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"DEVELOPMENT OF A MODEL OF HUMANIZED MOUSE TO STUDY THE CROSS-TALK BETWEEN TUMOR AND IMMUNE SYSTEM AND OF A THERAPEUTIC APPROACH FOR PANCREATIC CANCER WITH HUMAN DENDRITIC CELLS LOADED WITH POST-APOPTOTIC TUMORS"

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To my mother, my father and Fabio

"None of this had even a hope of any practical application in my life. But 10 years later, when we were designing the first Macintosh computer, it all came back to me. And we designed it all into the Mac. It was the first computer with beautiful typography. If I had never dropped in on that single course in college, the Mac would have never had multiple typefaces or proportionally spaced fonts. And since Windows just copied the Mac, its likely that no personal computer would have them.

If I had never dropped out, I would have never dropped in on this calligraphy class, and personal computers might not have the wonderful typography that they do. Of course it was impossible to connect the dots looking forward when I was in college. But it was very, very clear looking backwards 10 years later.

Again, you can't connect the dots looking forward; you can only connect them looking backwards. So you have to trust that the dots will somehow connect in your future. You have to trust in something–your gut, destiny, life, karma, whatever. This approach has never let me down, and it has made all the difference in my life."

Steve Jobs, from the 2005 Stanford Commencement Address

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Abbreviations list

5-FU = 5-Fluorouracil

ABD= ATPase domain

ACT = adoptive cell transfer

Ann V = Annexin V

APCs = Antigen Presenting Cells

ATP = adenosine-3-phosphate

ARG-1= arginase-1

BCR = B cell receptor

BM = Bone marrow

CDRs = complementary-determining regions

CDs= cluster of differentiation

cGy = centy-Gray

Cox-2= cyclooxygenase-2

CRT = chemoradiotherapy

CTLA-4= cytotoxic T-lymphocytes-associated antigen 4

CTLs = Cytotoxic T Lymphocytes

DAMPs = danger-associated molecular patterns

DAPI = 4',6-diamidino-2-phenylindole

DCs = Dendritic cells

EFS = event-free survival

ELISA = Enzymed-Linked Immunoassorbent Assay

ER = endoplasmatic reticulum

FACS = fluorescence-activated cell sorting

FasL = Fas Ligand

FBS = Fetal bovine serum

GEM = gemcitabine

GM-CSF = granulocyte-macrophage stimulating factor

GvHD = graft versus host desease

H&E = hematoxylin/eosin

H-2 = hystocompatibility-2

HIFU = high-intensity focused ultrasound

HLA = human leucocytes antigens

HMGB-1 = high mobility group box-1

HSPs = Heat Shock Proteins

HT = hyperthermia

i.h = intra-hepatical

i.p = intraperitoneal

i.v = endovenous

i.v. = intravenous

ICAM-1 = Intercellular Adhesion Molecule 1

iDCs = immature Dendritic cells

IFN-γ = interferon-γ

Ig = immunoglobulin

IHC = immunohistochemistry

I_i = invariant chain

IL- = interleukin

KO = knock out

LPS= lipopolysaccharide

M-CSF= macrophage-colony stimulating factor

mDCs = mature Dendritic cells

MDSCs= myeloid derived suppressor cells

MHC-I, -II = Major Histocompatibilty Complex-I, -II

MMP = matrix metalloproteinases

NK = Natural Killer cells

NOS= nitric-oxide synthase

OS = overall survival

PaCa = Pancreatic cancer

PAMPs = Pathogens Associated Molecular Patterns

PanIN = pancreatic intraepithelial neoplasia

PB = peripheral blood

PBD = Peptide Binding Domain

PBMCs (hu) = human peripheral blood mononuclear cells

PBS = Phosphate-buffered Saline

PDCs= plasmacytoid dendritic cells

PDAC = Pancreatic Ductal Adenocarcinoma

PGE_2 = Prostaglandin E_2

PI = Propidium Iodide

Rag2 = recombinase activated gene 2

RFA = Radiofrequency

s.c. = subcutaneously

SCID= severe combined immunodeficiency

TAAs = Tumor Associated Antigens

TAP= transporter associated with antigen processing

TCR = T cell receptor

TGF-β = Transforming Growth Factor-β

T_h1, 2, 17 = T helper cell-1, -2, -17

TNF-α, -β = Tumor Necrosis Factor-α, -β

TRANCE= TNF-related association-induced cytokine

T_{reg} = T regulatory cells

VEGF = vascular endothelial growth factor

w. t. = wild type

Summary

Pancreatic cancer has a poor prognosis and the 5-years survival rate is less than 1%, with a median survival of 4–6 months. For this reason, new therapies are needed and immunotherapy might be an option.

The development of a preclinical model to study in vivo the cross-talk between tumor and immune system can be crucial. The initial aim of this study was the reconstitution of newborn immunodeficient mice with intrahepatically injection of bone marrow-derived leukocyte precursor CD34+ cells from pancreatic cancer patients, in order to obtain patient-specific humanized mice, to be implanted subcutaneously or orthotopically with pancreatic tumor cells from the same patient; such a system should allow to study *in vivo* the cross-talk between tumor and immune system and to evaluate immunotherapeutic approaches in a "patient-specific setting". Unfortunately, burocratic problems hampered so far the acquisition of bone marrow-derived leukocyte precursor CD34+ cells from pancreatic cancer patients as well as from healthy donors. Therefore, we reprogrammed the the study and:

1- we established protocols of delivery of human PBMCs to C57BL/6-Rag2$^{-/-}$ γ chain$^{-/-}$ immunodeficient mice, starting from either newborn or adult mice;

2- we developed an adoptive cell transfer (ACT) therapy based on human T lymphocytes activated *in vitro* by autologous DCs loaded with necrotic pancreatic tumor cells after hyperthermia or UVC treatment, to be delivered to C57BL/6-Rag2$^{-/-}$ γ chain$^{-/-}$ mice bearing the same human pancreatic tumor from which the necrotic cells used for DC loading were derived.

As expected, the reconstitution we obtained using human PBMCs regarded only the human T and B cells compartment, and not cells of the innate immunity. The differences observed in mice reconstituted by injection of human PBMCs at newborn versus mice injected at adult age concerned mortality and the presence of T cells in peripheral blood. Mice injected at newborn age showed very high mortality following injection (67%) and relatively low percentage of reconstituted animals (45% of alive animals). Nevertheless reconstituted mice presented human lymphoid cells either circulating in the blood or in lymphoid organs. On the contrary, mice injected with human PBMCs at adult age did not show any mortality effect, but the amounts of circulating human lymphoid cells in the

blood was very low, even if engrafment of human cells in lymphoid organs was similar to that found in mice injected at newborn and adult age.

The second part of work presented is focused on the development of an immunotherapeutic approach for pancreatic cancer. The immunogenic potential of dying tumor cells is receiving great attention on account of both its importance in enhancing T-cell directed immunotherapy and its indication of the best immunogenic source for *ex vivo* TAA DCs loading (Lake et al., 2005; Nowak et al., 2006). DCs are potent antigen-presenting cells and, as recently shown, can be activated by DAMPs, such as HMGB-1 and HSP-70, that are highly expressed after hyperthermia treatment of cancer cells and that induce anti-tumor immunity (Shi et al., 2006; Bianchi et al., 2007; Chen et al., 2009). Antigen presentation by DCs is essential to effective antitumor T cells responses in cancer patients, but it depends on DCs origin, maturation state and the environmental cytokine milieu.

For this reason, we focused our attention on the activation of a tumor-protective immune response using human PaCa-44, PT-45, PANC-1 and PANC-2 pancreatic tumor cell lines.

We developed an immunotherapeutic strategy based on human dendritic cells from healthy blood donors loaded with cancer cells in secondary necrosis due to incubation at 56°C or exposure to UVC.

This immunotherapeutic approach appears promising, especially using UVC exposure as tumor death treatment. The treatments induced apoptosis followed by secondary necrosis and the release in the supernatant of levels of DAMPs much higher than without treatment. DAMPs are responsible for stimulation of DCs to antigen uptake and maturation, and consequently, for an increased immunogenity of tumor cells, as shown by the expression of higher levels of maturation markers such as CD80, CD83 and MHC-II. Secondary necrosis also resulted in an improvement of uptake efficiency by DCs for all the four different pancreatic cancer cell lines. The specific immune response obtained when T cells from the same donor as DCs were added to activated DCs according to standard protocols, was variable between different pancreatic cancer cell lines and it was also blood donor-dependent: a specific immune response was obtained *in vitro* only against PaCa-44 and PT-45 cancer cell lines, and not against PANC-1 and PANC-2 cell lines. These results were also confirmed *in vivo* in a protocol of curative immunotherapy using PaCa-44 and PT-45 cancer cells only, even if the low number of T-lymphocytes injected and of animals for each groups are a crucial points for the assessment of a functional preclinical model.

Riassunto

Il cancro al pancreas ha una prognosi scarsa e la percentuale di pazienti affetti da questa patologia che sopravvivono per 5 anni è inferiore all'1%, con una sopravvivenza media che oscilla tra i 4 e i 6 mesi. Ecco perché sono necessarie nuove terapie, e l'immunoterapia potrebbe rivelarsi un approccio terapeutico valido e promettente.

Lo sviluppo di un modello preclinico per studiare *in vivo* l'interazione tra il tumore e il sistema immunitario può essere cruciale. L'obiettivo iniziale di questo studio era la ricostituzione di un sistema immunitario umano in topi immunodeficienti appena nati, mediante un'iniezione intraepatica di cellule staminali ematopoietiche CD34+ derivate dal midollo osseo di pazienti affetti da cancro al pancreas, al fine di ottenere topi umanizzati corrispondenti al paziente, da sottoporre a impianto ortotopico o sottocutaneo di cellule tumorali pancreatiche prelevate dal medesimo paziente. Tale sistema dovrebbe consentire di studiare in vivo l'interazione tra il tumore e il sistema immunitario e di valutare gli approcci immunoterapeutici più validi a seconda del diverso paziente.

Sfortunatamente, ad oggi, i problemi burocratici hanno impedito l'acquisizione di cellule precursori CD34+ derivate dal midollo osseo sia di pazienti affetti da cancro al pancreas sia di donatori sani. Per cui abbiamo riprogrammato il lavoro e:

1) abbiamo messo a punto dei protocolli di ricostituzione di un sistema immunitario umano in topi immunodeficienti C57BL/6-Rag2$^{-/-}$ γ chain$^{-/-}$ appena nati o adulti, utilizzando cellule mononucleate di sangue periferico di donatori sani (PBMCs)

2) abbiamo sviluppato un modello di 'trasferimento adottivo cellulare' (ACT) basato su linfociti T umani attivati *in vitro* da cellule dendritiche autologhe caricate con cellule necrotiche di adenocarcinoma pancreatico dopo ipertermia o trattamento con UVC. Questi linfociti T attivati *in vitro* sono stati trasferiti a topi C57BL/6-Rag2$^{-/-}$ γchain$^{-/-}$ precedentemente inoculati con la stessa linea cellulare tumorale dal quale sono state derivate le cellule necrotiche usate nella stimolazione *in vitro* delle cellule dendritiche.

Come previsto, la ricostituzione che abbiamo ottenuto usando cellule mononucleate di sangue periferico ha riguardato solo il compartimento delle cellule T e B e non le cellule dell'immunità naturale. Le differenze osservate nei topi ricostituiti mediante iniezione di PBMCs umane alla nascita rispetto ai topi iniettati in età adulta ha riguardato la mortalità e

la presenza di cellule T nel sangue periferico. I topi iniettati alla nascita hanno mostrato un'alta mortalità dopo il trattamento (67%) e una percentuale relativamente bassa di animali ricostituiti (45% sul totale degli animali rimasti in vita). Tuttavia i topi ricostituiti da neonati hanno presentato cellule linfoidi umane in circolo nel sangue periferico e negli organi linfoidi. Al contrario, i topi iniettati da adulti con PBMCs di donatori sani hanno mostrato un'ottima sopravvivenza dopo il trattamento, ma la quantità di cellule linfoidi umane in circolo nel sangue periferico si è rivelata molto bassa; tuttavia, la presenza di cellule umane negli organi linfoidi era simile a quella riscontrata nei topi iniettati da neonati.

La seconda parte del lavoro presentato si è focalizzata sullo sviluppo di un approccio immunoterapeutico per il cancro al pancreas. Il potenziale immunogenico delle cellule tumorali necrotiche sta ricevendo grande attenzione, in considerazione sia della sua importanza nell'aumento delle cellule T citotossiche specifiche per l'immunoterapia tumorale, sia della sua importanza come migliore fonte di antigeni tumorali (TAA) per le cellule dendritiche (Lake et al., 2005; Nowak et al., 2006). Le cellule dendritiche sono cellule in grado di presentare efficacemente un antigene a linfociti T e, come mostrato di recente, possono essere attivate da molecole DAMPs come l'HMGB-1 e l'HSP-70, che sono altamente espresse sulla superficie di cellule tumorali necrotizzate soprattutto dopo trattamento ipertermico e che inducono l'immunità antitumorale (Shi et al., 2006; Bianchi et al., 2007; Chen et al., 2009). La presentazione antigenica mediante cellule dendritiche è essenziale per ottenere risposte effettive delle cellule antitumorali T in pazienti malati di cancro, ma essa dipende dall'origine, dallo stato di maturazione e dal *milieu* citochinico.

Per questo motivo abbiamo focalizzato la nostra attenzione sull'attivazione di una risposta immunitaria che protegga dal tumore, usando PaCa-44, PT-45, PANC-1 e PANC-2, tutte linee cellulari umane di tumore al pancreas.

Abbiamo sviluppato una strategia immunoterapeutica basata su cellule dendritiche umane ottenute dal sangue di donatori sani, caricate con cellule tumorali necrotizzate dopo incubazione a 56°C o esposizione agli UVC.

Questo approccio immunoterapeutico pare promettente, soprattutto qualora si ricorra all'esposizione agli UVC come trattamento per necrotizzare il tumore. I trattamenti hanno indotto l'apoptosi seguita da necrosi secondaria delle cellule tumorali e il rilascio nel surnatante di livelli di DAMPs più alti di quelli riscontrati senza trattamenti.

Le DAMPs sono responsabili della stimolazione delle cellule dendritiche e, conseguentemente, dell'aumento del potere immunogenico delle cellule tumorali necrotiche, come mostrato dai più alti livelli dei marcatori della maturazione CD80, CD83 e MHC-II espressi dalle cellule dendritiche in presenza delle cellule tumorali necrotiche. La necrosi secondaria ha avuto come risultato anche un miglioramento dell'efficienza di fagocitosi di materiale necrotico da parte delle cellule dendritiche per tutte e quattro le linee cellulari utilizzate. La specifica risposta immunitaria ottenuta quando le cellule T di un donatore sono state aggiunte a cellule dendritiche autologhe attivate in base ai protocolli da noi standardizzati, è cambiata da una linea cellulare all'altra e a seconda del donatore utilizzato. Una risposta immunitaria specifica è stata ottenuta in vitro solo per le linee cellulari PaCa-44 e PT-45, e non contro le linee cellulari PANC-1 e PANC-2. Questi risultati sono stati confermati anche *in vivo* in un protocollo di immunoterapia curativa usando solo le cellule tumorali PaCa-44 e PT-45, nonostante il numero esiguo di linfociti-T iniettati per singolo animale ed il numero di animali a disposizione per ciascun gruppo sperimentale siano stati e restino tuttora un punto cruciale nello sviluppo del modello preclinico.

I. Introduction

Chapter 1: Pancreatic cancer

1.1.1. Pancreas anatomy and physiology

Pancreas is a retroperitoneal organ of endodermal derivation involved in glucose homeostasis and protein and carbohydrate digestion. Pancreas is located deep in the abdomen and it has a close anatomical relationship with the duodenum and the common bile duct. Anatomically, pancreas can be divided into three parts: head (caput), body (corpus) and tail (cauda). Functionally, it is a composite exocrine-endocrine gland.
The major components of the pancreatic parenchyma are (**Figure1**):

1. Acinar and duct cells (80% of the tissue mass of the organ), that are organized in a branching network. The acinar cells are functional units along the duct network and they synthetize and secrete zymogens into the ductal lumen in response to cues from the stomach and duodenum (Hezel A.F et al., 2006). Close to ducts, there are the central-acinar cells; the duct system begins within the acini themselves (Grapin-Botton A., 2005) . The interlobular ducts are the largest ones and they drain directly into the duct of Wirsung, the main pancreatic duct. Taken togheter, they represent the exocrine portion of pancreas.

2. Four specialized endocrine cell types (α, β, δ and PP), gathered together into clusters called islets of Langherhans (about 2% of pancreas volume). They are involved in the regulation of metabolism and glucose homeostasis through the secretion of hormones into the blood-stream, such as insulin (from β cells), glucagon (from α cells), somatostatin (from δ cells) and pancreatic polypeptide (from PP cells). They represent the endocrine portion of the gland.

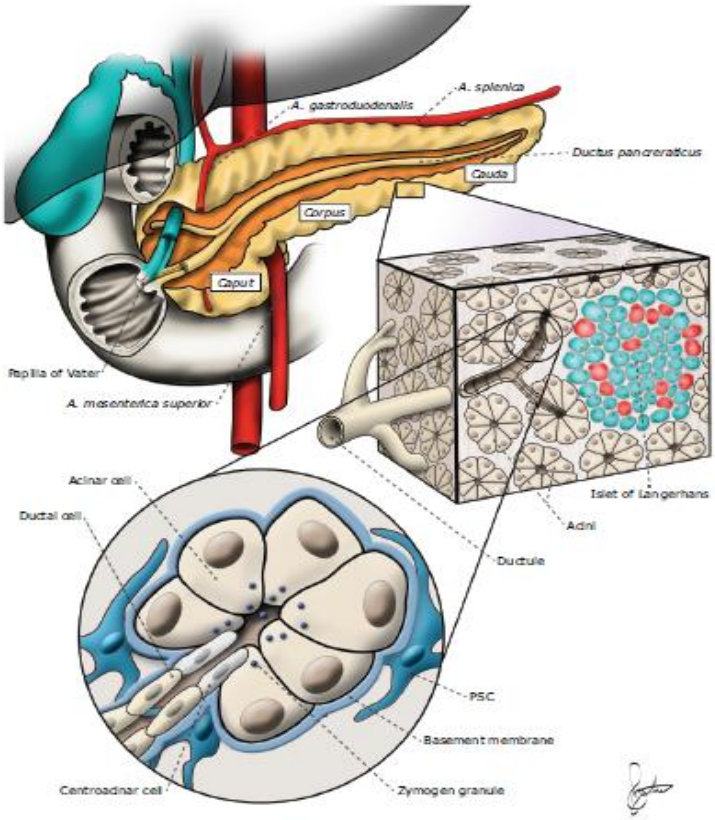


Figure 1 Pancreas. Overall anatomy of pancreas and components of pancreatic parenchyma: islets of Langherhans, acini and ducts. © *G. Andersson*, 2010

Mirroring the physiologic and cellular diversity of pancreas gives an idea of the spectrum of distinct pancreatic malignancies, that possess histological and molecular features recalling the characteristics of the normal cellular costituents (Hezel A.F et al., 2006).

1.1.2 Pancreatic cancer: classification

"Pancreatic cancer" is a broad term to describe as many as 20 different types of tumor that occur in pancreas. The phenotypic classification of pancreatic neoplasms is based on their cellular lineage of origin (acinar, ductal and endocrine phenotype). Based on this classification, we can distinguish exocrine and endocrine pancreatic tumors.

Exocrine tumors represent the majority of pancreatic neoplasms (more than 90%), and can be ductal or acinar.

Exocrine tumors: ductal tumors

Pancreatic Ductal AdenoCarcinoma (PDAC) is the most common pancreatic cancer and accounts for >85% of cases (Warshaw and Fernandez del Castillo 1992; Li D. et al., 2003; Hezel A.F et al., 2006). Microscopically, they are well differentiated, showing tubular structures, cystic formations and papillar structures. Despite the well differentiated appearance, the ductal adenocarcinoma is characterized by a highly aggressive, rapid and infiltrative growth pattern. A quite unique feature is the extensive stroma, referred to as the desmoplastic reaction (most of the tumor volume), that surrounds and infiltrates clusters of cancer cells. A ductal adenocarcinoma often develops through different premalignant states called Pancreatic Intraepithelial Neoplasia (PanIN), that will be described later in this chapter. Beside the PanINs, there are cystic lesions with premalignant potential, especially mucinous cysts.

Less common tumors with a ductal phenotype are the variants of ductal adenocarcinoma, intraductal papillary mucinous neoplasm (IPMNs, including colloid carcinoma), mucinous cystic neoplasm (MCNs), medullary carcinoma and other rare tumors (Kloppel G. et al., 2004). They show malignant potential and up to one third has an associated invasive cancer (Tanaka et al., 2006). The cystic lesions frequently are associated with clinical manifestations, such as abdominal pain, nausea and jaundice, whereas PanINs are often asymptomatic.

IPMNs are constituted of proliferative intraductal neoplastic cells with papillary characteristics that produce mucin. IPMNs derived from the main duct are more prone to harbor invasive cell components compared to IPMNs found in the branch ducts (Tanaka et al., 2006).

MCNs are most common in perimenopausal women and they are characterized by thick-walled multilobular cysts, often without comunication with the ductal part (Thompson et al., 1999).

Therefore, the ductal adenocarcinomas are the most common types of pancreas cancer and they have the most clinical relevance; for these reasons, the expression “pancreatic cancer” in common language is often used when referring to the ductal adenocarcinomas.

Exocrine tumors: acinar tumors

Less than 1% of all pancreatic cancers derive from the acinar cell lineage. Acinar cell carcinoma (ACC) lacks the desmoplastic stroma found in ductal adenocarcinoma and is often large in volume at the time of diagnosis. Most of the ACCs produce digestive enzymes such as trypsin and lipase (Klimstra et al., 1992). In some cases, the enzymes secreted cause fat necrosis due to lipase hypersecretion. The median survival of ACCs is approximately 19 months, which makes acinar cell carcinoma more aggressive than endocrine tumors (PENs) but less aggressive if compared to ductal adenocarcinomas (Holen et al., 2002).

Endocrine tumors

Endocrine tumors are only about 1% of all cases and they affect the hormone-producing cells of pancreas.

The most common endocrine tumors are the Pancreatic Endocrine Neoplasms (PENs) (Heitz et al., 1982). PENs are low-grade malignancies and arise from the islets of Langherans; only 50% of PENs really secrete hormones and they are called "functional" (Kloppel & Heitz, 1988).

