastatic effect (Saito et al., 1998). However, the CBA results confirm ongoing anti-tumour responses in the subcutaneous and to a lesser extent in the spontaneous mPAC tumour. Mainly IFN-γ, MCP-1, TNF-α and IL-6 are involved in these processes.

To analyse the relative expression of immune relevant genes, mRNAs of the spontaneous mPAC and the subcutaneous mPAC regressor tumour stroma were determined by real time PCR. TRAIL, an apoptosis inducing ligand, is expressed at a higher level in the spontaneous mPAC explants than in the subcutaneous counterpart. The expression of TRAIL by T cells and monocytes induces apoptosis of TRAIL-receptor activated T cells and tumour cells. Interestingly, recent publications reveal that tumour cells are able to express TRAIL via tumour-derived microvesicles, which results in T cell death (Huber et al., 2005; Morse et al., 2005). If the spontaneous mPAC tumour cells express TRAIL and are themselves resistant to TRAIL, this characteristic could explain the low anti-tumour immune response and the progressive growth of the tumour. If this is the case, the subcutaneous mPAC regressor tumour cells, which are derived from spontaneous mPAC tumour, must have lost or downregulated their TRAIL expression.

The death-inducing FasL has the same functional properties as TRAIL. For the induction of apoptosis via FasL the target cell must express the Fas receptor (Fas). Many tumours express the FasL which results in suppression of the anti-tumour immune response by apoptosis of immune cells, a mechanism called counterattack (Andreola et al., 2002; Bennett et al., 1998). The subcutaneous mPAC regressor tumour and the spontaneous mPAC tumour do not express Fas, indicating that the Fas/FasL apoptotic pathway is not relevant in this murine pancreatic tumour model. The higher expression of IL-6 and MCP-1 in subcutaneous mPAC regressor tumour detected in the CBA assay could be confirmed, although to lower extent, by the real time PCR. The chemokine IP-10 is more strongly expressed in subcutaneous compared to spontaneous mPAC tumour, which might be responsible for the high expression of IFN-γ in the subcutaneous mPAC regressor tumour rejection process. The CBA and the real time PCR results show a significant expression of IP-10 and MCP-1 in the regressor stroma. Because IP-10 is an important inducer of IFN-γ and MCP-1 is chemotactic for monocytes, IFN-γ expressing cells and macrophages most probably play a major role in the subcutaneous pancreatic tumour regression.

In summary, the expression of relevant cytokines and chemokines in the tumour stroma of the subcutaneous and spontaneous PAC indicates an anti-tumour immune response in both models. A significant difference was seen in the expression of IFN-γ, TNF-α, MCP-1, and IL-6, which could suggest a stronger immune response in the transplantable PAC.

4.1.2 Characterization of the tumour-specific cellular immune responses in subcutaneous and spontaneous mPAC tumours

Knowing the cytokine expression pattern in the tumour stroma, immunofluorescence and immunohistochemistry staining of tumour sections were performed to specify the cells, which are responsible for the particular increased cytokine expression (3.1.2). In the subcutaneous mPAC regressor and spontaneous mPAC tumour, $CD4^+$ and $CD8^+$ tumour-infiltrating lymphocytes (TILs) could be detected. $CD4^+$ cells were the predominantly infiltrating cells in both tumours, whereas significantly less $CD8^+$ cells were found in spontaneous mPAC tumour. These observations were confirmed by counts of three independent experiments. These results show that both T cell subtypes migrate into both tumours and could influence the tumourgenesis of the pancreatic cancer. In order to further quantify the number of TILs, single-cell suspensions were prepared from tumours, stained and analysed by FACS. The FACS results confirmed the histological results and identified 10- to 20-fold more $CD8^+$ T cell populations in the subcutaneous mPAC regressor tumour. These results predict a $CD8^+$ T cell anti-tumour response of the subcutaneous tumour accompanied by strong $CD4^+$ T cell infiltration. Interestingly, the TCR-associated CD3 molecule, which is important for the cell surface expression of the antigen-binding chains of T cells, is downregulated or not expressed in a number of $CD4^+$ and $CD8^+$ infiltrating cells. $CD4^+$ and $CD8^+$ cells in the spleen (splenocytes), lymph nodes, and blood express CD3 (data not shown).

Different types of TILs can have a negative or positive influence on the anti-tumour response. Homing and activity markers categorize the functionality of TILs. For this purpose, well established spontaneous pancreatic tumours from EL-TGF-α x $Trp53^{-/-}$ mice and six-day old subcutaneous tumours from immune competent C57BL/6 wt mice were isolated; tumour-infiltrating cells were harvested and analysed by surface and intracellular FACS. The subcutaneous mPAC regressor tumour harbours a nine-fold higher number of $CD4^+CD25^+$ T cells, in contrast to spontaneous mPAC tumour. This result suggests that $CD4^+CD25^+$ cells could play an important role in the anti-tumour immune response of the rejected subcutaneous mPAC tumours. Although mPAC regressor tumour cells do not express MHC class II, it is known that $CD4^+$ T cells can function as an effector cell and eliminate tumour cells by a delayed-type hypersensitivity-like reaction, during which they attract and activate innate effector cells, such as macrophages or NK cells (Greenberg, 1991). However, in most of the tumour models, $CD4^+$ T lymphocytes are necessary for the anti-tumour immune response of CTLs (Bourgeois et al., 2002; Schoenberger et al., 1998).

On the other hand, T regulatory (T_{reg}) cells, which also express the CD4 and CD25 receptors, are able to suppress the anti-tumour immune responses (Onizuka et al., 1999; Shimizu et al., 1999). In order to determine the ratio of activated $CD4^+CD25^+$ cells versus regulatory $CD4^+CD25^+$ cells, tumour-infiltrating $CD4^+CD25^+$ T cells from subcutaneous and spontaneous mPAC tumours were stained intracellularly with an antibody against the specific T_{reg} cell transcription factor Foxp3. A high percentage of T_{reg} cells were discovered in both tumours with no significant differences. This high yield of T_{reg} cells in the subcutaneous mPAC regressor tumour is surprising, knowing that T_{reg} cells function as immune suppressors of tumour-specific CTLs (Chen et al., 2005). In addition, $CD4^+CD25^+$ cells from the spleen and / or draining lymph node(s) of subcutaneous and spontaneous tumour bearing mice have shown a normal percentage of

T_{reg} cells compared to C57BL/6 naïve mice. Although the percentage of T_{reg} cells is high in both tumours, the immune suppressive effect of T_{reg} cells does not seem to be important for the rejection of the subcutaneous mPAC regressor tumour. Progressively growing tumours often have a high percentage of infiltrating T_{reg} cells, referring to their immune suppressive effect (Curiel et al., 2004).

Adoptive T cell experiments were done to investigate, whether a difference in T cell homing could explain the difference observed in spontaneous and subcutaneous mPAC tumours, as suggested by others and as observed in MET mice (Garbi et al., 2004; Onrust et al., 1996). Indeed, a significantly higher number of transferred T cells were found in subcutaneous mPAC regressor tumours. This shows that T cell homing is clearly different between subcutaneous mPAC regressor and spontaneous mPAC tumours, which leads to the different immune response observed (3.1.3). One possibility might be that the tumour environment in the spontaneous mPAC tumour does not provide inflammatory stimuli for the cells to home and become effector cells. In addition, transferred T cells were stained with the leukocyte adhesion molecule CD62L (L-selectin), which mediates the migration into lymphatic tissue, such as lymph nodes, and which is highly expressed on the surface of naïve and resting circulating T_H cells. Before and after T cell infiltration into mPAC tumours, cells were analysed for CD62L expression. Although splenocytes of subcutaneous and spontaneous mPAC tumour bearing mice show a high percentage of $CD62L^+$ cells of the total T lymphocytes that were transferred, only infiltrated T lymphocytes of spontaneous tumours that were transferred still express the homing receptor. This indicates, TILs from spontaneous tumour are not activated (Jung et al., 1988; Kishimoto et al., 1989). In addition, previous studies have shown that tumours are able to develop an intrinsic resistance to leukocyte infiltration, which might impair tumour-specific immune responses (Ganss et al., 2002). CD62L seems to be downregulated after infiltration into the subcutaneous regressor tumour, which indicates activation.

In order to analyse systemic immune responses, the antigen specificity of CTLs in both pancreatic tumour models were studied. EL-TGF-α x $Trp53^{-/-}$ transgenic and C57BL/6 wt mice were vaccinated with mPAC regressor tumour cells and the IFN-γ secretion of $CD8^+$ cells were determined after restimulation. Tumour-specific CTL responses could be detected in both mice bearing mPAC regressor tumour or the spontaneous mPAC tumour. This shows that CTLs have an important effector role in this pancreatic murine model.

4.1.3 Characterization of tumour-specific humoral immune response against subcutaneous and spontaneous mPAC tumours

Patients with pancreatic cancer can develop tumour-specific antibody responses (Nakatsura et al., 2002). Therefore, to further validate the immunologic significance of the described tumour model, sera from EL-TGF-α x $Trp53^{-/-}$ mice and mice that were subcutaneously injected with mPAC regressor tumour cells were analysed. Although a humoral immune response against mPAC regressor tumour cells could be detected in both mice, the antibody response in the subcutaneous mPAC regressor tumour was stronger than in the spontaneous mPAC tumour (3.1.4). This result shows that plasma cells are involved in the anti-tumour immune response of both pancreatic tumours. The increased progressive growth of the mPAC regressor tumour in $RAG^{-/-}$ mice compared to the nude mice supports the influence of plasma cells (Garbe et al., 2006). The

analysis of the Ig isotype reveals that the spontaneous mPAC tumour mouse develops mainly an IgG2b antibody isotype, which is involved in the classical pathway of complement activation and associated with T_H1 responses. The subcutaneous tumour mice harbour IgG1 isotype antibodies, which also induce the classical pathway of the complement system, but induce macrophage and the T_H2 pathway (Gately et al., 1998). The immunoglobulin immune responses also confirm the IL-6 detected in the subcutaneous and spontaneous tumour stroma, which is known to stimulate the humoral immune response (see above).

Both mPAC tumour models show clearly a cellular and humoral tumour-specific immune response. However, the anti-tumour immune responses in the subcutaneous mPAC tumour are stronger and TILs are active, in contrast to TILs of the spontaneous mPAC tumour. The difference might be that the tumour stroma of the spontaneous mPAC tumour does not provide inflammatory stimuli for the cells to home and become effector cells. It also has been shown that tumours secrete inhibitory factors, such as TGF-beta and prostaglandine (PGE)-2, which further hamper the effect of effector cells. In addition, these results show that B cells, T_H cells, and macrophage seem to support the strong specific anti-tumour response of CTLs against the subcutaneous mPAC tumour. However, the anti-tumour response against the spontaneous mPAC tumour is much weaker, which could explain the progressive tumour growth. These results further show that transplantable subcutaneous tumours, which have long been integral to tumour immunology research, are a poor model to understand tumour-specific immune responses; therefore, spontaneous tumour mouse models should rather be used for preclinical testing of possible new therapeutic approaches.

4.2 Variant of the mPAC regressor tumour can evade the host immune responses

The mPAC regressor tumour cell line is rejected in immune competent mice, but grows progressively, even though with a short delay, when injected into $RAG^{-/-}$ mice. As a consequence of malignant transformation, malignant cells acquire genetic instability; within the tumour phenotypic, variants may arise that exhibit selective growth advantages (Nowell, 1976). These variants can escape immunological or other homeostatic control mechanisms of the host and become the precursors of a newly emerging subpopulation that now become dominant (Urban et al., 1982).

The tumour variant mPAC progressor tumour did arise spontaneously in an immune competent C57BL/6 wt mouse after rejection of the mPAC regressor tumour. Injection of mPAC progressor tumour cells produced progressively growing tumours in 100% of the immune competent syngenic mice. The establishment of the new cell line mPAC progressor tumour provided the opportunity to analyse possible tumour escape mechanisms in a rapidly growing transplantation tumour model.

To confirm the same origin of the regressor and progressor mPAC tumour cell lines, DNA analysis for the specific transgenic characterizations were performed. Both cell lines have the same genetic background as the transgenic EL-TGF-α x $Trp53^{-/-}$ mice (3.2). The mPAC progressor tumour cell line does not express the MHC class I molecule. Even when the MHC class I molecule was transfected into this tumour cell line, all tumours grew progressively. This reveals that loss or downregulation of MHC class I is not the mechanism that led to the immune escape of mPAC progressor tumour, as it has been shown in other tumour models (Garrido et al., 1993; Hicklin et al., 1999).

In order to investigate, whether mPAC regressor and mPAC progressor tumours share the same CTL antigen, *in vitro* and *in vivo* experiments were performed. Triggering the immune system of C57BL/6 wt mice by subcutaneous injection of mPAC regressor tumour cells led to impaired growth of a second challenge with mPAC regressor tumour cells, which emphasizes again the immunogenic phenotype of the original mPAC regressor tumour cell line. But the challenge with mPAC regressor tumour cells did not prevent the progressive growth of the variant mPAC progressor (data not shown). The immunological unresponsiveness of the mPAC regressor-primed mice towards challenge with mPAC progressor tumour cells suggests that mPAC regressor and mPAC progressor cells do not share the same tumour antigens on their surface. Other studies have shown with comparable experiments that the regressive and progressive variants of the same tumour are immunologically related (Caignard et al., 1985). Moreover, mice already bearing the progressive growing variant mPAC tumour, did not tolerate the growth of mPAC regressor tumour (data not shown).

