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# IMMUNOMODULATORY IMPACT OF INTERFERON-ALPHA IN COMBINATION WITH CHEMORADIATION OF PANCREATIC ADENOCARCINOMA (CapRI)

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**CONTENTS**

**ABBREVIATION LIST**

5-FU – 5-Fluorouracil

ADCC – antibody-dependent cellular cytotoxicity

APC – antigen presenting cells

BCA – Bicinchoninic acid

BFA – Brefeldin A

CAD – caspase activated deoxyribonuclease

CapRI – adjuvant treatment of pancreatic adenocarcinoma with chemoradioimmunotherapy

CD – clusters of differentiation

CDDP – Cisplatin

CMA – Concanamycin A

CR – chemoradiation

CTL – cytotoxic T cells

DC – dendritic cells

DISC – death inducing signalling complex

EBV – Epstein-Barr virus

EGF-R – endothelial growth factor receptor

ELISA – enzyme-linked immunosorbent assay

ELISPOT – enzyme-linked immunospot

EMSA – electrophoretic mobility shift assay

ER – endoplasmic reticulum

FADD – Fas associated death domain

FasL – Fas ligand

FITC – fluorescein isothiocyanate

Gy – gray

HLA – human leukocyte antigen

IFN – interferon

IFN-$\alpha$ - interferon-alpha

IKK – I$\kappa$B kinase

IL – interleukin

KIRs – killer inhibitory receptors

LFA – lymphocyte function-associated antigen

MACS – magnetic activated cell sorting

MHC – major histocompatibility complex

MTT - 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide

NCRs – natural cytotoxicity receptors

NEMO – NF-κB essential modulator

NK cells – natural killer cells

PBL – peripheral blood lymphocytes

PBMC – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PI – propidium iodide

RHD – Rel homology domain

RT – radiotherapy

SRBCs – sheep red blood cells

STATs - signal-transducing activators of transcription

TNF – tumor necrosis factor

VEGF – vascular endothelial growth factor

# 1. INTRODUCTION

## 1.1 Pancreatic cancer – epidemiology and prognosis

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States and the sixth in Europe, with more than 200,000 deaths annually worldwide.

There is a combination of environmental, lifestyle and genetic factors influencing the risk for pancreatic cancer. Tobacco smoking is recognized as a major risk factor, while alcohol use, coffee consumption and exposure to organochlorine or hydrocarbon solvents were associated with increased risk for pancreatic cancer. The risk increases also with increased intake of fats, animal proteins, complex carbohydrates, products containing high levels of nitrite compounds as well as with the increase of the amount of calories consumed. Although in most cases the cause is unknown, about 5 to 10% of the pancreatic cancer cases have a large genetic component. It was reported a 3 to 5-fold higher risk among individuals that have first-degree relatives with pancreatic cancer. Several genetic syndromes have been associated with pancreatic cancer: familial breast cancer (BRCA2 mutations), familial atypical multiple mole melanoma syndrome, hereditary pancreatitis, hereditary non-polyposis colorectal carcinoma, Peutz-Jeghers syndrome. These syndromes account only for a small fraction of the familial pancreatic cancer. A small percent (3-4%) of pancreatic cancers may be attributed to chronic pancreatitis. A history of diabetes mellitus type II developed 10 or more years prior to pancreatic cancer diagnosis can be causally related to the pancreatic tumor. This association may be the result of prolonged exposure to elevated postload glucose concentration and hyperinsulinemia. Overweight and obese individuals have also an increased risk for pancreatic cancer (Michaud, 2004).

More than 95% of the pancreatic cancers are of exocrine origin (adenocarcinoma). Carcinoma of the exocrine pancreas has an especially poor prognosis. The five-year survival rate is less than 1% with a median survival of 4-6 months. Even after surgical intervention with a curative intention, the two-year survival rate is in specialized centers at best 25% (Raraty et al. 2002).

Before becoming invasive, pancreatic ductal adenocarcinoma undergoes noninvasive stages including pancreatic intraepithelial neoplasia (PanINs).

In early stages pancreatic carcinoma is usually asymptomatic unless the tumor arises in the pancreatic head near the intrapancreatic portion of the bile duct, obstructing it and producing

jaundice. In advanced phases, the symptoms associated with pancreatic carcinoma include pain due to the tumor invasion of the celiac and mesenteric plexus; symptoms of pancreatic exocrine insufficiency (malabsorption, steatorrhea) as well as fatigue, weight loss, anorexia. Most patients with pancreatic cancer have hyperglycemia.

### 1.2 Adjuvant treatment of pancreatic adenocarcinoma

The best treatment for pancreatic adenocarcinoma is surgical resection, but, at the moment of diagnosis, it is possible in only 10-15% of the patients. Most treatment failures are due to local recurrence and/or hepatic metastases which occur within one or two years after surgery. Chemo- and/or radiotherapy used as adjuvant (postoperative) treatment may improve long-term survival.

Various chemo- and/or radiation regimens have been tested in small studies for adjuvant treatment of pancreatic carcinoma. Most of them use 5-FU or gemcitabine as chemotherapeutic agent, sometimes in combination with other agents such as CDDP. These protocols are often combined with radiation (Tsai et al. 2003).

5-Fluorouracil (5-FU) is a fluorinated pyrimidine differing from the normal RNA substrate, uracil, by a fluorinated number 5 carbon. Its mechanism of action consists in blocking the methylation reaction of deoxyuridylic acid to thymidylic acid, interfering with DNA synthesis. This creates a thymine deficiency that provides unbalanced growth and cell death.

Cisplatin (CDDP) is a platinum compound whose cytotoxic action is produced by inhibition of the DNA precursors, and, to a lesser extent, of RNA and protein synthesis. Its major mechanism of action is the intrastrand crosslinking of DNA due to platinum. Cisplatin does not possess significant cell-cycle dependency; therefore its toxicity is similar in all phases of the cell cycle.

Radiotherapy (RT) is delivered mainly with high-energy photons (gamma-rays or X-rays) and charged particles (electrons). Gamma-rays originate from excited nuclei and are produced by radioactive sources, whereas X-rays are produced by electron energy transitions within the atom or through the deceleration of high-kinetic energy electrons using linear accelerators. The most commonly used therapeutic charged particles are the electrons produced by a linear accelerator. The unit of measurement for the quantification of radiation is the “absorbed dose” which is the energy absorbed per unit mass. It represents the energy deposited by secondary charged particles in the medium. The unit of absorbed dose is the gray (Gy) which is defined

as the absorption of 1 joule per kilogram. Radiation randomly interacts with molecules within the cell. The cellular response to radiation consists of DNA damage (single- and double-strand breaks in the sugar-phosphate backbone of the DNA molecule, cross-links between DNA strands and chromosomal proteins). Radiation damage is primarily manifested by the loss of cellular reproductive capacity, but also alternatively some cell types are killed through apoptosis induction. Both 5-FU and cisplatin act as radiosensitizers.

The ESPAC-1 trial is the only trial to date that enrolled a large number of patients (n=550). The trial tested the hypothesis that chemoradiotherapy (40 Gy and weekly 5-FU) with or without 6 months additional chemotherapy (5FU, 425mg/m², day 1-5 and folinic acid, 20mg/m², d1-5, repeated monthly) provided an improvement in survival benefits compared to no adjuvant treatment. In a 2×2 factorial design, the 5-year survival rate for patients receiving chemoradiation was 10.0% and 19.6% without, and 21.1% for patients receiving chemotherapy and 8.4% without. The authors concluded from this that radiochemotherapy shows only limited success (Neoptolemos et al. 2004).

Investigators from the Virginia Mason Clinic have recently published data from a phase II trial where cisplatin (CDDP), 5-fluorouracil (5-FU), interferon alpha-2b (IFN-α), and external-beam radiation (RT) were administered following pancreaticoduodenectomy. They have treated 43 patients with mainly stage III tumors. 84% had positive lymph nodes and 19% had positive resection margins. After a mean follow-up of 31.9 months, 67% of the patients were still alive. The actuarial overall survival rates for one, two and five years were 95%, 64%, and 55%, respectively. Due to the toxicity of this combination treatment 42% patients were hospitalized during treatment, but there were no treatment-related deaths (Picozzi et al. 2003). Our research group termed this regimen CapRI for *a*djuvant treatment of *p*ancreatic adenocarcinoma with *C*hemo*R*adio*I*mmunotherapy.

### 1.3 Role of interferon-alpha (IFN-α) in the combined modality treatment of pancreatic adenocarcinoma

Interferons are a family of molecules that include more than 15 expressed genes and a number of pseudogenes coded on chromosome 9 (except IFN-γ). There are two types of interferons: type I includes IFN-α, IFN-β and IFN-ω, while type II is represented by IFN-γ.

IFN-α belongs to the group of type I interferons, which are already used in cancer therapy (e.g. malignant melanoma, renal cell carcinoma, hairy cell leukemia, chronic myeloid

leukemia; Belardelli et al. 2002; Kirkwood, 2002). IFN-α is produced by monocytes/macrophages, lymphoblastoid cells, fibroblasts and plasmacytoid dendritic cells (Gutterman, 1994). Several other cell types are known to produce type I interferons after viral infections. IFN-α binds to the IFN-α/β receptor CD118; binding to the EBV-receptor CD21 is also described (Delcayre et al. 1991). The IFN receptor is coupled to a Janus-family tyrosine kinase, which phosphorylates signal-transducing activators of transcription (STATs), which translocate to the nucleus where they activate the transcription of several different genes inducing the synthesis of host cell proteins that contribute to the inhibition of viral replication (Domanski et al. 1995; Mogensen et al. 1999).

In addition to its anti-viral properties, IFN-α exhibits several other features that might be of interest, especially for the use in combination treatments of cancer. Some of these features are: a) direct inhibitory effects on tumor cell growth (Pfeffer et al. 1998; Iacopino et al. 1996); b) radio- and chemosensitizing effects described for 5-FU, cisplatin and dacarbazin (Kurzrock R et al. 1991; Holsti et al. 1987); c) anti-angiogenic properties (Decatris et al. 2002; Solorzano et al. 2003; Wang et al. 2003); d) enhancement of immunogenicity of tumors. This phenomenon is provoked by an increase of MHC class I expression which enhance immune recognition (Pfeffer et al. 1998) and e) modulation of the immune system: IFN-α plays an essential role in the differentiation, maturation and function of dendritic cells (DC), enhances the survival of T cells by expression of anti-apoptotic genes, induces the generation of $CD8^+$ memory cells, enhances macrophage activities, and activates natural killer (NK) cells thus releasing cytokines (Pfeffer et al. 1998; Marrak et al. 1999; Matikainen et al. 1999; Paquette et al. 1998).

Previously, our research group has tested the impact of direct effects, radio- and chemosensitizing properties as well as anti-angiogenic features of IFN-α in the CapRI-regimen and found only moderate influence by these mechanisms (Ma J et al. 2005).

### 1.4 Antitumoral immune mechanisms

There are two types of immune responses: the innate immune response, and a specific immune response known as adaptive immune response. Innate immunity involves granulocytes (also called polymorphonuclear leucocytes), macrophages, natural killer (NK) cells and the complement system. Adaptive immune responses depend upon lymphocytes and dendritic cells (DC).

There are two major types of lymphocytes: B lymphocytes (B cells) which mature in the bone marrow and when activated differentiate into plasma cells that secrete antibodies; and T lymphocytes (T cells) which mature in the thymus and are divided in two main classes. One class differentiates upon activation into cytotoxic T cells (CTL) which kill the recognized target cell, while the second class of T cells differentiates into helper T cells which activate other cells such as B cells and macrophages or have regulatory properties. Antigen recognition by T cells is made through the T cell receptor which recognizes the antigen in the form of a complex of a foreign peptide bound to a major histocompatibility complex (MHC) molecule on the surface of the target cell. The two classes of T cells are distinguished through the expression of cell surface proteins CD4 (for helper T cells) and CD8 (for cytotoxic T cells) which play the role of co-receptors. As co-receptors in the antigen recognition, CD4 binds to the MHC class II molecule and CD8 to the MHC class I. MHC class I molecules collect peptides derived from proteins synthesized in the cytosol and are thus important in anti-tumoral immunity. MHC class II molecules bind peptides derived from proteins internalized into intracellular vesicles. Mature recirculating T cells which have not yet encountered their specific antigens are known as naïve T cells. They are induced to proliferate and differentiate into armed effector T cells the first time when they encounter their specific antigen in the form of a peptide:MHC complex on the surface of an activated antigen-presenting cell (APC). The most important antigen-presenting cells are the dendritic cells.