The functional PENs are named differently, depending on the hormone secreted (i.e. insulinoma, glucagonoma etc.) and the result is a dramatic increase in the amounts of hormones produced with a variety of symptoms: for istance, patients with insulinoma often show extreme hypoglycemia.

1.1.3 Epidemiology and risk factors of pancreatic cancer

Pancreatic cancer is one of the most lethal human cancers and a major unsolved health problem. PDAC ranks 8th in a worldwide ranking of cancer deaths (Lowenfels AB et al., 2006); but it ranks 13th respect to incidence, as it is considered a relatively rare tumor. PDAC is characteristically a tumor of elderly individuals: 80% of cases occurs in patients between 60 and 80 years of age, while patients below 40 years are rare (Luttges J et al., 2004). The disease is also more prevalent in males than in females (Raimondi S. et al., 2009).

Pancreatic cancer patients have poor 5-year survival rate (for only 5%) and the median survival is less than 6 months. One of the main problems with pancreatic cancer is late onset, with hindering of clinical research. Jaundice, resulting from obstruction of biliary ducts, is often the first symptom, although it generally appears after the tumor has metastasized. Many other symptoms are non-specific as abdominal pain, nausea, vomiting, itchy skin and loss of weight and appetite (Lillemoe KD et al., 2000; Sakorafas et al., 2000). Thus, diagnosis is difficult and close to 100% of patients with pancreatic cancer already show metastasis at the time of diagnosis.

The incidence rate of pancreatic cancer differs among countries suggesting that environmental or lifestyle risk factors may be part of etiology, even if only a few factors are still known. Early epidemiologic studies suggest that carcinogens from tobacco or other environmental sources might enter the bile and reflux through the pancreatic duct, causing pancreatic cancer (Wynder EL et al., 1973). This hypothesis could explain the excess of incidence of pancreatic neoplasms in the head of pancreas.

Current data confirm three types of risk factors: cigarette smoking, diabetes and chronic pancreatitis (Li D et al., 2004).

a) Smoking increased of 2-3 times the risk of pancreatic cancer in current smokers compared to people who have never smoked (de Braud F et al., 2004). Exposure to environmental smoking (passive smoking) causes a non-significant increase of the risk. The effect of smoking is related to the mutagens present in the metabolites of tobacco (Lowenfels et al., 2006).

b) Type II diabetes and pancreatic cancer also have an interesting but unclear relationship (Huxley et al., 2005). Often, patients with pancreatic cancer develop diabetes and patients with diabetes have an increased risk of developing cancer. This indicates that there are etiological associations to dietary factors and glucose metabolism.

c) Pancreatitis is linked with pancreatic cancer (Lowenfels et al., 1993; Bansal P et al., 1995). Even though the causal relationship between pancreatitis and pancreatic neoplasms is not well understood, the most likely hypothesis is that a constant inflammatory reaction in pancreas leads to transformative events in pancreatic cells. The most common cause of pancreatitis is alcohol consumption (Genkinger et al., 2009) but there could also be genetic risk factors: patients with hereditary pancreatitis experience show a 53-fold increased incidence of pancreatic cancer (Bardeesy N. et al.,

2002). The link between chronic pancreatitis and pancreatic cancer has not been fully investigated.

1.1.4 Malignant transformation and genetic alterations

Pancreatic intraepithelial neoplasms (PanINs) are precursors of invasive pancreatic cancer that develops from ductal epithelium, and they accumulate histological and genetic abnormalities in their progression towards an invasive management.

The PanINs nomenclature has been standardized by Hruban et al. in 2001: they are divided into different stages, from PanIN-1A to PanIN-3 (**Figure 2A**).

The normal duct has a low-columnar epithelium. At the PanIN-1A stage, the epithelium becomes taller, with still basally located non-atypical nuclei, and it shows a papillary or pseudo-stratified structure in PanIN-1B. In PanIN-2, cells lose their polarity and lesions manifest architecturally as flat or papillary lesions with higher degree of nuclear atypia.

In stage PanIN-3 (also called "carcinoma *in situ*"), clusters of epithelial cells are budding off and can be found in the lumen. These cells are characterized by nuclear atypia, prominent nucleoli and mitotic activity (Baumgard M et al., 2005; Takaori K et al., 2004; Hruban RH et al., 2000).

Tissue microarrays have been used to identify genetic alterations occurring in the different PanIN stages (Maitra et al., 2003). It has been shown that molecular alterations in the PanINs are not random but, on the contrary, they follow a specific sequence during PanIN development, which is fundamental for the malignant transformation. The genetic basis in pancreatic cancer is extremely complex and heterogeneous (Jones et al., 2008).

The molecular changes can be stratified into groups of early, intermediate and late alterations, summarized in **Figure 2B**.

Interestingly, the activation of K-ras mutations and the telomere lenght shortening are nearly ubiquitous and occur early in the transformation process, indicating the importance of these molecules in the pathogenesis of pancreatic cancer (Bardeesy & DePinho, 2002).K-ras mutations are the first molecular abnormality detectable in PanIN and they can be considered a "signature" of pancreatic cancer. Telomerase activity is significantly higher in invasive lesions compared to normal adjacent structures.

Nevertheless, it is peculiar that this high activity is preceded by shortage of telomeres, that have been documented in all types of pre-invasive lesions (Heek NT et al., 2002). The second step include p16, p53, DPC4 and BRCA2 genes inactivation.

Furthermore, recent studies indicate that cell damage and inflammation, which occur during acute or chronic pancreatitis, result in an acinar-to ductal metaplasia (acinar cells during tissue regeneration show duct-like morphology). In the normal pancreas, this metaplasia is transient but, if K-ras is mutated, the acinar-to-ductal metaplasia can become persistent and develop PanINs lesions (Morrison et al., 2010).

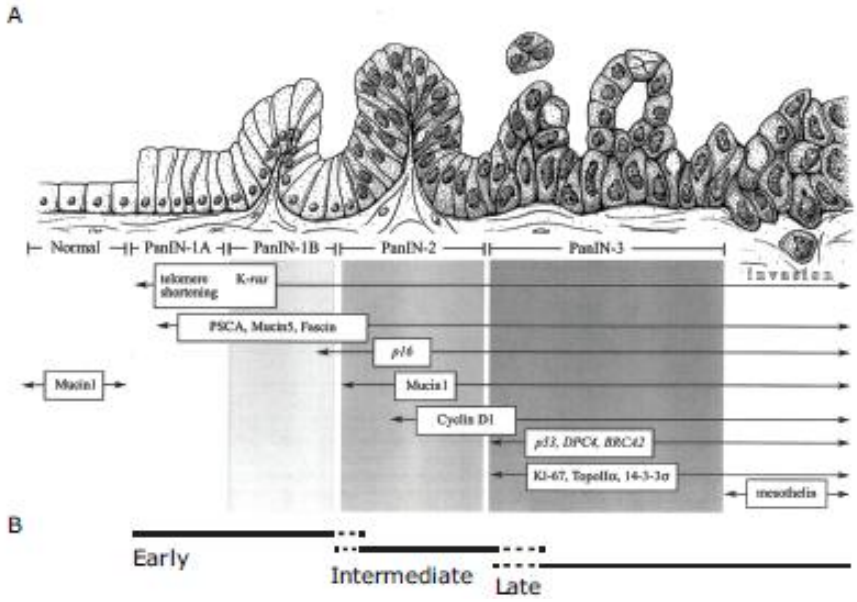


Figure 2. **A**: Morphological appearance and genetic alterations in different PanIN-stages (Maitra et al., 2003). **B**: Molecular changes stratified as early, intermediate or late.

1.1.5 Biological models for Pancreatic Cancer

Two model systems for studying pancreatic adenocarcinoma have been developed: *in vivo* models, that employ animal models, and *in vitro* models, that use cell lines and pathological specimens. Each of these models has advantages and disadvantages. Animal models offer the possibility to study both temporal and spatial events, but the biological properties of pancreatic tumors in these models are different from humans and so interpretation of these findings need to be viewed cautiously.

On the other hand, tumor cell lines are useful for evaluating responses of tumors to drugs in preclinical studies and harbour all the genetic damages that lead to transformation and metastasis. The disadvantage is that it is not possible to determine the temporal order at which such defects have occurred.

Tumor specimens are useful to understand premalignant lesions and spatial arrangement of tumors that occur in tumor progression, but the events that precede and follow the time point at which the specimen was taken, cannot be determined.

So it is necessary to use all these systems to better understand pancreatic cancerogenesis.

Mouse models: xenograft

Since their introduction in 1966 (Flanagan S.P., 1966), nude mice have gained popularity as a model to study human tumor biology because they are able to host cancer cells. They are also used to examine the effects of therapeutic drugs or radiation particularly in cancers difficult to manage, such as pancreatic cancer.

Implantation of tumor cells in the subcutaneous tissue has limited clinical value since spontaneous metastasis in parenchymatous organs are rarely observed. Subcutaneous tumors in donor animals never metastasized and resembled encapsulated benign tumors rather than infiltrative malignancies, even if derived from undifferentiated or poorly differentiated cell lines.

Orthotopic models, in which tumor cells are directly injected in murine pancreas, are considered more appropriate to mimic the clinical situation, because they allow the growth of cellular clones with metastatic potential. However, this technique is strongly operator-dependent and frequently there is a simultaneous metastatic spread of cancer cells into the intra-abdominal organs during injection procedure.

The orthotopic implantation of fragments has been proposed as a superior technique compared to cell injection (Hotz HG et al., 2003). Dislocation of tumor fragments from implantation site was not observed, but the metastatic potential is only about 50 % versus the 100% observed in human disease.

Mouse models: genetically engineered mice (GEM)

Genetically engineered mice (GEM) have provided a successful *in vivo* system to study the biological impact of oncogenic mutations (Weiss WA, van Dyke et al, 2002). GEM

have the potential to identify early markers of disease and provide better preclinical models for therapeutic initiatives.

Human PDACs show mutational activation of K-ras gene and inactivation of Ink4a, Arf and p53 tumor suppressor genes.

In the Kras Ink4a/Arf engineered model, all PDACs retain p53 function, indicating that loss of p53 is not an obligatory step for tumor progression. A notable feature of these models is the maintenance of the wild-type SMAD4 expression in all tumors. However, these data suffer of some important differences in reciprocal interactions of Arf, p53 and Ink4a between mice and humans (Aguirre AJ et al., 2003; Rangarajan et al., 2004).

Human PDACs are characterized by widespread chromosomal instability. Its origin include a number of defects involving the mitotic spindle apparatus, various cell cycle checkpoint pathway, telomeres dysfunction, increased ROS and defects in DNA repair (Hezel AF et al., 2006). Genomic analysis using CGH-arrays and spectral karyotyping (SKY) have found that all the evaluated PDAC models show evidence of a global genomic alteration, suggesting that at least some of the mechanisms driving genomic instability are active in these mouse models. Anyway, the absolute number of chromosomal structural aberrations in the current collection of GEM appears less than that observed in humans.

So, even if mouse model are helpful, it remains to be determined whether there are cross-species differences in the oncogenic pathways leading to development of PDAC.

In vitro models

The establishment of a large bank of pancreatic cancer cell lines from a wide panel of human cancer specimens may reflect the diversity of tumor phenotypes and could provide adequate models for studying pancreatic cancer disease heterogeneity. Recently, cancer studies have acknowledged the active roles that tumor stroma can play in carcinogenesis, focusing on the abnormal communication between tumor cells and their microenvironment.

The intense stromal interaction between cancer cells and orthotopic tumor-derived fibroblasts is a predominant characteristic of pancreatic cancer even if it is not well understood. So, co-culture experiments with or without a transwell chamber can be helpful to clarify these interactions with microenvironment.

However, these *in vitro* experiments do not consider the role of the extracellular matrix. Recently, experimental models have been developed to provide the *in vitro* stroma microenvironment for cellular interaction: collagen gel-based co-culture models represent a valid model to study cellular interactions (Che Z.M. et al., 2006).

1.1.6 Treatments of Pancreatic Cancer

Treatment strategies for pancreatic cancer have evolved in the last years and new drugs have been introduced, but unfortunately there is no curative treatment and palliative care is the most likely outcome. Even a small benefit from a new treatment is important for pancreatic cancer patients.

Early disease

Tumor at stage I or II can be candidate for surgery, whereas stage III and IV tumors are considered unresectable.

For localized tumors, surgery is the treatment of choice, but only 20% of the patients have a resectable disease at the time of diagnosis (Bilimoria et al. 2007). Depending on the localization, different surgical strategies are used: the cephalic pancreato-duodenectomy, called Whipple procedure, is preferred for tumors of the head of pancreas, distal pancreatectomy in the cauda and total pancreatectomy for large cystic lesions. Only in total pancreatectomy the whole pancreas is removed and both hormones and digestive enzymes must be substituted lifelong.

As a complement to surgery, adjuvant chemotherapy, given post-surgery, improves the progression-free survival (Neoptolemos et al., 2004; Regine et al., 2008), but the overall survival is only marginally affected (Oettle et al., 2007).

Locally advanced and systemic disease

For the rest of the patients with locally advanced or systemic disease, the treatment of choice is palliative chemotherapy. However, the active treatment is beneficial because the survival in patients with locally advanced disease is improved with chemotherapy (Sultana et al., 2007).

Gemcitabine (GEM) is a nucleoside (cytidine) analogue that incorporates into the DNA and blocks DNA replication; 5-fluorouracil (5-FU) is an inhibitor of the thymidylate synthetase. These are the two chemotherapeutic agents most evaluated in pancreatic cancer. Randomized trials have shown gemcitabine to be more effective than fluorouracil when administered intravenously (Burris et al., 1997), making gemcitabine the gold standard drug for the last decade.

Even though pancreatic cancers show an active neo-angiogenesis and an over-expression of Vascular Endothelial Growth Factor (VEGF), anti-angiogenetic approaches have been tried with MMP-inhibitors and anti-VEGF agents, but they failed (Saif, 2006). A large phase III trial has been recently published comparing gemcitabine with a combination of gemcitabine and bevacizumab, a monoclonal antibody that targets and blocks VEGF. This trial did not improve patients survival (Kindler H.L et al., 2005; Kindler H.L et al., 2010).

Also endostatin, an anti-angiogenic agent derived from type XVIII collagen, has been tested: interestingly, association of endostatin with gemcitabine had better effects than gemcitabine or endostatin in monotherapy in an animal xenograft model of human pancreatic cancer (Wang et al., 2010),but no results from clinical trials are available.

Many studies have been performed attempting to inhibit the cyclooxygenase 2 (Cox-2) in pancreatic cancer. Cyclooxygenase 2 (Cox-2) is an enzyme that produces prostaglandins that can lead to inflammatory promotion of cellular transformation. Its expression in pancreatic cancer has been correlated with a poorer prognosis (Juuti et al., 2006).

1.1.7 Survival

The overall median survival in pancreatic cancer is between 4 and 5 months (Bilimoria et al., 2007), and the major causes of death are peritoneal dissemination or liver metastasis (Saif, 2006). Bilimoria et al have studied the survival in pancreatic cancer in relation to stage, and compared resected to non resected tumors in a large patient population with over 121.000 pancreatic cancer cases. Their results show that resection of a tumor at a low stage improves dramatically the survival, but the gain in survival

diminishes at higher stages. Patients with a low stage (small tumor with no signs of disseminated disease) only show a median survival of 24,1 months after surgery.
Long-term survival only occurs in 10-20% of the patients that undergo surgery with curative intent (without chemotherapy) (Yeo et al., 1995; Goldstein et al., 2004).
Pancreatic cancer produces metastasis early and we do not have the instruments to correctly classify the disease today. During the past decades, the 5-year survival rate has only improved marginally, and today it is about 5% in the Nordic countries (Storm et al., 2007), in Europe (Sant et al., 2009) and in the United States (Bilimoria et al., 2007; Jemal et al., 2008) This makes pancreatic cancer the tumor type with the lowest long-term survival rate among all the most common malignancies. Thus new therapies are needed and immunotherapy could be an option**.**

Chapter 2: Features of immune system

1.2.1 Innate and adaptive immunity

The immune system has evolved numerous strategies to counteract pathogens that we encounter throughout our lifetimes. The immune system can be divided into innate and adaptive.
The innate (natural) response is non-specific and it is the first-line of defense against different pathogens. Innate immunity is a multi-pronged attack by specialized cells and proteins, including acute phase proteins, the complement system, inflammatory mediators (such as IFNs) and antimicrobial peptide as defensins. Phagocytic cells (neutrophils and macrophages) engulf and then kill microorganisms and foreign molecules, whilst natural killer cells (NKs) patrol the cell surface of cells they encounter for “self” molecules of the major histocompatibility complex (MHC) and kill any cells lacking these molecules (Lyunggren et al., 1990; Smyth et al., 2002).
The adaptive (acquired) immune response, which is initially slower, eventually mounts a specific response that clears the infection and culminates in immunological memory. The main cells of the adaptive immune response are B and t lymphocytes. Adaptive immunity is elicited when T and B lymphocytes specifically recognize the antigen and become activated. Activation of T lymphocytes occurs via the T cell receptor (TCR)-

mediated recognition of an antigenic peptide presented by MHC molecules expressed on the surface of an antigen presenting cell (APC), which collect antigens from its surroundings, processes into peptides, assembles the peptides to MHC molecules and finally exposes the MHC-antigenic peptide complexes on cell surface.. There are two major types of MHC molecules, named MHC class I (MHC-I) and MHC class II (MHC-II) molecules, playing different roles in antigen presentation to T cells.

B lymphocytes also bind antigens utilizing an antigen-specific receptor called the B cell receptor (BCR), a cell-surface immunoglobulin molecule. After the initial lymphocytes activation, adaptive immunity leads to a memory response, where secondary exposure to the same antigen produces a faster and stronger response. The adaptive immune system can be divided into a humoral and a cell-mediated arm. Humoral adaptive immunity consists of antibody-producing B cells and cell-mediated adaptive immunity is mainly mediated by $CD4^+$ T-helper cells (Th) and $CD8^+$ cytotoxic T cells (CTLs).

1.2.2 T lymphocytes and T-cell receptor (TCR)

The TCR is a heterodimer consisting of two transmembrane polypeptide chains, designated α and β, covalently linked to each other by disulfide bonds. Similarly to immunoglobulin heavy and light chains, each α- and β-chain consists of one Ig-like N-terminal variable domain (V), one Ig-like constant domain (C), a hydrophobic transmembrane region and a short cytoplasmic region. The V regions of α- and β-chains contain short stretches of amino acids where the variability between different TCRs is concentrated, and these form the hypervariable or Complementary-Determining Regions (CDRs). Three CDRs of the α-chain are juxtaposed to three similar regions of β-chain to form the part of the TCR that specifically recognizes peptide-MHC complexes.

The TCR complex is not produced in a secreted form and it does not perform effector functions on its own, but initiates signals that activate the effector functions of T cells. Peptide-MHC complexes recognition relies on the CDRs loops of both TCR α- and β-chains. The affinity of TCR for peptide-MHC complexes is low and it is the likely reason for the need of accessory molecules to stabilize T cell adhesion to APCs, thus allowing biological responses to be initiated.

The TCR α- and β-chains display very short intracytoplasmic tails, unable to transduce signals inside the cell, and so they are non-covalently associated to the CD3 complex and to ζ proteins; when the TCR recognizes antigen, these associated proteins transduce the signals that lead to T cell activation (**Figure 3**).

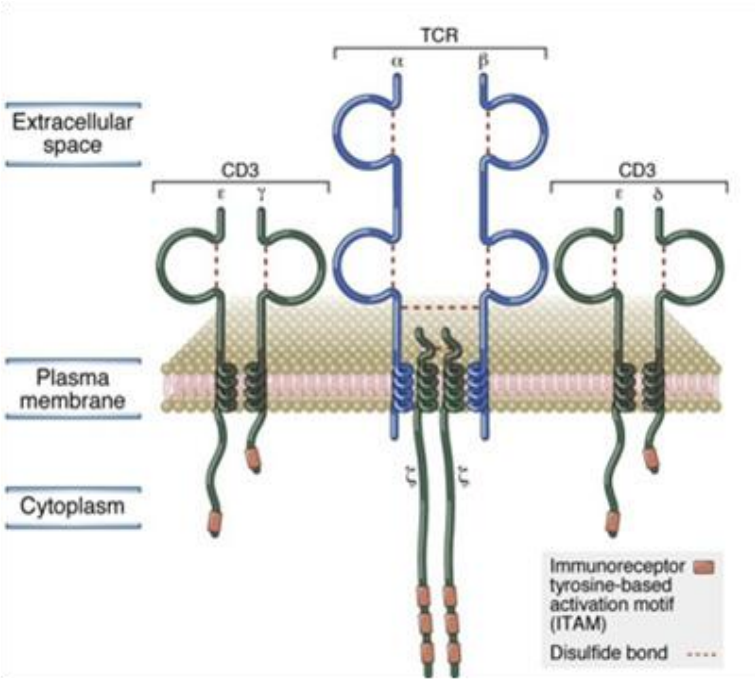


Figure 3: schematic representation of a TCR complex with the α and β chains of the MHC-interacting receptor associated to the γ, δ, ε chains (CD3) and to ζ proteins (Abbas AK *et al*. 2010).

In addition to the TCR, T cells express other integral membrane proteins that play crucial roles in the responses of these cells: the accessory molecules.
These molecules bind other molecules (ligands) that are present on the surfaces of other cells and in the extracellular matrix; they are non-polymorphic and invariant; they transduce biochemical signals to the interior of the T cell that are important in regulating functional responses; they increase the strength of adhesion between T cells and APCs and they are important for the homing and retention of T cells to tissues.

- The co-receptors CD4 and CD8, once bound to the non-polymorphic regions of MHC molecules, transduce signals that, together with signals delivered by the TCR complex, initiate T cell activation. Depending on the type of TCR-

associated co-receptor CD4 or CD8, T lymphocytes can be divided into two main groups: T $CD4^+$ and T $CD8^+$. The co-receptor found on cell surface can also be considered a functional marker: CD4 binds to the β2 domain and to non-polymorphic portions of the β1 domain of the MHC-II β chain, and $CD4^+$ T cells interact with cells expressing MHC-II, i.e. mainly professional APCs, presenting antigens phagocytosed from the external environment. CD8 binds to the α3 domain of the MHC-I α chain, and $CD8^+$ T cells interact with cells expressing MHC-I, i.e. professional APCs but also any type of nucleated cell, presenting cytosolic antigens.

- CD40L (CD154) is one of the most potent costimulatory molecule and interacts with its cognate receptor, CD40, on the APCs. It is responsible for a reciprocal activation, because it is able to upregulate costimulatory and adhesion molecules on the APCs.