To compare the stroma of both mPAC tumours, cytokine expressions of explanted tumours were analysed by CBA assay and real time PCR. Although there was no significant difference in IL-12 expression of the mPAC regressor compared to the spontaneous mPAC tumour, the IL-12 expression of the mPAC progressor tumour is significantly lower. The enhanced IL-12 expression in the mPAC regressor tumour stroma supports the specific antibody response of isotype IgG1 and a role of $CD4^{+}$ T_H1 cells (Trinchieri, 1995). All other cytokine expressions did not show a significant difference between the two tumours. The expression of the apoptosis-inducing cytokine

TRAIL was significantly higher in the regressor tumour. This indicates a T cell or macrophage induced apoptosis process of mPAC regressor tumour cells. Like the spontaneous tumour, the stroma cells of the progressor tumour do not express IP-10 and only express a minor amount of MCP-1.

The immunosuppressive cytokine TGF-β is known to be expressed at high levels in malignancies and it is often related to poor prognosis (Gorsch et al., 1992). However, a high number of T_{reg} cells exists in the mPAC regressor tumour, which is most probably the source of the detected TGF-β (Chen et al., 2005; Nakamura et al., 2001). The question why the mPAC regressor but not the progressor tumour is rejected still needs to be answered. The growth of the progressor seems not to be exclusively dependent on the immunosuppressive effects of TGF-β and IL-10, because both cytokines are expressed at higher levels in the regressor tumour stroma. VEGF favours the angiogenesis in tumours, which results in enhanced tumour growth (Senger et al., 1993). The relatively high expression in the mPAC regressor tumour stroma and the low expression in the mPAC progressor tumour stroma, suggest that VEGF has no essential influence on the tumourgenesis in these murine models.

Immunohistochemistry and FACS analysis has revealed a high amount of $CD4^+$ and $CD8^+$ cells in the mPAC regressor tumour. In contrast, low infiltrations of both T cell types are found in the mPAC progressor tumour. Therefore, $CD8^+$ cells, in particular CTLs, could make the difference in the tumour kinetics of the mPAC regressor and progressor. Although the immunohistochemistry results indicate a similar infiltration rate of $CD4^+$ cells in both tumours, FACS analysis is believed to be more sensitive and reliable, showing more infiltrating $CD4^+$ cells in the mPAC regressor tumour.

Infiltrating macrophages and / or NK cells (both expressing $CD11b^+$) could be detected in large quantities in mPAC regressor tumour. These cells were present at the periphery and centre of the mPAC regressor tumour, as compared to the mPAC progressor tumour. The centre of the mPAC regressor tumour displays a necrotic phenotype of tumour cells with infiltration of $CD11b^+$ cells. FACS analysis proved that most of the infiltrating $CD11b^+$ cells are IA/IE^+, consequently, macrophages. The immunohistochemistry analysis is consistent with FACS data, which illustrate a higher number of macrophages in the mPAC regressor tumour. Recent publications have shown that macrophages can support but also inhibit a tumour-specific immune response, which seems to be dependent on the involved T cells (Ibe et al., 2001). Macrophages can function as APCs, which present the processed antigen to $CD4^+$ cells. This fact could explain the high infiltration of $CD4^+$ cells into the regressor tumour.

In order to analyze if the infiltrating $CD4^+$ cells in the mPAC progressor tumour were activated, $CD4^+$ TILs were stained with CD40L. CD40L is known to have an important function in co-stimulatory interactions with APCs (Noelle et al., 1992). Surprisingly, there is no significant difference in the amount of $CD40L^+$ T cells in both tumours. Once again, it was shown that $CD4^+$ cells are not the main effector cells in these mPAC tumour models.

Focused on CTLs as main effector cells, *in vitro* generated mPAC regressor tumour-specific CTLs were adoptively transferred into regressor and progressor tumour bearing mice. TILs were analysed. The lower infiltration rate of specific CTLs into progressor tumours could be explained by downregulation or loss of a specific mPAC tumour antigen on the mPAC progressor tumour cells.

Functional activity of tumour-specific T lymphocytes was detected in mPAC regressor tumour bearing mice by IFN-γ expression. Progressor tumour bearing mice did not show any relevant IFN-γ expression of $CD8^+$ but for $CD4^+$ cells. Cross experiments (vaccination with regressor and restimulation with progressor or *vice versa*) did not develop a CD8, but a CD4 anti-tumour immune response. This indicates that IFN-γ expressing $CD4^+$ (T_H1) cells are not able to inhibit the mPAC progressor tumour growth and that the mPAC progressor tumour does not initiate a CTL anti-tumour immune response.

In summary, regressor tumour bearing mice develop mPAC tumour-specific CTLs, in contrast to progressor tumour bearing mice. Both mice develop a specific IFN-γ anti-tumour immune response. Tumour specific CTLs make the difference between progressive growth or regression of mPAC tumours. These mPAC regressor tumour-specific CTLs express IFN-γ after vaccination with mPAC regressor tumour cells, which support the essential role IFN-γ for the rejection of many tumours (Blankenstein and Qin, 2003; Qin and Blankenstein, 2000; Qin et al., 2003; van Elsas et al., 2001; Winter et al., 1999; Wu et al., 2004). In order to confirm the importance of IFN-γ in the rejection of the mPAC regressor tumour, mPAC regressor tumour cells were injected into IFN-γ knockout mice (C57BL/6 IFN-$\gamma^{-/-}$). In these mice, the regressor tumour grew progressively, which indicates that IFN-γ is an important cytokine in the rejection of mPAC regressor tumour cells.

The detected humoral immune response against mPAC regressor tumour cells could not protect or inhibit the outgrowth of the mPAC progressor tumours. Since no soluble immune suppression and counterattack factors are expressed by the mPAC progressor tumour, most likely an antigen loss or downregulation might explain the mPAC progressor tumour outgrowth.

4.3 Chronic pancreatitis can favour the tumour growth and suppress the tumour-specific immune response

The factors involved in the induction of chronic pancreatitis are mutations and environmental factors. The mutations target the mechanisms protecting the pancreas from premature trypsinogen activation, which causes injury and finally leads to pancreatic autodigestion and an inflammatory response. These mutations are hereditary and can cause chronic pancreatitis in early childhood. Alcohol and tobacco smoking are the main environmental factors, which cause chronic pancreatitis. In addition, a modulated immune system can favour chronic inflammation or fibrosis of the pancreas (Etemad and Whitcomb, 2001; Whitcomb, 2004). However, recent studies reveal that patients with chronic pancreatitis have multiple factors (Etemad and Whitcomb, 2001). The likelihood of having genetic susceptibility to pancreatic injury, plus the necessary environmental factors to repeatedly trigger pancreatitis, plus an immune response leading to chronic inflammation and fibrosis, rather than healing, is small and it emphasizes the observation that chronic pancreatitis is relatively uncommon in humans and it is difficult to induce in animals. However, it is suggested that chronic pancreatitis creates a milieu in the pancreas which favours the development of pancreatic cancer.

The aim of this study was to investigate the relation between chronic pancreatitis and the induction and progression of pancreatic cancer. Thereby, this study focused on the effect of chronic pancreatitis on the anti-tumour immune responses to the mPAC regressor tumour. In addition, the pathomorphological tumour development was analysed in EL-TGF-α x $Trp53^{-/-}$ transgenic mice, suffering from chronic pancreatitis.

Regressor tumour bearing mice with chronic pancreatitis have shown a reduced CD8- and CD4- IFN-γ response, as compared to mice bearing the regressor tumour but not having chronic pancreatitis (3.3.2). Although, these results are preliminary, chronic pancreatitis seems to reduce the immune responses against the mPAC regressor tumour. *In vivo* analyses of mice bearing the mPAC regressor tumour and suffering from chronic pancreatitis have shown that one out of five mice developed a progressively growing tumour after 60 days (data not shown). This confirms partially the suppression of the tumour-specific immune responses by chronic inflammation. Because EL-TGF-α x $Trp53^{-/-}$ mice did not develop a sufficient tumour-specific immune response by CTL, only the pathomorphology of the developed spontaneous tumour could be studied, while the twelve week old EL-TGF-α x $Trp53^{-/-}$ mice suffered from chronic pancreatitis. Only one out of three mice showed a more undifferentiated histology of the pancreas tumour, indicating an acceleration of the spontaneous tumour development.

These experiments, although preliminary, demonstrate in a murine model for human pancreatic adenocarcinoma, that chronic inflammation in the pancreas can decrease the tumour-specific immune response.

4.4 Cyclophosphamide can stimulate the anti-tumour immune response against subcutaneous pancreatic adenocarcinoma in a T cell-dependent manner

The tumour stroma is believed to play a crucial role in the tumourgenesis (Kalluri, 2003; Ibe et al., 2001; Lutsiak et al., 2005). Often, immune cells are part of the stroma cells and support tumour growth. These cells differ from their normal counterparts. Manipulation of the stroma of progressively growing murine tumours has revealed a successful strategy to reject tumours (Ibe et al., 2001; Lutsiak et al., 2005). Therefore, the tumour stroma may also serve as a target for immune intervention. In addition, the localization of the tumour may contribute to the tumour development and progression. Thus, the inability of the circulating T cells to reach the tumour site could lead to ignorance of the presence of the tumour (Ochsenbein et al., 1999; Ochsenbein et al., 2001).

In order to address the role of the tumour stroma in the murine pancreatic tumour model, the mPAC regressor and progressor tumour cell lines were injected into different sites of the mouse. Intravenous (i.v.) and liver injection of the mPAC progressor tumour cells lead to a progressively growth in the lung and liver, respectively. In contrast, no mPAC regressor tumour growth could be detected in the same organs. Both tumour cell lines did not grow after intraperitoneal (i.p.) injection. These results indicate that the mPAC regressor tumour cell line can be targeted by the immune system independent of their localization. In contrast, mPAC progressor tumour cells seem to grow in each organ. Because these tumour cells do not grow after i.p. injection, it could be concluded that mPAC tumour cells must be embedded into a matrix of nonmalignant tissue in order to develop a solid tumour.

In order to analyse if the developed mPAC regressor tumour in the nude mice (T cell-deficient) grew due to possible *in vivo* changes of the regressor tumour cells (selection; mutation), the progressively growing mPAC regressor tumour in the nude mice was isolated and single cell suspensions of the tumour were subcutaneously injected into immune competent C57BL/6 wt mice. The same process was done with a spontaneous tumour. Interestingly, the regressor tumour cells isolated from the nude mice now grew progressively in immune competent C57BL/6 wt mice. In contrast, single cell suspensions of spontaneous tumours did not grow. This indicates that the mPAC regressor tumour cell line can undergo changes *in vivo* under non-immunological pressure, which can convert the tumour into a progressor tumour. By injecting tumour pieces instead of single cell suspensions, the same results were obtained.

Cyclophosphamide is a chemotherapeutic drug, which is used as a cytostatic for different types of cancer. In contrast to clinical applications, where they used a high dose of cyclophosphamide to suppress the immune system, recent studies have shown that a low dose of this drug stimulates the immune system (Bass and Mastrangelo, 1998; Ibe et al., 2001).

Cyclophosphamide had no influence on the progressive *in vitro* growth of mPAC regressor or progressor tumour cells (3.4.4). The titration of this drug revealed that one i.p. injection of 200 mg/kg BW results in the strongest inhibition of the subcutaneous mPAC progressor tumour. The concentration of cyclophosphamide used was optimal

and consistent with what has been shown in other studies (Ibe et al., 2001; Lutsiak et al., 2005). In order to investigate, whether cyclophosphamide is a stimulator of the anti-tumour immune response against the mPAC progressor tumour, C57BL/6 wt and nude mice bearing the progressor tumour were injected with cyclophosphamide (3.4.5). Interestingly, the progressor tumour in T cell-deficient mice grew normally without a significant delay. In contrast, the growth of the mPAC progressor tumour in C57BL/6 wt mice was inhibited. This indicates that the anti-tumour function of cyclophosphamide against mPAC is dependent on T cells. Recent studies suggest that cyclophosphamide inhibits the proliferation of T_{reg} cells, which could explain the delay in mPAC progressor tumour growth (Lutsiak et al., 2005). The mPAC progressor tumour contains a high percentage of T_{reg} cells in its tumour stroma, which could be responsible for its progressive growth. Other researchers believe that TIMs can be manipulated in the tumour stroma by means of cyclophosphamide treatment. Thereby, macrophages change their cytokine expression pattern from IL-10 to IFN-γ, which is known to be a strong anti-tumour effector cytokine (Ibe et al., 2001). In the tumour stroma of the mPAC progressor, both TIMs and the immune suppressive cytokine IL-10 were expressed. Together with the results of the cyclophosphamide experiments, it could be concluded, that the mPAC progressor tumour growth is due to the suppressive effect of IL-10, which is expressed by TIMs. On the other hand, T_{reg} cells, which can suppress the tumour-specific immune response, also express IL-10. By means of the inhibitor effect of cyclophosphamide on T_{reg} cell proliferation, IL-10 expression would decrease and the tumour-specific immune response would be increased. At this stage of the study, it is not clear which pathway is possible for this murine mPAC tumour model and further studies are required.