A third lineage of lymphoid cells is represented by the natural killer (NK) cells which comprises approximately 15% of all circulating lymphocytes. They develop in the bone marrow from the common lymphoid progenitor cell and circulate in the blood as large lymphocytes with distinctive cytotoxic granules. NK cells lack antigen specific receptors, are part of the innate immune system and are able to recognise and kill a limited range of tumor cells or virus-infected cells. They are activated in response to interferons or macrophage-derived cytokines. NK cells have two types of surface receptors that control their cytotoxic activity. The activating receptors that trigger killing by NK cells are represented by NKG2D and the natural cytotoxicity receptors (NCRs): NKp30 and NKp46. A second set of receptors inhibit activation and prevent NK cells from killing normal host cells. They belong to the immunoglobulin superfamily and are called killer inhibitory receptors (KIRs). These inhibitory receptors are specific for MHC class I alleles which explains why NK cells selectively kill target cells with low MHC class I expression. Another possible mechanism through which NK cells recognize their targets is by recognizing alterations in MHC class I expression.

The majority of circulating NK cells are characterised by the $CD56^{+}CD16^{+}$ surface phenotype. There were described reciprocal NK cells – dendritic cells (DC) interactions which regulate the quality and the intensity of innate immune responses (Moretta, 2005). Human NK cells can be divided into two subsets based on their cell-surface density of CD56: $CD56^{bright}$ and $CD56^{dim}$. The $CD56^{dim}$ subset is more naturally cytotoxic and expresses higher levels of KIRs and CD16. $CD56^{bright}$ subset has the capacity to produce abundant cytokines following activation of monocytes, but has low natural cytotoxicity and is $CD16^{dim}$ or $CD16^{-}$ (Cooper et al. 2001). It was shown that more than 95% of peripheral blood and 85% spleen NK cells are $CD56^{dim}CD16^{+}$ and express perforin, the natural cytotoxicity receptors (NCRs), as well as, in part, KIRs. NK cells in lymph nodes have mainly a $CD56^{bright}CD16^{-}$ phenotype and lack perforin, KIRs and all NCR expression (Ferlazzo et al. 2004). CD16 (FcγRIII) triggers NK cell recognition of antibody-opsonized target cells during antibody-dependent cellular cytotoxicity (ADCC).

Anti-tumoral immunity is based on the ability of cytotoxic T lymphocytes to recognize specific antigens on human tumors as the tumor rejection antigens which are peptides of cell proteins expressed by MHC class I molecules. A mechanism for tumor escape from the immune recognition is to lose the expression of a particular MHC class I molecule, perhaps through immunoselection by T cells specific for a peptide presented by that MHC class I molecule. When a tumor loses expression of all MHC class I molecules it cannot be recognized by cytotoxic T cells but it becomes more susceptible to NK cell killing (Janeway CA et al. 2001).

### 1.5 Mechanisms of apoptosis induction

Cell death typically follows one of two pathways: apoptosis (programmed cell death) or necrosis (accidental cell death).

Apoptosis (or programmed cell death) begins when a cell activates its own destruction by initiating a cascade of events. It is a form of cell death characterized by morphological changes: the cytoplasm begins to shrink following the cleavage of lamin and actin filaments. Nuclear condensation can also be observed following the breakdown of chromatin and nuclear structural proteins, and in many cases the nuclei of apoptotic cells take on a "horse-shoe" like appearance. Apoptotic cells also undergo plasma membrane changes that trigger the macrophage response, as the translocation of phosphatidylserine from the inner cytoplasmic membrane to the cell surface. Morphological changes of the membrane appear

towards the end of the apoptotic process as membrane blebs or blisters. Small vesicles called apoptotic bodies can also be observed.

Apoptosis involves the activation of a family of cysteine aspartate proteases (caspases) which pre-exist in unstimulated cells as inactive zymogens and are activated by proteolytic cleavage. These proteins breakdown or cleave key cellular substrates that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus into characteristic 200-base-pair fragments. The result of these biochemical changes is the appearance of morphological changes in the cell.

There are two major pathways in inducing the apoptosis: the extrinsic pathway involving the death receptors (members of the tumor necrosis factor receptor family engaged by their ligands) and the intrinsic pathway (mitochondrial).

Apoptotic signalling through the extrinsic pathway is triggered by the engagement of members of the tumor necrosis factor (TNF) receptor family (TNFR1, Fas/CD95, DR3, DR4, DR5, and DR6) by their ligands (TNFα, lymphotoxin α, FasL, Apo3L, and TNF-related apoptosis-inducing ligand), resulting in receptor trimerization and activation of procaspase-8 and -10. DNA damage or cell stress leading to the activation of p53 or the Bcl-2 family proapoptotic proteins such as Bax and Bak initiate the intrinsic pathway and induce the mitochondrial release of apoptogenic molecules such as cytochrome c, apoptosis-inducing factor, or Smac/DIABLO (second mitochondria-derived activator of caspases/direct inhibitors of apoptosis [IAP]-binding protein with low pI). Cytochrome c binds to the apoptotic protease-activating factor-1, and this dimer forms the apoptosome complex with procaspase-9. Interconnection exists between the extrinsic and intrinsic pathways, since caspase-8 proteolysis through the extrinsic pathway leads to cleavage and activation of Bid, a proapoptotic member of the Bcl-2 family. Once cleaved, truncated Bid incorporates into the mitochondrial membrane and induces cytochrome c release, which in turns leads to apoptosome formation and thus the intrinsic apoptotic pathway.

Necrosis by contrast is not self initiated and results from an injury to the cell. The characteristics of necrosis are a loss of membrane integrity, inflammation, and significantly less DNA degradation as compared with apoptosis.

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells kill their targets by programming them to undergo apoptosis. They exert cytotoxicity via two distinct pathways: the perforin/granzyme and the Fas (CD95, Apo-1)/Fas ligand (FasL) pathway.

Perforins and granzymes are preformed cytotoxic enzymes contained in specialized lytic granules which are released in a calcium-dependent manner upon recognition of antigen on the surface of a target cell. After their release from the granules, the perforins polymerize to form transmembrane pores in the target cells, allowing the entry of another class of cytotoxic proteins contained in the lytic granules, the granzymes, resulting in apoptosis of the target cell. The granzymes are proteases with a role in triggering apoptosis in the target cell. Granzyme B can cleave caspase CPP-32 which activates CAD (caspase-activated deoxyribonuclease) by cleaving an inhibitory protein (ICAD). CAD is believed to be the final effector of DNA degradation in apoptosis (Janeway CA et al. 2001). Granzyme B was proved as a major effector of target cell lysis by NK cells (Mahrus et al. 2005).

Fas is a death receptor, member of the TNF receptor family, expressed on the cell surface of most tissues. Ligation with its natural ligand, FasL, expressed on activated cytotoxic T lymphocytes and NK cells results in formation of a death inducing signalling complex (DISC) formed by trimerization of Fas followed by recruitment of an adaptor molecule (Fas associated death domain – FADD) and pro-caspase 8. Pro-caspase 8 then becomes activated leading to a protease cascade ending with the activation of CAD which enters the nucleus and cleaves the DNA. Fas-based cytotoxicity depends on de novo synthesis of RNA and protein and is probably dependent on vacuolar organelles such as Golgi apparatus (Waterhouse et al. 2002; Janeway CA et al. 2001).

Perforin/granzyme pathway can be selectively inhibited using Concanamycin A (CMA), a macrolide which acts as a specific inhibitor of vacuolar type $H^+$-ATPase. Vacuolar type $H^+$-ATPase acidifies vacuolar organelles such as lysosomes and Golgi apparatus, and acidification plays a critical role in maintaining the integrity of vacuolar organelles. Increased pH of the lytic granules accelerates the degradation of perforin (Waterhouse et al. 2004; Kataoka et al. 1996).

Fas/FasL pathway can be selectively inhibited using Brefeldin A (BFA). Brefeldin A is a macrocyclic lactone and acts as an inhibitor of intracellular glycoprotein transport through the prevention of Endoplasmic Reticulum (ER) – Golgi protein trafficking. Brefeldin A also affects the morphology and functional properties of lysosomes (Kataoka et al. 1996).

## 1.6 Pathways of nuclear transcription factor NF-κB activation and its role in carcinogenesis

Transcription factors are proteins able to bind specifically to short DNA sequences located in gene promoters and to interact directly or indirectly with the transcription mechanism modulating the transcription level of target genes.

The nuclear transcription factor NF-κB is implicated in regulation of apoptosis (acting mainly as an antiapoptotic factor), drug resistance, carcinogenesis and inflammation (through the activation of genes that contribute to adaptive immunity and the secretion of proinflammatory cytokines).

NF-κB is a dimeric complex of various subunits that belong to the Rel family which comprises two groups of proteins: those that do not require proteolytic processing and those that require proteolytic processing. The first group consists of RelA (also known as p65), RelB and c-Rel. The second group includes NF-κB1 (also known as p105) which is processed to produce the mature p50, and NF-κB2 (also known as p100) which is processed to produce the mature p52. Due to the presence of a strong transcriptional activation domain, RelA is responsible for most of NF-κB transcriptional activity. NF-κB proteins share a Rel homology domain (RHD) which allows DNA binding, dimerization and nuclear localization. NFκB resides normally in the cytoplasm were it is sequestrated by a family of inhibitors (IκB). Upon stimulation by various activators (e.g. cytokines, lipopolysaccharide, growth factors, stress inducers, chemotherapeutic agents) IκB is phosphorylated and NFκB is free to enter the nucleus. Activation of NF-κB through IκB phosphorylation is dependent on the activation of IκB kinases (IKK). The IKK complex is composed of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit IKKγ also called NF-κB essential modulator (NEMO). There are two pathways for the activation of NF-κB: a classical pathway with a bias towards inflammation and an alternative pathway with a bias towards differentiation, architecture and proliferation within the B cell compartment. The classical pathway is activated by stimuli such as TNF-α, IL-1 or lipopolysaccharide which involves the phosphorylation of p105 by the IKKβ subunit and results in nuclear translocation of RelA-p50. The alternative pathway is activated by TNF family members as CD40L, lymphotoxin B; it involves the phosphorylation of p100 by IKKα and the nuclear translocation of RelB-p52. IKKγ (NEMO) is absolutely required for the NF-κB activation through the classical pathway, but not for the alternative pathway (Delhalle et al. 2004).

NF-κB has an antiapoptotic role through different mechanisms: a) transcriptional activation of many antiapoptotic genes whose products are implicated in blocking the apoptotic cascade (e.g. IAPs, c-FLIP, Bcl-2 family proteins); b) repression of several proapoptotic genes (e.g. bax); c) interference with the proapoptotic function of p53.

NF-κB activity confers an increased resistance of cancer cells to chemotherapeutic agents (Arlt et al. 2002; Arlt et al. 2003). It regulates genes that modulate cellular drug uptake or elimination, such as multidrug resistance-1 (Bentires-Alj et al. 2003).

### 1.7 Aims of the study

The purpose of this work is to investigate whether the addition of IFN-α to chemoradiotherapy has a direct cytotoxic effect on pancreatic carcinoma cell lines influencing the proliferation rate and inducing apoptosis, as well as to establish the impact of the immunomodulatory effect of IFN-α in chemoradioimmunotherapy of pancreatic carcinoma. The role IFN-α prestimulation on the cytotoxic activity of different types of lymphocytes will be analyzed, investigating whether this is a direct effect of IFN-α on the lymphocytes or an indirect one by monocyte activation and which are the mechanisms used by the lymphocytes to induce apoptosis in the tumor cells.

Further, the influence of chemo-, radiotherapy, their combinations with IFN-α and chemoradioimmunotherapy on the susceptibility of pancreatic carcinoma cells to the cytotoxic activity of IFN-α prestimulated lymphocytes will be analyzed.

The influence of IFN-α on the immunogenicity of pancreatic carcinoma cell lines and the hypothesis of NF-κB inhibition following IFN-α treatment will be also investigated.