- CD28 is a membrane protein that transduces signals that function together with signals delivered by TCR complex to activate T cells and recognizes B7 molecules on APCs.

- A second receptor for B7 molecules is CTLA-4, expressed on recently activated $CD4^+$ and $CD8^+$ T cells. Its function is to inhibit T cell activation by counteracting signals delivered by CD28.

T cell proliferation in response to antigen recognition is mediated primarily by an autocrine growth pathway, in which the responding T cells secrete their own growth-promoting cytokines (particularly IL-2) and also express cell surface receptors for these cytokines. Some of the progeny of antigen-stimulated T cells develop into long-lived, functionally quiescent memory cells, which are responsible for enhanced and accelerated secondary immune responses on subsequent exposures to the same antigen.
The main cytokine produced by T cells is IL-2, that functions as a growth factor for the T cells. Under different activation conditions, naïve T cells that are stimulated by antigens may differentiate into subsets that secrete distinct sets of cytokines and

perform different effector functions (Lyunggren et al., 1990; Smyth et al., 2002) (see 1.2.5).

1.2.3 Antigen presenting cells: dendritic cells (DCs)

Dendritic cells (DCs) are professional antigen presenting cells (APCs) of hemopoietic origin with potent effects on primary T cell differentiation and activation and thus are of central relevance to immune responses and vaccine development (Thery et al., 2001; Banchereau et al., 2000). DCs are present in lymphoid organs, in the epithelia of the skin,gastrointestinal and respiratory tracts (the main portals through which microbes can enter), and in most parenchymal organs.

Although many cells can function as APCs such as macrophages, B cells and some epithelial cells, DCs are by far the most potent APCs given their unique ability to stimulate naive T cells (Guermonprez et al., 2002).

DCs form a physical link between the periphery and the secondary lymphoid organs, acting as sentinels for “dangerous” antigens in the peripheral tissues and then migrating to secondary lymphoid organs, in the areas where naive T cells are located, to transmit information about the nature of the pathogen and the infected tissues.

At the immature stage, DCs exhibit potent endocytic activity and express various receptors mediating endocytosis of particulate antigens, debris from pathogens and dead cells.

They take up antigen via non-specific receptors for Pathogens Associated Molecular Patterns (PAMPs). Internalized antigens are then processed into peptides in specific vescicular compartments and associated to MHC-II molecules. More often in DCs than in B cells or macrophages, portions of internalized antigen may also exit the endocytic vesicles and enter the cytosolic pathway of antigen processing and presentation, to be presented in association with MHC-I molecules (cross-priming).

During the process of maturation, mostly associated with their migration from the periphery to lymphoid organs, DCs shift from an antigen-capturing mode to a T cells-sensitizing mode. The maturation process is induced by microbial products (LPS, CpG oligonucleotides), inflammatory chemokines (TNF-α, IL-1), T cells (through CD40/CD40L interaction) and stress (necrosis, transplantation) (Smyth et al., 2001).

DCs maturation induces a down-regulation of antigen uptake and processing capacity and an up-regulation of immunostimulatory properties like expression of B7-1 and B7-2 co-stimulatory molecules and MHC-II molecules.

Two types of DCs precursors have been identified in human peripheral blood: monocytes, which differentiate into immature myeloid DCs (MDCs), upon exposure to GM-CSF and to IL-4, and plasmacytoid cells, that display features of the lymphoid lineage and require IL-3 for their development into plasmacytoid DCs (PDCs) (Rissoan et al., 1999; Liu et al., 2001).

Upon activation, mature MDCs produce large amounts of IL-12 and preferentially induce naive $CD4^+$ T cells to differentiate into Th1 cells, whereas mature PDCs produce low level of IL-12 and induce the development of Th2 cells (Moser et al., 2000; Belz et al., 2002) or immunosuppressive T cells (Moseman et al., 2004).

So, the functional properties of DCs are affected by their state of maturation when encountering T cells. For example, repetitive stimulation of alloreactive T cells with mature $CD83^+$ human monocyte-derived DCs induces predominantly Th1 responses.

By contrast, repetitive stimulation with immature $CD83^-$ DCs favors the emergence of non-proliferating, IL-10 producing T cells (Jin et al., 2004), the human counterpart of a recently described population of immunoregolatory T cells involved in maintaining peripheral tolerance (Jonuleit et al., 2000; Fontenot et al., 2004; Sakaguchi 2004).

1.2.4 Antigen presentation pathway

MHC molecules reveal which proteins are produced by the cell in a particular time point and which proteins are taken up from its surrounding milieu. In this way, they provide a means for T cells to inspect this antigenic repertoire and detect peptides derived from intra- or extra-cellular pathogens.

Peptides originating from endogenous proteins are loaded onto MHC-I molecules and are displayed to $CD8^+$ T cells, while exogenous antigens are primarily loaded onto MHC-II molecules to be presented to $CD4^+$ T cells.

The MHC-I molecule, consisting of 2 polypeptide chains, namely constant β_2 microglobulin and variable α chain, is present on almost every nucleated cell. Upon ubiquitination, endogenous proteins are delivered to the proteasome in the cytosol,

where they are degraded into peptides. The peptides are then translocated to the endoplasmic reticulum (ER) via a molecular complex named "transporter associated with antigen processing (TAP)" and eventually bound to MHC-I within the peptide-binding cleft (York et al., 1996). MHC-I-bound peptides are tipically 8-12 amino acids long (Tong et al., 2004).

Finally, the MHC-I-loaded molecule travels via the Golgi to the cell surface where the peptide/MHC complex is displayed to $CD8^+$ T cells.

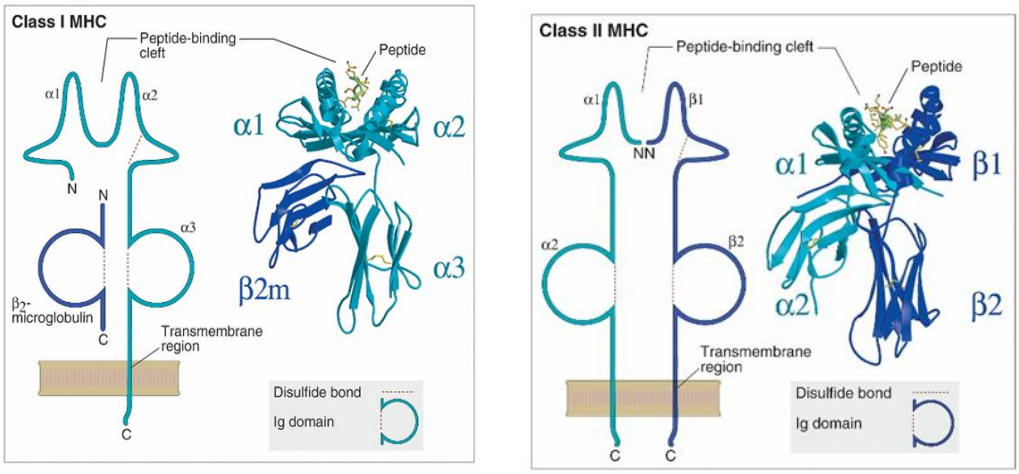
Interferon-γ (IFN-γ) can induce upregulation of the three proteasomal subunits LMP2, LMP7 and MECL1 (Akiyama et al., 1994). This so-called immunoproteasome is distinct from the standard proteasome: it produces different sets of peptides, due to altered cleavage patterns. While immature DCs express both the immunoproteasome and standard proteasome in equal parts, mature DCs express only immunoproteasome (Macagno et al., 1999).

At first glance, it seems that the immunoproteasome might be more efficient in antigen presentation than the standard proteasome, since its cleavage patterns yield peptides with the anchor residues that bind to MHC-I most efficiently. Nevertheless, some epitopes cannot be processed by the immunoproteasome and this means that T cells directed against these epitopes will not be activated by mature DCs and therefore remain quiescent. For tumor antigens, the immunoproteasome is more efficient, while the standard proteasome is more effective in processing other epitopes. This has strong implications for immunotherapy, both for cancer and infectious disease, regarding the optimal immunization protocol for a particular antigen (Van den Eynde et al., 2001; Morel et al., 2000).

The MHC-II molecule, which consists of an α and β chain, both polymorphic, is present on APCs such as DCs, macrophages and B cells (Wang et al., 2001). Exogenous antigens can be taken up via macropinocytosis (non-specific uptake of extracellular fluid), phagocytosis (receptor-mediated attachment and engulfment) or receptor-mediated endocytosis (binding to clathrin-coated pits) (Brode et al., 2004). After uptake by one of these processes, exogenous proteins are transported by early endosomes, that eventually become late endosomes. In the late endosome, the process of acidification activates proteases to generate peptides.

MHC II molecules are produced in the ER, but subsequently enter vescicles that fuse with late endosomes, so the MHC molecules can bind peptide (Wang et al., 2001). MHC II bound peptides are generally 9-25 amino acids in length, and even longer (Tong et al., 2004). The antigen presentation pathways are represented in **Figure 4**.

A)



B)

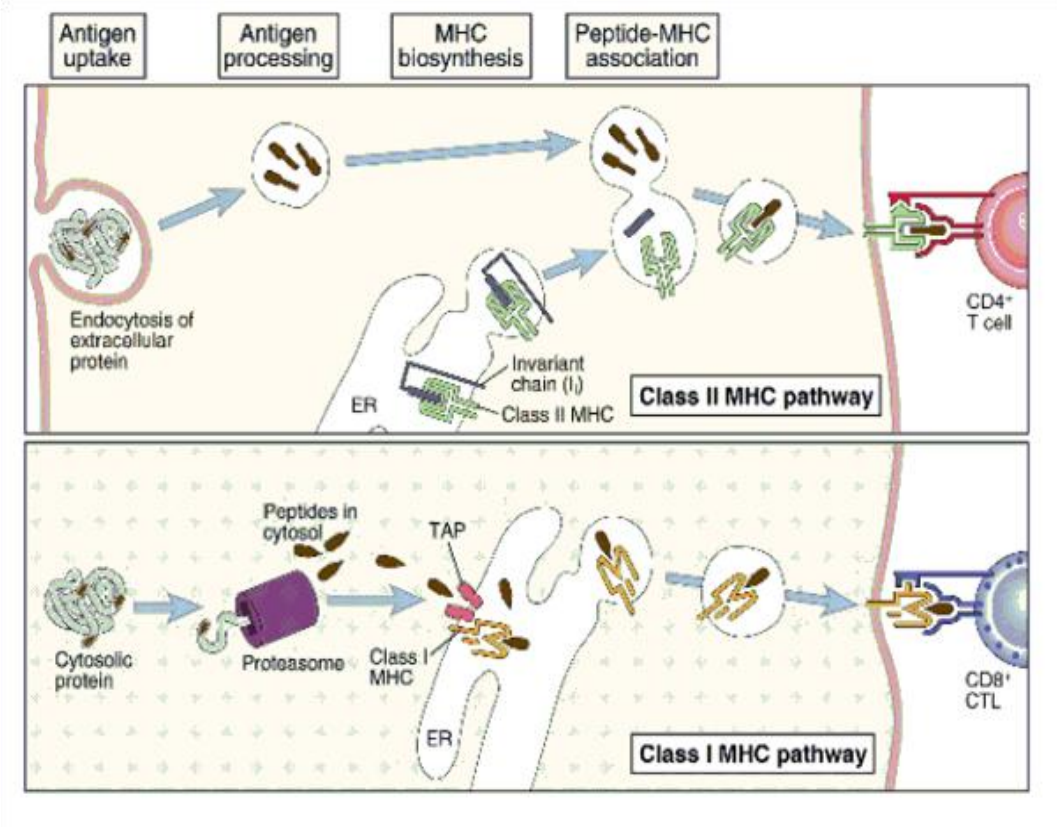


Figure 4: MHC-I and –II molecules and antigen presentation pathways. A) Schematic representation of an MHC-I and -II molecules. B) Exogenous (for MHC-II) and endogenous (for MHC-I) antigen presentation pathways. (Abbas AK *et al*. 2010).

1.2.5 The immunological synapse

Immature DCs are sentinels of the immune system that travel through the body sampling the antigenic milieu, and therefore they have an enhanced capacity to take up antigens.

Upon maturation, DCs upregulate homing receptors (e.g. CCR7) and migrate to draining lymph nodes (Allavena et al., 2000). At this point, their enhanced ability to take up antigens is replaced by enhanced capabilities to provide T cell costimulation.

In lymphoid tissues, naive T cells travel through surveying other cells in search of an antigen they are specific for. According to the two signals hypothesis (Lafferty et al., 1975), a T cell is efficiently activated when it recognizes its specific MHC/peptide complex via its TCR. This primary signal is followed by a second signal, delivered when costimulatory receptors on the APC are bound to their cognate ligands on the T cell. Without this costimulatory signal, the T cell enters a state of anergy (Jenkins et al., 1987).

There are two prominent families of costimulatory molecules which provide the second signal in T cell activation: 1) members of the immunoglobulin superfamily, such as CD28 and ICOS; 2) members of the TNF superfamily, such as CD40L (CD154) and TNF-related activation-induced cytokine (TRANCE). One of the most potent costimulatory molecules is CD40L, which interacts with its receptor, CD40, on the APC. This costimulatory signal not only activate the T cell, but reciprocal activation occurs since the CD40-CD40L interaction upregulates costimulatory and adhesion molecules on the APC, such as CD80, CD83, CD86, ICAM I and LFA3 (van Kooten et al., 1997; Cella et al., 1996).

There are also other members of these two families which inhibit T cell responses. One inhibitory molecule of T cells is the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), that binds with high affinity CD80 and CD86 ligands on APC (as CD28). These interactions are represented in **Figure 5**.

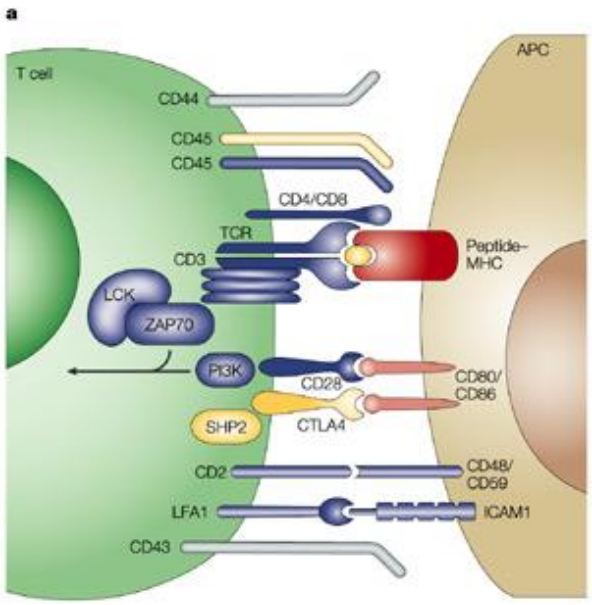


Figure 5. The immunological synapse. For optimal T cell activation, signal 1 (TCR-MHC/peptide) and signal 2 (costimulation) must be provided. Signals from molecules such as CTLA-4 inhibit activation.

1.2.6 T-cell effector function

$CD4^+$ T cells, often referred to as **T helper cells**, recognize antigens derived from extracellular sources phagocytosed, processed and presented through the MHC II-peptide complexes on APCs. The effector response of $CD4^+$ T cells consists in the secretion of a very large number of different cytokines, which play a fundamental role in the modulation of the effector functions of all the cells involved in an immune response.

The activated naive $CD4^+$ T cells (T_h0) can differentiate into four major subsets, which differ for the cytokines they secrete:

- **T_h1**: they secrete IFN-γ, IL-2 (the essential T cell growth factor) and TNF-β (tumor necrosis factor-β) that activate $CD8^+$ T cells and NK cells. T_h1 differentiation pathway is the response to microbes that infect or activate macrophages and to those that activate NK cells; the main inducer of T_h1 responses is IL-12 which is produced after TLR-ligation on DCs (Lanzavecchia and Sallusto, 2001).

- **T_h2**: they secrete IL-4, IL-5 and IL-13 that influence B cell class switch to IgE and activate eosinophils. T_h2 differentiation occurs in the response to helminths and allergens, which cause chronic T cell stimulation; the key determinant of T_h2 cell differentiation is IL-4 (Tham et al., 2002).

- **T_h17**: they secrete the IL-17 family of cytokines and IL-22. They promote inflammatory responses, particularly at mucosal sites. IL-17 stimulates many cells of the innate immune system, as well as other types of cells, to synthesize IL-1, IL-6 and TNF-α, that also result in inflammation.

- **T_{reg}**: they inhibit or suppress the differentiation and the function of other subsets of $CD4^+$ T cells and other cell types. T_{reg} cells, which generally express CD25, suppress immune responses directed at both self-molecules and foreign antigens and secrete TGF-β (transforming growth factor-β) and IL-10.

The cytokines present in the microenvironment where the activation of a $CD4^+$ T cell occurs, drive the differentiation of the naive $CD4^+$ T cell towards a particular subset and are generally derived from cells of the innate immune system, particularly from DCs. T_h1 cells develop in the presence of IL-12; T_h2 cells develop in the presence of IL-4; T_h17 cells develop in the presence of TGF-β and IL-21, while T_{reg} cells develop in the presence of TGF-β.

Naive CD8 T cells differentiate into **cytotoxic T lymphocytes (CTLs)** whose main function is to eliminate virus infected and, in some cases, tumor cells. As for $CD4^+$ T cells, they need co-stimulation in order to be activated upon antigen recognition.
The classical view of how this costimulation works was described as a three-cell interaction, where the $CD4^+$ and $CD8^+$ T cells simultaneously recognize antigens presented on the same APC. After activation, the T_h1 cells secrete cytokines such as IL-2 which stimulate CD8 activation.
However, since CTL responses can be induced also in the absence of T_h1 cells (Deeths et al., 1999), another model has been proposed, divided in two steps:

1) the Th1 cells interact with APCs by MHC-II/peptide complex, becoming activated and able to condition the APCs;
2) conditioned APCs acquire the competence to activate $CD8^+$ T cells directly through up-regulation of CD80 and CD86.

While Th cells mainly act by influencing the action of other immune cells, CTLs have the capacity to directly kill cells expressing viral or cancer antigens.

Once activated, the $CD8^+$ T cell initiates killing by attaching to the target cell; paired adhesion molecules expressed on the T cell and target cell surfaces help to maintain contact between the cells for several hours. The CTL contains granules with proteins with cytotoxic functions and expresses the surface molecule Fas ligand (FasL). After attaching to the target cell, the CTL modifies its internal structure so that these granules are driven close to the area of contact with the target cell and releases the contents of the granules by exocytosis. The major constituents of the granules involved in target cell killing are perforins, that form pores in the target cell membrane, and granzymes, serine proteases that pass into the target cell through the pores inducing apoptosis. The interaction of the molecule FasL on the $CD8^+$ T cell with Fas, a surface molecule expressed on many host cells, activates the apoptosis of the target cell via a sequential activation of the caspases of the target cell. Once the CTL has initiated these killing pathways, it detaches from the dying cell to attack and kill another target.

Once the antigen has been eliminated or the infectious agents cleared, 99% of the pool of activated and effector cells dies. This leaves a surviving population of memory T cell that is still expanded, compared to the original naive cell clone size. The memory T cell that survive the phase of contraction of the clone size are generally long-lived, frequently with a lifetime of years. They are involved in protective responses after a subsequent exposure to the same antigen (Coico et al., 2009; Abbas et al., 2010).

Chapter 3: Tumor immunology

1.3.1. Immunosurveillance and cancer immunoediting

Cancer arises from the uncontrolled proliferation and spread of clones of transformed cells.

In 2001, the six hallmarks of cancer cells were defined as (Cancerfonden and Socialstyrelsen, 2001):

1. cancer cells are self-sufficient in acquiring growth signals;
2. they are insensitive to anti-growth signals;
3. they are resistant to apoptosis;
4. they have limitless replicative potential;
5. they are able to sustain angiogenesis;
6. they are able to invade surrounding tissues and metastasize.

These points are sufficient for cancer growth, but several steps of tumor progression are supported by altered stromal cells as fibroblasts, endothelial cells and leukocytes.

Classical cancer treatments, such as surgery, chemotherapy, radiation and hormonal treatment, not only lead to severe side effects but are also mainly effective only in early stage cancer. Therefore, there is a great medical need for alternative treatment strategies.

The possibility that cancer may be eradicated by specific immune responses has been the impulse for many studies.

Lewis Thomas and Macfarlane Burnet postulated the “Immunosurveillance Theory”, which states that a main function of the immune system is to seek out and destroy cancers as they arise (Burnett et al., 1970). This theory emerged from data showing that syngeneic mice could be immunized and reject methyl-cholantrene- and virally-induced tumors (Malberg et al., 2006), indicating that tumor antigens exist and can be detected by the immune system. The immunosurveillance theory can be validated showing that immunodeficiency results in an increased incidence of cancer. An increase in cancer incidence is also observed in immunosuppressed transplant recipients (Ratliff et al.,

1993). However, despite immunosurveillance, cancers develop in immunocompetent hosts.

It was recently suggested that inability of the immune system to eradicate tumors or avoidance of immunesurveillance is the seventh hallmark of cancer (Dunn G.P. et al., 2002 and 2004; Smyth M.J. et al., 2004; Zitvogel L. et al., 2006).

The term "cancer immunoediting" was coined to explain this phenomenon (Dunn G.P. et al., 2002). The cancer immunoediting model describes three phases (**Figure 7**):

1. *elimination* phase (immunosurveillance): the immune system targets and destroys antigen bearing tumor cells;
2. *equilibrium* phase: the immune system applies immunologic pressure, which controls tumor growth, but not fully eliminates tumor cells. During this phase, tumor cells acquire mutations that render them less sensitive to immune attack;
3. *escape* phase: immune resistant tumor cells expand to a detectable tumor.