Abbreviations

7-AAD	7-Amino-actinomycin D
ADCC	Antibody-dependent cell mediated cytotoxicity
AICD	Activation-induced cell death
APAAP	Alkaline phosphatase-anti-alkaline phosphatase
APC	Allophycocyanin
APC	Antigen presenting cell
APO2L	Apoptosis inducing ligand
ARF	Alternative reading frame
BCR	B cell antigen receptor
bp	Base pairs
BRCA	Breast cancer
BSA	Bovine serum albumin
BW	Body weight
°C	Celsius
CAF	Cancer-associated fibroblast
cAMP	Cyclic adenosine-mono-phosphate
CBA	Cytometric bead array
CD	Cluster of differentiation
Cdk	Cyclin-dependent kinase
CEA	Carcinoembryonic antigen
CIS	Carcinoma *in situ*
CK	Cytokeratin
CpG	Cytosine-phosphorothioate-guanine
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
Cy	Cyclophosphamide
Cy2	Cyanin2
Cy3	Cyanin3
D	Diversity
DAPI	4´, 6-diamidin-2´-phenylindol
DC	Dendritic cell

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleotide
DPC	Deleted in pancreatic carcinoma
D.P.X.	p-Xylene-bis (N-pyridinium bromide) (neutral mounting medium)
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
EL	Elastase
EL4	Murine thymoma cell line
ERK	Extracellular-regulated kinase
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanat
FLIP	FLICE (caspase-8) inhibitory protein
FMuLV	Friend murine leukaemia virus
Foxp3	Fork head box p3
GFP	Green fluorescent protein
gp	Glycoprotein
GTP	Guanosine triphospate
H&E	Haematoxylin & Eosin
HER	Human epidermal growth factor receptor
hGH	Human growth hormone
HLA	Human leukocyte antigen
HPV16	Human papilloma virus
HSP	Heat-shock protein
IFN	Interferon
ICOS	Inducible co-stimulator
IDO	Indoleamine 2,3-dioxygenase
Ig	Immunoglobulin

IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IL	Interleukin
INK4	Inhibitor of Cdk4
i.p.	Intraperitoneal
IP-10	Interferon-γ inducing protein-10
IPMN	Intraductal papillary mucinous neoplasm
i.v.	Intravenous
ivp	*in vivo* passaged
J	Joining
Jnk	Jun N-terminal kinase
Kb	Kilobase
kg	Kilogram
K-*ras*	Kirsten-*ras*
L	Ligand
l	Litre
LAM	Leukocyte adhesion molecule
LOH	Loss of heterozygosity
mAb	Monoclonal antibody
MAGE	Melanoma antigen
MAP	Mitogen-activated protein
MCA	Methylencholanthrene
MCN	Mucinous cystic neoplasm
MCP	Monocyte chemoattractant protein
mg	Milligram
µg	Microgram
MHC	Major histocompatibility complex
MIC	MHC class I chain related glycoprotein
mIg	Membrane immunoglobulin
MKK	MAP kinase-kinase
ml	Millilitre
MMP	Matrix metalloproteinase
mPAC	Murine pancreatic adenocarcinoma
mRNA	Messenger ribonucleic acid

MTS	Multiple tumour suppressor
MUC	Mucin
MV	Membrane vesicles
NaCl	Sodium chloride
NaN_3	Sodium azide
NFAT	Nuclear factor of activated T cells
ng	Nanogram
NK	Natural killer
NKT	Natural killer T
NSCLC	Non-small cell lung carcinoma
OD	Optical density
ODN	Oligodesoxynucleotides
o.n.	Over night
OVA	Ovalbumin
OVAC	Ovarian carcinoma cell line
PAC	Pancreatic adenocarcinoma
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	R-phycoerythrin
PerCP	Peridinin chlorophyll protein
PFA	Paraformaldehyde
pg	Picogram
Pgp-1	P-glycoprotein-1
PSA	Prostate-specific antigen
RAG	Recombinase activating gene
RAP	Repressor activator protein
Rb	Retinoblastoma
RMA	Rauscher murine leukaemia virus (MuLV)-induced T cell lymphoma cell line
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Reverse transcription

RT-PCR	Reverse transcription dependent polymerase chain reaction
s.c.	Subcutaneous
SCID	Several combined immunodeficiency
SD	Standard deviation
SDS	Sodiumdodecylsulfate
SEB	Staphylococcal enterotoxin B
SEREX	Serological analysis of recombinant cDNA expression library
SMAD	Small mother against decapentaplegic
STAT	Signal transducer and activator of transcription
STK	Serine / threonine kinase
SV40	Simian virus 40
TAA	Tumour-associated antigen
TAM	Tumour associated macrophage
TAP	Transporter for antigen presentation
TBS	Tris-buffered saline
TCR	T cell antigen receptor
TEC	Tumour endothelial cells
TGF	Transforming growth factor
T_H	T_{Helper}
TIL	Tumour-infiltrating lymphocyte
TIM	Tumour-infiltrating macrophage
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis-factor apoptosis-inducing ligand
T_{reg}	T regulatory
Trp	Transformation related protein
U	Unit
ULBP	UL16-binding protein
V	Variable
VEGF	Vascular endothelial growth factor
wt	Wild type
ZAP	Zeta-associated protein

References

Adami,J., Gabel,H., Lindelof,B., Ekstrom,K., Rydh,B., Glimelius,B., Ekbom,A., Adami,H.O., and Granath,F. (2003). Cancer risk following organ transplantation: a nationwide cohort study in Sweden. Br. J. Cancer *89*, 1221-1227.

Aguirre,A.J., Bardeesy,N., Sinha,M., Lopez,L., Tuveson,D.A., Horner,J., Redston,M.S., and DePinho,R.A. (2003). Activated Kras and Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma. Genes Dev. *17*, 3112-3126.

Ahmad,M., Rees,R.C., and Ali,S.A. (2004). Escape from immunotherapy: possible mechanisms that influence tumor regression/progression. Cancer Immunol. Immunother. *53*, 844-854.

Albert,M.L., Sauter,B., and Bhardwaj,N. (1998). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature *392*, 86-89.

Algarra,I., Garcia-Lora,A., Cabrera,T., Ruiz-Cabello,F., and Garrido,F. (2004). The selection of tumor variants with altered expression of classical and nonclassical MHC class I molecules: implications for tumor immune escape. Cancer Immunol. Immunother. *53*, 904-910.

Almoguera,C., Shibata,D., Forrester,K., Martin,J., Arnheim,N., and Perucho,M. (1988). Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell *53*, 549-554.

Andre,F., Schartz,N.E., Chaput,N., Flament,C., Raposo,G., Amigorena,S., Angevin,E., and Zitvogel,L. (2002). Tumor-derived exosomes: a new source of tumor rejection antigens. Vaccine *20 Suppl 4*, A28-A31.

Andreola,G., Rivoltini,L., Castelli,C., Huber,V., Perego,P., Deho,P., Squarcina,P., Accornero,P., Lozupone,F., Lugini,L., Stringaro,A., Molinari,A., Arancia,G., Gentile,M., Parmiani,G., and Fais,S. (2002). Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. J. Exp. Med. *195*, 1303-1316.

Arai,H., Chan,S.Y., Bishop,D.K., and Nabel,G.J. (1997). Inhibition of the alloantibody response by CD95 ligand. Nat. Med. *3*, 843-848.

Balkwill,F., Charles,K.A., and Mantovani,A. (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. Cancer Cell *7*, 211-217.

Balkwill,F. and Mantovani,A. (2001). Inflammation and cancer: back to Virchow? Lancet *357*, 539-545.

Bardeesy,N. and DePinho,R.A. (2002). Pancreatic cancer biology and genetics. Nat. Rev. Cancer *2*, 897-909.

Barton,C.M., Staddon,S.L., Hughes,C.M., Hall,P.A., O'Sullivan,C., Kloppel,G., Theis,B., Russell,R.C., Neoptolemos,J., Williamson,R.C., and . (1991). Abnormalities of the p53 tumour suppressor gene in human pancreatic cancer. Br. J. Cancer *64*, 1076-1082.

Bass,K.K. and Mastrangelo,M.J. (1998). Immunopotentiation with low-dose cyclophosphamide in the active specific immunotherapy of cancer. Cancer Immunol. Immunother. *47*, 1-12.

Bennett,M.W., O'Connell,J., O'Sullivan,G.C., Brady,C., Roche,D., Collins,J.K., and Shanahan,F. (1998). The Fas counterattack in vivo: apoptotic depletion of tumor-infiltrating lymphocytes associated with Fas ligand expression by human esophageal carcinoma. J. Immunol. *160*, 5669-5675.

Blankenstein,T. and Qin,Z. (2003). The role of IFN-gamma in tumor transplantation immunity and inhibition of chemical carcinogenesis. Curr. Opin. Immunol. *15*, 148-154.

Bolt,A.B., Papanikolaou,A., Delker,D.A., Wang,Q.S., and Rosenberg,D.W. (2000). Azoxymethane induces KI-ras activation in the tumor resistant AKR/J mouse colon. Mol. Carcinog. *27*, 210-218.

Boon,T. (1993). Tumor antigens recognized by cytolytic T lymphocytes: present perspectives for specific immunotherapy. Int. J. Cancer *54*, 177-180.

Bourgeois,C., Rocha,B., and Tanchot,C. (2002). A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. Science *297*, 2060-2063.

Brat,D.J., Lillemoe,K.D., Yeo,C.J., Warfield,P.B., and Hruban,R.H. (1998). Progression of pancreatic intraductal neoplasias to infiltrating adenocarcinoma of the pancreas. Am. J. Surg. Pathol. *22*, 163-169.

Brigati,C., Noonan,D.M., Albini,A., and Benelli,R. (2002). Tumors and inflammatory infiltrates: friends or foes? Clin. Exp. Metastasis *19*, 247-258.

Brugge,W.R., Lauwers,G.Y., Sahani,D., Fernandez-del Castillo,C., and Warshaw,A.L. (2004). Cystic neoplasms of the pancreas. N. Engl. J. Med. *351*, 1218-1226.

Brunda,M.J. (1994). Interleukin-12. J. Leukoc. Biol. *55*, 280-288.

Brunda,M.J., Luistro,L., Warrier,R.R., Wright,R.B., Hubbard,B.R., Murphy,M., Wolf,S.F., and Gately,M.K. (1993). Antitumor and antimetastatic activity of interleukin 12 against murine tumors. J. Exp. Med. *178*, 1223-1230.

Burnet,F.M. (1957). Cancer—a biological approach. Brit. Med. J. *1*, 841-847.

Burnet,F.M. (1970). The concept of immunological surveillance. Prog. Exp. Tumor Res. *13*, 1-27.

Caignard,A., Martin,M.S., Michel,M.F., and Martin,F. (1985). Interaction between two cellular subpopulations of a rat colonic carcinoma when inoculated to the syngeneic host. Int. J. Cancer *36*, 273-279.

Carreno,B.M. and Collins,M. (2002). The B7 Family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Annu. Rev. Immunol. *20*, 29-53.

Carreno,B.M. and Collins,M. (2003). BTLA: a new inhibitory receptor with a B7-like ligand. Trends Immunol. *24*, 524-527.

Casey,G., Yamanaka,Y., Friess,H., Kobrin,M.S., Lopez,M.E., Buchler,M., Beger,H.G., and Korc,M. (1993). p53 mutations are common in pancreatic cancer and are absent in chronic pancreatitis. Cancer Lett. *69*, 151-160.

Cerboni,C., Mousavi-Jazi,M., Wakiguchi,H., Carbone,E., Karre,K., and Soderstrom,K. (2001). Synergistic effect of IFN-gamma and human cytomegalovirus protein UL40 in the HLA-E-dependent protection from NK cell-mediated cytotoxicity. Eur. J. Immunol. *31*, 2926-2935.

Chailley-Heu,B., Rambaud,C., Barlier-Mur,A.M., Galateau-Salle,F., Perret,C., Capron,F., and Lacaze-Masmonteil,T. (2001). A model of pulmonary adenocarcinoma in transgenic mice expressing the simian virus 40 T antigen driven by the rat Calbindin-D9K (CaBP9K) promoter. J. Pathol. *195*, 482-489.

Chaux,P., Lethe,B., Van Snick,J., Corthals,J., Schultz,E.S., Cambiaso,C.L., Boon,T., and van der Bruggen,P. (2001). A MAGE-1 peptide recognized on HLA-DR15 by CD4(+) T cells. Eur. J. Immunol. *31*, 1910-1916.

Chen,M.L., Pittet,M.J., Gorelik,L., Flavell,R.A., Weissleder,R., von Boehmer,H., and Khazaie,K. (2005). Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. Proc. Natl. Acad. Sci. U. S. A *102*, 419-424.

Chen,Y.T., Scanlan,M.J., Sahin,U., Tureci,O., Gure,A.O., Tsang,S., Williamson,B., Stockert,E., Pfreundschuh,M., and Old,L.J. (1997). A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proc. Natl. Acad. Sci. U. S. A *94*, 1914-1918.