## 2. MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Chemicals

| | |
|---|---|
| Amersham (Braunschweig) | [$\gamma^{32}$-P]ATP (3,000 Ci/mmol at 10 Ci/ml); Repel-Silane ES |
| BD Pharmingen (Heidelberg) | AnnexinV-FITC/PI kit; Human Granzyme B ELISPOT Set |
| Biochrom (Berlin) | Fetal Bovine Serum (FBS); Trypan Blue 0,5% |
| Biomedica (Vienna, Austria) | "EZ4U" kit (nonradioactive cell proliferation and cytotoxicity assay) |
| Bristol-Myers Squibb (München) | Cisplatin (PLATINEX®) |
| CarlRoth (Karlsruhe) | HEPES; Sodium Chloride |
| Essex Pharma (München) | Interferon-α (Intron A) |
| Grüssing (Filsum) | Magnesium Chloride |
| Gry-Pharma (Kirchzarten) | 5-Fluorouracil |
| Hartmann Analytic (Braunschweig) | [Cr51] Sodium Chromate (2 mCi / 400µl) |
| Mallinckrodt Baker (Deventer, The Nederlands) | Sodium Acetate Trihydrate |
| Merck (Darmstadt) | Acetic Acide, Potassium Chloride |
| Miltenyi (Bergisch Gladbach) | MACS CD56 Micro Beads; MACS LS Separation Columns |
| PAA Laboratories (Cölbe) | Bovine Serum Albumine (BSA); Dulbecco's phosphate buffered saline (PBS); HEPES Buffer Solution; Lymphozyten Separationsmedium; RPMI 1640 with L-Glutamine; Trypsin/EDTA |
| Packard Bioscience (Groningen, The Nederlands) | Ultima Gold (scintillation fluid) |
| Roche (Mannheim) | Neuraminidase from Vibrio cholerae; Leupeptin |
| Serva Electrophoresis (Heidelberg) | Tween® 20 |
| Sigma (Deisenhofen) | 1,4 Dithio-DL-treitol (DTT); 3-Amino 9-Ethyl Carbamazole (AEC); Acrylamide; Ammonium Chloride; Amonium Phosphate; Bicinchoninic Acid (BCA) Protein Assay Kit; Boric Acid; Brefeldin A; Concanamycin A; Dimethyl Sulphoxide 99%(DMSO); Ethylen diamine tetraacetic acid (EDTA); Ethylene glycol tetraacetic acid (EGTA); Glycerol; Hydrogen peroxide 30% solution in water; IGEPAL®; Methylene – BisAcrylamide; N,N – |

| | |
|---|---|
| Sigma (Deisenhofen) | Dimethylformamide; Phenylmethylsulfonyl Fluoride (PMSF);<br>PKH-2 Green Fluorescent Cell Linker Kit; PKH-26 Red Fluorescent Cell Linker Kit; Potassium Hydrogencarbonate; Potassium Hydroxid; TEMED (N,N,N‘,N‘ – Tetramethylethylendiamine);<br>Tris Base (tris[hydroxymethy]aminomethane) |

### 2.1.2 Antibodies

| | |
|---|---|
| BD Pharmingen (Heidelberg) | Isotype-control anti-mouse $IgG_1$-FITC/PE;<br>anti HLA-ABC-FITC; anti HLA-DR-PE; anti CD3-PE; anti CD16-FITC; anti CD19-FITC; anti CD21-FITC; anti CD56(N-CAM)-PE; anti CD95 (Fas)-FITC; CD178 (Fas ligand) |
| Beckman-Coulter (Krefeld) | Goat F(ab’)2 fragment anti-mouse IgG (H+L)-PE |
| PBL Biomedical Laboratories (Piscataway, USA) | anti CD118 (IFN $\alpha$, $\beta$ receptor) |

### 2.1.3 Nucleotides

| | |
|---|---|
| Pharmacia (Freiburg) | Poly(dI/dC) |
| Promega (Heidelberg) | NF-κB consensus oligonucleotide<br>5'-AGTTGAGGGGACTTTCCCAGGC-3' |

### 2.1.4 Pancreatic carcinoma cell lines

| | |
|---|---|
| ATCC (USA) | AsPC-1<br>MIA PaCa-2<br>PANC-1 |
| DSMZ (Germany) | CAPAN-2<br>DAN-G<br>KCI-MOH1<br>PA-CLS 52 |
| | PK-9 was obtained from Makoto Sunamura, Department of Surgery, Tohoku University School of Medicine, Sendai, Japan |

### 2.1.5 Blood preparations

| | |
|---|---|
| Blutbank Mannheim | Buffy-coat preparations |
| Sigma (Deisenhofen) | Red blood cells from sheep |

### 2.1.6 Solutions and buffers

| | |
|---|---|
| | BSA 1% in PBS, sterile filtered through 0.22 μm filter |
| Erythrolysis Buffer | Ammonium Chloride 8.29 g/L; Potassium Hydrogencarbonate 1.0 g/L; EDTA 0.0371 g/L in double distilled water, filtered through 0.22 μm filter |
| TBE 10× | Tris Base 108 g/L; Boric Acid 55g/L; EDTA 20mM pH 8.0 in double distilled water |
| Total Protein Extract (TOTEX) Buffer | HEPES 20 mM; Sodium Chloride 0.35 M; Glycerol 20%; IGEPAL® 1%; Magnesium Chloride 1 mM; EDTA 0.5 mM; EGTA 0.5 mM; Leupeptin 10 mg/L; DTT 0.5 mM; PMSF 0.2 mM |

### 2.1.7 Culture medium

| | |
|---|---|
| RPMI-Medium | Fetal Calf Serum 10% (heat-inactivated 30 min at 56°C); Penicillin $10^5$ U/L; Streptomycin $10^5$ μg/L in RPMI 1640 with L-Glutamine |
| HEPES-Medium | Fetal Calf Serum 10% (heat-inactivated 30 min at 56°C); Penicillin $10^5$ U/L; Streptomycin $10^5$ μg/L; HEPES 25 mM in RPMI 1640 with L-Glutamine |
| Freezing medium | DMSO 20%; Fetal Calf Serum 40%; RPMI-medium 20% |

### 2.1.8 Other materials

| | |
|---|---|
| Amersham (Little Chalfont, UK) | "Hyperfilm MP" High performance autoradiography film |
| Whatman (Maidstone, UK) | DE81 ion exchange DEAE cellulose paper |

## 2.2 Methods

### 2.2.1 Cell culture

Pancreatic carcinoma cells were incubated at 37°C under 5% $CO_2$ in a humidified atmosphere (HERAcell, Heraeus, Hanau). Adherent cells were harvested by trypsinization, washed and centrifuged at 400×g for 7 minutes at room temperature (Centrifuge 5810R, Eppendorf AG, Hamburg). The pellet was resuspended in fresh medium and cultured in 75 $cm^2$ flasks. The pancreatic carcinoma cell lines were cultivated in RPMI-medium. They were split 1:5 twice a week.

The cell lines were preserved frozen at -80°C in cryotubes as $4\times10^6$ cells per tube in 1.4 ml mixture 1:1 RPMI-medium: Freezing medium.

Cell culture procedures were performed under sterile conditions using a laminar airflow (HERAsafe KS 12, Kendro Laboratory Products, Hanau).

### 2.2.2 Treatment of the pancreatic carcinoma cell lines

Pancreatic carcinoma cells were cultured in 25 $cm^2$ flasks at a density of $5\times10^5$ cells/flask in RPMI medium and incubated at 37°C under 5% $CO_2$ in a humidified atmosphere for 24 hours before starting the treatment.

Tumor cells were treated for 5 days either with 5-fluorouracil (5-FU) 65 µg/ml continuously, day 1-5; cisplatin (CDDP) 3 µg/ml on day 1, for 60 minutes; radiation 1.8 Gy/day (Gammacell 1000 Irradiator, MDS Nordion, Canada), day 1-5; interferon alpha (IFN-α) 1,000 U/ml, day 1, 3, and 5; chemoradiation (CR) consisting of 5-FU, CDDP and radiation or the entire chemoradioimmunotherapy combination (CapRI).

### 2.2.3 Preparation of lymphocytes

#### *2.2.3.1 Preparation of peripheral blood lymphocytes (PBLs)*

Peripheral blood mononuclear cells (PBMCs) were separated from buffy-coats by Ficoll density gradient centrifugation as the cells from the interphase. They were seeded in six well-plates at a density of $5\times10^6$ cells/ml in HEPES-medium at 37°C under 5% $CO_2$ in a humidified atmosphere and were allowed to adhere for one hour. The peripheral blood lymphocytes (PBLs) were isolated as the nonadherent cells and were seeded in six well-plates at a density of $2.5\times10^6$ cells/ml.

*2.2.3.2 Preparation of T cells and non-T cells*

T cells were separated trough a rosetting assay using neuraminidase treated sheep red blood cells (SRBCs) as follows: SRBCs were incubated for one hour at 37°C with $4\times10^{-3}$ units/ml neuraminidase from Vibrio cholerae and afterwards resuspended as 5 ml centrifuge-packed SRBCs in 50 ml RPMI 1640 with 10% fetal bovine serum. PBLs suspended at a density of $10^6$ cells/ml in PBS were mixed 10:1 with the neuraminidase treated SRBC suspension, centrifuged at 400×g for 5 minutes and incubated on ice for one hour to form E-rosettes, then gently resuspended and a Ficoll density gradient centrifugation was performed. The peletted fraction containing E+ rosette forming cells (T cells) underwent erythrolysis with $NH_4Cl$ solution. The cells harvested from the interphase were termed non-T cells.

The rosetting of T cells by sheep erythrocytes is mediated through the interaction of the cell surface glycoprotein CD2 on the T cells with T11TS molecule on sheep erythrocytes. T11TS is homologous to the lymphocyte function-associated antigen-3 (LFA-3, CD58) which is a receptor for CD2.

*2.2.3.3 Separation of NK cells*

NK cells were separated from the PBL fraction through positive selection using the MACS technique according to the manufacturer's instructions. The cells were labeled with anti-CD56 beads and enriched on a LS separation column.

Effector cells were stimulated with 1,000 U/ml IFN-α overnight when indicated. If not indicated differently, the whole PBMC fraction was stimulated and PBLs were separated prior to the experiments.

### 2.2.4 Flow cytometric analysis

Flow cytometry is the characterization of single cells as they pass at high speed through a laser beam. The laser acting as a light source develops parameters of light scatter as well as exciting the fluorescent molecules used to label the cell. Cells are characterized individually by their physical and/or chemical properties.

Cell type and the quantity of antigens expressed by each cell can be distinguished using antibodies which will bind specifically cell surface antigen epitopes, tagged with flourochromes.

An Epics®XL.MCL flow cytometer (Beckman-Coulter, Krefeld, Germany) was used. Human pancreatic carcinoma cell lines were stained using monoclonal antibodies against HLA-ABC, HLA-DR, CD95 (Fas), Fas ligand. Lymphocytes were stained using monoclonal antibodies against CD3 (T cells), CD16 and CD56 (NK cells), CD19 (B cells), CD21 and CD118 (interferon receptors). Negative control was represented by cells stained with corresponding isotype control antibody. Gates were set to under 2% for the negative controls. Mean fluorescence was normalized to mean fluorescence of the control antibodies.

### 2.2.5 MTT assay for cell proliferation

MTT assay is a nonradioactive method used for quantitative determination of cellular proliferation and activation. The assay is based on the cleavage of the tetrazolium salt MTT into formazan by the "succinate-tetrazolium reductase" system which belongs to the respiratory chain of the mitochondria and is active only in metabolically intact cells. The proliferation rate is determined as the amount of turnover of yellow tetrazolium salt into its red formazan derivative.

The "EZ4U" kit was used according to the manufacturer's instructions. Tumor cells were seeded at a density of 2,000 cells per well in triplicate in a 96-well plate. The proliferation rate was determined after 5 hours of incubation. Absorbance at 450 nm with 620 nm as a reference was measured with an ELISA reader; the absorbance of a medium blank was subtracted. The proliferation index was calculated by setting the proliferation of the untreated cells to 1.

### 2.2.6 Determination of apoptosis, cell death and cell count

Cell death typically follows one of two pathways: apoptosis (programmed cell death) or necrosis. Cells undergoing programmed cell death may translocate phosphatidylserine from their inner cytoplasmic membrane and express it on the cell surface. In the presence of $Ca^{2+}$ Annexin will bind to phosphatidylserine. Propidium Iodide (PI) discriminates between apoptosis and necrosis on the basis of dye exclusion. Propidium iodide (PI) intercalates into double-stranded nucleic acids. It can penetrate the membranes of dying or dead cells, but is excluded by viable cells. In combination AnnexinV-FITC/PI is a membrane integrity assay. While necrotic cells lose membrane integrity early during their death process, apoptotic cells may have compromised membranes in later fazes of their death. Therefore in advanced apoptosis cells with breached outer membrane may uptake PI and bind Annexin-FITC as well because the inner cytoplasmic membrane with phosphatidylserine is available for labeling.

Trypan blue is a dye used to determine the viability of the cells. Living cells exclude the dye while dead cells take it.

On the fifth day of treatment the cells were harvested. The amount of viable cells was evaluated on a Neubauer improved counting chamber (PaulMarienfeld, Lauda-Königshofen) by trypan blue exclusion. 20µl cell suspension was mixed with 180 µl trypan blue 0.5% diluted 1:10.

For investigation of the apoptosis and necrosis cells were stained using the AnnexinV-FITC/PI kit according to the manufacturer's instructions, and further analyzed using an Epics®XL.MCL flow cytometer (Beckman-Coulter, Krefeld, Germany). Apoptosis was defined as AnnexinV positive cells, late apoptosis as AnnexinV and PI double positive cells, necrosis as PI positive cells. Unstained cells were used to set up controls.

### 2.2.7 Cytotoxicity assays for untreated pancreatic carcinoma cells

The cytotoxic activity of the lymphocytes was evaluated using a standard chromium release assay.

Chromium release assay uses target cells labeled with radioactive Chromium as Sodium Chromate ($Na_2{}^{51}CrO_4$), afterwards exposed to the effector cells. The release of radioactive Chromium into the culture medium is proportional with the number of target cells killed.