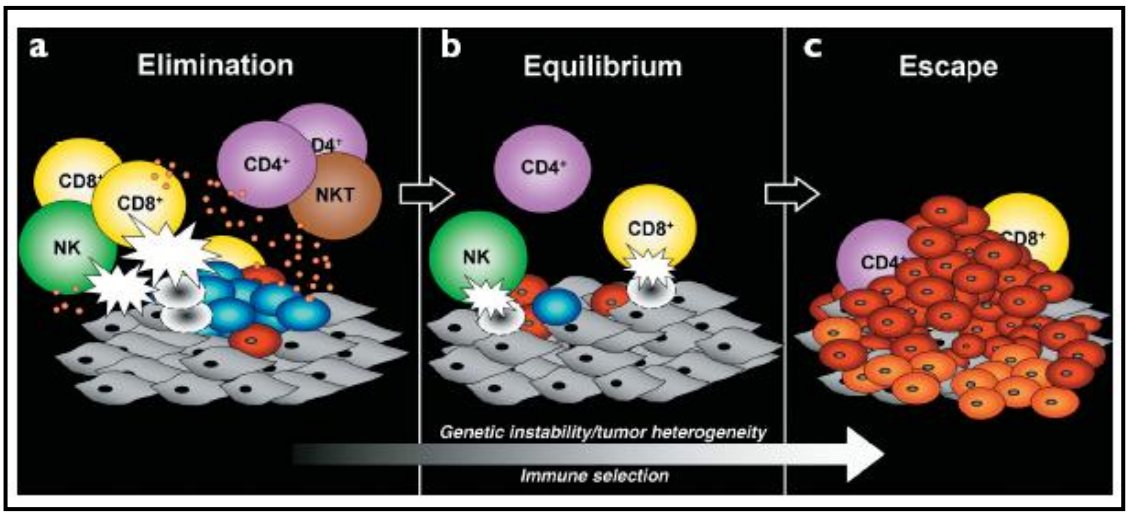


Figure 7. Cancer immunoediting encompasses three process**. a)** Elimination; **b)** Equilibrium; **c)** Escape. In **a)** and **b)**, developing tumor cells (blue), tumor cell variants (red), and underlying stroma and non-transformed cells (grey) are shown; in **c)** additional tumor variant (orange) that have formed as result of equilibrium process are shown. Different lymphocyte populations are as marked. The small orange circles represent cytokines and the white flashes represent cytotoxic activity of lymphocytes against tumor cells (Dunn G.P et al., 2002).

1.3.2. Tumor antigens

A tumor antigen is the specific target against which the immune response is generated after vaccination. Several tumor antigens , recognized by T cells, have been identified and used in clinical trials (Lee G.K et al., 2002). Tumor antigens can be categorized into four groups: (a) antigens unique to an individual patient's tumor; (b) antigens common to a histologically similar group of tumors; (c) tissue-differentiation antigens; and (d) ubiquitous antigens expressed by normal and malignant cells (Berzofsky J.A et al., 2004).

Due to their genetic instability, tumor cells do not express common proteins; but they can express tumor associated antigens (TAAs), that have low or limited expression in non-malignant cells.. An extensive listing of the known TAAs is available, and more are being discovered (Renkvist et al., 2001). They have mainly been found in melanomas and melanocytes as Melan-A (Coulie P.G. et al., 1994) and tyrosinase (Brichard V. et al., 1993), but also in other epithelial tissues, such as prostate specific antigen (PSA) (Corman J.M. et al., 1998). Other TAAs, such as HER-2/neu (Fisk B. et al., 1995), hTERT (Minev B. et al., 2000) and survivin (Andersen M.H. et al., 2001), are overexpressed in tumors but also expressed at lower levels in normal tissues.

Another group of tumor antigens are tumor-specific antigens and they are uniquely expressed by tumor cells. Tumor specific antigens are relatively rare and among these are:

1. antigens that arise from point mutations in oncogenes or tumor suppressor genes, such as *Ras* in pancreatic and colorectal cancer (Gjertsen M.K. et al., 1998) or *p53* in lung cancer (Yanuck M. et al., 1993). These antigens expose new and potentially immunogenic epitopes, which have never been encountered by the host's immune system and thus they can potentially be recognized by T cells;
2. antigens belonging to the cancer testis family, which are the results of re-activation of genes that are normally silent in adult tissues, such as antigens of MAGE, BAGE and GAGE families (Novellino L. et al., 2005);
3. antigens derived from virally-induced tumors, that constitute almost 20% of all human cancers (Stewart B.W. 2003), for example the E7 antigen from HPV (which causes cervical carcinoma), (Eiben G.L. et al., 2003) and the EBNA2

antigen derived from Epstein-Barr virus (which is associated with B cell lymphomas) (Taylor G.S. et al., 2004).

Sometimes, the use of the whole tumor as source of antigens is the gold standard in tumors where no strong antigens are available (Matera and Garetto, 2009).

1.3.3 Tumor escape

Tumors evade immune targeting also by actively suppressing immune responses. Mechanisms such as antigen loss, expression of inhibitory molecules, lack of costimulation, MHC-I downregulation, may explain the failure of an endogenous immune response in tumor control (Shreiber H. Et al., 2002).

- Inhibitory cell populations: Tolerogenic DCs, ,regulatory T cells and myeloid derived suppressor cells (MDSCs)

Tumor derived IL-10 and TGF-β have several suppressive effects on antitumor immune responses (Mocellin S. et al., 2004; Teicher B.A. et al., 2007). In addition to their direct effects on T cells, such as inhibition of IL-2 production (Thomas D.A. et al., 2005), secretion of IL-10 and TGF-β, was reported to suppress differentiation and function of local DCs (Moore K.W. et al., 2005; Mou H.B. et al., 2004). The result is the presence of tolerogenic DCs in the tumor microenvironment, that leads to ineffective T cell priming and induction of T cells anergy (Steinbrick K. et al., 1999). Other factors contributing to the accumulation of tolerogenic DCs in tumors are macrophage-colony stimulating factors (M-CSF) and IL-6 (Gabrilovich D. et al., 2004). IL-10, TGF-β and tolerogenic DCs have also been shown to promote the conversion of naive $CD4^+$ T cells to T reg cells (Chen W. et al., 2003; Ghiringhelli F. et al., 2005). On the other hand, T reg cells are commonly detected in tumors and can be described as a T cell population, which mediate suppression of a large portion of TAA-specific T cells (Curiel T.J. et al, 2004). Tumour progression is often associated with altered haematopoiesis, which leads to the accumulation of myeloid cells at the tumour site, in the blood, secondary lymphoid organs, and bone marrow (Bronte V et al., 2005; Serafini P et al., 2006). These cells, named myeloid-derived suppressor cells (MDSCs) modify the tumour microenvironment through key enzymes of L-Arginine metabolism: nitric-oxide synthase (NOS) and arginase 1 (ARG1) (Bronte V et al., 2005; Gallina, G et al., 2005).

The depletion of L-Arg in the microenvironment leads to inhibition of T cell proliferation through the down-regulation of CD3zeta-chain expression in T lymphocytes (Rodriguez PC et al., 2002), thus suppressing the anti-tumor immune response.

- Loss of antigen presentation and resistance to apoptosis

The exact mechanism that controls the downregulation of tumor antigens are not known in most cases; however, the propagation of antigen loss variants may be facilitated by epitope immunodominance. The phenomenon of immunodominance may be thought of as the preferential immunodetection of one or a few epitopes among many expressed on a given target. The theory of immunodominance, as it relates to tumor escape, predicts that one of the ways that antigen loss variants within a tumor are shielded from immune pressure is that parental tumor cells carrying the immunodominant epitope serve as a red flag for immune attack, thereby diverting attention from the tumor variants. Once the parental cells are eliminated, a new hierarchy is established among the variant subpopulations, and formerly immunorecessive epitopes become dominant (Khong H.T. et al., 2002). A tumor variant that has lost the restricting MHC class I allele while retaining the immunodominant antigen could cross-present this antigen to $CD8^+$ CTLs by DCs and maintain an immunodominant response to a “phantom” target at the expenses of more appropriate and effective responses to other antigens (Seung S. et al., 1993; Khong H.T. et al., 2002). Different mechanisms can lead to the total or partial loss of MHC-I expression, that can be produced at any step required for MHC-I expression, from transcription to transport to cell surface (Garcia-Lora et al., 2003). Most tumors seem to grow in a non inflammatory microenvironment that does not conduct to immune activation. Recognition of tumor antigens by DCs under these conditions will not lead to DCs activation and maturation. In addition, lack of expression of costimulatory molecules by tumor cells may lead to T cell anergy and suboptimal activation of NK cells.

Chapter 4: Apoptosis, Necrosis and Hyperthermia

Homeostasis is maintained in multicellular organisms by a balance between cell proliferation and cell death. Several types of cell death have been described: apoptosis, cell death associated with autophagy, necrosis, and many other different types (Kumar V. et al., 2005). Apoptosis and necrosis are the two most studied types of cell death:.

1.4.1 Apoptosis and necrosis

Apoptosis is a pathway of cell death induced by a tightly regulated intracellular program: dying cells activate enzymes that degrade the nuclear DNA and nuclear and cytoplasmic proteins. Plasma membrane remains intact, but its structure is altered, thus becoming an avid target for phagocytosis. Dead cells are rapidly cleared, before their contents have leaked out, and therefore cell death by this pathway does not elicit an inflammatory reaction in the host. Apoptosis occurs physiologically in many situations, for example to eliminate unwanted or potentially harmful cells and cells that have outlived their usefulness. It is also a pathologic event when cells are damaged beyond repair, especially when the damage affects DNA.

The apoptotic pathway presents a series of morphologic and biochemical characteristics, such as:

- cell shrinkage: the cell is smaller in size, the cytoplasm is dense and the organelles, although relatively normal, are more tightly packed;
- chromatin condensation: the chromatin aggregates peripherally, under the nuclear membrane, into dense masses of various shapes and sizes. The nucleus itself may break up, producing two or more fragments;
- formation of cytoplasmic blebs and apoptotic bodies: the apoptotic cell first shows extensive surface blebbing, then undergoes fragmentation into membrane-bound apoptotic bodies composed of cytoplasm and tightly packed organelles, with or without nuclear fragments;
- phagocytosis of apoptotic cells or cell bodies, usually by macrophages: the apoptotic bodies are rapidly degraded within lysosomes, and the adjacent healthy cells migrate or proliferate to replace the space occupied by the now deleted apoptotic cell (Kumar V. et al., 2005).

Necrosis refers to a spectrum of morphologic changes that follow cell death in living tissue, largely resulting from the progressive degradative action of enzymes on the lethally injured cells. As commonly used, necrosis is the gross and histologic correlate of cell death occurring in the setting of irreversible exogenous injury. Necrotic cells are unable to maintain membrane integrity and their contents often leak out: this may elicit inflammation in the surrounding tissue. The morphologic appearance of necrosis is the result of denaturation of intracellular proteins and enzymatic digestion of the cell. The necrotic cells show the following features:

- increased eosinophilia attributable to loss of the normal basophilia imparted by the RNA in the cytoplasm;
- a more glassy homogeneous appearance than that of normal cells and the cytoplasm becomes vacuolated;
- non-specific breakdown of the DNA: the basophilia of the chromatin may fade (karyolysis); nuclear shrinkage and increased baspophilia (pyknosis); DNA condenses into a solid, shrunken basophilic mass (karyorrhexis). With the passage of time the nucleus of the necrotic cell totally disappears (Kumar V. et al., 2005).

Apoptotic process can progress to secondary necrosis in situations where clearance by scavengers does not operate and so the complete apoptotic program can fully evolve. This is the case of some physiological situations where apoptotic cells are shed into ducts or into territories topologically outside the organism (like the gut or the airways lumen) or in the lumina of the acini, where the chances of encountering scavengers are small. Moreover, extensive secondary necrosis has been described in multicellular animals in situations of massive apoptosis that surmounts the available scavenging capacity, and when this capacity is directly affected by detrimental effects on scavenger cells or by processes that affect molecules involved in phagocytosis of apoptotic cells. The above observations support the conclusion that the complete apoptotic process in multicellular animals is genetically controlled by an intrinsic program that includes an autolytic termination by secondary necrosis, which makes that process self-sufficient leading to self-elimination when scavengers are not available. The above observations also show that there is an obvious and important functional limitation: depending on an assisting scavenger cell, this mechanism fails if that cell is not available or is defective,

in which case apoptosis will proceed to its completion, leading to the secondary necrotic outcome. One mechanism for the genesis of pathogenic consequences of extensive or persistent apoptotic secondary necrosis is the leakage of cytotoxic, pro-inflammatory and immunogenic molecules by the autolysing cells. These molecules include Danger Associated Molecular Pattern (DAMPs), and their release may be beneficial in regards to immunogenic cell death in tumors. However, necrosis-associated release of DAMPs often has pathological pro-inflammatory and immunogenic consequences, contributing to the pathology frequently associated with extensive or persistent secondary necrosis. Release of lysosomal proteolytic enzymes and the oxidative burst occurring during secondary necrosis may participate in the generation of DAMPs by modifying original cell components, that are later released due to the eventual rupture of the plasma membrane. Among pro-inflammatory and immunogenic DAMPs are proteases, nucleosomes consisting of double-stranded DNA and histones, S100 calgranulin proteins, high mobility group box-1 (HMGB-1) protein, proteolitically processed autoantigens, and urate crystals. Secondary necrotic cells can be phagocytosed by scavengers *in vivo* and this phagocytosis often is pro-inflammatory and immunogenic, and thus may represent another mechanism for the genesis of pathogenic consequences of secondary necrosis. On the other hand, secondary necrosis affecting tumor cells has recently gained an additional relevance due to its recognition as a process with likely beneficial implications in anticancer therapies by facilitating the activation of the immune system and consequently the clearance of tumor cells.

Anticancer chemotherapy and radiotherapy are largely mediated by apoptosis. It has been reported that, after treatment with some chemotherapeutic agents or ionizing irradiation, tumor cells may become highly immunogenic when injected into immunocompetent mice (Kepp O. et al., 2009). Several observations have been accumulated suggesting that such immunogenicity is associated to the progression to secondary necrosis of therapy-induced apoptotic tumor cell death and to the release by secondary necrotic tumor cells of the DAMP molecule HMGB-1: tumor cells treated *in vitro* with some apoptogenic chemotherapeutic agents or radiotherapy release HMGB-1 protein, and secondary necrosis was suggested to be the mechanism for that release. As discussed elsewhere (Brusa D. et al., 2008), clearance of apoptotic cells may be insufficient during therapy-induced apoptotic tumor cell death *in vivo*, resulting in the accumulation of secondary necrotic cells.

It has been found that secondary necrotic tumor cells stimulate DCs (Buttiglieri S. et al., 2003), and thus are immunogenic. The presentation of tumor antigens by mature DCs leads to $CD4^+$ and $CD8^+$ T cell activation. HMGB-1 released by secondary necrotic tumor cells is recognized by DCs which prime $CD4^+$ and $CD8^+$ T cells (Silva M.T. et al., 2010).

1.4.2 Hyperthermia and Heat Shock Proteins (HSPs)

The use of hyperthermia in the therapy of cancer has been an object of clinical interest for many years. Hyperthermia seems to be particularly effective in combination with radiotherapy and/or radioimmunotherapy. The molecular mechanism by which hyperthermia leads to radiosensitization is not clear; however, activation of early response genes, heat shock factors, and subsequently Heat Shock Proteins (HSPs) has been proposed to play a role. HSPs constitute a superfamily of distinct proteins that are operationally named according to their molecular mass. Most HSPs are expressed constitutively and are further induced under stress conditions, including temperature increase. HSPs are chaperones that assist newly synthesized polypeptides during protein folding and translocation to intracellular compartments and are important in the clearance of unfolded or improperly folded proteins. In the context of immune systems, HSPs transfer antigenic peptides, thereby facilitating peptide presentation to $CD8^+$ T cells (Shi H. et al., 2006).

Mammalian HSPs have been classified into five families according to their molecular size: HSP100, HSP90, HSP70, HSP60 and small HSPs (15-30 kDa) including HSP27. Each family of HSPs is composed of members that are expressed either constitutively or regulated inductively, and that are targeted to different sub-cellular compartments. HSP27 and HSP70 are the most strongly and universally induced chaperones. Under normal conditions, HSP70 proteins function as ATP-dependent molecular chaperones that assist the folding of newly synthesized polypeptides, the assembly of multi-protein complexes and the transport of proteins across cellular membranes. HSP70 contains two distinct functional regions: a peptide binding domain (PBD) and the amino-terminal ATPase domain (ABD). Under stressful conditions, elevated HSP70 levels allow cells

to cope with increased concentrations of unfolded or denatured proteins (Garrido C. et al., 2006).

HMGB-1 is a relatively small protein of 215 amino acid residues. Structurally, the protein is organized into three distinct domains: two tandem HMG box domains (A box and B box), which are spaced by a short flexible linker, and a 30 amino acid-long acidic C-terminal tail. As structural units, HMG boxes are well conserved throughout evolution and are characterized by three α-helices, which are arranged in an L-shaped configuration. In the nucleus, HMGB-1 is a non-histone DNA-binding protein and serves as a structural component to facilitate the assembly of nucleoprotein complexes. The intracellular as well as extracellular function of HMGB-1 is further regulated by post-translational modification of the protein (Sims G.P. et al., 2010). HMGB-1 can also be secreted by different cells, like monocytes/macrophages, and plays important roles in inflammation and tumor metastasis (Muller S. et al., 2001); it is also passively released by necrotic cells, though not by apoptotic cells, triggering inflammation (Youn J.H. et al., 2006). In fact, once released, HMGB-1 is able to activate several cells involved in the immune response (i.e. DCs) or inflammatory reactions, and can act as a cytokine itself (Muller S. et al., 2001).

Chapter 5: Cancer immunotherapy

1.5.1 Active and passive immunotherapy

Active specific cancer immunotherapies, or “cancer vaccines”, aim at the induction of tumor antigen-specific immune responses, both cellular and humoral, and also at the generation of immunological memory. Using non-specific immune stimulators, such as cytokines (IFN-α, IL-2 or CpG- Kempf R.A. et al., 1986; Lotem M. et al., 2004; Kochenderfer J.N. et al., 2006), tumor-specific immune responses can be further enhanced. Despite the strong antitumor immune responses observed in animal models, limited responses have been observed in clinical trials.

Passive cancer immunotherapy administers ready-made effector molecules or cells (tumor antigen-specific antibodies or effector T cells).

In contrast to active cancer immunotherapy, the passive approach does not always require an active participation by the patient's immune system. It is short-lived and relies on repeated administration of the effectors.

Whilst the majority of the active immunotherapeutic approaches are still in preclinical evaluation or in early phase of clinical trials, several of the antibody-based therapies have resulted in very successful and clinically available agents, such as trastuzumab(that targets the Her-2 antigen expressed on breast cancer cells (Goldenberg M.M. et al., 1999), or the anti-CD20 antibody rituximab (used in B lymphoma treatment (Anderson D.R. et al., 1997).

Another technique of passive specific immunotherapy is adoptive transfer of effector T cells. This involves isolation of patient T cells, either from the tumor or from the blood, that after *ex vivo* processing are re-administered to the patient. This approach demonstrated clinical responses in melanoma patients (Mackensen A. et al., 2006).

A schematic overview of active and passive cancer immunotherapy is illustrated in **Figure 6**.

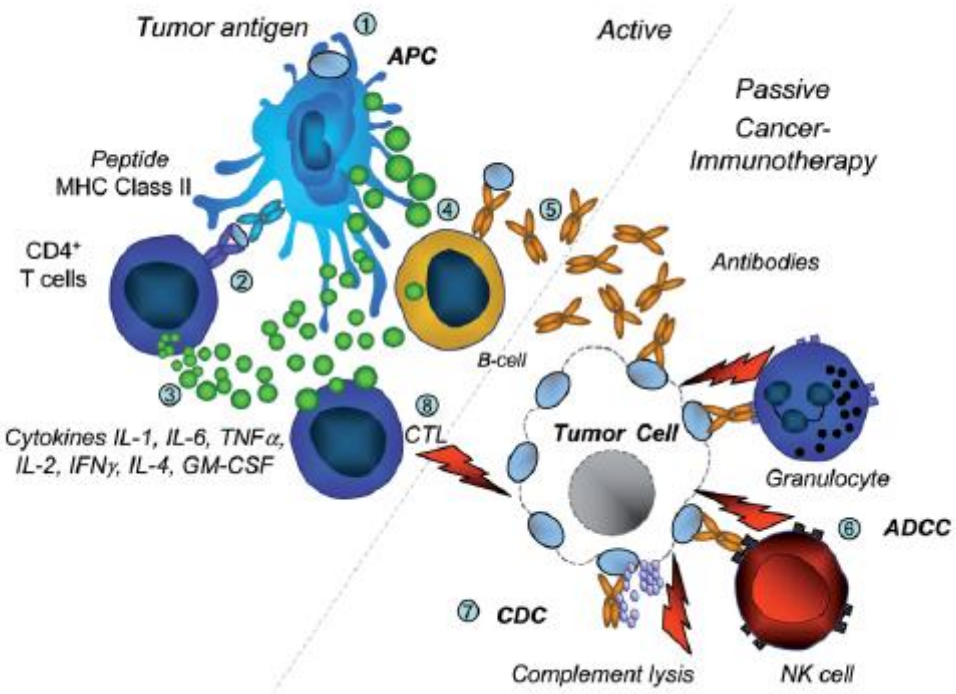


Figure 6. Active and passive cancer immunotherapy. Active cancer immunotherapy comprises tumor antigen uptake by APCs (1), epitope (peptide) presentation to $CD4^+$ T cells (2), cytokine release (3), B cell activation (4) and antibody production (5), leading to lysis of tumor cells including different passive

alternatives like activation of immune effectors cells-ADCC (6), activation of complement cascade-CDC (7) or unspecific attack by cytotoxic T lymphocytes (8). Schuster et al., 2006.

1.5.2 Pancreatic cancer immunotherapy

In many cancers it may be difficult to generate tumor reactive T cells due to the immunosuppressive factors produced by tumor cells. The expression of FasL or RCAS-1, which either induce apoptosis or inhibition of T cell proliferation, was reported in pancreatic cancers (Pernick NL. et al., 2002). RCAS-1 was found expressed in most pancreatic cancers and high expression is correlated with poor survival.

Some antigens were found to be recognized by $CD8^+$ T cells. Using sera from pancreatic cancer patients, Kawakami et al. have isolated 4 pancreatic cancer antigens: KU-PAN-1, KU-TES-1 and two DNA mismatch repair enzymes.

Passive immunotherapy with high doses of activated antitumor lymphocytes can be insufficient to induce an immune response able to cause tumor regression in immunocompromised patients with a large tumor burden. The most important problems of adoptive transfer *in vivo* are the maintenance and the accumulation of T cells in tumor tissues. However, it is now possible to generate tumor-reactive T cells from the PBMCs of cancer patients by *in vitro* stimulation, using identified tumor antigens (Rivoltini L. et al, 1995). Tumor reactive T cells from patients pre-immunized with tumor antigens were generated more efficiently, suggesting that combined use of active and passive immunotherapy could be the best solution.