Cho,Y., Miyamoto,M., Kato,K., Fukunaga,A., Shichinohe,T., Kawarada,Y., Hida,Y., Oshikiri,T., Kurokawa,T., Suzuoki,M., Nakakubo,Y., Hiraoka,K., Murakami,S., Shinohara,T., Itoh,T., Okushiba,S., Kondo,S., and Katoh,H. (2003). CD4+ and CD8+ T cells cooperate to improve prognosis of patients with esophageal squamous cell carcinoma. Cancer Res. *63*, 1555-1559.

Clarke,P., Mann,J., Simpson,J.F., Rickard-Dickson,K., and Primus,F.J. (1998). Mice transgenic for human carcinoembryonic antigen as a model for immunotherapy. Cancer Res. *58*, 1469-1477.

Coussens,L.M. and Werb,Z. (2002). Inflammation and cancer. Nature *420*, 860-867.

Cubilla,A.L. and Fitzgerald,P.J. (1976). Morphological lesions associated with human primary invasive nonendocrine pancreas cancer. Cancer Res. *36*, 2690-2698.

Curiel,T.J., Coukos,G., Zou,L., Alvarez,X., Cheng,P., Mottram,P., Evdemon-Hogan,M., Conejo-Garcia,J.R., Zhang,L., Burow,M., Zhu,Y., Wei,S., Kryczek,I., Daniel,B., Gordon,A., Myers,L., Lackner,A., Disis,M.L., Knutson,K.L., Chen,L., and Zou,W. (2004). Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat. Med. *10*, 942-949.

Darnell,R.B. and Posner,J.B. (2003). Observing the invisible: successful tumor immunity in humans. Nat. Immunol. *4*, 201.

De Plaen,E., Lurquin,C., Van Pel,A., Mariame,B., Szikora,J.P., Wolfel,T., Sibille,C., Chomez,P., and Boon,T. (1988). Immunogenic (tum-) variants of mouse tumor P815: cloning of the gene of tum- antigen P91A and identification of the tum- mutation. Proc. Natl. Acad. Sci. U. S. A *85*, 2274-2278.

De Smedt,T., Van Mechelen,M., De Becker,G., Urbain,J., Leo,O., and Moser,M. (1997). Effect of interleukin-10 on dendritic cell maturation and function. Eur. J. Immunol. *27*, 1229-1235.

Derynck,R., Gelbart,W.M., Harland,R.M., Heldin,C.H., Kern,S.E., Massague,J., Melton,D.A., Mlodzik,M., Padgett,R.W., Roberts,A.B., Smith,J., Thomsen,G.H., Vogelstein,B., and Wang,X.F. (1996). Nomenclature: vertebrate mediators of TGFbeta family signals. Cell *87*, 173.

DiGiuseppe,J.A., Hruban,R.H., Goodman,S.N., Polak,M., van den Berg,F.M., Allison,D.C., Cameron,J.L., and Offerhaus,G.J. (1994a). Overexpression of p53 protein in adenocarcinoma of the pancreas. Am. J. Clin. Pathol. *101*, 684-688.

DiGiuseppe,J.A., Hruban,R.H., Offerhaus,G.J., Clement,M.J., van den Berg,F.M., Cameron,J.L., and van Mansfeld,A.D. (1994b). Detection of K-ras mutations in mucinous pancreatic duct hyperplasia from a patient with a family history of pancreatic carcinoma. Am. J. Pathol. *144*, 889-895.

Dudley,M.E. and Rosenberg,S.A. (2003). Adoptive-cell-transfer therapy for the treatment of patients with cancer. Nat. Rev. Cancer *3*, 666-675.

Dunn,G.P., Bruce,A.T., Ikeda,H., Old,L.J., and Schreiber,R.D. (2002). Cancer immunoediting: from immunosurveillance to tumor escape. Nat. Immunol. *3*, 991-998.

Dunn,G.P., Old,L.J., and Schreiber,R.D. (2004a). The immunobiology of cancer immunosurveillance and immunoediting. Immunity *21*, 137-148.

Dunn,G.P., Old,L.J., and Schreiber,R.D. (2004b). The three Es of cancer immunoediting. Annu. Rev. Immunol. *22*, 329-360.

Egeblad,M. and Werb,Z. (2002). New functions for the matrix metalloproteinases in cancer progression. Nat. Rev. Cancer *2*, 161-174.

Ehrlich,P. (1909). Ueber den jetzigen stand der Karzinomforschung. Ned. Tijdschr. Geneeskd. 273-290.

Ericsson,C., Seregard,S., Bartolazzi,A., Levitskaya,E., Ferrone,S., Kiessling,R., and Larsson,O. (2001). Association of HLA class I and class II antigen expression and mortality in uveal melanoma. Invest Ophthalmol. Vis. Sci. *42*, 2153-2156.

Etemad,B. and Whitcomb,D.C. (2001). Chronic pancreatitis: diagnosis, classification, and new genetic developments. Gastroenterology *120*, 682-707.

Fontana,A., Frei,K., Bodmer,S., Hofer,E., Schreier,M.H., Palladino,M.A., Jr., and Zinkernagel,R.M. (1989). Transforming growth factor-beta inhibits the generation of cytotoxic T cells in virus-infected mice. J. Immunol. *143*, 3230-3234.

Galandrini,R., Cernetti,C., Albi,N., Dembech,C., Terenzi,A., Grignani,F., and Velardi,A. (1991). Interleukin-6 is constitutively produced by human CTL clones and is required to maintain their cytolytic function. Cell Immunol. *138*, 11-23.

Ganss,R., Arnold,B., and Hammerling,G.J. (2004). Mini-review: overcoming tumor-intrinsic resistance to immune effector function. Eur. J. Immunol. *34*, 2635-2641.

Ganss,R., Ryschich,E., Klar,E., Arnold,B., and Hammerling,G.J. (2002). Combination of T-cell therapy and trigger of inflammation induces remodelling of the vasculature and tumor eradication. Cancer Res. *62*, 1462-1470.

Garbe,A.I., Vermeer,B., Gamrekelashvili,J., von Wasielewski,R., Greten,F.R., Westendorf,A.M., Buer,J., Schmid,R.M., Manns,M.P., Korangy,F., and Greten,T.F. (2006). Genetically induced pancreatic adenocarcinoma is highly immunogenic and causes spontaneous tumor-specific immune response. Cancer Res. *66*, 508-516.

Garbi,N., Arnold,B., Gordon,S., Hammerling,G.J., and Ganss,R. (2004). CpG motifs as proinflammatory factors render autochthonous tumors permissive for infiltration and destruction. J. Immunol. *172*, 5861-5869.

Garcia-Lora,A., Algarra,I., Gaforio,J.J., Ruiz-Cabello,F., and Garrido,F. (2001). Immunoselection by T lymphocytes generates repeated MHC class I-deficient metastatic tumor variants. Int. J. Cancer *91*, 109-119.

Garcia-Lora,A., Martinez,M., Algarra,I., Gaforio,J.J., and Garrido,F. (2003). MHC class I-deficient metastatic tumor variants immunoselected by T lymphocytes originate from the coordinated downregulation of APM components. Int. J. Cancer *106*, 521-527.

Garrido,F., Cabrera,T., Concha,A., Glew,S., Ruiz-Cabello,F., and Stern,P.L. (1993). Natural history of HLA expression during tumour development. Immunol. Today *14*, 491-499.

Gately,M.K., Renzetti,L.M., Magram,J., Stern,A.S., Adorini,L., Gubler,U., and Presky,D.H. (1998). The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu. Rev. Immunol. *16*, 495-521.

Giardiello,F.M., Welsh,S.B., Hamilton,S.R., Offerhaus,G.J., Gittelsohn,A.M., Booker,S.V., Krush,A.J., Yardley,J.H., and Luk,G.D. (1987). Increased risk of cancer in the Peutz-Jeghers syndrome. N. Engl. J. Med. *316*, 1511-1514.

Gingrich,J.R., Barrios,R.J., Morton,R.A., Boyce,B.F., DeMayo,F.J., Finegold,M.J., Angelopoulou,R., Rosen,J.M., and Greenberg,N.M. (1996). Metastatic prostate cancer in a transgenic mouse. Cancer Res. *56*, 4096-4102.

Glasner,S., Memoli,V., and Longnecker,D.S. (1992). Characterization of the ELSV transgenic mouse model of pancreatic carcinoma. Histology type of large and small tumors. Am. J. Pathol. *140*, 1237-1245.

Godfrey,D.I., Hammond,K.J., Poulton,L.D., Smyth,M.J., and Baxter,A.G. (2000). NKT cells: facts, functions and fallacies. Immunol. Today *21*, 573-583.

Goggins,M., Schutte,M., Lu,J., Moskaluk,C.A., Weinstein,C.L., Petersen,G.M., Yeo,C.J., Jackson,C.E., Lynch,H.T., Hruban,R.H., and Kern,S.E. (1996). Germline BRCA2 gene mutations in patients with apparently sporadic pancreatic carcinomas. Cancer Res. *56*, 5360-5364.

Gorelik,L. and Flavell,R.A. (2001). Immune-mediated eradication of tumors through the blockade of transforming growth factor-beta signaling in T cells. Nat. Med. *7*, 1118-1122.

Gorer,P.A. (1950). Studies in antibody response of mice to tumour inoculation. Br. J. Cancer *4*, 372-379.

Gorsch,S.M., Memoli,V.A., Stukel,T.A., Gold,L.I., and Arrick,B.A. (1992). Immunohistochemical staining for transforming growth factor beta 1 associates with disease progression in human breast cancer. Cancer Res. *52*, 6949-6952.

Grau,A.M., Zhang,L., Wang,W., Ruan,S., Evans,D.B., Abbruzzese,J.L., Zhang,W., and Chiao,P.J. (1997). Induction of p21waf1 expression and growth inhibition by transforming growth factor beta involve the tumor suppressor gene DPC4 in human pancreatic adenocarcinoma cells. Cancer Res. *57*, 3929-3934.

Greenberg,P.D. (1991). Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. Adv. Immunol. *49*, 281-355.

Greten,T.F. and Jaffee,E.M. (1999). Cancer vaccines. J. Clin. Oncol. *17*, 1047-1060.

Grippo,P.J., Nowlin,P.S., Demeure,M.J., Longnecker,D.S., and Sandgren,E.P. (2003). Preinvasive pancreatic neoplasia of ductal phenotype induced by acinar cell targeting of mutant Kras in transgenic mice. Cancer Res. *63*, 2016-2019.

Gross,L. (1943). Intradermal immunization of C3H mice against a sarcoma that originated in an animal of the same line. Cancer Res. *3*, 326-333.

Haanen,J.B., Baars,A., Gomez,R., Weder,P., Smits,M., de Gruijl,T.D., von Blomberg,B.M., Bloemena,E., Scheper,R.J., van Ham,S.M., Pinedo,H.M., and van den Eertwegh,A.J. (2006). Melanoma-specific tumor-infiltrating lymphocytes but not circulating melanoma-specific T cells may predict survival in resected advanced-stage melanoma patients. Cancer Immunol. Immunother. *55*, 451-458.

Hahn,S.A., Schutte,M., Hoque,A.T., Moskaluk,C.A., da Costa,L.T., Rozenblum,E., Weinstein,C.L., Fischer,A., Yeo,C.J., Hruban,R.H., and Kern,S.E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science *271*, 350-353.

Hanahan,D. (1985). Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature *315*, 115-122.

Hanahan,D. and Weinberg,R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Harris,D.P., Haynes,L., Sayles,P.C., Duso,D.K., Eaton,S.M., Lepak,N.M., Johnson,L.L., Swain,S.L., and Lund,F.E. (2000). Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat. Immunol. *1*, 475-482.

Harris,R.A., Eichholtz,T.J., Hiles,I.D., Page,M.J., and O'Hare,M.J. (1999). New model of ErbB-2 over-expression in human mammary luminal epithelial cells. Int. J. Cancer *80*, 477-484.

Hezel,A.F., Kimmelman,A.C., Stanger,B.Z., Bardeesy,N., and DePinho,R.A. (2006). Genetics and biology of pancreatic ductal adenocarcinoma. Genes Dev. *20*, 1218-1249.

Hicklin,D.J., Marincola,F.M., and Ferrone,S. (1999). HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. Mol. Med. Today *5*, 178-186.

Hofmeister,V., Vetter,C., Schrama,D., Brocker,E.B., and Becker,J.C. (2006). Tumor stroma-associated antigens for anti-cancer immunotherapy. Cancer Immunol. Immunother. *55*, 481-494.

Hoglund,P., Ljunggren,H.G., Ohlen,C., Ahrlund-Richter,L., Scangos,G., Bieberich,C., Jay,G., Klein,G., and Karre,K. (1988). Natural resistance against lymphoma grafts conveyed by H-2Dd transgene to C57BL mice. J. Exp. Med. *168*, 1469-1474.

Hong,S., Wilson,M.T., Serizawa,I., Wu,L., Singh,N., Naidenko,O.V., Miura,T., Haba,T., Scherer,D.C., Wei,J., Kronenberg,M., Koezuka,Y., and Van Kaer,L. (2001). The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. Nat. Med. *7*, 1052-1056.

Hook,L.M., Agafonova,Y., Ross,S.R., Turner,S.J., and Golovkina,T.V. (2000). Genetics of mouse mammary tumor virus-induced mammary tumors: linkage of tumor induction to the gag gene. J. Virol. *74*, 8876-8883.