Tumor cells were labeled with 100 µCi $^{51}Cr$ for two hours. After washing, 10,000 target cells per well were incubated with effector cells at different effector to target cell ratios (E:T ratio) over four hours. The supernatant was collected and counts per minute were determined (Packard, Dreieich, Germany). Each experiment was performed in triplicate and the mean value was calculated. Maximum release was obtained by incubating the target cells with an anionic detergent (0.1% IGEPAL®). The negative control (spontaneous release) was represented by target cells without effector cells. The ratio between maximal and spontaneous release was generally >5. Cytotoxicity calculations were performed using the following formula:

$$\text{Percent cytotoxicity} = \frac{\text{experimental release - spontaneous release of target cells}}{\text{maximal release - spontaneous release of target cells}}$$

### 2.2.8 Cytotoxicity assays for pretreated pancreatic carcinoma cells

*2.2.8.1 Flow cytometric based cytotoxicity assay*

Tumor cells pretreated with chemotherapy could not be investigated in a standard chromium release assay because they showed too high spontaneous release of chromium or were not suitable for labeling. Therefore, a flow cytometric based cytotoxicity assay was developed. Treated and untreated tumor cells were stained with fluorescent dyes, either PKH-2 (green) or PKH-26 (red), mixed 1:1 and incubated over four hours with effector cells (E:T ratio of 80:1). The percentage of red and green tumor cells was then analyzed by flow cytometry. Since the tumor cell lysis of untreated tumor cells at this given effector to target cell ratio is known, the approximately cell lysis of pretreated tumor cells could be calculated using the following formula:

$$lysis_{pretreated\ cells}\ [\%] \approx \left(b - \frac{e \times a \times (100\% - c)}{d \times 100\%}\right) \times \frac{100\%}{b}, \text{ with}$$

$a$ = percentage of untreated cells in control mix (without PBL) [%], and

$b$ = percentage of treated cells in control mix (without PBL) [%], and

$c$ = known lysis of untreated cells at given E:T ratio (from $^{51}$Cr-release assay) [%], and

$d$ = percentage of remaining untreated cells after four hour incubation with PBL [%];

$e$ = percentage of remaining treated cells after four hour incubation with PBL [%],

in which $a + b \approx 100\%$ and $d + e \approx 100\%$.

*2.2.8.2 MTT based cytotoxicity assay*

Pancreatic carcinoma cells were treated over four days with either chemoradiation (CR) or chemoradioimmunotherapy (CapRI). On the fourth day of treatment the cells were harvested and plated in 96-well plates as $4 \times 10^3$ cells/well. After overnight adherence step, the pancreatic carcinoma cells were co-cultured with lymphocytes as $32 \times 10^4$ lymphocytes/well. After 4 hours co-culture the plates were washed with fresh cell culture medium and cell proliferation was assessed through a MTT assay using the "EZ4U" kit as described. The proliferation rate was determined by photometric measurement after 65 hours of incubation. Wells with untreated cells, treated cells alone and lymphocytes alone were used as controls and were subtracted.

### 2.2.9 Blockage of apoptosis induction mechanisms

To determine which mechanism is involved in inducing the apoptosis of target cells, the perforin and/or Fas-mediated pathway, effector cells ($2.5 \times 10^6$ in 2.5ml of RPMI-1640) were pre-treated for two hours at 37°C with either 100 nM of Concanamycin A (CMA), which causes the degradation of perforin-containing granules, or 10 μM of Brefeldin A (BFA) which disrupts Fas/FasL-mediated interactions. The cells were analyzed afterwards in a cytotoxicity assay.

### 2.2.10 Granzyme B ELISPOT

The enzyme-linked immunospot (ELISPOT) assay is used for detecting and enumerating individual cells which secrete a particular protein. The assay is based on the sandwich enzyme-linked immunosorbent assay (ELISA). The wells of the ELISPOT plate are coated with antibody specific for the protein that is being assayed for. The protein molecules are released locally around each effector cell and are "captured" by the specific antibody. A second antibody that is also specific for the protein of interest is added; this antibody is coupled to an enzyme that is capable of converting a substrate into an insoluble coloured product forming spots of colour.

The Human Granzyme B ELISPOT Set was used according to the manufacturer's instructions. The effector cells (NK cells and PBLs, unstimulated or stimulated with IFN-α 1,000 U/ml) were co-cultured with human pancreatic carcinoma cells over four hours, in quadruplicates, in the 96 well plates provided by the manufacturer. The effector cells were seeded at a density of 45,000 cells per well, while the tumor cells were seeded at a density of 560 cells per well. The plates were read using the KS ELISPOT System, release 4.1 (Carl Zeiss Light Microscopy, Göttingen, Germany). Wells without cells, effector cells alone and tumor cells alone served as controls and were subtracted from the results.

### 2.2.11 Electrophoretic mobility shift assay for NF-κB binding activity

Transcription factors are proteins able to bind specifically to short DNA consensus sequences located in gene promoters modulating the expression level of the target genes. The DNA-binding activity of the transcription factor NF-κB was analyzed using the electrophoretic mobility shift assay.

Electrophoretic mobility shift assay (EMSA) is a technique used to determine protein:DNA interactions. EMSA can be used qualitatively to identify sequence-specific DNA-binding

proteins. It is based on the observation that protein:DNA complexes migrate more slowly than free DNA molecules when subjected to non-denaturing TBE-polyacrylamide gel electrophoresis. Because the rate of DNA migration is shifted or retarded upon protein binding, the assay is also referred to as a gel shift or gel retardation assay. In this technique, cell extracts are incubated with a radioactive double stranded oligonucleotidic probe containing the binding site of the transcription factor. Nonspecific competitor DNA such as poly(dI-dC) is included in the binding reaction to minimize the binding of nonspecific proteins to the labeled target DNA. These repetitive polymers provide an excess of nonspecific sites to adsorb proteins that will bind to any general DNA sequence. Samples are then resolved by native polyacrylamide gel electrophoresis followed by autoradiography. Active transcription factors bind to their consensus sequence, which is visualized by a retarded band. By competition, the retarded band will disappear if the protein extract is exposed to an excess of non radioactive oligonucleotide containing the same consensus sequence as the radioactive probe; this can be used for ascertainment of the binding specificity.

*2.2.11.1 Nuclear protein extraction*

Pancreatic carcinoma cells were cultured in 75 $cm^2$ flasks at a density of $2\times10^6$ cells/flask. The cells were treated as follows: a) either 5-FU 65 μg/ml or increasing concentrations of IFN-α (1,000 U/ml, 5,000 U/ml, 10,000 U/ml) or combinations between 5-FU and increasing concentrations of IFN-α for 30h; b) either single agents 5-FU 65 μg/ml, cisplatin (CDDP) 3 μg/ml for 1h, radiation 10 Gy, IFN-α 10,000 U/ml or their combination with IFN-α 10,000 U/ml, or the entire combination therapy (CapRI) for 30h.

Following treatment, nuclear proteins were harvested in the total protein extract (TOTEX) buffer. Protein concentration was measured using the bicinchoninic acid (BCA) assay which relies on the formation of a $Cu^{2+}$-protein complex under alkaline conditions, followed by reduction of the $Cu^{2+}$ to $Cu^{1+}$. Bicinchoninic acid is a highly specific chromogenic reagent for $Cu^{1+}$, forming a purple-blue complex with an absorbance maximum at 562 nm. The absorbance is directly proportional to protein concentration.

Nuclear protein (10 μg) was used in the electrophoretic mobility shift assay.

*2.2.11.2 Labeling of the oligonucleotide*

Before the experiment, NF-κB consensus sequence 5'-AGTTGAGGGGACTTTCCCAGGC-3' (binding region underlined) was end-labeled with polynucleotide kinase in the presence of [$\gamma$-$^{32}$P]-dATP to a specific activity >$5\times10^7$ cpm/μg DNA.

*2.2.11.3 Electrophoretic mobility shift assay*

Binding of NF-κB to 1 ng of radiolabeled NF-κB consensus oligonucleotide was performed for 20 min at room temperature in 10 mmol/l Hepes, pH 7.5, 0.5 mmol/l EDTA, 100 mmol/l KCl, 2 mmol/l dithiothreitol, 2% glycerol, 4% Ficoll, 0.25% NP-40, 1 mg/ml BSA and 0.1 μg/ml poly (dI/dC). Protein DNA complexes were separated from the unbound DNA probe by electrophoresis at 200V (Bio-Rad Power Pac 200, Bio-Rad, Munich, Germany) in 5% polyacrylamide gels containing 3.25% glycerol and 0.5 × Tris-Borate-EDTA (TBE). Specificity of binding was ascertained by competition with a 160-fold molar excess of unlabeled consensus oligonucleotides. Gels were dried on DE81 ion-exchange chromatography paper and afterwards exposed to autoradiographic films for 12 – 96 hours at –80°C. The autoradiographic bands were analysed by densitometry using a computerized-image analyzer (GS-700 Imaging Densitometer, Bio-Rad, Munich, Germany).

**2.2.12 Statistical analysis**

Non parametrical analysis (Wilcoxon test) and paired t test on SPSS 11.5 were used to analyze statistical significance where appropriate. A *p*-value < 0.05 was considered as significant.

## 3. RESULTS

In previous work of Dr. Jian-Hua Ma and the author was investigated the direct effect of IFN-α alone or in combination with chemoradiotherapy on pancreatic carcinoma cell lines. As these data obtained by our research group are already published (Ma J et al. 2005) and are also presented in detail in the thesis of Dr. Jian-Hua Ma, "Enhancement of Chemotherapeutic and Radio-Immunotherapeutic Sensitivity on Human Pancreatic Cancer Cells due to Interferon-alpha (CapRI Scheme in Vitro)" (Ma J 2004), this work will not focus on them furthermore. Here is mentioned just a short summary:

Results obtained by AnnexinV/PI stain and cell count showed that IFN-α is able to decrease the cell survival index having direct inhibitory properties, and some synergistic influence in combination treatments, especially when added to radiotherapy. While treatment with 5-FU and its combinations produced a significant reduction in the proliferation rate and VEGF secretion, IFN-α was able to prevent the increase in proliferation rate and VEGF secretion of CDDP resistant cells. Regarding the immunophenotyping of the treated tumor cells there was a decrease in MHC class I expression after 5-FU treatment which could be prevented by addition of IFN-α. Mean expression of MHC class I increased after IFN-α treatment. Furthermore, high expression of MHC molecules, CD118, EGF-R and Fas was predictive for a good response. As these results showed that IFN-α has only limited chemo- and radiosensitizing effects, we hypothesized that the main mechanism of action of IFN-α in chemoradioimmunotherapy (CapRI) is the immunomodulation. Lymphocytes stimulated overnight with IFN-α had an increased proliferation rate, a higher expression of FasL and an increased cytotoxic activity.

In the present work was further investigated which fraction of the peripheral blood lymphocytes is responsible for the increased cytotoxic activity after IFN-α stimulation, whether IFN-α exerts a direct effect on different types of lymphocytes or an indirect one by monocyte activation, as well as their mechanisms of apoptosis induction. Chemoradiation (CR) was compared with chemoradioimmunotherapy (CapRI) regarding proliferation and apoptosis induction in the pancreatic carcinoma cells, as well as cytotoxic effect of lymphocytes. The effect of IFN-α on NF-κB binding activity in pancreatic carcinoma cell lines was also investigated.

### 3.1 Comparison between chemoradiation and chemoradioimmunotherapy regarding the proliferation rate and apoptosis induction

IFN-α has a moderate direct cytotoxic effect on human pancreatic carcinoma cell lines and increases the apoptotic effect of 5-fluorouracil and cisplatin. Here it was tested whether IFN-α has a direct cytotoxic impact when it is used in combination with chemoradiotherapy. When comparing the effect of chemoradiation (5-FU+CDDP+radiation) with the effect of chemoradioimmunotherapy (5-FU+CDDP+radiation+IFN-α) regarding the proliferation rate of the surviving cells (Fig. 1A) and the apoptosis induction (Fig. 1B) there were no statistically significant differences between the two treatments.