For example, the use of autologous DCs pulsed with tumor antigens as an active immunotherapy to generate a cytotoxic immune response. Several studies have documented the generation of antitumor CTL cells after vaccination with tumor antigen-loaded DCs (Takahashi H. et al., 1993). Immunization with DCs pulsed with hTERT or p53 resulted in better antitumor effects than direct peptide administration (Tokunaga et al., 2005). In immunization trials using DCs pulsed with tumor lysates or synthetic peptides, tumor regression was observed in patients with prostate cancer, colon cancer and B-cell lymphoma (Nestle et al., 1998). A decrease of soluble tumor markers in the serum was observed in a patient with a pancreatic neuroendocrine tumor, following immunization with DCs pulsed with autologous tumor lysates (Schott et al., 2001).

Chapter 6: Humanized mice for preclinical research

Small animal models have been very helpful to investigate immunological mechanisms but certain limitations are unavoidable because of species-specific differences to humans.

Mice bearing severe immunodeficiecies (SCID) and harbouring human cells or tissues are frequently referred to as "humanized mice". They are a promising tool for study complex mechanisms in human biology and they at least may partly overcome these species-specific differences. Mice bearing human immune systems have been developed to investigate immune-mediated disease pathogenesis (Melkus et al., 2006; Shultz et al., 2007) , allogeneic tissue rejection and tolerance *in vivo* (Pober et al., 2003).

However, humanized mice remain a difficult field of research and a relevant model for preclinical evaluation of human T cells *in vivo* is currently lacking.

1.6.1 Protocols of "humanization": aspects and problems

Two main ways of "humanization" have been developed: the SCID-hu model, which consists of engrafting immunocompromised mice with human fetal tissues, and the hu-PBL-SCID model, which is derived from intraperitoneal injection of human peripheral blood lymphocytes (PBLs).

About 20 years ago, the original "SCID-hu" model was developed by McCune et al., using C.B-17-SCID mice (an immunocompromised mouse strain which has not essential adaptive immune system) as recipients for human hematopoietic tissues, including fetal liver, bone and or thymus originating from human fetuses (McCune et al., 1988; Namikawa et al., 1990; Kyoizumi et al., 1992). Engrafted human hematopoietic tissue resulted in low levels of human T and B cells that were able to produce a primary antibody response when autologous fetal skin, serving as an additional source of DCs, was co-engrafted along with thymus, bone morrow and lymph node (Carballido et al., 2000). The low numbers of mature human cells that repopulate the lymphoid tissues of SCID-hu mice makes this model inappropriate to study the mature functional human immune system (Krowka et al., 1991).

However, adoptive transfer of peripheral blood mononuclear cells (PBMCs) in the same mouse strain (hu-PBL-SCID model) supported low levels of engrafment of T, B and DCs (Mosier et al., 1988). Despite the numerous studies performed in the hu-PBL SCID model, results dealing with reconstitution of SCID mice by human engrafted PBLs were not consensual in terms of number and characteristic of human cells repopulating mice, depending both on screened organs and time of engrafment (Mosier et al., 1988; Tary-Lehmann et al., 1992; Martino et al., 1993). Moreover, a still controversial point is the existence of a graft-*versus*-host (GvH) reaction in these chimeras, supported by histological and functional studies (Murphy et al., 1992; Hoffmann-Fezer et al., 1993). This is one of the most critical point of the "humanized mouse models": if this xenoreaction effectively occurred, it should influence the human T-cell repertoire.
However, C.B-17-SCID animals are still significantly resistant to adoptive transfer of PBLs; indeed, further studies showed that resistance to adoptive transfer of human cells was reduced in animal treated with anti-asialo GM1, which depletes NK cells or in C.B-17-SCID animals that have the "beige" mutation that impairs NK-cell function (Murray et al., 1994; Christianson et al., 1996).
Technical advances, such as the transfer of isolated hematopoietic stem cells (HSCs) in newborn or adult C.B-17-SCID and NOD-SCID mice (Lapidot et al., 1992; Pflumio et al., 1996) and the development of more severely immunodeficient mouse strains lacking adaptive and innate immune responses, as Rag2 $^{-/-}$ γ-chain $^{-/-}$ mice (Goldman et al., 1998; Mazurier et al., 1999), have enhanced the applicability of humanized mice reducing the risk of GvH reactions and increasing the level of human cells engrafted.

1.6.2 Rag2 $^{-/-}$ γ-chain $^{-/-}$ mice

Rag2 $^{-/-}$ γ-chain $^{-/-}$ mice were developed in 1998 by Schultz et al. In these mice the homologous genes coding for the "recombination activating gene 2" (RAG2), involved in the recombination of V(D)J sequences of the T and B cells receptors) are deleted on both homologous chromosomes, as well as the common γ-chain genes encoding the common cytokine receptor γ chain. The results of these double knock out is a drastically absence of B, T cells and NK cells functions. Compared to other SCID mice, they have the advantage of a relatively stable phenotype without the development of spontaneous

tumors over time (van Rijn et al., 2006). In 2004, Traggiai et al injected intra-hepatically newborn Rag2 $^{-/-}$ γ-chain $^{-/-}$ mice of the BALB/c background with human cord-blood hematopoietic stem cells and, after 4, 8 and 12 weeks, they monitored engraftment and reconstitution of injected mice. They reconstituted a human adaptive and functional immune system *in vivo* with no evidence of GvHD *(*Traggiai et al., 2004*)*.

On the other hand, intravenous injection of huPBMCs containing 15 million fresh T cells into adult Rag2 $^{-/-}$ γ-chain $^{-/-}$ mice of C57BL/6 background leads to such a high chimerism that lethal GvHD reactions occur within 3 weeks in greater than 80% of mice (van Rijn et al.,2003). However, Van Rijn et al showed that this model is characterized by an antigen-driven process, with xenoreactivity as the stimulus for shaping the human T-cell repertoire (van Rijn et al., 2006).

So far, despite some unsolved problems , the huPBMC- Rag2 $^{-/-}$ γ-chain $^{-/-}$ model can be considered the most sensitive and clinically relevant model for the *in vivo* analysis of human T cells.

II. Aims of the study

Pancreatic cancer is one of the most aggressive malignant cancers, due to advanced stage at diagnosis, early systemic dissemination and poor response to chemotherapy or radiation therapy. The failure of conventional therapeutic options has motivated the search for immunotherapeutic approaches. In fact, some correlations between pancreatic cancer and immune system have been described: local and systemic alterations of effector T cell responses have been recognized in pancreatic cancer patients and pancreatic cancer cells produce immunomodulatory cytokines such as TGF-β, with immunosuppressive effects.

The initial aim of this study was the reconstitution of newborn immunodeficient mice with intrahepatically injection of bone marrow-derived leukocyte precursor CD34+ cells from pancreatic cancer patients, in order to obtain patient-specific humanized mice, to be implanted subcutaneously or orthotopically with pancreatic tumor cells from the same patient. This setting should allow to study *in vivo* the cross-talk between tumor and immune system and to evaluate immunotherapeutic approaches much better than in *in vitro* settings.

So far, we had some burocratic problems in the acquisition of bone marrow-derived leukocyte precursor CD34+ cells from pancreatic cancer patients as well as from healthy donors. Therefore, waiting for bone marrow, we reprogrammed the aims of the study as:

1- the establishment of protocols of delivery of human PBMCs to Rag2 -/- γ chain -/- immunodeficient mice, starting from either newborn or adult mice;

2- the development of an adoptive cell transfer (ACT) therapy based on human T lymphocytes activated *in vitro* by autologous DCs loaded with necrotic pancreatic tumor cells after hyperthermia or UVC treatment, to be delivered to Rag2 -/- γ chain -/- immunodeficient mice bearing the same human pancreatic tumor,,from which the necrotic cells used for DC loading were derived.

III. Materials and methods

3.1 Reconstitution of a human immune system in Rag2 $^{-/-}$ γ chain$^{-/-}$ mice

Rag2$^{-/-}$ γ-chain$^{-/-}$ mice were purchased from TACONIC Farm (Hudson, NY, USA) and a colony was propagated and maintained in the facility of University of Verona.

Reconstitution with human PBMC-derived cells from healthy donors was attempted in both newborn and adult (8 weeks of age) Rag2$^{-/-}$ γ-chain$^{-/-}$ mice.

Newborn animals (n=33) were γ-irradiated after birth (200 cGy) and, the day after, injected intrahepatically with 10-20 millions of PBMCs.

Adult animals (n=8) were divided in four groups, γ-irradiated (0-200-300 and 600 cGy) at day 0 and inoculated at day 1 by intraperitoneal injection (i.p.) with 50 millions PBMCs. Another group of adult animals (n=6), not irradiated, was injected i.p. with Percoll gradient-separated human lymphocytes from healthy donors.

The presence of cells of the human immune system was monitored:

- by FACS analysis on peripheral blood of mice 4 or 8 weeks after injection and
- at the end of the experiments, when mice were sacrified and organs were collected for FACS analysis and immunohistochemistry.

3.2 Cell lines

The human PaCa-44, PT-45, PANC-1 and PANC-2 cell lines, all derived from primary pancreatic adenocarcinoma and HLA-A*0201 homozygous have been largely studied by Prof. Aldo Scarpa and coworkers (Department of Pathology and Diagnostics, University of Verona, Italy), together with several other cell lines of similar origin also for the expression of immunogically relevant molecules (Scupoli MT et al., 1996).

Cells were maintained in RPMI-1640 (GIBCO INVITROGEN Corporation, San Diego, CA, USA) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS EUROCLONE, Pavia, Italy) and L-glutammine 1 mM (BIOCHROM AG, Cambridge, UK). Cell cultures were incubated in a humidified incubator at 37°C and 5% CO_2 atmosphere. To be propagated and for analysis, cells were detached by trypsin treatment

(0.05% trypsin at 37°C for 5 minutes, GIBCO INVITROGEN Corporation, San Diego, CA, USA) for use in culture.

3.3 Immunofluorescence and cytofluorimetric analysis

PaCa-44, PT-45, PANC-1 and PANC-2 cells were monitored by direct and indirect immunofluorescence and flow cytometry on a FACSCalibur (BD PHARMINGEN, Milan, Italy) for the maintenance of surface expression of MHC-I and –II, co-stimulatory and adhesion molecules, using the following specific monoclonal antibodies: B9.12.1, specific for human MHC-I monomorphic determinants; D1.12, specific for the MHC-II molecule HLA-DR (Ref Scupoli et al.); anti-human CD54-PE (BD-PHARMINGEN, Milan, Italy), specific for the human ICAM-1 (Intercellular Adhesion Molecule-1); anti-human CD58-FITC (BD-PHARMINGEN, Milan, Italy), specific for human LFA-3 (lymphocyte function-associated antigen 3); anti-human CD80-PE (BIOLEGEND, San Diego, CA, USA), specific for the human B7-1 molecule, and anti-human CD86-PE (BIOLEGEND, San Diego, CA, USA), specific for human B7-2 molecule.

FACS analysis of cells from peripheral blood of mice and of cells from cross-priming experiments was performed with anti-CD45-APC (pan-leukocyte marker), anti-CD3-PE-Cy7 (for T cells), anti-CD8-FITC (for $CD8^+$ T cells), anti-CD4-APC (for $CD4^+$ T cells), anti-CD14-PE (for monocytes), anti-CD19-FITC (for B cells) and anti-CD56-FITC (for NK cells). All these antibodies were purchased from BIOLEGEND, San Diego, CA, USA.

Human DCs were stained with anti-CD1a-FITC, anti-CD14-PE, anti-CD80-PE or -APC and anti-CD83-FITC (BIOLEGEND, San Diego, CA, USA). Specific control isotypes were used in direct immunofluorescence (BIOLEGEND, San Diego, CA, USA).

10’000 events were acquired for each sample processed. Results were analyzed using the DIVA Software (BD Biosciences, Bedford, MA, USA) and images were processed by the Flow Cytometry Analysis Software FlowJo.

3.4 Killing of tumor cells and analysis of tumor cell death parameters

PaCa-44, PT-45, PANC-1 and PANC-2 cells received the following treatments: γ-irradiation with a ^{137}Cs γ-irradiator source (13000 cGy), 56°C by direct immersion of collecting tubes with the cells into a temperature-controlled water bath for 15, 30, 60 or 120 minutes, UVC (3.6J/cm^2) using an UVC lamp PLS 9W/2P, W 2.3 (PHILIPS, Milan, Italy) for 10, 20, 30 or 60 minutes, or Gemcitabine (1μM) (LILLY, Indianapolis, IN, USA) for 72 hours.

After 4 or 20 hours from the above treatments, 1 x 10^6 tumor cells were stained with Annexin V(Ann V)-FITC and Propidium iodide (PI)-PE (ROCHE Diagnostics Corporation, Indianapolis, IN, USA) and they were analyzed by flow cytometry.

3.5 Analysis of DAMPs and TGF-β molecules release

At the indicated times, cells were centrifuged and the supernatants were collected and frozen at -20°C. For lysates, 1 x 10^6 alive tumor cells were treated with RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% NP-40) and protease inhibitors (SIGMA-ALDRICH, St. Louis, MO, USA) and then they were frozen at -80°C.

The release of the HSP-70 and HMGB-1 DAMPs molecules and TGF-β was evaluated on collected supernatants by enzyme-linked immunoassorbent assay (ELISA) kits, according to manufacturer's instructions. The range of sensitivity of the HSP-70 assay was 4.68-150 ng/ml.(R&D Systems, Minneapolis, MN, USA); the range of sensitivity of the HMGB-1 assay was 2.5-80 ng/ml (IBL INTERNATIONAL GMBH, Hamburg, Germany); the range of sensitivity of the TGF-β immunoassay was 31.2-2'000 pg/ml (R&D Systems, Minneapolis, MN, USA)

3.6 Generation of immature Dendritic Cells (iDCs)

Immature DCs were generated from Buffy coats of HLA-A2$^+$ blood bank donors of Policlinico G.B. Rossi, Verona, Italy, according to standard protocol (Markowicz, S.,

and E. G. Engleman. 1990). Peripheral blood mononuclear cells (PBMCs) were isolated by standard density gradient centrifugation (Ficoll Paque^{TM}-PLUS, GE Healthcare, Chalfont St. Giles, UK). They were washed twice with Phosphate-Buffered Saline (PBS) and then monocytes and lymphocytes were separated by a density Percoll^{TM} gradient centrifugation (GE Healthcare, Chalfont St. Giles, UK). Monocytes were plated in 24-well tissue culture plates (GREINER BIO-ONE Labortechnik, Frickenhausen, Germany) at a concentration of 3-3,5 x 10^6 cells in 1 ml of RPMI 1640 medium supplemented with 2% FBS and 1mM L-glutammine. After 1 hour, not adherent cells were separated from the rest of mononuclear cells by gentle washing. The remaining (adherent) cells were cultured for 5 days with RPMI 1640 medium supplemented with 10% FBS and 1 mM L-glutammine, in the presence of human granulocyte/macrophage colony-stimulating factor GM-CSF (final concentration: 50 ng/mL) and IL-4 (final concentration: 20 ng/mL) (PEPROTECH, Rocky Hill, NJ, USA).After 5 days they were collected and used as immature DCs.

3.7 Maturation assay of human DCs co-cultured with treated tumor cells

Four hours after the treatments indicated above, PaCa-44, PT-45, PANC-1 and PANC-2 cells were mixed with iDCs (2:1 ratio) and after 24 hours of incubation, DCs were exposed to a new medium containing the maturative cytokines TNF-α (final concentration: 10 ng/ml), IL-1β (final concentration: 1 ng/ml), IL-6 (final concentration: 10 ng/ml) (PEPROTECH, Rocky Hill, NJ, USA) and PGE_2 (final concentration: 1μg/ml) (SIGMA-ALDRICH, St. Louis, MO, USA). The effect of incubating the tumor with iDCs was studied before and 48 hours after the addition of maturative cytokines by FACS analysis using anti-human CD80-PE, anti-human CD83-FITC direct antibodies and anti-human MHC-II primary antibody (see 3.2). A total of 10’000 cells were acquired for each sample.

3.8 Uptake of dying tumor cells by iDCs

PaCa-44, PT-45, PANC-1 and PANC-2 cells were red-stained with PKH26-PE (SIGMA-ALDRICH, St. Louis, MO, USA) according to the manufacturer's instructions, before receiving the above killing treatments. Four hours later, tumor cells were incubated with iDCs (2:1 ratio) in human GM-CSF and human IL-4-containing medium at 37°C (see 3.5). After 24 hours, the mixed culture was stained with anti-CD80-APC (see 3.2). Phagocytosis of tumor cells by iDCs was assessed by flow cytometric analysis as the percentage of double-stained (PE^+/APC^+) cells on a total of 10'000 events.

For microscopy analysis of tumor engulfment, DCs and tumor cells cultured and stained as described above (for DCs, we used anti-human CD1aFITC-see 3.3) were mounted on a coverslip in Acqueous Gel Mounting (SIGMA-ALDRICH, St. Louis, MO, USA) and the fluorescence was assessed by FITC and TRITC filters on a fluorescence microscope (Olympus BX41) equipped with a Leica DFC320 camera and Leica Qwin Software. Images were processed by Adobe Photoshop 7.0.

3.9 Cross-priming of T lymphocytes with autologous tumor-loaded DCs

PaCa-44, PT-45, PANC-1 and PANC-2 tumor cells, not-treated or treated at 56°C or with UVC, were mixed with iDCs (2:1 ratio) and after 24 hours of incubation, DCs were exposed to a new medium containing the maturative cytokines TNF-α, IL-1β, IL-6 and PGE_2 (see 3.7). The day before the first stimulation, lymphocytes of the same donor were thawed and plated in a large flask with complete medium in presence of human IL-2 (final concentration: 20U/ml). After 48 hours, mDCs were added to autologous T lymphocytes at a ratio of 1:10 in 24-well tissue culture plates. Human IL-2 (final concentration: 20U/ml) was added to the cultures after 24 hours and the procedure has been repeated weekly, for up to two total stimulations. For cross-priming cultures, we used RPMI 1640 medium supplemented with 10% FBS, 1 mM L-glutammine and 10 ml/L Penicillyn/Streptomycin (SIGMA-ALDRICH, St. Louis, MO, USA).

Generation of specific anti-tumor response was evaluated five days after the last stimulation as release of human IFN-γ in ELISA assay and in measure of CTL-specific cytotoxicity in standard ^{51}Cr release assays.

3.10 Human IFN-γ ELISA assay

Supernatants of cross-priming co-cultures were collected 5 days after the last stimulation and the release of human IFN-γ was assessed in ELISA assay (R&D Systems, Minneapolis, MN, USA). Briefly, 100 µl of samples were incubated in a coated Microtiter Plate with the detection antibody for 2 hours; then we added of streptavidin-HRP solution for 30 minutes and we measured the absorbance at 450 nm (reference-wavelength: 650 nm). The range of sensitivity of the assay was 15.6-1000 pg/ml.

3.11 ^{51}Cromium release cytotoxicity assays

The cytotoxicity of lymphocytes from cross-priming co-cultures were measured in a standard ^{51}Cr release assay. Due to the very low cytotoxic activity elicited in the initial steps of the immunization protocol, a 50:1 effector:target ratio was chosen as the reference ratio for all the cytotoxicity experiments performed.

Briefly, cross-priming co-cultured cells were harvested and used as effectors against wild type (w.t.) PaCa-44, PT-45, PANC-1 and PANC-2 target cells labelled with ^{51}Cr Na Chromate (PERKINELMER Life Sciences Inc. Boston, MA, USA), in a 4 hours-incubation standard ^{51}Cr release assay. When indicated, targets were kept at 4°C for 20 minutes with the anti-human MHC class I blocking mAb W6/32 (SEROTEC, DBA Italia s.r.l., Milan, Italy), 25µg/ml, before being used as targets.

The ^{51}Cr release was measured in the supernatants of co-cultures; the percentage of specific lysis was calculated according to the formula: % Specific lysis = [(experimental cpm - spontaneous cpm) / (maximal cpm - spontaneous cpm) x 100]. CPM was the acronym for counts per million.

3.12 Adoptive cell transfer (ACT) of *in vitro* activated T cells

$Rag2^{-/-}$ γ-$chain^{-/-}$ mice (n=4/group) were injected subcutaneously (s.c.) with alive PaCa-44 or PT-45 tumor cells (2,5 x 10^6 cells/mouse) and 4 days later they were intravenously (i.v.) or intraperitoneally (i.p.) transferred with human lymphocytes (5 x 10^6cells/mouse) stimulated twice *in vitro* by cross-priming with DCs loaded with the corresponding necrotic tumor cell as reported above (See 3.9). Mice were injected i.p. with recombinant human IL-2 (30'000U/mouse) twice a day for three consecutive days for i.v. ACT and once for i.p. ACT, according to current protocols (Dolcetti et al., 2010).

Healthy state of mice and tumor progression were evaluated three times a week; when tumor diameter was 1-1,5 cm, mice were sacrified and peripheral blood, tumor and organs were collected for FACS analysis (see 3.3) and immunohistochemistry assays. If signs of pain and fatigue became evident in the animals earlier, they were sacrified immediately. We evaluated tumor volumes considering tumor cells as growing s.c. as a sphere (cm^3). All *in vivo* experiments are part of a Research Protocol already notified to the Italian Ministry of Health on April 16th 2008, Protocol n.22 /2008.

3.13 Immunohistochemistry of organs and PaCa-44 and PT-45 tumor tissues of mice

Tumor and organs of control and treated mice were fixed in buffered formalin (Mondial, Italia) for 24h and then they were paraffin-embedded following standard protocols (Chilosi et al., 2003). Serial sections of 2,5-3 µm thickness were cut with microtome and immunostained with hematoxilin & eosin (H&E) or a monoclonal antibody recognizing human CD45+ cells (Dako, Milan, Italy). When this marker was positive, human markers for CD3+ cells, CD4+ cells, CD8+ cells, CD20+cells , CD138+ cells, Ki67 and Cytokeratin-8 were also used (Dako, Milan, Italy).

All samples were processed using a sensitive avidin-streptavidin-peroxidase technique (Biogen San Ramon, CA, USA) in an automated staining system (GenoMX i6000, BioGenex).

3.14 Statistical analysis

Data are expressed as mean value of three different experiments ± standard deviation (SD). One-way ANOVA and Holm-Sidak test for multiple comparison were used to compare parametric variables among multiple groups, and, finally, Student's t test was used to compare parametric variables between two groups. The Shapiro-Wilk test was preferred to test normal distribution of a numeric variable. In any case, differences were considered statistically significant for *P* values ≤ 0.05.

For *in vivo* experiments, tumor incidence was defined as the time from the inoculation of the tumor to the appearance of a palpable mass. Overall Survival (OS) was defined as the time from the inoculation of the tumor to death of the animals. Tumor growth rate was defined as the time from the tumor onset to a mass diameter of 1-1,5 cm.