Hopert,A., Uphoff,C.C., Wirth,M., Hauser,H., and Drexler,H.G. (1993). Specificity and sensitivity of polymerase chain reaction (PCR) in comparison with other methods for the detection of mycoplasma contamination in cell lines. J. Immunol. Methods *164*, 91-100.

Hruban,R.H., Adsay,N.V., Albores-Saavedra,J., Compton,C., Garrett,E.S., Goodman,S.N., Kern,S.E., Klimstra,D.S., Kloppel,G., Longnecker,D.S., Luttges,J., and Offerhaus,G.J. (2001a). Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. Am. J. Surg. Pathol. *25*, 579-586.

Hruban,R.H., Iacobuzio-Donahue,C., Wilentz,R.E., Goggins,M., and Kern,S.E. (2001b). Molecular pathology of pancreatic cancer. Cancer J. *7*, 251-258.

Hruban,R.H., Wilentz,R.E., Goggins,M., Offerhaus,G.J., Yeo,C.J., and Kern,S.E. (1999). Pathology of incipient pancreatic cancer. Ann. Oncol. *10 Suppl 4*, 9-11.

Hu,Y.X., Watanabe,H., Ohtsubo,K., Yamaguchi,Y., Ha,A., Okai,T., and Sawabu,N. (1997). Frequent loss of p16 expression and its correlation with clinicopathological parameters in pancreatic carcinoma. Clin. Cancer Res. *3*, 1473-1477.

Huang,A.Y., Golumbek,P., Ahmadzadeh,M., Jaffee,E., Pardoll,D., and Levitsky,H. (1994). Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. Science *264*, 961-965.

Huang,L., Goodrow,T.L., Zhang,S.Y., Klein-Szanto,A.J., Chang,H., and Ruggeri,B.A. (1996a). Deletion and mutation analyses of the P16/MTS-1 tumor suppressor gene in human ductal pancreatic cancer reveals a higher frequency of abnormalities in tumor-derived cell lines than in primary ductal adenocarcinomas. Cancer Res. *56*, 1137-1141.

Huang,L., Lang,D., Geradts,J., Obara,T., Klein-Szanto,A.J., Lynch,H.T., and Ruggeri,B.A. (1996b). Molecular and immunochemical analyses of RB1 and cyclin D1 in human ductal pancreatic carcinomas and cell lines. Mol. Carcinog. *15*, 85-95.

Huber,V., Fais,S., Iero,M., Lugini,L., Canese,P., Squarcina,P., Zaccheddu,A., Colone,M., Arancia,G., Gentile,M., Seregni,E., Valenti,R., Ballabio,G., Belli,F., Leo,E., Parmiani,G., and Rivoltini,L. (2005).

Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. Gastroenterology *128*, 1796-1804.

Hutchinson,J.N. and Muller,W.J. (2000). Transgenic mouse models of human breast cancer. Oncogene *19*, 6130-6137.

Ibe,S., Qin,Z., Schuler,T., Preiss,S., and Blankenstein,T. (2001). Tumor rejection by disturbing tumor stroma cell interactions. J. Exp. Med. *194*, 1549-1559.

Igney,F.H., Behrens,C.K., and Krammer,P.H. (2000). Tumor counterattack--concept and reality. Eur. J. Immunol. *30*, 725-731.

Igney,F.H., Behrens,C.K., and Krammer,P.H. (2005). CD95L mediates tumor counterattack in vitro but induces neutrophil-independent tumor rejection in vivo. Int. J. Cancer *113*, 78-87.

Jacks,T., Remington,L., Williams,B.O., Schmitt,E.M., Halachmi,S., Bronson,R.T., and Weinberg,R.A. (1994). Tumor spectrum analysis in p53-mutant mice. Curr. Biol. *4*, 1-7.

Jaffee,E.M., Hruban,R.H., Canto,M., and Kern,S.E. (2002). Focus on pancreas cancer. Cancer Cell *2*, 25-28.

Jaffee,E.M., Lazenby,A., Meurer,J., Marshall,F., Hauda,K.M., Counts,C., Hurwitz,H., Simons,J.W., Levitsky,H.I., and Pardoll,D.M. (1995). Use of murine models of cytokine-secreting tumor vaccines to study feasibility and toxicity issues critical to designing clinical trials. J. Immunother. Emphasis. Tumor Immunol. *18*, 1-9.

Jager,E., Jager,D., Karbach,J., Chen,Y.T., Ritter,G., Nagata,Y., Gnjatic,S., Stockert,E., Arand,M., Old,L.J., and Knuth,A. (2000). Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4*0101-0103 and recognized by CD4(+) T lymphocytes of patients with NY-ESO-1-expressing melanoma. J. Exp. Med. *191*, 625-630.

Jager,E., Jager,D., and Knuth,A. (1999). CTL-defined cancer vaccines: perspectives for active immunotherapeutic interventions in minimal residual disease. Cancer Metastasis Rev. *18*, 143-150.

Jager,E., Ringhoffer,M., Altmannsberger,M., Arand,M., Karbach,J., Jager,D., Oesch,F., and Knuth,A. (1997). Immunoselection in vivo: independent loss of MHC class I and melanocyte differentiation antigen expression in metastatic melanoma. Int. J. Cancer *71*, 142-147.

Jager,M.J., Hurks,H.M., Levitskaya,J., and Kiessling,R. (2002). HLA expression in uveal melanoma: there is no rule without some exception. Hum. Immunol. *63*, 444-451.

Jemal,A., Siegel,R., Ward,E., Murray,T., Xu,J., Smigal,C., and Thun,M.J. (2006). Cancer statistics, 2006. CA Cancer J. Clin. *56*, 106-130.

Jochheim,A., Hillemann,T., Kania,G., Scharf,J., Attaran,M., Manns,M.P., Wobus,A.M., and Ott,M. (2004). Quantitative gene expression profiling reveals a fetal hepatic phenotype of murine ES-derived hepatocytes. Int. J. Dev. Biol. *48*, 23-29.

Jones,R.L., Young,L.S., and Adams,D.H. (2000). Immunotherapy in hepatocellular carcinoma. Lancet *356*, 784-785.

Jung,T.M., Gallatin,W.M., Weissman,I.L., and Dailey,M.O. (1988). Down-regulation of homing receptors after T cell activation. J. Immunol. *141*, 4110-4117.

Kalluri,R. (2003). Basement membranes: structure, assembly and role in tumour angiogenesis. Nat. Rev. Cancer *3*, 422-433.

Kalluri,R. and Zeisberg,M. (2006). Fibroblasts in cancer. Nat. Rev. Cancer *6*, 392-401.

Kamb,A., Gruis,N.A., Weaver-Feldhaus,J., Liu,Q., Harshman,K., Tavtigian,S.V., Stockert,E., Day,R.S., III, Johnson,B.E., and Skolnick,M.H. (1994). A cell cycle regulator potentially involved in genesis of many tumor types. Science *264*, 436-440.

Karre,K., Ljunggren,H.G., Piontek,G., and Kiessling,R. (1986). Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature *319*, 675-678.

Kern,S., Hruban,R., Hollingsworth,M.A., Brand,R., Adrian,T.E., Jaffee,E., and Tempero,M.A. (2001). A white paper: the product of a pancreas cancer think tank. Cancer Res. *61*, 4923-4932.

Kern,S.E., Kinzler,K.W., Bruskin,A., Jarosz,D., Friedman,P., Prives,C., and Vogelstein,B. (1991). Identification of p53 as a sequence-specific DNA-binding protein. Science *252*, 1708-1711.

Kern,S.E., Pietenpol,J.A., Thiagalingam,S., Seymour,A., Kinzler,K.W., and Vogelstein,B. (1992). Oncogenic forms of p53 inhibit p53-regulated gene expression. Science *256*, 827-830.

Kiessling,R., Wasserman,K., Horiguchi,S., Kono,K., Sjoberg,J., Pisa,P., and Petersson,M. (1999). Tumor-induced immune dysfunction. Cancer Immunol. Immunother. *48*, 353-362.

Kim,J.V., Latouche,J.B., Riviere,I., and Sadelain,M. (2004). The ABCs of artificial antigen presentation. Nat. Biotechnol. *22*, 403-410.

Kim,J.W., Wieckowski,E., Taylor,D.D., Reichert,T.E., Watkins,S., and Whiteside,T.L. (2005). Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes. Clin. Cancer Res. *11*, 1010-1020.

Kishimoto,T.K., Jutila,M.A., Berg,E.L., and Butcher,E.C. (1989). Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. Science *245*, 1238-1241.

Klein,G. (1966). Tumor antigens. Annu. Rev. Microbiol. *20*, 223-252.

Klimstra,D.S. and Longnecker,D.S. (1994). K-ras mutations in pancreatic ductal proliferative lesions. Am. J. Pathol. *145*, 1547-1550.

Kondratiev,S., Sabo,E., Yakirevich,E., Lavie,O., and Resnick,M.B. (2004). Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma. Clin. Cancer Res. *10*, 4450-4456.

Kripke,M.L. (1974). Antigenicity of murine skin tumors induced by ultraviolet light. J. Natl. Cancer Inst. *53*, 1333-1336.

Kuper,H., Adami,H.O., and Trichopoulos,D. (2000). Infections as a major preventable cause of human cancer. J. Intern. Med. *248*, 171-183.

Kurte,M., Lopez,M., Aguirre,A., Escobar,A., Aguillon,J.C., Charo,J., Larsen,C.G., Kiessling,R., and Salazar-Onfray,F. (2004). A synthetic peptide homologous to functional domain of human IL-10 down-regulates expression of MHC class I and Transporter associated with Antigen Processing 1/2 in human melanoma cells. J. Immunol. *173*, 1731-1737.

Lamagna,C., Aurrand-Lions,M., and Imhof,B.A. (2006). Dual role of macrophages in tumor growth and angiogenesis. J. Leukoc. Biol. *80*, 705-713.

Lillemoe,K.D., Yeo,C.J., and Cameron,J.L. (2000). Pancreatic cancer: state-of-the-art care. CA Cancer J. Clin. *50*, 241-268.

Livingstone,L.R., White,A., Sprouse,J., Livanos,E., Jacks,T., and Tlsty,T.D. (1992). Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell *70*, 923-935.

Liyanage,U.K., Moore,T.T., Joo,H.G., Tanaka,Y., Herrmann,V., Doherty,G., Drebin,J.A., Strasberg,S.M., Eberlein,T.J., Goedegebuure,P.S., and Linehan,D.C. (2002). Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. J. Immunol. *169*, 2756-2761.

Ljunggren,H.G. and Karre,K. (1990). In search of the 'missing self': MHC molecules and NK cell recognition. Immunol. Today *11*, 237-244.

Lowe,S.W., Ruley,H.E., Jacks,T., and Housman,D.E. (1993). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell *74*, 957-967.

Lowenfels,A.B., Maisonneuve,P., Cavallini,G., Ammann,R.W., Lankisch,P.G., Andersen,J.R., Dimagno,E.P., Andren-Sandberg,A., and Domellof,L. (1993). Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. N. Engl. J. Med. *328*, 1433-1437.

Lundin,J., Nordling,S., von Boguslawsky,K., Roberts,P.J., and Haglund,C. (1996). Prognostic value of immunohistochemical expression of p53 in patients with pancreatic cancer. Oncology *53*, 104-111.

Lurquin,C., Van Pel,A., Mariame,B., De Plaen,E., Szikora,J.P., Janssens,C., Reddehase,M.J., Lejeune,J., and Boon,T. (1989). Structure of the gene of tum- transplantation antigen P91A: the mutated exon encodes a peptide recognized with Ld by cytolytic T cells. Cell *58*, 293-303.

Lutsiak,C.M.E., Semnani,R.T., DePascalis,R., Kashmiri,S.V.S., Schlom,J., and Sabzevari,H. (2005). Inhibition of CD4+CD25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. Blood *105*, 2862-2868.

Lyman,M.A., Aung,S., Biggs,J.A., and Sherman,L.A. (2004). A spontaneously arising pancreatic tumor does not promote the differentiation of naive CD8+ T lymphocytes into effector CTL. J. Immunol. *172*, 6558-6567.

Mackay,C.R. (1993). Immunological memory. Adv. Immunol. *53*, 217-265.

Maitra,A., Fukushima,N., Takaori,K., and Hruban,R.H. (2005). Precursors to invasive pancreatic cancer. Adv. Anat. Pathol. *12*, 81-91.

Malkinson,A.M. (2001). Primary lung tumors in mice as an aid for understanding, preventing, and treating human adenocarcinoma of the lung. Lung Cancer *32*, 265-279.

Malmberg,K.J. (2004). Effective immunotherapy against cancer: a question of overcoming immune suppression and immune escape? Cancer Immunol. Immunother. *53*, 879-892.

Mantovani,A., Bottazzi,B., Colotta,F., Sozzani,S., and Ruco,L. (1992). The origin and function of tumor-associated macrophages. Immunol. Today *13*, 265-270.

Mantovani,A., Sozzani,S., Locati,M., Allavena,P., and Sica,A. (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol. *23*, 549-555.