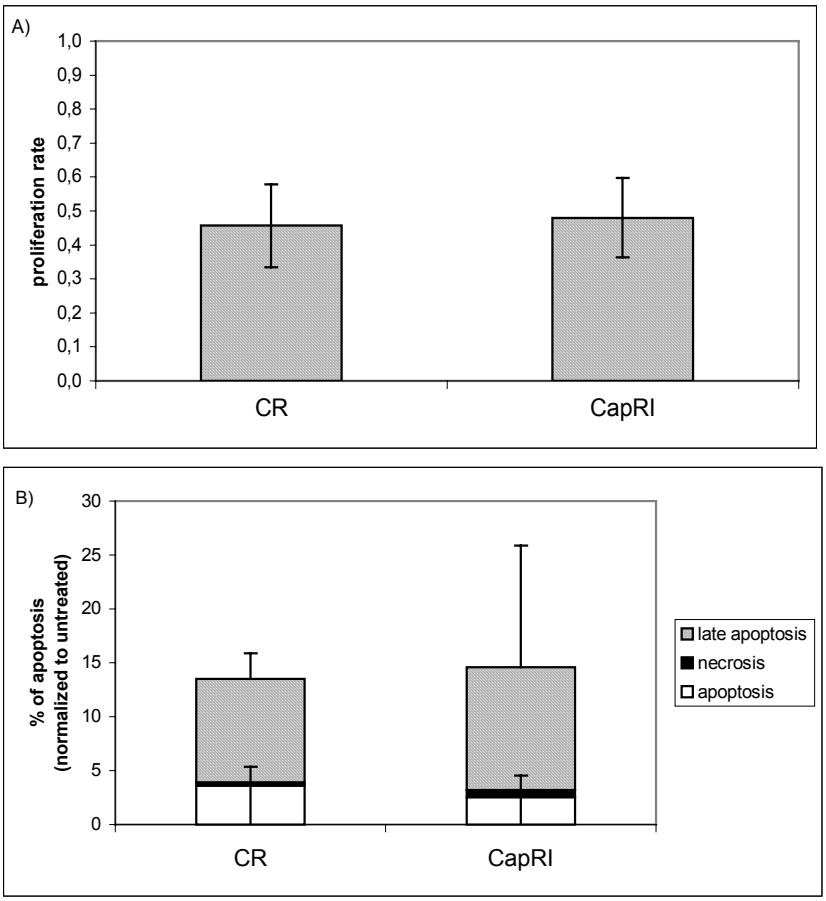


**Figure 1A/B:** Comparison between chemoradiotherapy (CR) and chemoradioimmunotherapy (CapRI) on pancreatic carcinoma cells

A) Comparison between chemoradiotherapy and chemoradioimmunotherapy regarding the proliferation rate. B) Comparison between chemoradiotherapy (CR) and chemoradioimmunotherapy (CapRI) regarding apoptosis induction. Data are shown as mean ± standard deviation from eight cell lines each with three separate experiments.

### 3.2 The influence of IFN-α on lymphocytes

Hypothesizing that the role of IFN-α in multimodality treatment is an immunological one, here was tested the influence of IFN-α on the cytotoxic effect of lymphocytes.

The cytotoxic activity of PBLs from healthy donors was first tested against untreated pancreatic carcinoma cell lines. Unstimulated PBLs showed no significant cytotoxic effect (12.5 ± 6.3% at an effector to target ratio of 80:1). IFN-α stimulated PBLs showed a significantly enhanced cytotoxicity (34.3 ± 5.5%; $p<0.02$, Fig. 2A). As the adherent fraction of the mononuclear cells was present during incubation, its influence was investigated. Therefore, total PBMC fraction or just the PBLs were stimulated overnight with IFN-α and their cytotoxic effect was investigated. There was no significant difference between these two groups (Fig. 2B).

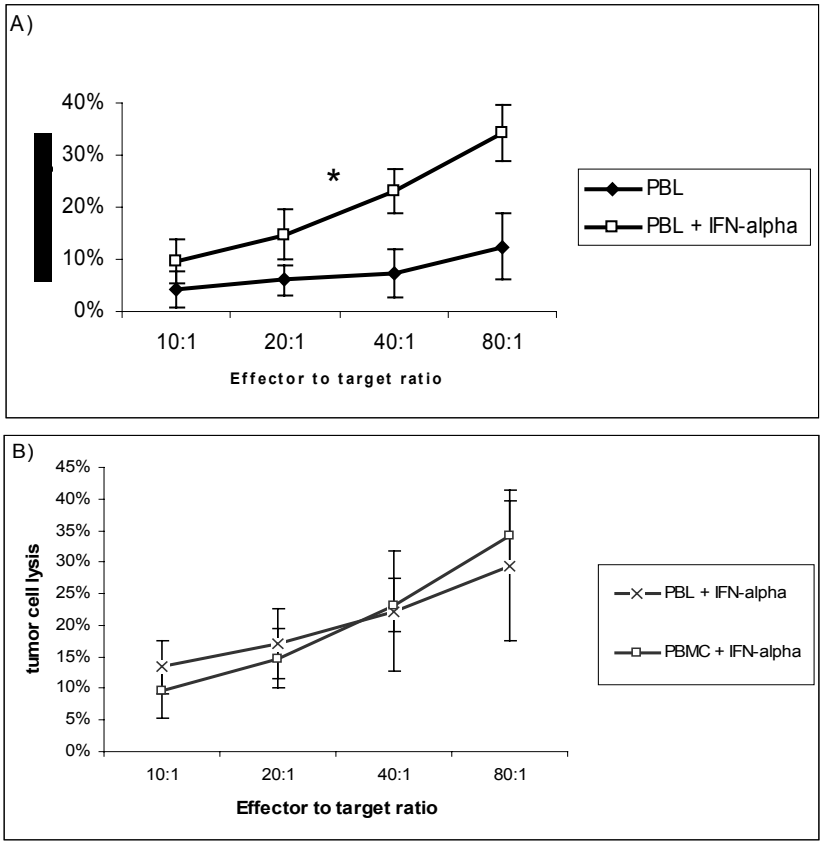


**Figure 2A/B:** Cytotoxic effect of PBLs against untreated pancreatic carcinoma cell lines

A) Cytotoxicity of PBLs after IFN-α stimulation. Data are shown as mean ± standard deviation from eight cell lines each with three separate experiments. These data were obtained with the contribution of Dr. Jian-Hua Ma and are also presented in his doctoral thesis "Enhancement of Chemotherapeutic and Radio-Immunotherapeutic Sensitivity on Human Pancreatic Cancer Cells due to Interferon-alpha (CapRI Scheme in Vitro)" as figure 9 (page 39). B) PBLs were stimulated overnight with IFN-α with (PBMCs) or in the absence (PBLs) of the monocyte fraction. Data are shown as mean ± standard deviation from eight cell lines each with three separate experiments. An asterisk indicates statistical significance ($p<0.05$).

Further, T cells were separated and their cytotoxic effect was compared with the cytotoxic effect of the non T cells. 83.5 ± 5.3% of the enriched T cell fraction was positive for CD3. Cells in the negative fraction expressed surface antigens as follows: 62.1 ± 8.1% $CD16^+$ cells; 59.7 ± 2.2% $CD56^+$ cells; 35.4 ± 8.1% $NKG2D^+$ cells; 44.2 ± 6.2% $CD161^+$ cells; 24.2 ± 6.9% $CD19^+$ cells; 3.7 ± 0.7% $CD3^+$ cells, while the IFN-receptor CD118 was expressed at a rate of 45.9 ± 8.9% (data not shown).

Unstimulated, as well as IFN-α stimulated T cells, failed completely to lyse pancreatic tumor cells (lysis < 5%); while T cell-depleted lymphocytes (i.e. mostly NK cells) had a significant cytotoxic activity without any stimulation (lysis 23.0 ± 25.9% at an effector to target ratio of 80:1). The cytotoxic effect was significantly increased after preincubation with IFN-α (42.7 ± 31.6% at an effector to target ratio of 80:1; Fig. 3A).

To investigate whether this was a direct effect on NK cells or an indirect one by monocyte activation, the influence of overnight co-incubation with monocytes upon the non-T cell fraction was assessed, but the effect failed to be significant (Fig 3B).

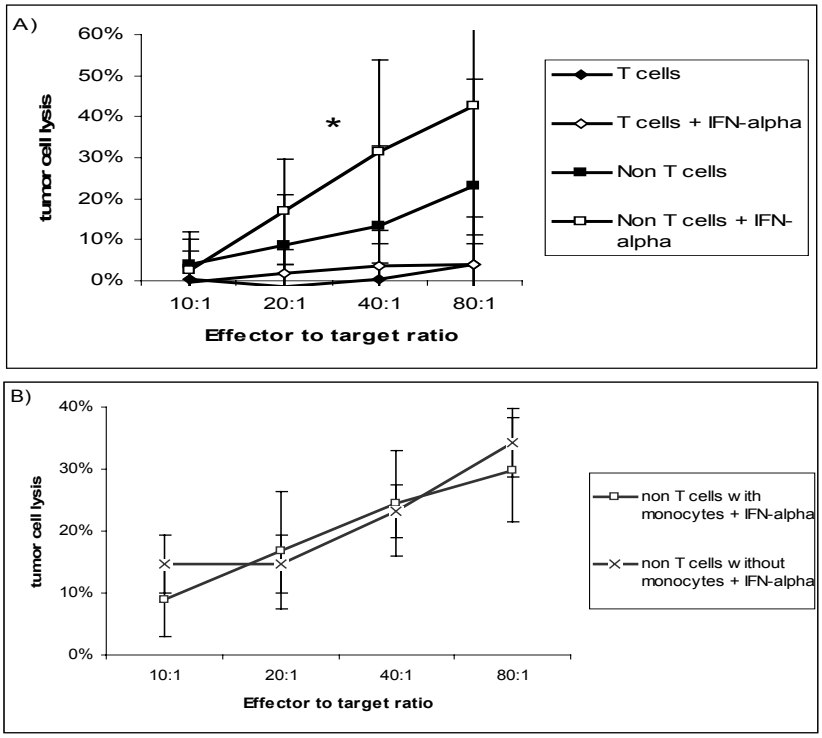


**Figure 3A/B:** Cytotoxicity of immunological subpopulations against tumor cells

A) PBLs from healthy donors were separated into a T cell and a Non-T cell fraction by a rosetting procedure and stimulated overnight with IFN-α. The next day their cytotoxic activity against the pancreatic carcinoma cell lines was tested in a standard chromium release assay at increasing effector to target ratios. B) Non-T cell fraction was stimulated overnight in the presence or in the absence of the monocytes. Data are shown as mean ± standard deviation from eight cell lines each with three separate experiments. An asterisk indicates statistical significance ($p$<0.05).

### 3.3 Analysis of the killing mechanism

In order to assess the way of killing, NK cells and PBLs were investigated using a granzyme B release ELISPOT assay. NK cells were enriched through CD56 positive selection using the MACS technique (purity 87.4 ± 3.6%). Lymphocytes were tested against six pancreatic carcinoma cell lines (ratio 80:1). Granzyme B spots were detected after a four-hour incubation period. Unstimulated as well as IFN-α prestimulated PBLs showed no remarkable increase in granzyme B release (1-2 spots/45,000 cells) compared to PBLs without addition of tumor cells. NK cells even without stimulation showed after co-culture with tumor cells a significant increase in granzyme B release compared to PBLs (2.8 ± 1.0 spot, $p<0.03$; Fig. 4A). A strong increase in numbers of spots could be achieved with IFN-α stimulated NK cells (41.2 ± 6.0 spots/45,000 cells; $p<0.001$).

In order to establish the importance of Fas/FasL interaction and that of the perforin pathway in the apoptosis induction by effector cells, degradation of perforin-containing granules using concanamycin A and disruption of Fas/FasL interactions using brefeldin A were studied. Disruption of Fas/FasL interactions between T cell-depleted lymphocytes and tumor cells resulted in a statistically significant decrease of cytotoxicity. The cell lysis at an effector to target ratio of 80:1 dropped from 51.3 ± 8.1% to 8.8 ± 1.3%, $p<0.02$. Degradation of perforin-containing granules reduced the cytotoxic activity of T cell depleted PBLs to 21.2 ± 3.9% lysis at an E:T ratio of 80:1, but this was not statistically significant ($p=0.06$; Fig. 4B).