Tumor incidence and OS curves were calculated according to Kaplan and Meier, and differences between murine subgroups were tested using the log-rank test.

P value ≤ 0.05 was considered as statistically significant.

Statistical analyses were performed using STATA IC v.10.1 (StataCorp, College Station, TX) for Microsoft Windows®.

IV. Results

SECTION I

Attempts to reconstitute a human immune system in Rag2$^{-/-}$γ-chain$^{-/-}$ immunodeficient mice using peripheral blood mononuclear cells (PBMCs)

To study the cross-talk between human cancer and immune system and to test *in vivo* an immunotherapeutic strategy using DCs loaded with hyperthermia-treated cancer cells, we tried to set up the conditions for obtaining a "humanized mouse model" displaying many features of human immune system**.** The goal was to reconstitute the human immune system of pancreatic cancer (PaCa) patients in severely immunodeficient mice, by means of intrahepatically delivery to newborns of hematopoietic CD34+ human bone marrow-derived immune precursor cells. These mice would be the ideal "container" for the xenograft of PaCa derived from the same patients who donated the bone marrow, in order to obtain a mouse model allowing to study the tumor-immune system cross-talk.

In the animal facility of the University of Verona, we have generated a colony of Rag2 $^{-/-}$γ-chain$^{-/-}$immunodeficient mice (double knockout mice of the C57BL/6J x C57BL/10SgSnAi background), with severe combined immunodeficiency (SCID) (hereafter named SCID mice), starting with 5 females and 5 males purchased from Taconic animal models (New York's River Valley, NY USA), and it took some months due to a 25% mortality in newborns (in pathogen free conditions and not treated). To improve engrafment, according to literature, SCID mice should be irradiated before injection of human cells, to eliminate a residual host immune activity due to the presence of NK, NKT, cells of the myelo-monocyte lineage and to create room for the grafting of human cells. Therefore SCID mice were exposed to sublethal irradiation 24 hours after birth with a Cesium 137 source, and it took time also to set up the irradiation protocol. The effects of different times of exposure to the Cesium 137 source were monitored by FACS analysis on irradiated mice at 4 weeks after irradiation, compared to untreated, littermate control mice.

Since initially we could not obtain bone marrow from healthy donors as well as from PaCa patients, we carried out experiments of reconstitution with peripheral blood mononuclear cells (PBMCs) from healthy donors, described here.

Two different protocols of reconstitution with human PBMCs, using either newborn or adult Rag2$^{-/-}$ γ-chain$^{-/-}$ immunodeficient mice were attempted:

i) PBMCs inoculation in sublethally irradiated newborn animals. Newborns were intrahepatically injected with 10-20 millions PBMCs 24 hours after sublethal irradiation.

ii) PBMCs inoculation in not-irradiated or sublethally irradiated adult animals, 8-9 weeks old. Animals were injected intraperitoneally (i.p.) with 50 millions PBMCs.

4.1 Newborn Rag2$^{-/-}$ γ-chain$^{-/-}$ immunodeficient mice

Newborn Rag2$^{-/-}$ γ-chain$^{-/-}$ immunodeficient animals (n=33) were sublethally irradiated one day after birth and injected intrahepatically with 10-20 millions of human PBMCs the following day.

Among treated animals, 67% (22/33) died between 15-20 days after intrahepatically injection; in 45% (5/11) of live treated animals the 27.1±0.5% of total blood cells expressed human CD45 at high levels 4 weeks after injection, and this phenotype lasted about 6 months. These human cells were CD3$^+$ (31.3±0.3% of total blood cells) CD4$^+$ (14.3±3.4% of total blood cells) and CD8$^+$ (17.4±2.5% of total blood cells). No CD19$^+$, CD56$^+$ or CD14$^+$ cells were detectable in the blood (**Figure 8**).

Immunohistochemistry showed relevant differences only in the spleen and lung of treated mice *versus* untreated (**Figure 9** and **10**): the presence of human CD20$^+$ B cells and CD3$^+$, CD4$^+$ and CD8$^+$ T cells infiltrates into the spleen and lung was assessed in all treated mice, expecially near blood vessels. These cells were also CD138$^+$, a marker of mature B cells. These lymphocytes were polyclonal, as shown by k and λ markers (data not shown), and theywere highly proliferative as shown by an increase in the number of Ki67$^+$ cells.

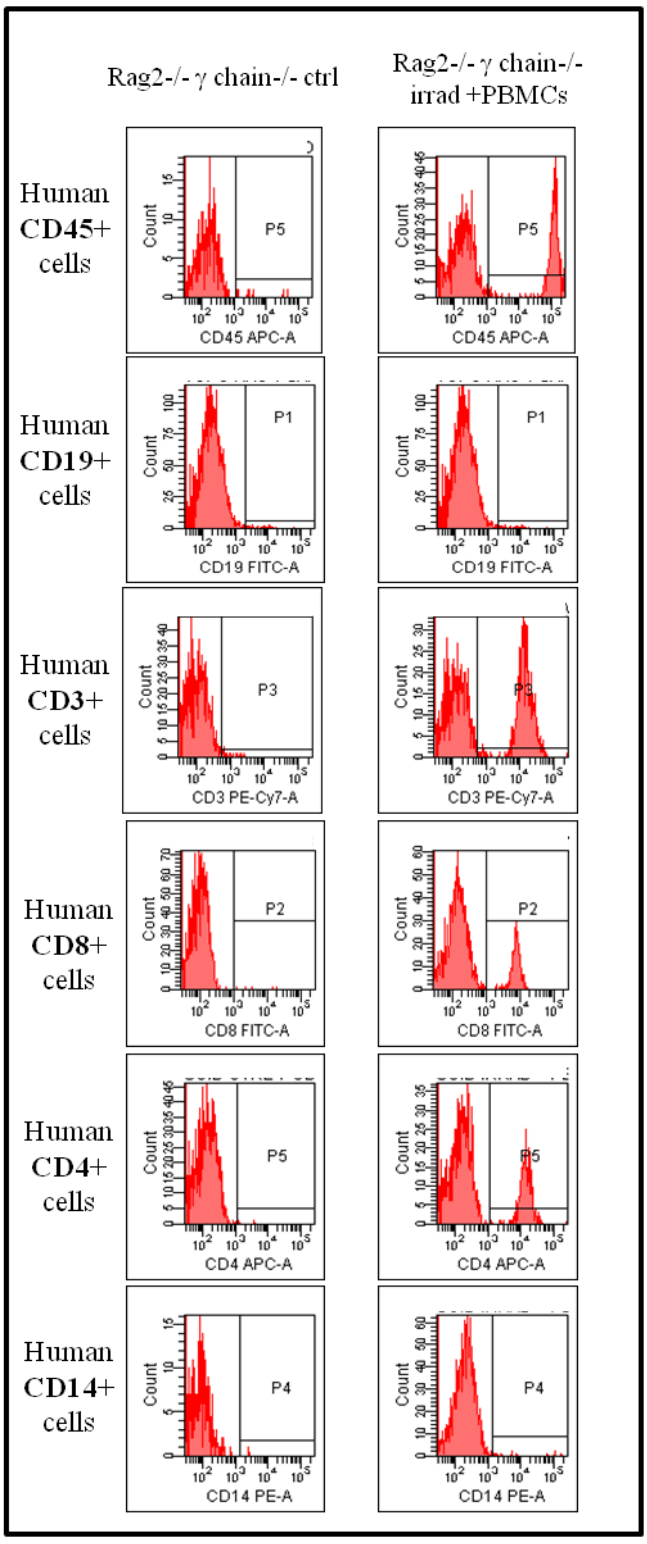


Figure 8: Cytofluorimetric analysis of peripheral blood of Rag2$^{-/-}$ γ-chain$^{-/-}$ mice 4 weeks after intrahepatic PBMCs injection at the age of newborn: untreated mouse (left column) and mouse γ-irradiated and injected intrahepatically with PBMCs (right column). Histograms show a representative case of treated and reconstituted newborn Rag2$^{-/-}$ γ-chain$^{-/-}$ mice compared to untreated. Fluorescence values are expressed in absorbance units (a.u.).

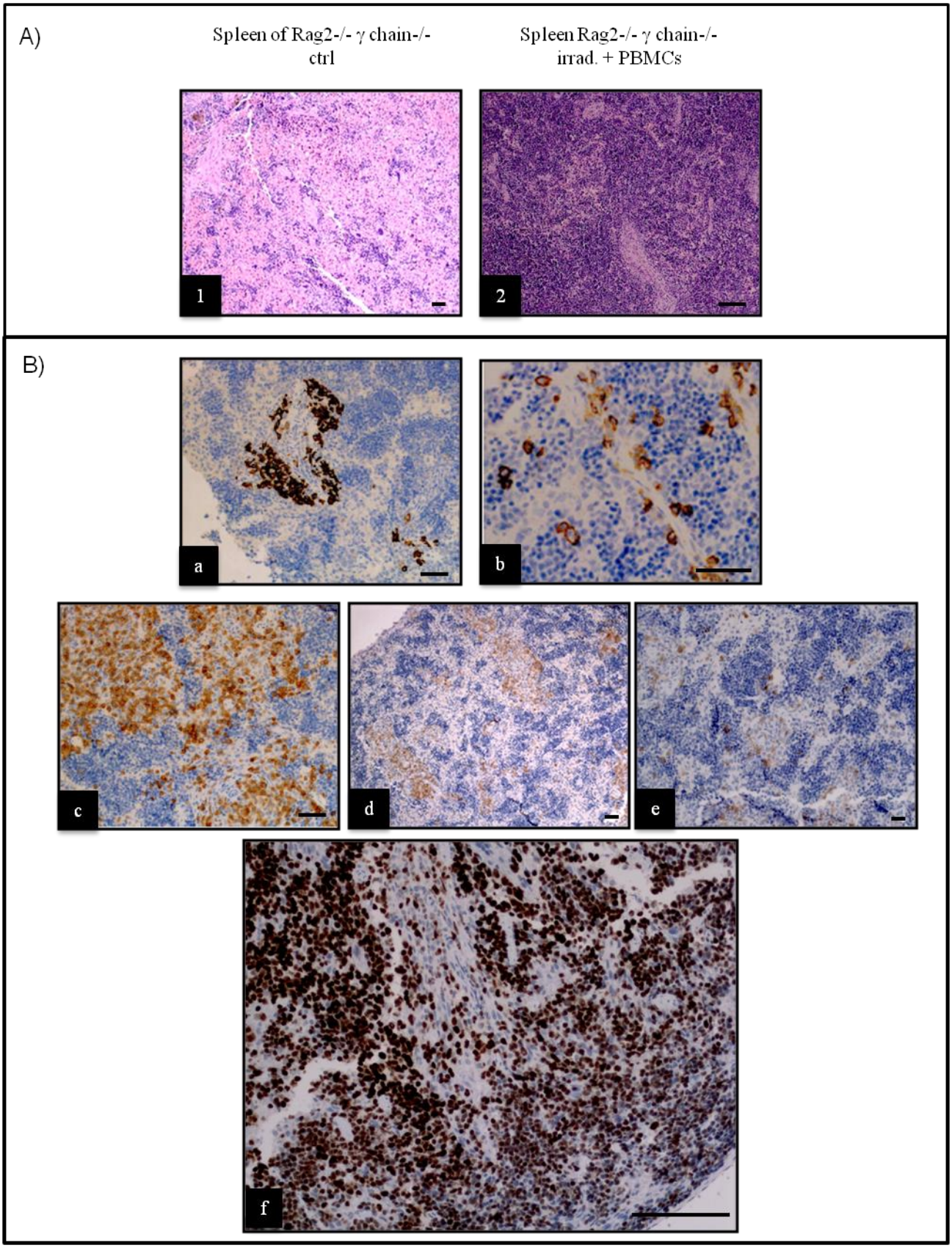


Figure 9: Spleen from a representative case among Rag2$^{-/-}$γ-chain$^{-/-}$mice γ-irradiated and injected intrahepatically with PBMCs at newborn age, four weeks after injection. **A)** 1-2: H&E staining of spleen specimen from: untreated mouse (1; 100× magnification) and treated mouse (2; 200× magnification) **B)**

(a-f) immunohistochemical analysis of spleen from a treated mouse (2): staining with CD20 (a; 200× magnification), CD138 (b; 400× magnification), CD3 (c; 200× magnification), CD4 (d; 100× magnification), CD8 (e; 100× magnification) and Ki67 (f; 400× magnification) markers. Bars represent 250 μm.

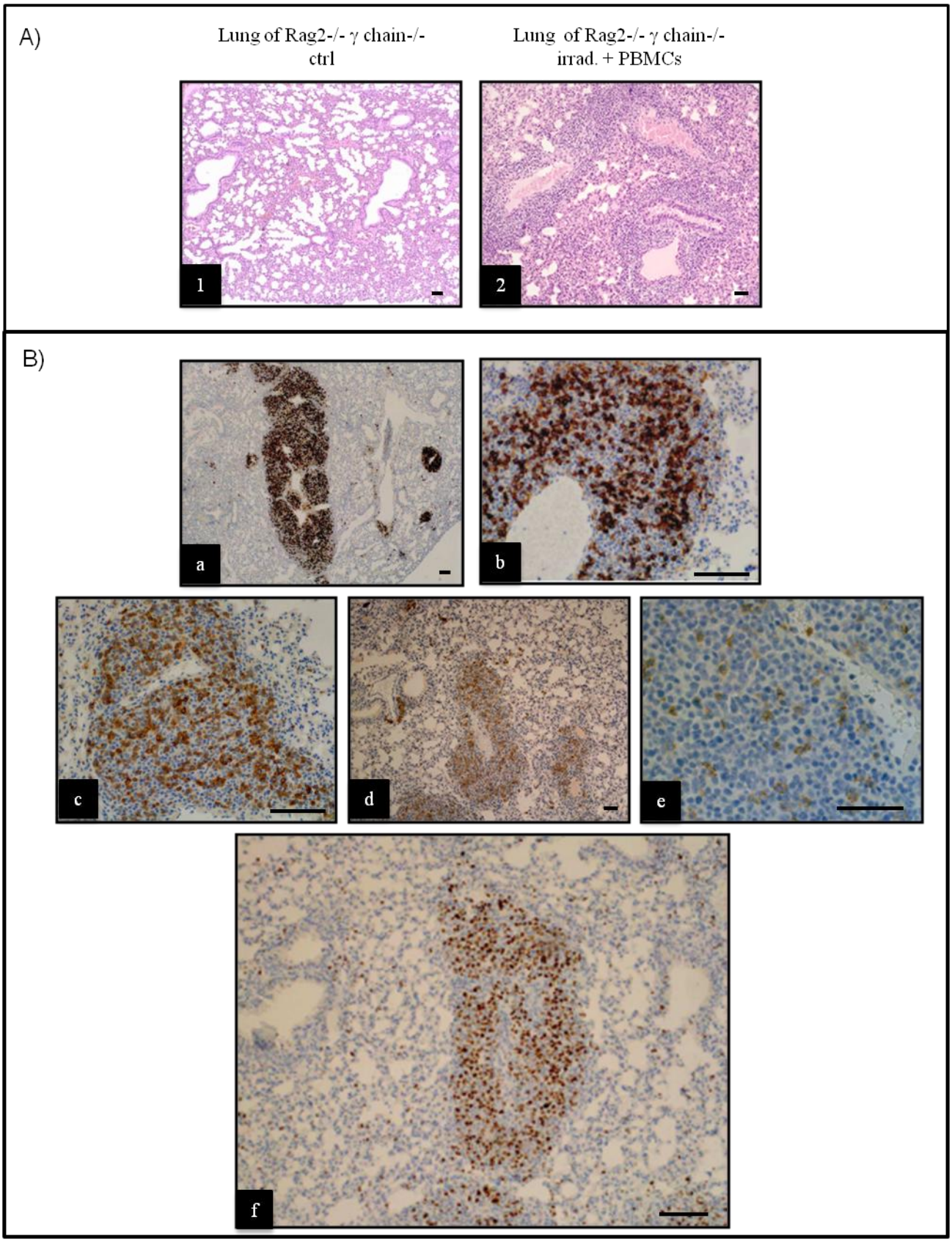


Figure 10: Lung from a representative case among Rag2$^{-/-}$γ-chain$^{-/-}$mice γ-irradiated and injected intrahepatically with PBMCs at newborn age, four weeks after injection: **A)** H&E staining of lung specimen from (1-2): untreated mouse (1; 40× magnification) and treated mouse (2; 100× magnification);

B) (a-f)Immunohistochemical analysis of lung from a treated mouse (2) with: CD20 (a; 40× magnification), CD138 (b; 400× magnification), CD3 (c; 400× magnification), CD4 (d; 100× magnification), CD8 (e; 400× magnification) and Ki67 (f; 200× magnification) markers. Bars represent 250 μm for 100×, 200× and 400× magnifications and 500 μm for 20× and 40× magnifications. The figure shows a representative case of different reconstituted newborn $Rag2^{-/-}$ γ-$chain^{-/-}$ mice.

We finally evaluated the presence of Graft *versus* Host Disease (GvHD) in treated mice by direct observation of animals during the experiments and by immunohistochemical analysis of different organs (data not shown). Only one treated mouse displayed clear symptoms of Graft *versus* Host Disease (GvHD) (i.e.: severe weight loss, hunched posture, ruffled furr and reduced mobility). All the other treated animals did not develop detectable symptoms. Accordingly, immunohistochemistry analysis did not indicate any infiltrate of human inflammatory cells in skin, gut and liver. Unfortunately, this analysis could not be performed in the single symptomatic mouse, due to its premature and sudden death not allowing collection of material for histology).

4.2 Adult $Rag2^{-/-}$ γ-$chain^{-/-}$ immunodeficient mice

Three months-old adult mice (n=8) were injected i.p. with -50 millions of human PBMCs. Among them, one group (n=6) was γ-irradiated (200-300-600 Gy) the day before PBMCs injection, whilst a second group (n=2) was not γ-irradiated. γ-irradiation with 600 Gy resulted lethal, whilst the other doses did not. γ-irradiation did not appear fundamental for reconstitution: no differences in engrafment of human cells were found in γ-irradiated mice compared to mice not γ-irradiated. All the PBMCs-injected adult animals showed low mortality after reconstitution. Another group of adult animals (n=6) was injected i.p. with Percoll gradient-separated human lymphocytes from healthy donors.

Four weeks after injection, the presence of cells of the human immune system was evaluated in peripheral blood and organs of treated mice.

Peripheral blood of all treated mice showed very low levels of human $CD45^+$ cells (3.75±0.3% of total blood cells), $CD3^+$ cells (3.45±0.3% of total blood cells), $CD4^+$ cells (1.85 ±0.2% of total blood cells) and $CD8^+$ cells (1.55±0.6% of total blood cells).

No $CD19^+$, $CD56^+$ and $CD14^+$ cells were detectable (**Figure 11**). Immunohistochemistry of lymphoid and other organs showed relevant differencies only in spleen and lung of treated mice *versus* untreated mice (**Figure 12** and **13**): the presence of human $CD20^+$ B cells and $CD3^+$, $CD4^+$ and $CD8^+$ T cells infiltrates into the spleen and lung was assessed in all treated mice, expecially near blood vessels. $CD138^+$ B cells, more numerous in the lung than in the spleen, were polyclonal, as shown by k and λ markers (data not shown), and they were highly proliferative, as shown by an increase in the number of $Ki67^+$ cells (**Figure 11**)**.** Finally, reconstituted adult mice did not show evidences or symptoms of GvHD by direct observation of animals during the experiments and by immunohistochemical analysis of different organs (data not shown).

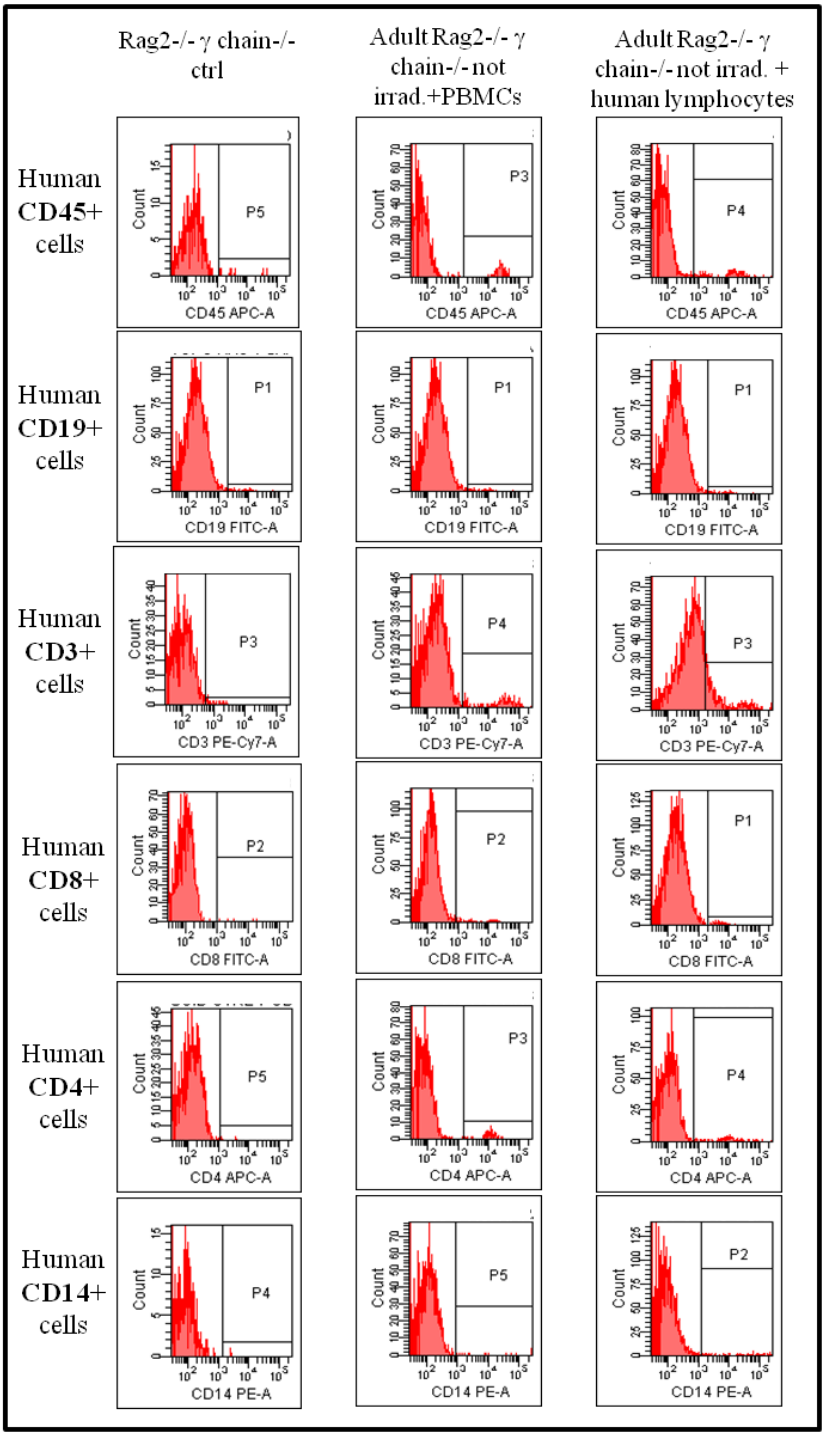


Figure 11: Cytofluorimetric analysis of peripheral blood of not-irradiated Rag2$^{-/-}$ γ-chain$^{-/-}$ mice 4 weeks after injection at adult age of human PBMCs or human Percoll-separated lymphocytes: untreated mouse (left column), mouse injected i.p. with PBMCs (central column) and mouse injected i.p. with lymphocytes only (right column). Histograms show a representative case of different treated newborn Rag2$^{-/-}$ γ-chain$^{-/-}$ mice. Fluorescence values are expressed in absorbance units (a.u.).