Marchand,M., Weynants,P., Rankin,E., Arienti,F., Belli,F., Parmiani,G., Cascinelli,N., Bourlond,A., Vanwijck,R., Humblet,Y., and . (1995). Tumor regression responses in melanoma patients treated with a peptide encoded by gene MAGE-3. Int. J. Cancer *63*, 883-885.

Martin,F., Caignard,A., Jeannin,J.F., Leclerc,A., and Martin,M. (1983). Selection by trypsin of two sublines of rat colon cancer cells forming progressive or regressive tumors. Int. J. Cancer *32*, 623-627.

Martinez-Lorenzo,M.J., Anel,A., Alava,M.A., Pineiro,A., Naval,J., Lasierra,P., and Larrad,L. (2004). The human melanoma cell line MelJuSo secretes bioactive FasL and APO2L/TRAIL on the surface of microvesicles. Possible contribution to tumor counterattack. Exp. Cell Res. *295*, 315-329.

Miyamoto,K., Miyake,S., and Yamamura,T. (2001). A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. Nature *413*, 531-534.

Mocellin,S., Marincola,F., Rossi,C.R., Nitti,D., and Lise,M. (2004). The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle. Cytokine Growth Factor Rev. *15*, 61-76.

Mohr,S., Leikauf,G.D., Keith,G., and Rihn,B.H. (2002). Microarrays as cancer keys: an array of possibilities. J. Clin. Oncol. *20*, 3165-3175.

Moretta,A., Bottino,C., Vitale,M., Pende,D., Biassoni,R., Mingari,M.C., and Moretta,L. (1996). Receptors for HLA class-I molecules in human natural killer cells. Annu. Rev. Immunol. *14*, 619-648.

Morse,M.A., Clay,T.M., Hobeika,A.C., Osada,T., Khan,S., Chui,S., Niedzwiecki,D., Panicali,D., Schlom,J., and Lyerly,H.K. (2005). Phase I study of immunization with dendritic cells modified with fowlpox encoding carcinoembryonic antigen and costimulatory molecules. Clin. Cancer Res. *11*, 3017-3024.

Moser,A.R., Dove,W.F., Roth,K.A., and Gordon,J.I. (1992). The Min (multiple intestinal neoplasia) mutation: its effect on gut epithelial cell differentiation and interaction with a modifier system. J. Cell Biol. *116*, 1517-1526.

Moustakas,A., Pardali,K., Gaal,A., and Heldin,C.H. (2002). Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. Immunol. Lett. *82*, 85-91.

Mukherjee,P., Ginardi,A.R., Madsen,C.S., Sterner,C.J., Adriance,M.C., Tevethia,M.J., and Gendler,S.J. (2000). Mice with spontaneous pancreatic cancer naturally develop MUC-1-specific CTLs that eradicate tumors when adoptively transferred. J. Immunol. *165*, 3451-3460.

Munder,M., Mallo,M., Eichmann,K., and ModolelI,M. (1998). Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: a novel pathway of autocrine macrophage activation. J. Exp. Med. *187*, 2103-2108.

Nakamura,K., Kitani,A., and Strober,W. (2001). Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. J. Exp. Med. *194*, 629-644.

Nakatsura,T., Senju,S., Itoh,M., Nishimune,Y., and Itoh,K. (2002). Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method. Eur. J. Immunol. *32*, 826-836.

Nastala,C.L., Edington,H.D., McKinney,T.G., Tahara,H., Nalesnik,M.A., Brunda,M.J., Gately,M.K., Wolf,S.F., Schreiber,R.D., Storkus,W.J., and . (1994). Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production. J. Immunol. *153*, 1697-1706.

Nguyen,L.T., Elford,A.R., Murakami,K., Garza,K.M., Schoenberger,S.P., Odermatt,B., Speiser,D.E., and Ohashi,P.S. (2002). Tumor growth enhances cross-presentation leading to limited T cell activation without tolerance. J. Exp. Med. *195*, 423-435.

Niehrs,C. (1996). Growth factors. Mad connection to the nucleus. Nature *381*, 561-562.

Noelle,R.J., Roy,M., Shepherd,D.M., Stamenkovic,I., Ledbetter,J.A., and Aruffo,A. (1992). A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells. Proc. Natl. Acad. Sci. U. S. A *89*, 6550-6554.

North,R.J. and Bursuker,I. (1984). Generation and decay of the immune response to a progressive fibrosarcoma. I. Ly-1+2- suppressor T cells down-regulate the generation of Ly-1-2+ effector T cells. J. Exp. Med. *159*, 1295-1311.

Nowell,P.C. (1976). The clonal evolution of tumor cell populations. Science *194*, 23-28.

Ochsenbein,A.F., Klenerman,P., Karrer,U., Ludewig,B., Pericin,M., Hengartner,H., and Zinkernagel,R.M. (1999). Immune surveillance against a solid tumor fails because of immunological ignorance. Proc. Natl. Acad. Sci. U. S. A *96*, 2233-2238.

Ochsenbein,A.F., Sierro,S., Odermatt,B., Pericin,M., Karrer,U., Hermans,J., Hemmi,S., Hengartner,H., and Zinkernagel,R.M. (2001). Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction. Nature *411*, 1058-1064.

Okabe,M., Ikawa,M., Kominami,K., Nakanishi,T., and Nishimune,Y. (1997). "Green mice" as a source of ubiquitous green cells. FEBS Lett *407*, 313-319.

Okada,K., Komuta,K., Hashimoto,S., Matsuzaki,S., Kanematsu,T., and Koji,T. (2000). Frequency of apoptosis of tumor-infiltrating lymphocytes induced by fas counterattack in human colorectal carcinoma and its correlation with prognosis. Clin. Cancer Res. *6*, 3560-3564.

Old,L.J. and Boyse,E.A. (1964). Immunology of experimental tumours. Annu. Rev. Med. *15*, 167-186.

Old,L.J., Boyse,E.A.C.D.A., and Carswell,E.A. (1962). Antigenic properties of chemically-induced tumors. Ann. N. Y. Acad. Sci. *101*, 80-106.

Onizuka,S., Tawara,I., Shimizu,J., Sakaguchi,S., Fujita,T., and Nakayama,E. (1999). Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. Cancer Res. *59*, 3128-3133.

Onrust,S.V., Hartl,P.M., Rosen,S.D., and Hanahan,D. (1996). Modulation of L-selectin ligand expression during an immune response accompanying tumorigenesis in transgenic mice. J. Clin. Invest *97*, 54-64.

Ornitz,D.M., Hammer,R.E., Messing,A., Palmiter,R.D., and Brinster,R.L. (1987). Pancreatic neoplasia induced by SV40 T-antigen expression in acinar cells of transgenic mice. Science *238*, 188-193.

Ornitz,D.M., Palmiter,R.D., Hammer,R.E., Brinster,R.L., Swift,G.H., and MacDonald,R.J. (1985). Specific expression of an elastase-human growth hormone fusion gene in pancreatic acinar cells of transgenic mice. Nature *313*, 600-602.

Ostrand-Rosenberg,S. (2004). Animal models of tumour immunity, immunotherapy and cancer vaccines. Curr. Opin. Immunol. *16*, 143-150.

Otto,K., Andersen,M.H., Eggert,A., Keikavoussi,P., Pedersen,L.O., Rath,J.C., Bock,M., Brocker,E.B., Straten,P.T., Kampgen,E., and Becker,J.C. (2005). Lack of toxicity of therapy-induced T cell responses against the universal tumour antigen survivin. Vaccine *23*, 884-889.

Ozcelik,H., Schmocker,B., Di Nicola,N., Shi,X.H., Langer,B., Moore,M., Taylor,B.R., Narod,S.A., Darlington,G., Andrulis,I.L., Gallinger,S., and Redston,M. (1997). Germline BRCA2 6174delT mutations in Ashkenazi Jewish pancreatic cancer patients. Nat. Genet. *16*, 17-18.

Paik,S., Shak,S., Tang,G., Kim,C., Baker,J., Cronin,M., Baehner,F.L., Walker,M.G., Watson,D., Park,T., Hiller,W., Fisher,E.R., Wickerham,D.L., Bryant,J., and Wolmark,N. (2004). A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N. Engl. J. Med. *351*, 2817-2826.

Pawelec,G. (2004). Tumour escape: antitumour effectors too much of a good thing? Cancer Immunol. Immunother. *53*, 262-274.

Pende,D., Rivera,P., Marcenaro,S., Chang,C.C., Biassoni,R., Conte,R., Kubin,M., Cosman,D., Ferrone,S., Moretta,L., and Moretta,A. (2002). Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. Cancer Res. *62*, 6178-6186.

Pisa,P., Halapi,E., Pisa,E.K., Gerdin,E., Hising,C., Bucht,A., Gerdin,B., and Kiessling,R. (1992). Selective expression of interleukin 10, interferon gamma, and granulocyte-macrophage colony-stimulating factor in ovarian cancer biopsies. Proc. Natl. Acad. Sci. U. S. A *89*, 7708-7712.

Qin,Z. and Blankenstein,T. (2000). CD4+ T cell--mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. Immunity. *12*, 677-686.

Qin,Z., Schwartzkopff,J., Pradera,F., Kammertoens,T., Seliger,B., Pircher,H., and Blankenstein,T. (2003). A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells. Cancer Res. *63*, 4095-4100.

Quaife,C.J., Pinkert,C.A., Ornitz,D.M., Palmiter,R.D., and Brinster,R.L. (1987). Pancreatic neoplasia induced by ras expression in acinar cells of transgenic mice. Cell *48*, 1023-1034.

Raccurt,M., Lobie,P.E., Moudilou,E., Garcia-Caballero,T., Frappart,L., Morel,G., and Mertani,H.C. (2002). High stromal and epithelial human gh gene expression is associated with proliferative disorders of the mammary gland. J. Endocrinol. *175*, 307-318.

Ravenswaay Claasen,H.H., Kluin,P.M., and Fleuren,G.J. (1992). Tumor infiltrating cells in human cancer. On the possible role of CD16+ macrophages in antitumor cytotoxicity. Lab Invest *67*, 166-174.

Reimer,T., Herrnring,C., Koczan,D., Richter,D., Gerber,B., Kabelitz,D., Friese,K., and Thiesen,H.J. (2000). FasL:Fas ratio--a prognostic factor in breast carcinomas. Cancer Res. *60*, 822-828.

Rivoltini,L., Radrizzani,M., Accornero,P., Squarcina,P., Chiodoni,C., Mazzocchi,A., Castelli,C., Tarsini,P., Viggiano,V., Belli,F., Colombo,M.P., and Parmiani,G. (1998). Human melanoma-reactive CD4+ and CD8+ CTL clones resist Fas ligand-induced apoptosis and use Fas/Fas ligand-independent mechanisms for tumor killing. J. Immunol. *161*, 1220-1230.

Robertson,M.J. and Ritz,J. (1996). Interleukin 12: Basic Biology and Potential Applications in Cancer Treatment. Oncologist. *1*, 88-97.

Roncarolo,M.G., Bacchetta,R., Bordignon,C., Narula,S., and Levings,M.K. (2001). Type 1 T regulatory cells. Immunol. Rev. *182*, 68-79.

Ronnov-Jessen,L., Petersen,O.W., and Bissell,M.J. (1996). Cellular changes involved in conversion of normal to malignant breast: importance of the stromal reaction. Physiol Rev. *76*, 69-125.

Rosenberg,S.A. (1996). Development of cancer immunotherapies based on identification of the genes encoding cancer regression antigens. J. Natl. Cancer Inst. *88*, 1635-1644.

Rosenberg,S.A. (1999). A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity. *10*, 281-287.

Rosenberg,S.A., Yang,J.C., Schwartzentruber,D.J., Hwu,P., Marincola,F.M., Topalian,S.L., Restifo,N.P., Dudley,M.E., Schwarz,S.L., Spiess,P.J., Wunderlich,J.R., Parkhurst,M.R., Kawakami,Y., Seipp,C.A., Einhorn,J.H., and White,D.E. (1998). Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat. Med. *4*, 321-327.

Rottenberg,M.E., Gigliotti-Rothfuchs,A., and Wigzell,H. (2002). The role of IFN-ã in the outcome of chlamydial infection. Curr. Opin. Immunol. *14*, 444-451.

Rowse,G.J., Tempero,R.M., VanLith,M.L., Hollingsworth,M.A., and Gendler,S.J. (1998). Tolerance and immunity to MUC1 in a human MUC1 transgenic murine model. Cancer Res. *58*, 315-321.

Rozenblum,E., Schutte,M., Goggins,M., Hahn,S.A., Panzer,S., Zahurak,M., Goodman,S.N., Sohn,T.A., Hruban,R.H., Yeo,C.J., and Kern,S.E. (1997). Tumor-suppressive pathways in pancreatic carcinoma. Cancer Res. *57*, 1731-1734.

Sadovnikova,E. and Stauss,H.J. (1996). Peptide-specific cytotoxic T lymphocytes restricted by nonself major histocompatibility complex class I molecules: reagents for tumor immunotherapy. Proc. Natl. Acad. Sci. U. S. A *93*, 13114-13118.

Saito,K., Ishikura,H., Kishimoto,T., Kawarada,Y., Yano,T., Takahashi,T., Kato,H., and Yoshiki,T. (1998). Interleukin-6 produced by pancreatic carcinoma cells enhances humoral immune responses against tumor cells: a possible event in tumor regression. Int. J. Cancer *75*, 284-289.