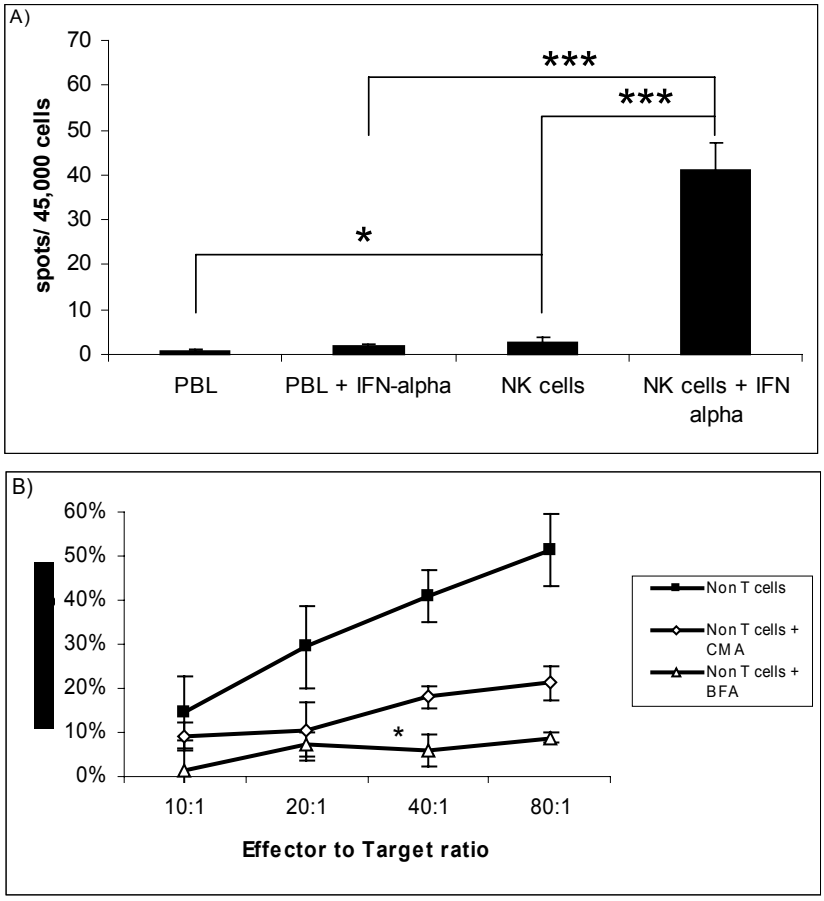


**Figure 4A/B:** Analysis of the killing mechanism

A) Granzyme B release of IFN-α prestimulated vs. unstimulated PBLs and NK cells co-cultured over 4 hours with untreated pancreatic carcinoma cells. Data are shown as mean ± standard error from six cell lines. An asterisk indicates statistical significance ($p$<0.05) three asterisks indicate a $p$<0.001. B) Inhibition of the cytotoxic effect of non T cells by disrupting the Fas/Fas-ligand mediated interaction using Brefeldin A (BFA) and by degradation of the perforin containing granules using Concanamycin A (CMA). Results were obtained using a $^{51}$Cr-release assay. PANC-1 cells were used as target. Data are shown as mean ± standard error from three separate experiments. An asterisk indicates statistical significance ($p$<0.05).

### 3.4 Cytotoxic activity of IFN-α prestimulated lymphocytes against pretreated tumor cells

Next was analyzed if treatment of tumor cells with chemo- and/or radiotherapy affects their susceptibility to the immunological effector cells. Pretreated tumor cells were incubated with IFN-α stimulated PBLs and cytolysis was determined in a flow cytometry-based cytotoxicity assay. Treatment with IFN-α, CDDP and/or radiation did not influence the susceptibility of tumor cells, whereas pretreatment with 5-FU or the whole CapRI-scheme led to an increase of cytolysis from 34% to approximately 70% ($p$<0.001; Fig. 5A).

The mechanism through which cytolysis is produced was assessed by inhibition of the perforin release (using Concanamycin A) and by blockage of the Fas/FasL interactions (using Brefeldin A). There was a blockage of additional cytolysis after addition of CMA. The cell lysis dropped from 63% to 36%, $p<0.01$. Blockage of Fas/FasL interactions resulted in reduction of cytolysis down to 44%, cell lysis of untreated cells was 40% ($p<0.01$; Fig. 5B). Since Fas/FasL interactions are mainly responsible for lysis of untreated tumor cells, it can be concluded that the gain in cytolysis after treatment with 5-FU is induced by an increased vulnerability to perforin.

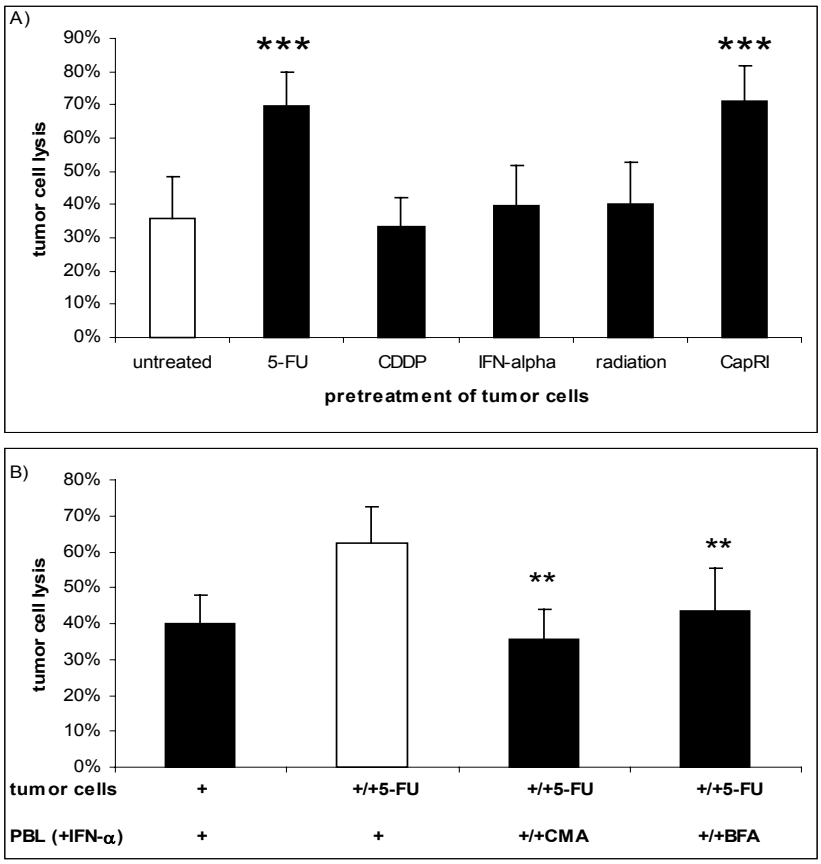


**Figure 5A/B:** Cytotoxicity against chemo- radiotherapy-pretreated tumor cells

A) Tumor cells were pretreated as indicated over five days. PBLs from healthy donors were stimulated overnight with IFN-α and tested for their cytotoxic activity against pretreated pancreatic carcinoma cell lines at an effector to target ratio of 80:1. Data are shown as mean ± standard error from eight cell lines each with three separate experiments. Three asterisks indicate statistical significance of $p<0.001$. These data were obtained with the contribution of Dr. Jian-Hua Ma and are also presented in his doctoral thesis "Enhancement of Chemotherapeutic and Radio-Immunotherapeutic Sensitivity on Human Pancreatic Cancer Cells due to Interferon-alpha (CapRI Scheme in Vitro)" as figure 10 (page 40). B) The pathway of apoptosis was investigated by degradation of perforin-containing granules or disruption of Fas/FasL interactions. PBLs were preincubated either with Concanamycin A (CMA) or Brefeldin A (BFA). 5-FU pretreated PANC-1 cells were used as targets. Data are shown as mean ± standard error from three separate experiments. An asterisk indicates statistical significance ($p<0.05$).

### 3.5 Cytotoxicity in the CapRI scheme versus chemoradiotherapy (CR)

Further, the importance of IFN-α in addition to chemoradiotherapy was investigated comparing the cytotoxic activity of unstimulated lymphocytes against chemoradiotreated tumor cells with the cytotoxic activity of IFN-α prestimulated lymphocytes against chemoradioimmunotreated tumor cells.

A flow cytometric based cytotoxicity assay was performed. The cytolytic effect of IFN-α prestimulated PBLs on chemoradioimmunotreated tumor cells (CapRI scheme) was compared with the cytolytic effect of unstimulated PBLs on chemoradiotreated tumor cells (CR). At an effector to target ratio of 80:1 there was an increase in the lytic effect from approximately 18% in the chemoradiotherapy scheme to 34% in the chemoradioimmunotherapy, but this failed to be statistically significant (Fig. 6).

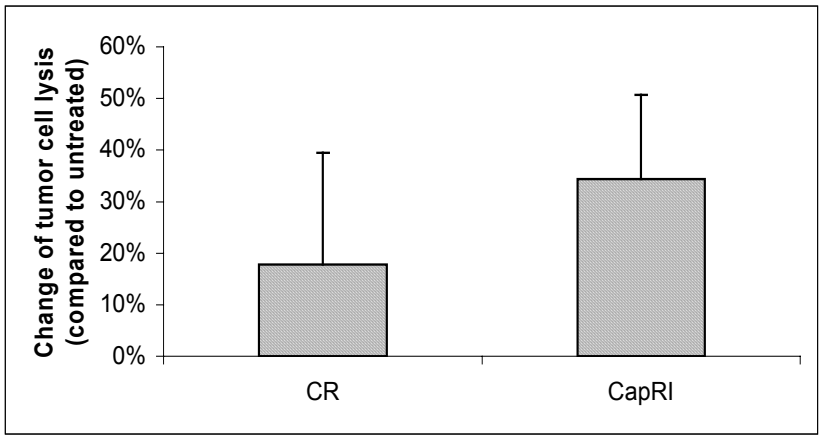


**Figure 6:** Flow cytometric based cytotoxicity in the chemoradiotherapy (CR) scheme vs. chemoradioimmunotherapy (CapRI) scheme

At a ratio effector to target cells of 80:1 chemoradiotreated cells were co-cultured over 4 hours with unstimulated lymphocytes, while chemoradioimmunotreated cells were co-cultured over 4 hours with IFN-α prestimulated lymphocytes. Data are shown as mean ± standard error from eight cell lines each with three separate experiments.

For further investigation, a MTT based cytotoxicity assay was performed. Chemoradiation (CR) treated cells were co-incubated with unstimulated lymphocytes; while chemoradioimmuno (CapRI)-treated cells were co-incubated with IFN-α prestimulated lymphocytes. After 4 hours of co-culture the proliferation rate was assessed using a MTT assay. While there was no significant difference between the proliferation rate after chemoradiotherapy compared to chemoradioimmunotherapy, when treated pancreatic carcinoma cells were co-incubated with PBLs, the proliferation rate in the CapRI scheme was

significantly lower than in the chemoradiotherapy scheme (-120.3 ± 36.1% vs. -75.2 ± 33.6%, p=0.0001; Fig. 7).

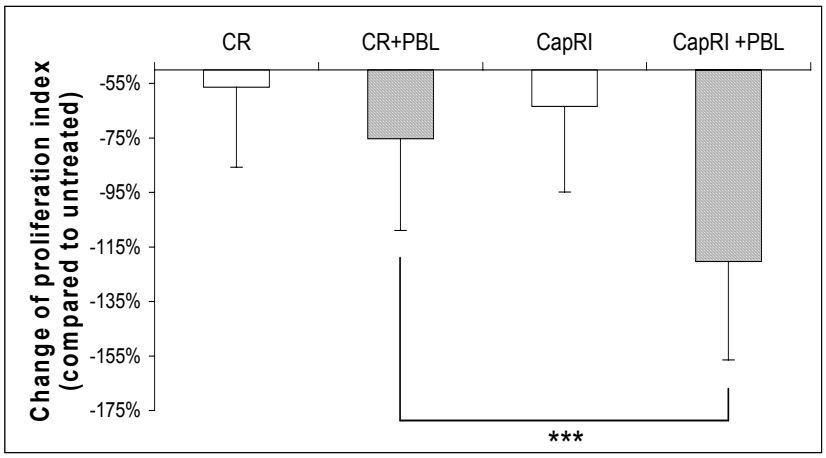


**Figure 7:** MTT based cytotoxicity in the chemoradiotherapy (CR) scheme vs. chemoradioimmunotherapy (CapRI) scheme

At a ratio effector to target cells of 80:1 chemoradiotreated cells were co-cultured over 4 hours with unstimulated lymphocytes, while chemoradioimmunotreated cells were co-cultured with IFN-α prestimulated lymphocytes. Data are shown as mean ± standard error from eight cell lines each with three separate experiments. Three asterisks indicate statistical significance of $p<0.0001$.

### 3.6 Influence of IFN-α on the immunogenicity of pancreatic carcinoma cells

The immunogenicity of the tumor cells and potential role of IFN-α were investigated looking for MHC and Fas/FasL expression. Seven out of eight investigated tumor cell lines had even in their untreated status a high expression of MHC molecules and IFN-α was not able to affect the percentage of positive cells. One cell line (PA-CLS 52) did not express the MHC molecules and this was also unaffected by the IFN-α treatment. However, tumor cells showed a significant increase in mean fluorescence of MHC class I (4.9 ± 4.8 vs.13.30 ± 7.0; $p<0.03$; Fig. 8A) after IFN-α treatment. Mean fluorescence of MHC class II was not significantly enhanced.

Treatment with IFN-α, 5-FU, CDDP and/or radiation showed no significant effect on Fas expression on tumor cells, although there was a tendency towards downregulation after 5-FU treatment. FasL expression was downregulated after 5-FU treatment; this was significant for treatment with the complete CapRI-scheme ($p< 0.05$; Fig. 8B).