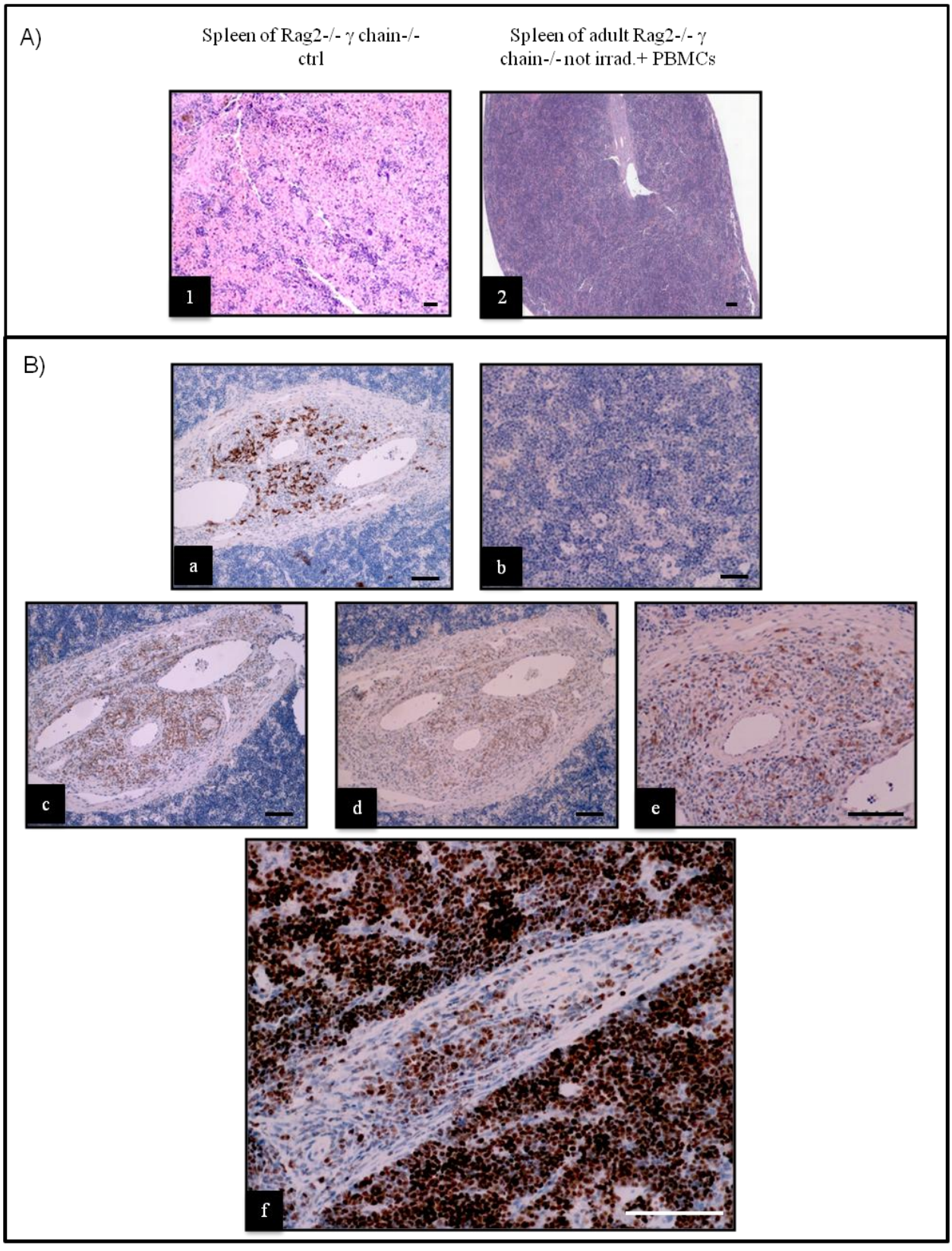


Figure 12: Spleen from a representative case among Rag2$^{-/-}$γ-chain$^{-/-}$mice injected i.p. with PBMCs at adult age, four weeks after injection. **A) (**1-2) H&E staining of spleen specimen from: untreated mouse (1; 100× magnification) and mouse injected i.p. with PBMCs (2; 40× magnification); **B)** (a-f) Immunohistochemical analysis of spleen from a treated mouse with human CD20 (a; 200×

magnification), CD138 (b; 200× magnification), CD3 (c; 200× magnification), CD4 (d; 200× magnification), CD8 (e; 400× magnification) and Ki67 (f; 400× magnification) markers. Bars represent 250 μm for 100×, 200× and 400× magnifications and 500 μm for 40× magnification.

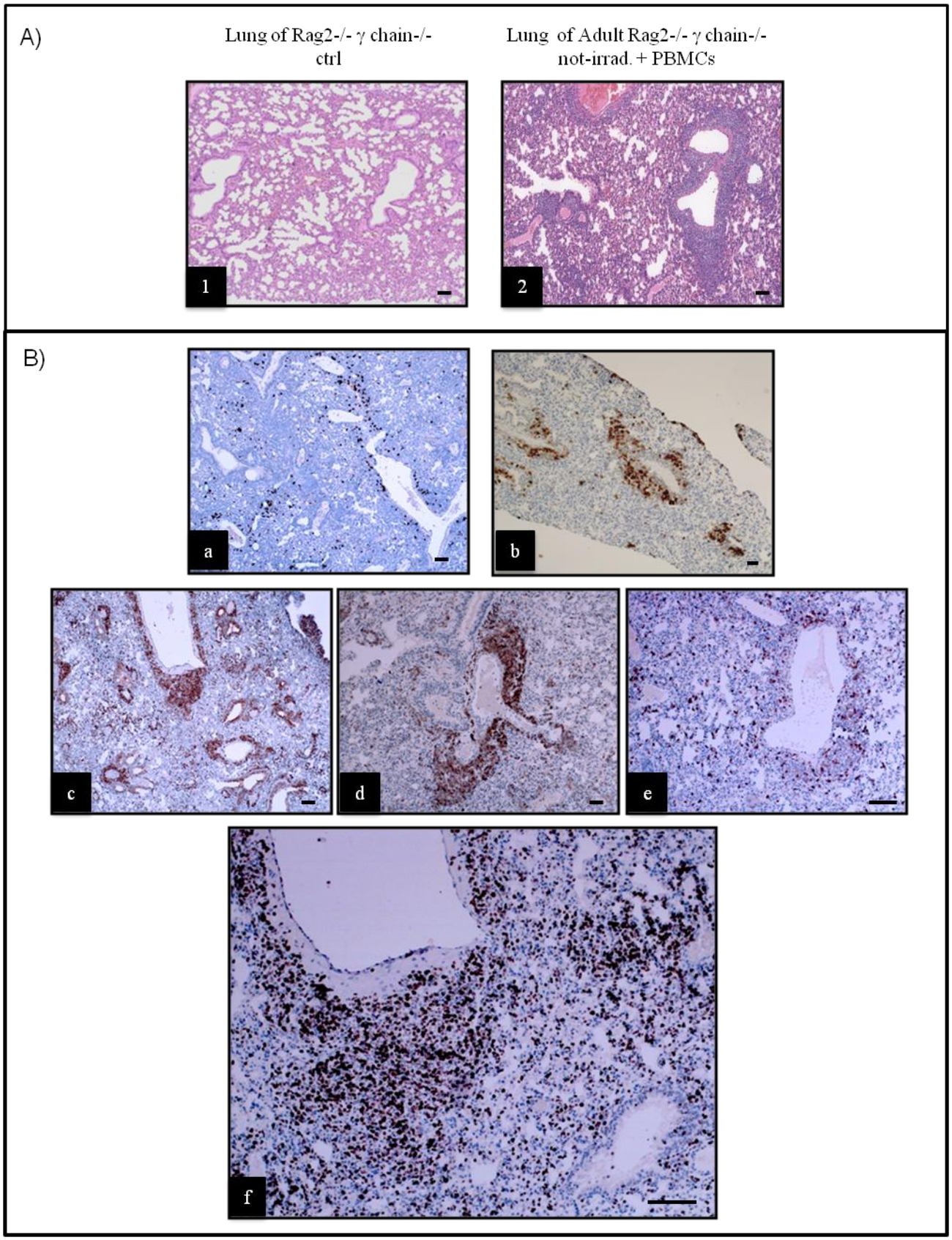


Figure 13: Lung from a representative case among Rag2$^{-/-}$γ-chain$^{-/-}$mice injected i.p. with PBMCs at adult age, four weeks after injection: **A)** (1-2) H&E staining of lung specimen from: not-treated mouse (1; 100× magnification) and mouse injected i.p. with PBMCs (2; 100× magnification); **B)** (a-f)

Immunohistochemical analysis of lung of a injected mouse with human CD20 (a; 100× magnification), CD138 (b; 40× magnification), CD3 (c; 100× magnification), CD4 (d; 100× magnification), CD8 (e; 200× magnification) and Ki67 (f; 200× magnification) markers. Bars represent 250 μm for 100× and 200× magnifications and 500 μm for 40× magnification.

IV. Results

SECTION II

4.3 Immunophenotype of PaCa-44, PT-45, PANC-1 and PANC-2 pancreatic cancer cells

The human primary pancreatic adenocarcinoma-derived PaCa-44, PT-45, PANC-1 and PANC-2 cell lines, HLA-A*0201 homozygous, were chosen for the present study because they express relevant amounts of the HLA-A*0201 molecule and thus could function as targets of CTLs from HLA-A*0201 donors (**Figure 14**).

Because important for the cross-talk with T cells (Yang S. and Schlom J. , 2009), the expression of MHC-II, adhesion and co-stimulatory molecules was also monitored. The ICAM-1 and LFA-3 adhesion molecules appeared differently expresses in these cell lines: PaCa-44 and PT-45 express only LFA-3, whilst PANC-1 and PANC-2 express also ICAM-I. On the contrary, MHC-II, B7-1 and B7-2 molecules were not expressed at all (**Figure 14**).

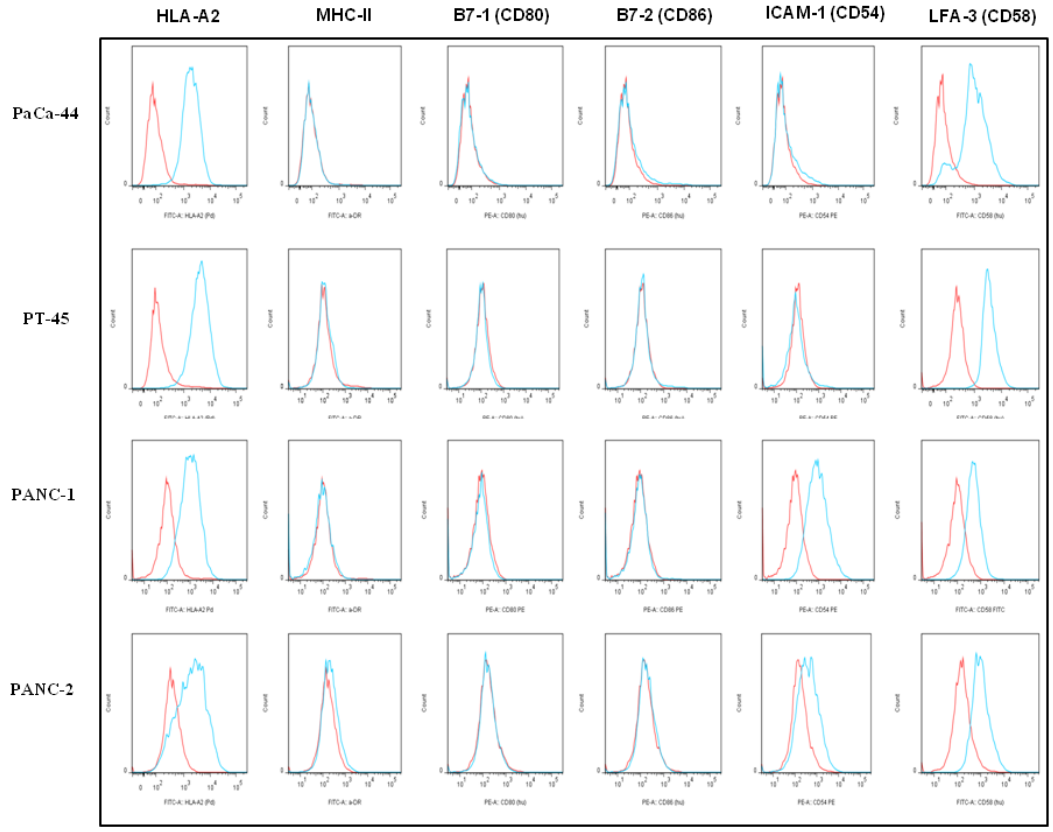


Figure 14: Cytofluorimetric analysis of PaCa-44, PT-45, PANC-1 and PANC-2 cancer cells after indirect immunofluorescence staining with monoclonal antibodies specific for HLA-A2 and MHC-II molecules, and after direct immunofluorescence staining with anti-human B7-1, B7-2, ICAM-1 and LFA-3 molecules, as indicated at the top of each line. The different cell lines are indicated to the left of each horizontal series of histograms. Red histograms indicate the fluorescence of isotype controls or CTRL FITC (fluorescence of each sample treated only with the secondary monoclonal antibody). Fluorescence values are expressed in absorbance units (a.u.). Images were analyzed by FlowJo application.

4.4 Hyperthermia induced apoptosis followed by secondary necrosis in PaCa-44, PT-45, PANC-1 and PANC-2 pancreatic cancer cells *in vitro*

As recently described by Brusa *et al.*, hyperthermia treatments at 56°C or by UVC lamp, inducing secondary necrosis, renders prostate tumor cells highly palatable to iDCs and able to activate T cell responses, because necrotic tumor cells release DAMPs molecules, like HSP70 and HMGB-1. To test if the conditions of hyperthermia treatments used *in vitro* by Brusa *et al.* could be applied also to pancreatic cancer, we performed hyperthermia experiments with the PaCa-44, PT-45, PANC-1 and PANC-2

human pancreatic tumor cell systems. The induced state of apoptosis-necrosis of tumor cells was evaluated by FACS analysis using Annexin-V–FITC and propidium iodide-PE staining kit (see 3.3). We could recognize alive cells (Ann V^-/PI^-), cells in primary necrosis (Ann V^-/PI^+), cells in early apoptosis (Ann V^+/PI^-), and cells in secondary necrosis (Ann V^+/PI^+) (**Figure 15A**).

The treatment of PaCa-44, PT-45, PANC-1 and PANC-2 cells in water bath at 56°C induced apoptosis followed by secondary necrosis (Ann V^+/PI^+) in about 80-90% of treated cells already after 15 minutes and there was no difference for all the other time points (30 minutes, 1 hour, 2 hour). These results were confirmed after 20 hours of incubation at 37°C (**Figure 15B**). Reported results of hyperthermic treated cells for all the time points were statistically significant compared to untreated cells, except for PANC-2 treated for 15 minutes and incubated at 37°C 4 hours after the treatment ($P < 0.01$; $P < 0.001$) (**Figure 15B**).

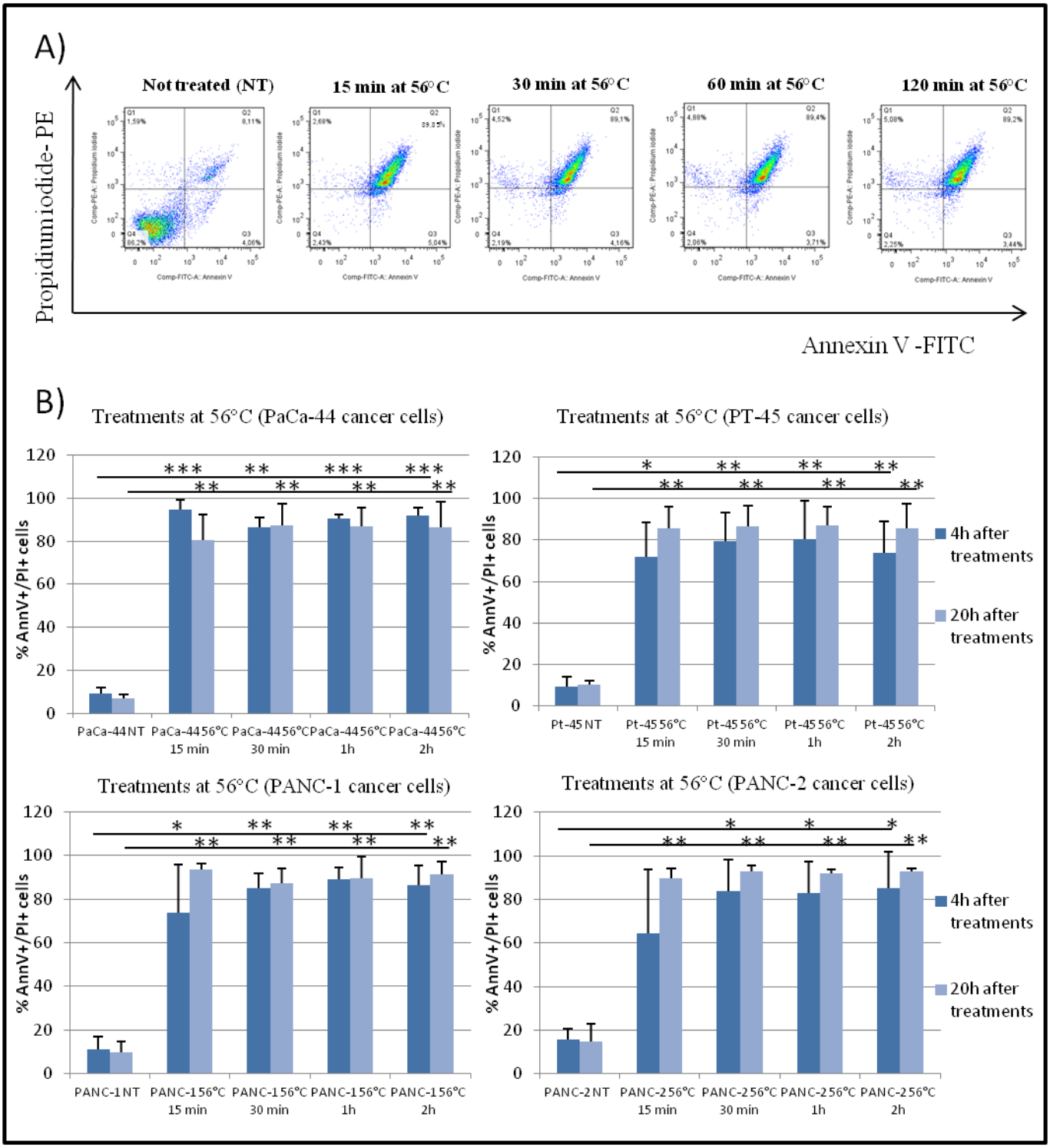


Figure 15: Treatments of PaCa-44, PT-45, PANC-1 and PANC-2 tumor cells at 56°C for 15, 30, 60 and 120 minutes. **A)** Cytofluorimetric analysis of PaCa-44 cells treated at 56°C at different time points and incubated 4 hours at 37°C after the treatments (images were analyzed by FlowJo application). The data shown are from one representative experiment out of three with similar results. **B)** Histograms with statistical analysis of all pancreatic cancer cell lines treated at 56 °C and incubated for 4 or 20 hours at 37°C before immunofluorescence staining with AnnV/PI kit. Each histogram indicates the mean value of three different experiments with standard deviation of the results. The differences between necrotic cells

after 56°C treatment and untreated cells were statistically significant (*= $P < 0.05$; **=$P < 0.01$; ***=$P < 0.001$).

In all the UVC treatments (time of exposure: 10, 20, 30 and 60 minutes) the fraction of necrotic cells ranged between 27% and 56%, depending on cell line and time of UVC exposure, and increased to 50-85% after 20 hours of incubation at 37°C (**Figure 16**). As shown, after UVC treatment and 4 hours incubation, there was an increase of $AnnV^+/PI^+$ cells, which was directly correlated with increasing UVC exposure time. This trend was maintained also 20 hour after treatments, except for PT-45 cells that displayed a high fraction of necrotic cells already after 10 minutes of UVC exposure, indicating that PT-45 cells are more sensitive to UVC exposure than the other tumor cell lines.

Statistical analysis confirmed the significance of the reported results for all cancer cell lines, especially for the longest UVC exposure time (30 minutes and 1 hour) after 4 hours incubation, and for all time of exposure after 20 hours of incubation ($P < 0.05$ for PANC-1; $P < 0.01$ for PaCa-44; $P < 0.001$ for PT-45 and PANC-2).

The exposure time of 1 hour for both treatments at 56°C and with UVC was chosen for the following experiments.