Sakaguchi,S., Sakaguchi,N., Asano,M., Itoh,M., and Toda,M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J. Immunol. *155*, 1151-1164.

Salih,H.R., Rammensee,H.G., and Steinle,A. (2002). Cutting edge: down-regulation of MICA on human tumors by proteolytic shedding. J. Immunol. *169*, 4098-4102.

Sandgren,E.P., Luetteke,N.C., Palmiter,R.D., Brinster,R.L., and Lee,D.C. (1990). Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. Cell *61*, 1121-1135.

Sandgren,E.P., Quaife,C.J., Paulovich,A.G., Palmiter,R.D., and Brinster,R.L. (1991). Pancreatic tumor pathogenesis reflects the causative genetic lesion. Proc. Natl. Acad. Sci. U. S. A *88*, 93-97.

Saran,A., Neveu,T., Covelli,V., Mouton,D., Pazzaglia,S., Rebessi,S., Doria,G., and Biozzi,G. (2000). Genetics of chemical carcinogenesis: analysis of bidirectional selective breeding inducing maximal resistance or maximal susceptibility to 2-stage skin tumorigenesis in the mouse. Int. J. Cancer *88*, 424-431.

Schoenberger,S.P., Toes,R.E., van der Voort,E.I., Offringa,R., and Melief,C.J. (1998). T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. Nature *393*, 480-483.

Schoppmann,S.F., Birner,P., Stockl,J., Kalt,R., Ullrich,R., Caucig,C., Kriehuber,E., Nagy,K., Alitalo,K., and Kerjaschki,D. (2002). Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. Am. J. Pathol. *161*, 947-956.

Schreiber H. and Rowley D.A. (1999). Basic Principles and Clinical Correlates; Inflammation: Inflammation and Cancer. (Philadelphia.: Lippincott Williams & Wilkins), pp. 1117-1129.

Schutte,M., Hruban,R.H., Geradts,J., Maynard,R., Hilgers,W., Rabindran,S.K., Moskaluk,C.A., Hahn,S.A., Schwarte-Waldhoff,I., Schmiegel,W., Baylin,S.B., Kern,S.E., and Herman,J.G. (1997). Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. Cancer Res. *57*, 3126-3130.

Schutte,M., Hruban,R.H., Hedrick,L., Cho,K.R., Nadasdy,G.M., Weinstein,C.L., Bova,G.S., Isaacs,W.B., Cairns,P., Nawroz,H., Sidransky,D., Casero,R.A., Jr., Meltzer,P.S., Hahn,S.A., and Kern,S.E. (1996). DPC4 gene in various tumor types. Cancer Res. *56*, 2527-2530.

Seino,K., Kayagaki,N., Okumura,K., and Yagita,H. (1997). Antitumor effect of locally produced CD95 ligand. Nat. Med. *3*, 165-170.

Senger,D.R., Van de,W.L., Brown,L.F., Nagy,J.A., Yeo,K.T., Yeo,T.K., Berse,B., Jackman,R.W., Dvorak,A.M., and Dvorak,H.F. (1993). Vascular permeability factor (VPF, VEGF) in tumor biology. Cancer Metastasis Rev. *12*, 303-324.

Seo,N., Tokura,Y., Takigawa,M., and Egawa,K. (1999). Depletion of IL-10- and TGF-beta-producing regulatory gamma delta T cells by administering a daunomycin-conjugated specific monoclonal antibody in early tumor lesions augments the activity of CTLs and NK cells. J. Immunol. *163*, 242-249.

Serrano,M., Lee,H., Chin,L., Cordon-Cardo,C., Beach,D., and DePinho,R.A. (1996). Role of the INK4a locus in tumor suppression and cell mortality. Cell *85*, 27-37.

Serrano,M., Lin,A.W., McCurrach,M.E., Beach,D., and Lowe,S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593-602.

Seymour,A.B., Hruban,R.H., Redston,M., Caldas,C., Powell,S.M., Kinzler,K.W., Yeo,C.J., and Kern,S.E. (1994). Allelotype of pancreatic adenocarcinoma. Cancer Res. *54*, 2761-2764.

Shankaran,V., Ikeda,H., Bruce,A.T., White,J.M., Swanson,P.E., Old,L.J., and Schreiber,R.D. (2001). IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature *410*, 1107-1111.

Sharif,S., Arreaza,G.A., Zucker,P., Mi,Q.S., Sondhi,J., Naidenko,O.V., Kronenberg,M., Koezuka,Y., Delovitch,T.L., Gombert,J.M., Leite-De-Moraes,M., Gouarin,C., Zhu,R., Hameg,A., Nakayama,T., Taniguchi,M., Lepault,F., Lehuen,A., Bach,J.F., and Herbelin,A. (2001). Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. Nat. Med. *7*, 1057-1062.

Shimizu,J., Yamazaki,S., and Sakaguchi,S. (1999). Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J. Immunol. *163*, 5211-5218.

Slingluff,C.L., Jr., Petroni,G.R., Yamshchikov,G.V., Hibbitts,S., Grosh,W.W., Chianese-Bullock,K.A., Bissonette,E.A., Barnd,D.L., Deacon,D.H., Patterson,J.W., Parekh,J., Neese,P.Y., Woodson,E.M., Wiernasz,C.J., and Merrill,P. (2004). Immunologic and clinical outcomes of vaccination with a multiepitope melanoma peptide vaccine plus low-dose interleukin-2 administered either concurrently or on a delayed schedule. J. Clin. Oncol. *22*, 4474-4485.

Smyth,M.J., Crowe,N.Y., and Godfrey,D.I. (2001a). NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. Int. Immunol. *13*, 459-463.

Smyth,M.J., Crowe,N.Y., Hayakawa,Y., Takeda,K., Yagita,H., and Godfrey,D.I. (2002). NKT cells - conductors of tumor immunity? Curr. Opin. Immunol. *14*, 165-171.

Smyth,M.J., Godfrey,D.I., and Trapani,J.A. (2001b). A fresh look at tumor immunosurveillance and immunotherapy. Nat. Immunol. *2*, 293-299.

Smyth,M.J., Thia,K.Y., Street,S.E., Cretney,E., Trapani,J.A., Taniguchi,M., Kawano,T., Pelikan,S.B., Crowe,N.Y., and Godfrey,D.I. (2000). Differential tumor surveillance by natural killer (NK) and NKT cells. J. Exp. Med. *191*, 661-668.

Sondak,V.K. and Sosman,J.A. (2003). Results of clinical trials with an allogenic melanoma tumor cell lysate vaccine: Melacine. Semin. Cancer Biol. *13*, 409-415.

Spiotto,M.T., Yu,P., Rowley,D.A., Nishimura,M.I., Meredith,S.C., Gajewski,T.F., Fu,Y.X., and Schreiber,H. (2002). Increasing tumor antigen expression overcomes "ignorance" to solid tumors via crosspresentation by bone marrow-derived stromal cells. Immunity. *17*, 737-747.

Steinman,R.M. and Dhodapkar,M. (2001). Active immunization against cancer with dendritic cells: the near future. Int. J. Cancer *94*, 459-473.

Stevens,T., Conwell,D.L., and Zuccaro,G. (2004). Pathogenesis of chronic pancreatitis: an evidence-based review of past theories and recent developments. Am. J. Gastroenterol. *99*, 2256-2270.

Su,G.H., Hilgers,W., Shekher,M.C., Tang,D.J., Yeo,C.J., Hruban,R.H., and Kern,S.E. (1998). Alterations in pancreatic, biliary, and breast carcinomas support MKK4 as a genetically targeted tumor suppressor gene. Cancer Res. *58*, 2339-2342.

Su,L.K., Kinzler,K.W., Vogelstein,B., Preisinger,A.C., Moser,A.R., Luongo,C., Gould,K.A., and Dove,W.F. (1992). Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science *256*, 668-670.

Su,Z., Dannull,J., Heiser,A., Yancey,D., Pruitt,S., Madden,J., Coleman,D., Niedzwiecki,D., Gilboa,E., and Vieweg,J. (2003). Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells. Cancer Res. *63*, 2127-2133.

Sutmuller,R.P., van Duivenvoorde,L.M., van Elsas,A., Schumacher,T.N., Wildenberg,M.E., Allison,J.P., Toes,R.E., Offringa,R., and Melief,C.J. (2001). Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. J. Exp. Med. *194*, 823-832.

Swain,S.L., Bradley,L.M., Croft,M., Tonkonogy,S., Atkins,G., Weinberg,A.D., Duncan,D.D., Hedrick,S.M., Dutton,R.W., and Huston,G. (1991). Helper T-cell subsets: phenotype, function and the role of lymphokines in regulating their development. Immunol. Rev. *123*, 115-144.

Swift,G.H., Hammer,R.E., MacDonald,R.J., and Brinster,R.L. (1984). Tissue-specific expression of the rat pancreatic elastase I gene in transgenic mice. Cell *38*, 639-646.

Tannenbaum,C.S., Wicker,N., Armstrong,D., Tubbs,R., Finke,J., Bukowski,R.M., and Hamilton,T.A. (1996). Cytokine and chemokine expression in tumors of mice receiving systemic therapy with IL-12. J. Immunol. *156*, 693-699.

Taylor,D.D., Chou,I.N., and Black,P.H. (1983). Isolation of plasma membrane fragments from cultured murine melanoma cells. Biochem. Biophys. Res. Commun. *113*, 470-476.

Taylor,D.D., Gercel-Taylor,C., Lyons,K.S., Stanson,J., and Whiteside,T.L. (2003). T-cell apoptosis and suppression of T-cell receptor/CD3-zeta by Fas ligand-containing membrane vesicles shed from ovarian tumors. Clin. Cancer Res. *9*, 5113-5119.

Tevethia,M.J., Bonneau,R.H., Griffith,J.W., and Mylin,L. (2006). A simian virus 40 large T-antigen segment containing amino acids 1 to 127 and expressed under the control of the rat elastase-1 promoter produces pancreatic acinar carcinomas in transgenic mice. J. Virol. *71*, 8157-8166.

thor Straten,P., Becker,J.C., Guldberg,P., and Zeuthen,J. (1999). In situ T cells in melanoma. Cancer Immunol. Immunother. *48*, 386-395.

Topalian,S.L., Gonzales,M.I., Parkhurst,M., Li,Y.F., Southwood,S., Sette,A., Rosenberg,S.A., and Robbins,P.F. (1996). Melanoma-specific CD4+ T cells recognize nonmutated HLA-DR-restricted tyrosinase epitopes. J. Exp. Med. *183*, 1965-1971.

Topalian,S.L., Rivoltini,L., Mancini,M., Markus,N.R., Robbins,P.F., Kawakami,Y., and Rosenberg,S.A. (1994). Human CD4+ T cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene. Proc. Natl. Acad. Sci. U. S. A *91*, 9461-9465.

Trefzer,U., Herberth,G., Wohlan,K., Milling,A., Thiemann,M., Sherev,T., Sparbier,K., Sterry,W., and Walden,P. (2004). Vaccination with hybrids of tumor and dendritic cells induces tumor-specific T-cell and clinical responses in melanoma stage III and IV patients. Int. J. Cancer *110*, 730-740.

Trinchieri,G. (1995). Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu. Rev. Immunol. *13*, 251-276.

Tsung,K., Dolan,J.P., Tsung,Y.L., and Norton,J.A. (2002). Macrophages as effector cells in interleukin 12-induced T cell-dependent tumor rejection. Cancer Res. *62*, 5069-5075.

Uphoff,C.C., Meyer,C., and Drexler,H.G. (2002). Elimination of mycoplasma from leukemia-lymphoma cell lines using antibiotics. Leukemia *16*, 284-288.

Urban,J.L., Burton,R.C., Holland,J.M., Kripke,M.L., and Schreiber H. (1982). Mechanisms of syngeneic tumor rejection. Susceptibility of host-selected progressor variants to various immunological effector cells. J. Exp. Med. *155*, 557-573.

van der Bruggen,P., Traversari,C., Chomez,P., Lurquin,C., De Plaen,E., Van den,E.B., Knuth,A., and Boon,T. (1991). A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science *254*, 1643-1647.

van der Bruggen,P., Zhang,Y., Chaux,P., Stroobant,V., Panichelli,C., Schultz,E.S., Chapiro,J., Van Den Eynde,B.J., Brasseur,F., and Boon,T. (2002). Tumor-specific shared antigenic peptides recognized by human T cells. Immunol. Rev. *188*, 51-64.

van Elsas,A., Sutmuller,R.P., Hurwitz,A.A., Ziskin,J., Villasenor,J., Medema,J.P., Overwijk,W.W., Restifo,N.P., Melief,C.J., Offringa,R., and Allison,J.P. (2001). Elucidating the autoimmune and antitumor effector mechanisms of a treatment based on cytotoxic T lymphocyte antigen-4 blockade in combination with a B16 melanoma vaccine: comparison of prophylaxis and therapy. J. Exp. Med. *194*, 481-489.

Wagner,M., Greten,F.R., Weber,C.K., Koschnick,S., Mattfeldt,T., Deppert,W., Kern,H., Adler,G., and Schmid,R.M. (2001). A murine tumor progression model for pancreatic cancer recapitulating the genetic alterations of the human disease. Genes Dev. *15*, 286-293.