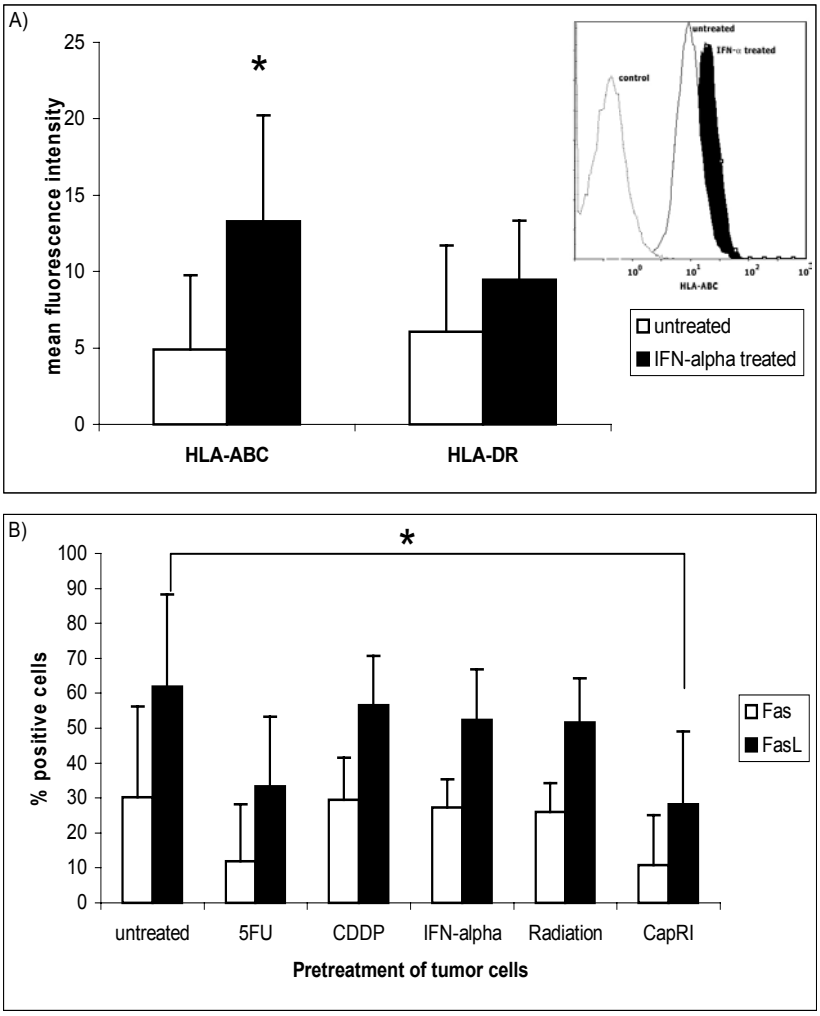


**Figure 8A/B:** Impact of IFN-α on the immunogenicity of tumor cells

A) Mean fluorescence of HLA-expression on tumor cells after five days of IFN-α stimulation with a representative overlay of flow cytometric analysis. B) Fas and FasL expression on tumor cells after five days of IFN-α stimulation or cytotostatic/radiation treatment. Data are shown as mean ± standard error from eight cell lines each with at least three separate experiments. An asterisk indicates statistical significance ($p<0.05$). These data were obtained with the contribution of Dr. Jian-Hua Ma and are also presented in his doctoral thesis "Enhancement of Chemotherapeutic and Radio-Immunotherapeutic Sensitivity on Human Pancreatic Cancer Cells due to Interferon-alpha (CapRI Scheme in Vitro)" as figure 11 A/B (page 41).

### 3.7 Cytotoxic activity of T cells against IFN-α treated tumor cells

As our group has recently shown, IFN-α is able to induce the immunoproteasome in pancreatic carcinoma cells (upregulation of LMP-2, LMP-7 and MECL); here was tested the impact of this switch on their susceptibility to T cell attacks. Unstimulated and IFN-α stimulated T cells had a weak cytotoxic effect on the untreated pancreatic carcinoma cells. Further, the effect of IFN-α on the susceptibility of tumor cells to HLA-matched T cells was investigated. IFN-α pretreated tumor cells were incubated with IFN-α stimulated T cells and cytolysis was determined in a standard chromium-release assay. T cells failed completely to

lyse untreated pancreatic carcinoma cells while IFN-α pretreated pancreatic carcinoma cells were moderately susceptible (14.3 ± 2.5% at an E:T ratio of 80:1; $p<0.001$; Fig. 9A).

The mechanism of apoptosis induction involved in the killing of the IFN-α treated cells by the IFN-α stimulated HLA-matched T cells was also investigated. The decrease in cytolysis after the inhibition of perforin-release using Concanamycin A was statistically significant – at an E:T ratio of 80:1 the cell lysis dropped from 16.9 ± 2.5% to 8.3 ± 2.8% ($p<0.03$). Disruption of Fas/FasL interactions after incubation with Brefeldin A inhibited killing completely, $p<0.01$ (Fig. 9B).

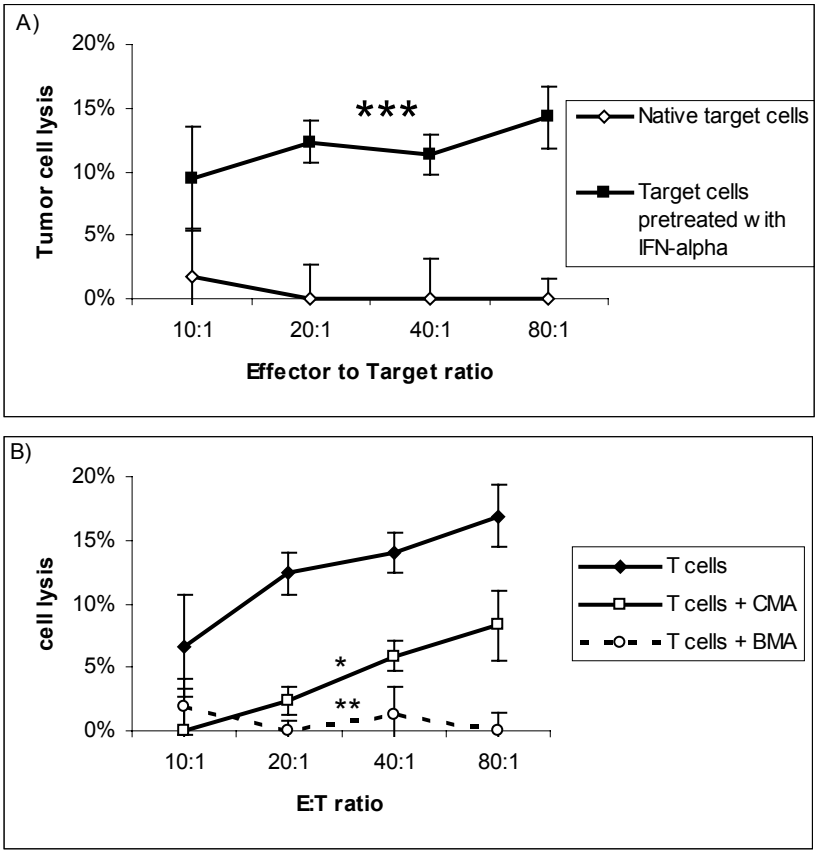


**Figure 9A/B:** Cytotoxicity of stimulated T cells against IFN-α treated tumor cells

A) T cells from a healthy donor (HLA $A2^+$) were separated and stimulated over night with IFN-α. HLA-matched tumor cells (Panc1, DAN-G) were stimulated with IFN-α as described. T cells were tested for their cytotoxic activity against stimulated and unstimulated pancreatic carcinoma cell lines at different E:T ratios in a standard chromium release assay. Data are shown as mean ± standard error from two cell lines each with at least three separate experiments. Three asterisks indicate statistical significance of $p<0.001$. B) The pathway of apoptosis induced by T cells was investigated by degradation of perforin-containing granules or disruption of Fas/FasL interactions. T cells were incubated either with Concanamycin A (CMA) or Brefeldin A (BFA) before they were used in a $^{51}$Cr-release assay. PANC-1 cells pretreated with IFN-α were used as targets. Data are shown as mean ± standard error from three separate experiments. An asterisk indicates statistical significance ($p<0.05$), two asterisks indicate $p<0.01$.

### 3.8 Effect of IFN-α on the NF-κB binding activity

Up-regulation of NF-κB is known to be a way of tumor-resistance in pancreatic carcinoma. Here was investigated whether IFN-α has any influence as a single agent as well as in combination with chemo- and radiotherapy. In order to evaluate the effect of IFN-α on the amount of active NF-κB complexes, DNA-binding activity of this transcription factor was assessed through electrophoretic mobility shift assays (EMSAs).

In one (PANC-1) out of the eight pancreatic carcinoma cell lines IFN-α treatment produced a decrease of the NF-κB binding activity when used as a single agent or in combination therapy. When using different concentrations of IFN-α in combination with 5-FU, the decrease in NF-κB binding activity correlated with the concentration of IFN-α (Table 1).

**Table 1:** The influence of increasing doses of IFN-α on NF-κB (change of the density bands in EMSA to untreated)

| Treatment | Change to untreated (%) |
|---|---|
| 5-FU | 371.9 |
| IFN-α 1,000 U/ml | -69.4 |
| IFN-α 5,000 U/ml | -45.9 |
| IFN-α 10,000 U/ml | -37.9 |
| 5-FU + IFN-α 1,000 U/ml | 161.6 |
| 5-FU + IFN-α 5,000 U/ml | 26.6 |
| 5-FU + IFN-α 10,000 U/ml | 21.1 |

Furthermore, the effect of single agents, their combinations with IFN-α, as well as the whole CapRI scheme were investigated on PANC-1 cells using 10,000 U/ml IFN-α, the dose which was the most efficient in combination with 5-FU. The effect of chemotherapy was an increase in NF-κB binding activity; addition of IFN-α, as well as the whole CapRI scheme had a decreasing effect (Fig. 10).

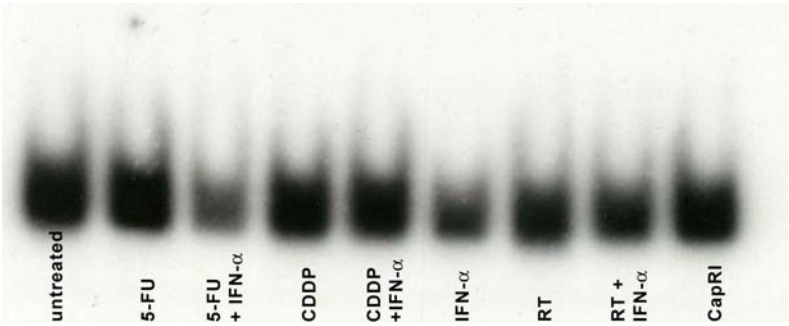


**Figure 10:** Influence of IFN-α in combination with different agents of the CapRI scheme upon the DNA-binding activity of NF-κB

NF-κB binding activity was assessed by electrophoretic mobility shift assay (EMSA) in PANC-1 cell line after different treatments. IFN-α concentration was 10,000 units/ml. Radiotherapy dose was 10 Gy.

5-FU alone led to an increase of NF-κB binding activity of 105%. Addition of IFN-α to 5-FU produced a significant decrease in NF-κB binding activity, which was below the level of untreated cells (-15.6%). The effect of IFN-α in this combination when compared with 5-FU as a single agent, was a 47.5% decrease. IFN-α as single agent therapy produced a decrease of 35.6% compared to untreated cells. The complete CapRI scheme decreased NF-κB binding activity to 19.1% compared to untreated cells (Table 2).

**Table 2:** The influence of IFN-α in combination with chemo-radiotherapy on the DNA-binding activity of NF-κB

NF-κB binding activity was assessed by electrophoretic mobility shift assays (EMSAs) in PANC-1 cell line after different treatments. IFN-α concentration was 10,000 units/ml. Radiotherapy dose was 10 Gy.

NF-κB bands were analyzed by computerized-image densitometry.

| Treatment | Change to untreated (%) |
|---|---|
| IFN-α | -35.6 |
| 5-FU | 105.1 |
| 5-FU + IFN-α | -15.6 |
| CDDP | 19.0 |
| CDDP + IFN-α | 3.4 |
| RT | -11.2 |
| RT + IFN-α | -22.7 |
| CapRI | -19.1 |

## 4. DISCUSSION

Carcinoma of the exocrine pancreas has an especially bad prognosis. Adjuvant treatment following resection prolongs the survival, but so far it has failed to produce long-lasting benefits. Results from a phase II trial, where chemoradiotherapy was combined with IFN-α, are very encouraging (55% 5-year survival compared to 10% after chemoradiation in the ESPAC-1 trial; Picozzi VJ et al. 2003).

Comparing the data from the phase II trial that used chemoradioimmunotherapy with the results of the ESPAC-1 trial, it can be hypothesized that IFN-α is the agent that turns a slightly effective treatment (radiochemotherapy) into a potent therapy. Several mechanisms are described that might explain why IFN-α could play a role in combination therapies: a) direct inhibitory effects on tumor cell growth (Pfeffer LM et al. 1998); b) radio- and chemosensitizing effects (Kurzrock R et al. 1991; Holsti LR et al. 1998); c) anti-angiogenic properties (Decatris M, 2002; Solorzano CC, 2003; Wang L, 2003); d) enhancement of immunogenicity of tumors (Pfeffer LM et al. 1998), and e) immunomodulatory effects (Belardelli F, 2002). Previous results obtained by our research group have shown that IFN-α has direct inhibitory properties with limited synergistic influence when added to chemo- or radiotherapy, and that the enhanced proliferation rate and VEGF secretion of cells re-growing after CDDP treatment is reverted when adding IFN-α (Ma J et al. 2005). These results may be partially responsible for the positive outcome of the CapRI scheme. Considering the immunomodulatory effects of IFN-α it can be hypothesized that the activation of the immune system by IFN-α plays a very important role in this treatment scheme.

When comparing the CapRI scheme with chemoradiotherapy there was no significant difference in the proliferation rate and apoptosis induction suggesting that the main mechanism through which IFN-α acts in the CapRI scheme might be the immunomodulatory one.

IFN-α is known to stimulate the immune-system by several strategies. Beside its direct activating effects on immunological effector cells, such as cytotoxic T cells and NK cells, IFN-α induces the differentiation and maturation of dendritic cells and enhances the immunogenicity of tumor cells upregulating the expression of MHC molecules. IFN-α also enhances the survival of T cells and is important for the proliferation and long-term survival of antigen-specific T cells (Belardelli F, 2002).

After overnight stimulation with IFN-α there was a significant increase in cytotoxic activity of PBLs against untreated pancreatic carcinoma cell lines. Tumor cells which were resistant to unstimulated PBLs could be lysed by IFN-α stimulated PBLs. It is known that IFN-α stimulates the differentiation and function of the dendritic cells, which play an important role in T cell activation (Paquette RL et al. 1998). Therefore the influence of co-culture with monocytes on the cytotoxic activity of IFN-α prestimulated PBLs was assessed. As stimulation in the absence of monocytes did not influence their cytotoxic activity, it is to be concluded that IFN-α had a direct effect in increasing the cytotoxic activity of lymphocytes against untreated pancreatic carcinoma cell lines.

Furthermore, it was investigated which fraction in the PBLs is responsible for the increased cytotoxicity upon IFN-α stimulation. Whether stimulated or not, T cells showed no significant cytotoxicity, while cytotoxic activity of the T-cell depleted lymphocyte fraction, consisting mainly of natural killer (NK) cells, was significant even in the absence of stimulation and increased significantly after overnight stimulation with IFN-α. These results confirm the data in the literature concerning the role of IFN-α in activating the natural killer cells (Belardelli F et al. 2002; Janeway CA et al. 2001). Although there is evidence regarding the role of dendritic cells in the activation of NK cells (Cooper MA et al. 2004; Ferlazzo G et al. 2004), this effect could not be proved as stimulation of the T cell depleted lymphocytes in the absence of monocytes did not influence their cytotoxicity.

When investigating the mechanism of apoptosis induction, the results showed that the cytotoxicity of PBLs was mediated mainly by Fas-induced apoptosis as well as by perforin release, but the latter failed to be statistically significant. Data in the literature stipulate that granzyme B is a major effector of target cell lysis by NK cells (Mahrus S et al. 2005). Therefore the release of granzyme B was investigated and the results confirmed these data, as NK cells even unstimulated had a significantly higher granzyme B release when compared with PBLs. This effect was significantly increased by overnight IFN-α stimulation.

Combination therapies as the CapRI protocol seem to be superior to mono-therapies not only through the addition of different strategies but by synergistic effects. IFN-α is well known to act as a radio- and a chemosensitizer and 5-FU and CDDP are used as well as radiosensitizers. When pretreated pancreatic carcinoma cell lines were used as target for IFN-α prestimulated immune cells the treatment with 5-FU made tumor cells more vulnerable. Analyzing the

mechanism of apoptosis induction, the treatment with 5-FU produced an increased vulnerability of the target cells to the perforin pathway.

Furthermore, IFN-α treatment enhances the immunogenicity of tumor cells. The density of MHC molecules on tumor cells increased significantly after treatment with IFN-α, confirming the data in the literature (Belardelli F et al. 2002).

FasL expression on tumor cells was not affected by IFN-α and decreased after 5-FU treatment. Fas expression on tumor cells was not significantly affected, although there was a strong tendency towards downregulation of Fas after 5-FU treatment. It was previously shown in the thesis of Dr. Jian-Hua Ma, "Enhancement of Chemotherapeutic and Radio-Immunotherapeutic Sensitivity on Human Pancreatic Cancer Cells due to Interferon-alpha (CapRI Scheme in Vitro)" (Ma J 2004), that after IFN-α stimulation lymphocytes express more Fas ligand (FasL). In the counterattack model of tumorigenesis, it has been proposed that tumors develop resistance to attack from FasL expressing cytotoxic T cells by downregulating Fas (immune escape); meanwhile, upregulating FasL they are able to induce apoptosis in Fas-expressing T cells (Kim R et al. 2004). Results obtained for the cell lines studied cannot support this model. The down-regulation of FasL on the surface of pretreated target cells may decrease apoptosis in the effector cells - this is one possible explanation for the enhanced susceptibility of tumor cells observed after 5-FU treatment. Supporting the importance of Fas regulation, T cells killed IFN-α pretreated tumor cells through the Fas/FasL pathway.

In general, T cells are able to enter pancreatic carcinoma, although the infiltrating cells often show reduced activation status (Kim R et al. 2004; von Bernstorff W et al. 2001). Various strategies are described to enhance the cytotoxicity of T cells and to direct them tumor-specific, e.g. co-culture with antigen-specific pulsed dendritic cells or direct injection of antigens which can be taken up by dendritic cells resulting in T cell stimulation (Emmrich J et al. 1998; Schmidt T et al. 2003; Hoos A et al. 2003; Stift A et al. 2003; Schnurr M et al. 2002). Although IFN-α stimulation of T cells alone failed to induce cytolytic activity, IFN-α pretreated tumor cells were more vulnerable to T cells.

The increase in T cell mediated cytolysis after IFN-α treatment of pancreatic carcinoma cells can be related with previous results obtained by our research group which have shown that treatment of pancreatic carcinoma cells with IFN-α produced a switch to the immunoproteasome in most of the investigated cell lines (Ma J 2004). It was reported that the

immunoproteasome plays an important role in determining the immunodominance hierarchy (Chen W et al. 2001; Morel S et al. 2002) so that it has direct impact on $CD8^+$ T cell responses by modifying the repertoire of responding $CD8^+$ T cells (Van den Eynde BJ et al. 2001).

Furthermore, the difference between the CapRI scheme and chemoradiotherapy was investigated regarding cytotoxicity in an experiment similar with the in vivo situation: IFN-α stimulated lymphocytes were used against chemoradioimmunotreated pancreatic carcinoma cells and unstimulated lymphocytes against chemoradiotreated target cells showing a significant increase of the cytotoxic effect in the CapRI scheme.

Many pancreatic carcinoma cell lines have an increased constitutive NF-κB activity (Arlt A et al. 2002). As NF-κB is an antiapoptotic factor implicated in carcinogenesis and resistance to chemotherapy, its inhibition seems important in improving the outcome of adjuvant treatment of pancreatic adenocarcinoma (Arlt A et al. 2003; Li Y et al. 2004). Various inhibitors can target different steps in the NF-κB signal transduction pathway: a) IKK inhibitors (non-steroidal anti-inflammatory drugs, cyclopentenone prostaglandins or natural compounds as the flavonoids) (Li et al. 2004; Sclabas GM et al. 2005; Muerkoster S et al. 2003); b) glucocorticoids (glucocorticoid receptors induce the synthesis of IκBα); c) proteasome inhibitors (e.g. peptide aldehydes, lactacystine, vinyl sulfone tripeptides, peptide boronic acids – bortezomib, natural compounds – eponemycin, epoxomycin); d) mesalamine (through inhibition of IL-1-driven p65 phosphorylation); e) oligonucleotides; f) peptides interfering with protein-protein interactions (Delhalle et al. 2004).

Previous reports have shown that IFN-α was able to suppress the antiapoptotic effect of NF-κB in a selection of primary cell cultures of renal cell carcinoma (Steiner T et al. 2001) and inhibited TNF-α-induced NF-κB in a human cervical cancer cell line (Suk K et al. 2001). Inhibition of inducible NF-κB activity reduces chemoresistance to 5-FU in human stomach cancer cell lines (Uetsuka H et al. 2003).

When investigating the effect of IFN-α on the DNA-binding activity of NF-κB, the results confirmed these data showing an induction of NF-κB by chemotherapy, 5-FU being the agent with the strongest inductive effect. In one pancreatic carcinoma cell line (PANC-1) IFN-α was able to revert this effect in a dose dependent manner, while the whole CapRI scheme showed a slight inhibitory effect. Therefore, it can be concluded that in certain pancreatic tumors IFN-α might act also through the inhibition of NF-κB.

In conclusion, NK cells can be activated by IFN-α against pancreatic carcinoma cells and pancreatic carcinoma cells are more susceptible to the immunological attacks after 5-FU treatment. As IFN-α is described to have direct effects on tumor cells and acts also synergistic with chemoradiation we could presume a complex network of enhancing interactions between the different agents used in the CapRI scheme.

## 5. SUMMARY

Data from a phase II trial combining chemoradiotherapy with IFN-α (CapRI scheme) for adjuvant treatment of pancreatic carcinoma are very encouraging. Hypothesizing that IFN-α is the agent which significantly improves radiochemotherapy this work focuses on the immunomodulatory effect of IFN-α in this regimen.

Eight human ductal pancreatic carcinoma cell lines were treated with the CapRI scheme (5-Fluorouracil, cisplatin, IFN-α and radiation). Peripheral blood lymphocytes, NK and T cells were preincubated with 1,000 U/ml IFN-α over 24 hours and tested in cytotoxicity assays against these cell lines and the mechanism of apoptosis induction was investigated, as well as the direct effect of IFN-α on pancreatic carcinoma cells regarding their immunogenicity and the influence on the DNA binding activity of the nuclear transcription factor NF-κB.

The results showed an increase in cytotoxic activity of peripheral blood lymphocytes after IFN-α treatment from 12.5% to 34.3% ($p<0.05$). This increase in cytotoxicity was due to the NK cells as shown after depletion of T cells (T cells 4% lysis, NK cells 42.7% lysis) and was mediated by Fas-induced apoptosis, as well as by perforin release. Pretreatment of tumor cells with 5-FU and its combinations showed a significant increase in the susceptibility of tumor cells to NK cells (untreated tumor cells 34.3%, CapRI scheme 69.1%). While there was no significant difference between the whole CapRI scheme and chemoradiotherapy regarding cell proliferation rate and apoptosis induction, the cytotoxic effect of IFN-α stimulated lymphocytes against CapRI-treated pancreatic carcinoma cells was significantly higher than the effect of unstimulated lymphocytes against chemoradiotreated tumor cells (CapRI scheme -120.3%, chemoradiotherapy -75.2%, $p=0.0001$).

IFN-α alone was able to increase the immunogenicity of pancreatic carcinoma cell lines increasing the mean expression of MHC class I in a significant manner which makes the tumor cells more susceptible to T cell cytotoxicity. In selected pancreatic carcinoma cell lines IFN-α might inhibit the DNA-binding activity of nuclear transcription factor NF-κB.

IFN-α activates NK cells against pancreatic carcinoma cells and 5-FU treatment makes tumor cells more susceptible. These mechanisms may be responsible for the improved clinical outcome of CapRI.

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## 7. CURRICULUM VITAE

PERSONAL INFORMATION

| | |
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| Name: | Patrut Emilia-Maria |
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| Mother: | Patrut Emilia (born Mirza) |

EDUCATION

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|---|---|
| 1990-1994 | High school for informatics, Cluj-Napoca, Romania |
| 30. June 1994 | High school graduation |

ACADEMIC PREPARATION

| | |
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| WS 1994/1995 | Beginning of medical training at the Faculty of Medicine, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca Romania |
| 16. September 2000 | Diploma examination (Medicine). Thesis: The role of hydrocolonic sonography in the diagnosis of colorectal diseases. |
| 26. September 2000 | License to practice medicine |

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The experiments concerning the cytotoxic activity of PBLs co-cultured with monocytes against untreated pancreatic carcinoma cells (chapter 3.2, figure 2A, page 30) and the cytotoxic activity of PBLs co-cultured with monocytes against pretreated pancreatic carcinoma cells (chapter 3.4, figure 5A, page 34) as well as mean fluorescence of HLA-expression, Fas and FasL expression on IFN-α treated pancreatic carcinoma cell lines (chapter 3.6, figure 8A/B, page 37), were performed with the contribution of Dr. Jian-Hua Ma and are presented also in his doctoral thesis “Enhancement of Chemotherapeutic and Radio-Immunotherapeutic Sensitivity on Human Pancreatic Cancer Cells due to Interferon-alpha (CapRI Scheme in Vitro)”.