Wagner,M., Luhrs,H., Kloppel,G., Adler,G., and Schmid,R.M. (1998). Malignant transformation of duct-like cells originating from acini in transforming growth factor transgenic mice. Gastroenterology *115*, 1254-1262.

Wahl,L.M. and Kleinman,H.K. (1998). Tumor-associated macrophages as targets for cancer therapy. J. Natl. Cancer Inst. *90*, 1583-1584.

Wei,C., Willis,R.A., Tilton,B.R., Looney,R.J., Lord,E.M., Barth,R.K., and Frelinger,J.G. (1997). Tissue-specific expression of the human prostate-specific antigen gene in transgenic mice: implications for tolerance and immunotherapy. Proc. Natl. Acad. Sci. U. S. A *94*, 6369-6374.

Wei,W.Z., Morris,G.P., and Kong,Y.C. (2004). Anti-tumor immunity and autoimmunity: a balancing act of regulatory T cells. Cancer Immunol. Immunother. *53*, 73-78.

Whitcomb,D.C. (2004). Value of genetic testing in the management of pancreatitis. Gut *53*, 1710-1717.

Whitcomb,D.C. and Pogue-Geile,K. (2002). Pancreatitis as a risk for pancreatic cancer. Gastroenterol. Clin. North Am. *31*, 663-678.

Whiteside,T.L. and Herberman,R.B. (1995). The role of natural killer cells in immune surveillance of cancer. Curr. Opin. Immunol. *7*, 704-710.

Wiendl,H., Mitsdoerffer,M., Hofmeister,V., Wischhusen,J., Bornemann,A., Meyermann,R., Weiss,E.H., Melms,A., and Weller,M. (2002). A functional role of HLA-G expression in human gliomas: an alternative strategy of immune escape. J. Immunol. *168*, 4772-4780.

Wigginton,J.M., Komschlies,K.L., Back,T.C., Franco,J.L., Brunda,M.J., and Wiltrout,R.H. (1996). Administration of interleukin 12 with pulse interleukin 2 and the rapid and complete eradication of murine renal carcinoma. J. Natl. Cancer Inst. *88*, 38-43.

Winter,H., Hu,H.M., Urba,W.J., and Fox,B.A. (1999). Tumor regression after adoptive transfer of effector T cells is independent of perforin or Fas ligand (APO-1L/CD95L). J. Immunol. *163*, 4462-4472.

Wirth,M., Berthold,E., Grashoff,M., Pfutzner,H., Schubert,U., and Hauser,H. (1994). Detection of mycoplasma contaminations by the polymerase chain reaction. Cytotechnology *16*, 67-77.

Wischhusen,J., Friese,M.A., Mittelbronn,M., Meyermann,R., and Weller,M. (2005). HLA-E protects glioma cells from NKG2D-mediated immune responses in vitro: implications for immune escape in vivo. J. Neuropathol. Exp. Neurol. *64*, 523-528.

Wolf,S.F., Temple,P.A., Kobayashi,M., Young,D., Dicig,M., Lowe,L., Dzialo,R., Fitz,L., Ferenz,C., Hewick,R.M., and . (1991). Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. J. Immunol. *146*, 3074-3081.

Wolfers,J., Lozier,A., Raposo,G., Regnault,A., Thery,C., Masurier,C., Flament,C., Pouzieux,S., Faure,F., Tursz,T., Angevin,E., Amigorena,S., and Zitvogel,L. (2001). Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. Nat. Med. *7*, 297-303.

Woo,E.Y., Yeh,H., Chu,C.S., Schlienger,K., Carroll,R.G., Riley,J.L., Kaiser,L.R., and June,C.H. (2002). Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. J. Immunol. *168*, 4272-4276.

Wu,T.H., Pabin,C.N., Qin,Z., Blankenstein,T., Philip,M., Dignam,J., Schreiber,K., and Schreiber,H. (2004). Long-term suppression of tumor growth by TNF requires a Stat1- and IFN regulatory factor 1-dependent IFN-gamma pathway but not IL-12 or IL-18. J. Immunol. *172*, 3243-3251.

Yeo,C.J., Cameron,J.L., Lillemoe,K.D., Sitzmann,J.V., Hruban,R.H., Goodman,S.N., Dooley,W.C., Coleman,J., and Pitt,H.A. (1995). Pancreaticoduodenectomy for cancer of the head of the pancreas. 201 patients. Ann. Surg. *221*, 721-731.

Zarour,H.M., DeLeo,A.B., and Storkus,W.J. (2003). Cancer Immunology - Tumor Antigens. In Holland & Frei Cancer Medicine, D.Kufe, R.Bast, B.Hait, W.Hong, R.Pollack, R.Weichselbaum, J.Holland, and E.Frei, eds. (Hamilton: BC Decker).

Zarour,H.M., Maillere,B., Brusic,V., Coval,K., Williams,E., Pouvelle-Moratille,S., Castelli,F., Land,S., Bennouna,J., Logan,T., and Kirkwood,J.M. (2002). NY-ESO-1 119-143 is a promiscuous major histocompatibility complex class II T-helper epitope recognized by Th1- and Th2-type tumor-reactive CD4+ T cells. Cancer Res. *62*, 213-218.

Zarour,H.M., Storkus,W.J., Brusic,V., Williams,E., and Kirkwood,J.M. (2000). NY-ESO-1 encodes DRB1*0401-restricted epitopes recognized by melanoma-reactive CD4+ T cells. Cancer Res. *60*, 4946-4952.

Zeng,G., Touloukian,C.E., Wang,X., Restifo,N.P., Rosenberg,S.A., and Wang,R.F. (2000). Identification of CD4+ T cell epitopes from NY-ESO-1 presented by HLA-DR molecules. J. Immunol. *165*, 1153-1159.

Zeng,G., Wang,X., Robbins,P.F., Rosenberg,S.A., and Wang,R.F. (2001). CD4(+) T cell recognition of MHC class II-restricted epitopes from NY-ESO-1 presented by a prevalent HLA DP4 allele: association with NY-ESO-1 antibody production. Proc. Natl. Acad. Sci. U. S. A *98*, 3964-3969.

Zou,J.P., Yamamoto,N., Fujii,T., Takenaka,H., Kobayashi,M., Herrmann,S.H., Wolf,S.F., Fujiwara,H., and Hamaoka,T. (1995). Systemic administration of rIL-12 induces complete tumor regression and protective immunity: response is correlated with a striking reversal of suppressed IFN-gamma production by anti-tumor T cells. Int. Immunol. *7*, 1135-1145.

Acknowledgement

This dissertation was performed in the work group Tumour Immunology of Prof. Dr. Tim Greten and Dr. Firouzeh Korangy, in the Department of Gastroenterology of the Medizinische Hochschule Hannover, Germany.

I would like to express my gratitude to Prof. Dr. Tim Greten, for his very good supervision and his confidence during this project. Throughout this project, I have been encouraged and offered valuable criticism, and continuous advice, concerning the technical aspects of this work by Prof. Dr. Tim Greten.

Further, I want to thank Dr. Firouzeh Korangy for her great support, supervision, fruitful discussions, and proof reading of my thesis. She was always available to me and willing to answer my questions concerning this work.

I would like to thank very much Prof. Dr. Walter Müller for being my second referee and Prof. Dr. Achim Gossler as third referee.

I am thankful that Prof. Dr. Michael Manns provided me a suitable workplace in the Department of Gastroenterology.

To all members and former members of the “AG Greten”-lab, I offer my great appreciation for their support, guidance, help, advice and smiles throughout my PhD study, and special thanks to my good colleagues Sonja and Fei.

I am very grateful to the members of the animal facility, especially to Rosi Mörstedt for her good advice, care, feeding, and cleaning of our mice.

In addition, I want to thank the AG Ott, AG Brunner and LEBAO group for sharing materials, equipment, and their interesting and valuable conversations.

Many thanks to Dr. Thomas Mair and Dr. Marcus Hauser of the Department of Biophysics, Otto-von-Guericke University of Magdeburg for fruitful discussions and for providing an office to me.

In particular, I would like to thank M.Sc. Dahlin and my Dad for their great support.

I am very grateful to my parents, my sister, my girlfriend Satenik and especially to my Oma for their love, unrestricted support and motivation throughout all the years.

Erklärung zur Dissertation

Hierdurch erkläre ich, dass die Dissertation „Tumour-specific immune responses and tumour stroma analysis in a murine model for pancreatic adenocarcinoma“ selbständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, 11. Januar 2007

_____

Benjamin Vermeer

Solemn declaration

I hereby solemnly declare that I composed the present thesis entitled "**Tumour-specific immune responses and tumour stroma analysis in a murine model for pancreatic adenocarcinoma**" independently and on my own responsibility. All means and institutions that were used have been cited completely. No other texts or aids were used other than those, which were duly cited.

Further, I affirm that this doctoral thesis was not used as the basis for my diploma nor for any similar examination.

I am aware of the procedure of the doctorate constitution.

Hannover, January 11, 2007

Benjamin Vermeer

Publication and Presentations

Publication

Garbe, A. I.,[1] **Vermeer, B.**,[1] Gamrekelashvili, J., von Wasielewski, R., Greten, F.R., Westendorf, A.M., Buer, J., Schmid, R.S., Manns, M.P., Korangy, F., and Greten, T.F.

"Genetically Induced Pancreatic Adenocarcinoma Is Highly Immunogenic and Causes Spontaneous Tumor-Specific Immune Responses", *Cancer Res*. 2006; 66(1): 508-16.

[1] A. Garbe and B. Vermeer contributed equally to this work.

Presentations at conferences

36th „Annual Meeting of the German Society of Immunology (DGfI)", Kiel, Germany, 21st - 24rd September 2005, „Genetically Induced Pancreatic Adenocarcinoma Is Highly Immunogenic and Causes Spontaneous Tumor-Specific Immune Responses" (poster)

„**Gemeinsame Jahrestagung der Deutschen, Österreichischen und Schweizerischen Gesellschaften für Hämatolgie und Onkologie**", Hannover, Germany, 1st – 5th of October 2005, „Genetically Induced Pancreatic Adenocarcinoma Is Highly Immunogenic and Causes Spontaneous Tumor-Specific Immune Responses" (poster)

Invited lecture

Otto-von-Guericke Universität Magdeburg, Department of Biophysics, „Immune Responses against Spontaneous Tumours in a Mouse Model for Pancreatic Cancer", Magdeburg, Germany, May 4, 2006 (oral presentation)

Curriculum Vitae

Name	Benjamin Vermeer
Address	Kronenstraße 28, 30161 Hannover, Germany
Date of birth	1972/08/21
Marital status	Not married
Nationality	Dutch

School

1982-1992

Gymnasium Martin-Luther-Schule, Marburg, Germany

Final degree: **Allgemeine Hochschulreife**

Study

1994-1999

Medical Biology at the Universiteit van Amsterdam, The Netherlands

1998-1999

Within the scope of the study, research project in molecular biology und immunology „Cloning and Expression of specific T_{Helper} Cell Markers“ at the „Institute for Child Health Research“, University of Western Australia, Perth, Australia

Final degree: **Master of Science** (M.Sc.)

Working experience

2000-2002

Research collaborator, Max-Planck-Institut für Molekulare Genetik, Berlin, Germany

2002-2004

Research collaborator, Deutsches Rheumaforschungs-zentrum, Berlin, Germany

Dissertation

Since 2004

Thesis „**Tumour-specific immune responses and tumour stroma analysis in a murine model for pancreatic adenocarcinoma**“ at the Gottfried Wilhelm Leibniz Universität Hannover and Medizinischen Hochschule Hannover, Germany Department of Tumourimmunology under the leadership of Prof. Dr. Tim Greten

Award

1999 research work for „Master of Science“ awarded with the “**Prof. Dr. Ph. H. Quanjer Preis**” of „Nederlands Astma Fonds“

Publication

Garbe A.I. and Vermeer B. (both first authors) et al. „Genetically Induced Pancreatic Adenocarcinoma Is Highly Immunogenic and Causes Spontaneous Tumor-Specific Immune Responses“, *Cancer Res*. 2006; 66(1): 508-16

Conferences

36th „Annual Meeting of the German Society of Immunology (DGfI)“, Kiel, Germany, 21st - 24rd September 2005, „Genetically Induced Pancreatic Adenocarcinoma Is Highly Immunogenic and Causes Spontaneous Tumor-Specific Immune Responses“ (poster)

„**Gemeinsame Jahrestagung der Deutschen, Österreichischen und Schweizerischen Gesellschaften für Hämatolgie und Onkologie**“, Hannover, Germany, 1st – 5th of October 2005, „Genetically Induced Pancreatic Adenocarcinoma Is Highly Immunogenic and Causes Spontaneous Tumor-Specific Immune Responses“ (poster)

Presentations

UniversitätsSpital Zürich, Department Experimental Rheumatology, „Expression of Specific T_{Helper} 1 und 2 Surface Markers in Human Rheumatoid- and Osteoarthritis", Zürich, **Switzerland**, November 3, 2003

Otto-von-Guericke Universität Magdeburg, Department Biophysics, „Immune Responses against Spontaneous Tumours in a Mouse Model for Pancreatic Cancer", Magdeburg, Germany, May 4, 2006

Hannover, January 11, 2007

Benjamin Vermeer