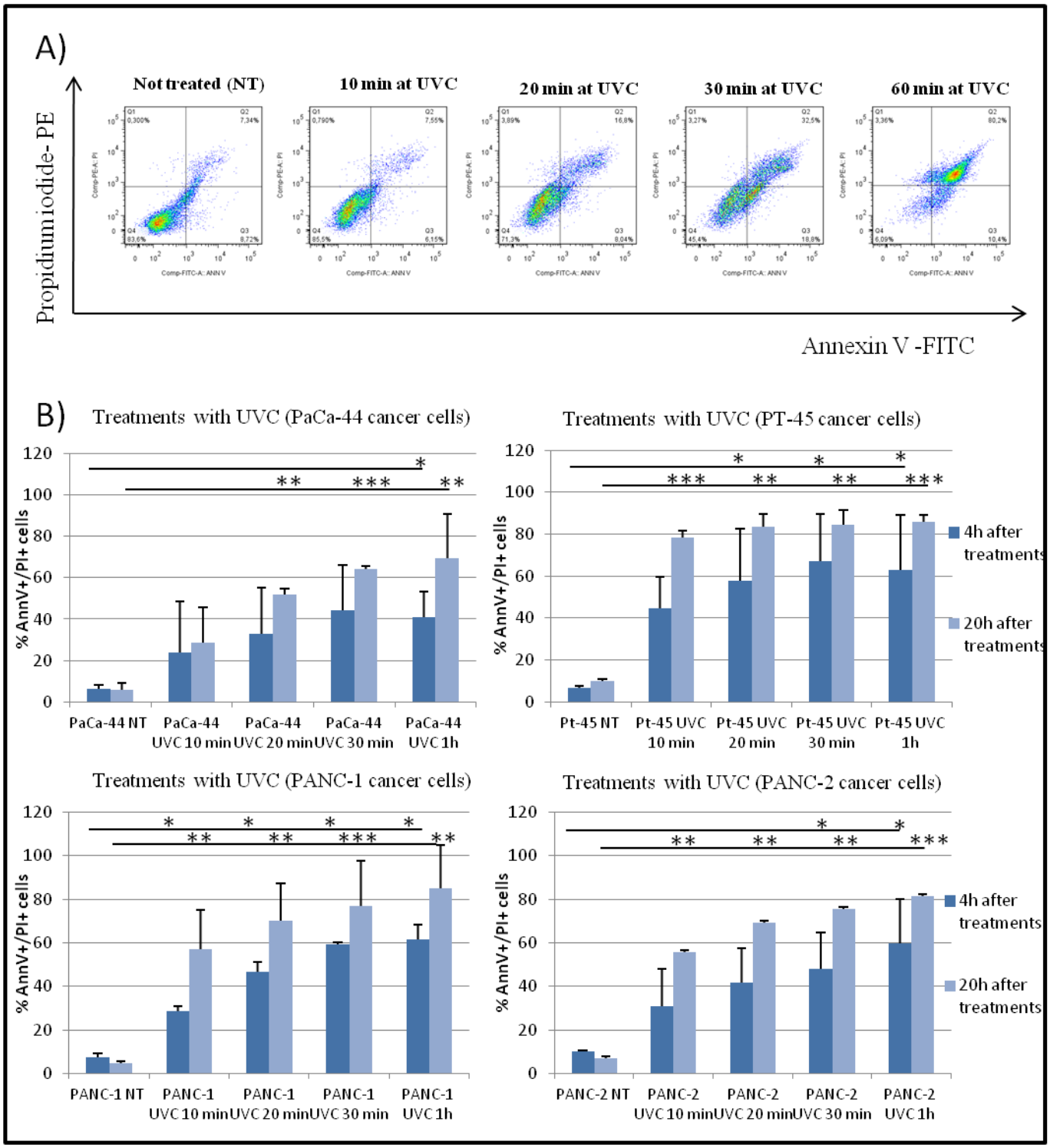


Figure 16: Treatments of PaCa-44, PT-45, PANC-1 and PANC-2 tumor cells by UVC lamp for 10, 20, 30 and 60 minutes. **A)** Cytofluorimetric analysis of PaCa-44 cells treated by UVC at different time points and incubated 4 hours at 37°C after the treatments (images were analysed by Flow Jo application). The data shown are from one representative experiment out of three with similar results. **B)** Histograms with statistical analysis of all pancreatic cancer cell lines treated by UVC and incubated for 4 or 20 hours at 37°C before immunofluorescence staining with AnnV/PI kit. Each histogram indicates the mean value of

three different experiments with standard deviation of the results. The differences between necrotic cells after UVC treatment and untreated cells are statistically significant (*= $P < 0.05$; **=$P < 0.01$; ***=$P < 0.001$).

Then tumor cell death was analyzed comparing 56°C and UVC treated cells with the same cancer cells γ-irradiated for 260 minutes (13000 cGy) or treated with Gemcitabine for 72 hours (final concentration: 1μM) (**Figure 17A**). In all pancreatic cancer cells, the treatment able to induce the highest fraction of necrotic cells was the hyperthermic one ($P < 0.01$ or $P < 0.001$ versus not-treated cells; $P < 0.05$ or $P < 0.01$ versus irradiated cells and cells treated with Gemcitabine for all cell lines) even if also the percentage of necrotic cells after exposure to UVC was significantly increased compared to untreated or irradiated cells or Gemcitabine-treated cells ($P < 0.05$ or $P < 0.01$for all cell lines), and it was comparable to 56°C incubation.

PaCa-44, PT-45, PANC-1 and PANC-2 cancer cells were resistant to γ-irradiation with no differences between 4 and 20 hours of incubation (**Figure 17B**).

Treatment with Gemcitabine (1μM) for 72 hours evidenced differences between cancer cell lines. PaCa-44 and PT-45 were more sensitive to this chemotherapeutic agent than PANC-1 and PANC-2: the fraction of treated necrotic cells significantly increased compared to untreated cells ($P < 0.05$ for PaCa-44; $P < 0.01$ for PT-45), although this fraction was significantly lower than that that found after hyperthermic treatment . For PaCa-44 and PT-45, Gemcitabine was also responsible of an increase of the fraction of early apoptotic $AnnV^+/PI^-$ cells, as reported in **Figure 17A**. On the other hand, PANC-1 and PANC-2 were resistant to the chemotherapeutic agent and the fraction of necrotic cells was comparable to untreated cells (**Figure 17B**).

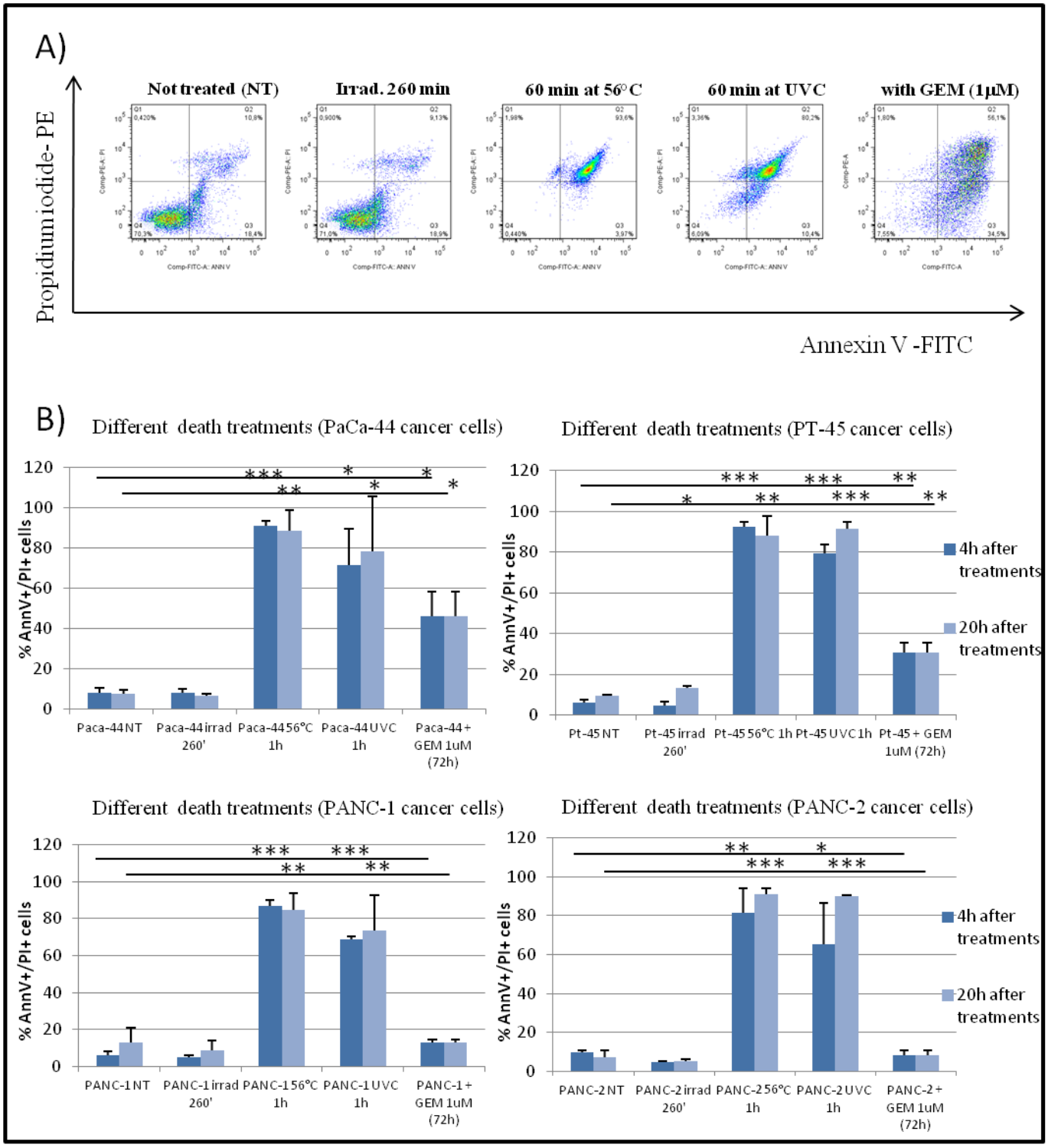


Figure 17: Treatments of PaCa-44, PT-45, PANC-1 and PANC-2 tumor cells by γ-irradiation source for 260 minutes (13000 cGy), at 56°C for 1 hour, by UVC for 1 hour and with Gemcitabine (final concentration: 1µM). **A)** Cytofluorimetric analysis of PaCa-44 cells treated with the different conditions reported above and incubated 4 hours at 37°C after the treatments (images were analysed by FlowJo application). The data shown are from one representative experiment out of three with similar results. **B)** Histograms with statistical analysis of all pancreatic cancer cell lines treated with the different conditions reported above and incubated 4 or 20 hours at 37°C before immunofluorescence staining with AnnV/PI

kit. Each histogram indicates the mean value of three different experiments with standard deviation of the results. The differences between necrotic cells after different treatments and untreated cells are statistically significant (*= $P < 0.05$; **=$P < 0.01$; ***=$P < 0.001$).

4.5 Hyperthermia at 56°C and UVC exposure treatments induced release of DAMP molecules (HMGB-1 and HSP-70) in PaCa-44, PT-45, PANC-1 and PANC-2 pancreatic cancer cells *in vitro*

We analyzed the release of DAMPs in the supernatants of treated and untreated PaCa-44, PT-45, PANC-1 and PANC-2 cells, after 4 or 20 hours of incubation at 37°C.

The levels of HMGB-1 and HSP-70, not present or present at poor levels in the supernatants of untreated cells, were especially increased after UVC exposure. The treatment at 56°C increased the release of HMGB-1, but not of HSP-70, as detected by ELISA specific assays (**Figure 18** and **Figure 19**).

As shown in **Figure 18,** there was a statistically significant HMGB-1 release after exposure of the tumor cells to UVC (about 100-200 ng/ml; $P < 0.01$ for PaCa-44 and PT-45 cells compared to untreated cells; about 100-150 ng/ml; $P < 0.05$ for PANC-1 and PANC-2 cell lines compared to untreated cells). Hyperthermia at 56°C induced an increase of HMGB-1 release, although this was not statistically significant.

When cells were treated at 56°C, there was no difference if they were incubated for 4 or 20 hours at 37°C; whereas, after UVC exposure, HMGB-1 release was increased after 20 hours of incubation.

The levels of HMGB-1 in the supernatants of γ-irradiated cells or cells treated with Gemcitabine were comparable to untreated cells. The highest amounts of HMGB-1 molecule were detected in cell lysates (about 270 ng/ml; $P < 0.01$ for PaCa-44, PT-45, PANC-1 and PANC-2 compared to untreated cells).

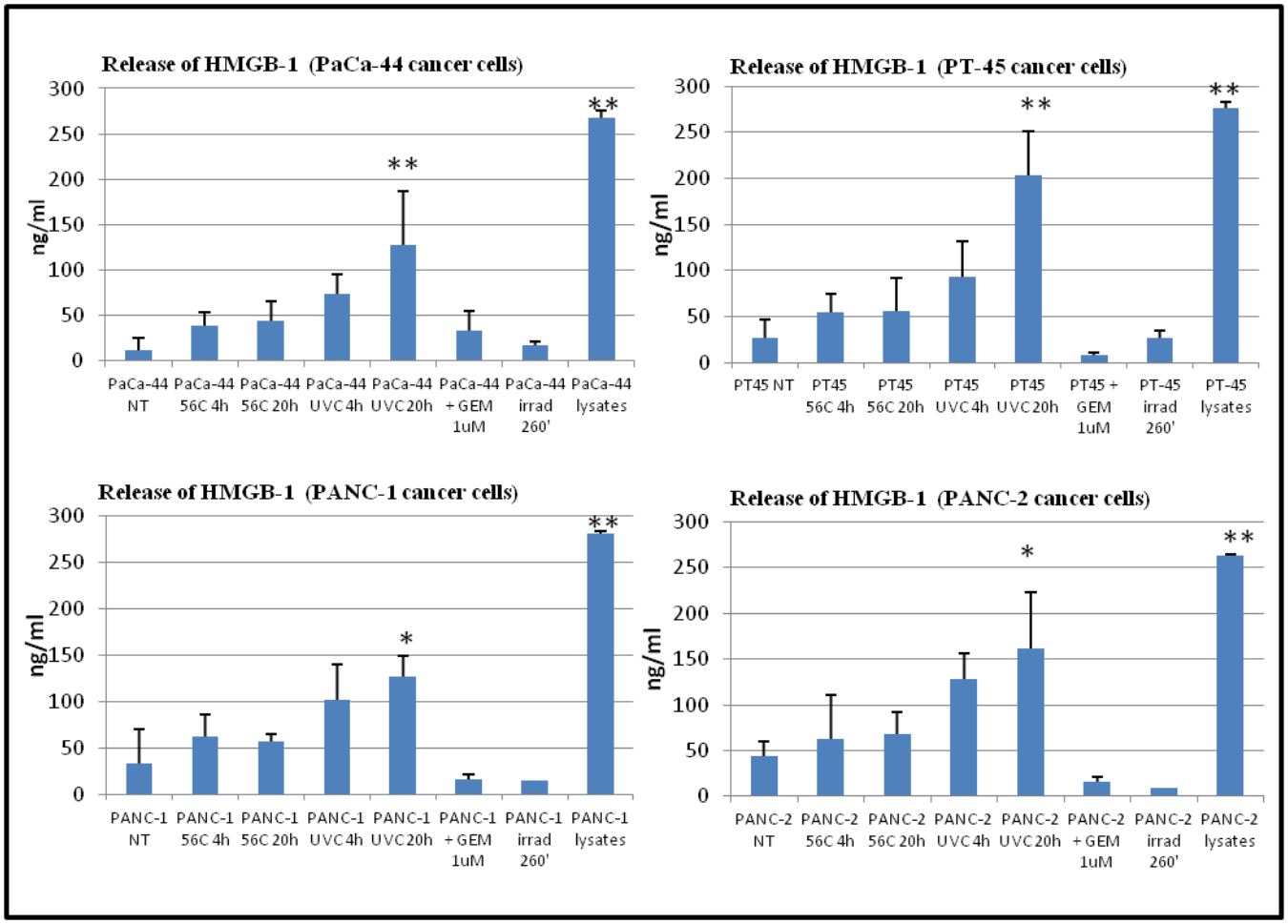


Figure 18: ELISA assay for the quantification of HMGB-1 protein in the supernatants or lysates of PaCa-44, PT-45, PANC-1 and PANC-2 cells. We evaluated untreated cells (NT) and cells treated at 56°C for 1 hour, with UVC for 1 hour, after γ-irradiation at 13000 cGy or treatment with Gemcitabine (1μM) for 72 hours. For 56°C and UVC treatments, we analyzed supernatants of cells incubated at 37°C for 4 or 20 hours after treatments. Cell lysates were used as positive controls. Each histogram indicates the mean value of three different experiments with standard deviation of the results. The statistically significant differences in HMGB-1 release between treated and untreated cells are indicated at the top of the histograms (*= $P < 0.05$; **=$P < 0.01$; ***=$P < 0.001$).

Figure 19 shows that HSP-70 was released in the supernatant only after UVC exposure (about 75-90 ng/ml; $P < 0.01$ for PaCa-44, PT-45, PANC-1 and PANC-2 cells compared to untreated cells; $P < 0.05$ for PANC-1, 4h after treatment compared to untreated cells), and after 20 hours of incubation at 37°C .

On the contrary, incubation at 56°C did not induce HSP-70 release after either 4 or 20 hours of incubation, in all four cell lines.

The levels of HSP-70 in supernatants of γ-irradiated cells or cells treated with Gemcitabine were comparable to untreated cells. Cell lysates released a statistically significant quantity of HSP-70 molecule for all cell lines (about 80-100 ng/ml; $P < 0.01$ for PaCa-44, PT-45, PANC-1 and PANC-2 compared to untreated cells).

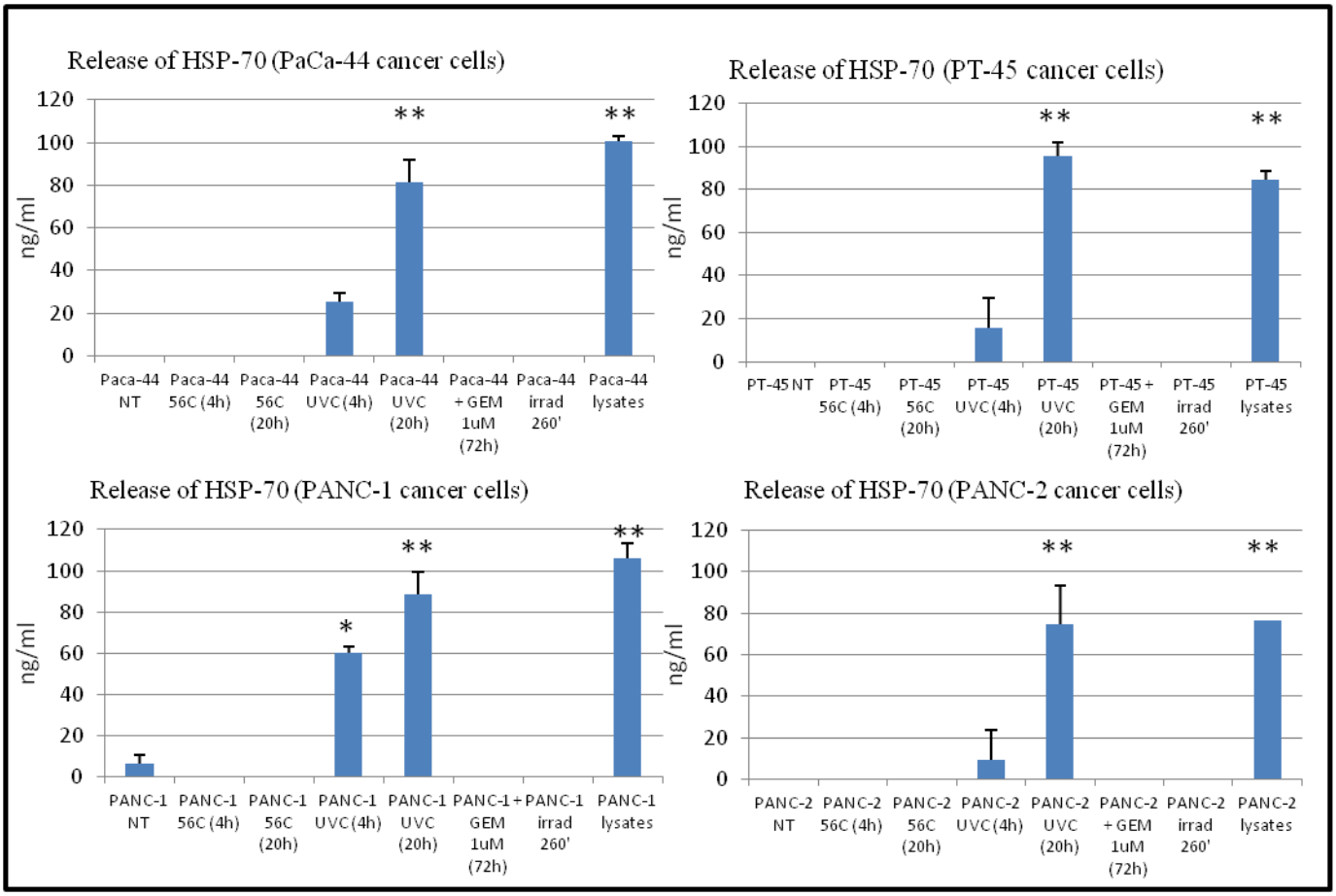


Figure 19: ELISA assay for the quantification of HSP-70 protein in the supernatants or lysates of PaCa-44, PT-45, PANC-1 and PANC-2 cells. We compared untreated cells (NT) to cells treated at 56°C for 1 hour, with UVC for 1 hour, γ-irradiation at 13000 cGy or with Gemcitabine (1μM) for 72 hours. For 56°C and UVC treatments, we analyzed supernatants of cells incubated at 37°C for 4 or 20 hours after treatments. Cell lysates were used as positive controls. Each histogram indicates the mean value of three different experiments with standard deviation of the results. The statistically significant differences in HSP-70 release between treated and untreated cells are indicated at the top of the histograms (*= $P < 0.05$; **=$P < 0.01$; ***=$P < 0.001$).

4.6 Hyperthermia reduced the release of TGF-β in PaCa-44, PT-45, PANC-1 and PANC-2 pancreatic cancer cells *in vitro*

Similarly to many cancer cells, pancreatic cancer cells secreted the immunosuppressive cytokine TGF-β (**Figure 20**): the ELISA assay detected 200-350 pg/ml of TGF-β in the colture medium from PaCa-44 and PANC-2 48 hrs cultures, and about 500-600 pg/ml in the colture medium from PT-45 and PANC-1 48 hrs cultures. TGF-β secretion appeared inhibited after γ-irradiation at 13000 cGy or incubation at 56°C or UVC exposure for 1 hour, but not after treatment with Gemcitabine. Cells treated with Gemcitabine released levels of TGF-β comparable to those of untreated cells. These results were statistically significant for PaCa-44 ($P < 0.05$ compared to untreated cells) and PANC-1 ($P < 0.05$ compared to untreated cells) but not for PT-45 and PANC-2: PT45 secretion showed high variability among experiments, and PANC-2 did not release relevant levels of TGF-β even when untreated, compared to the other cell lines.

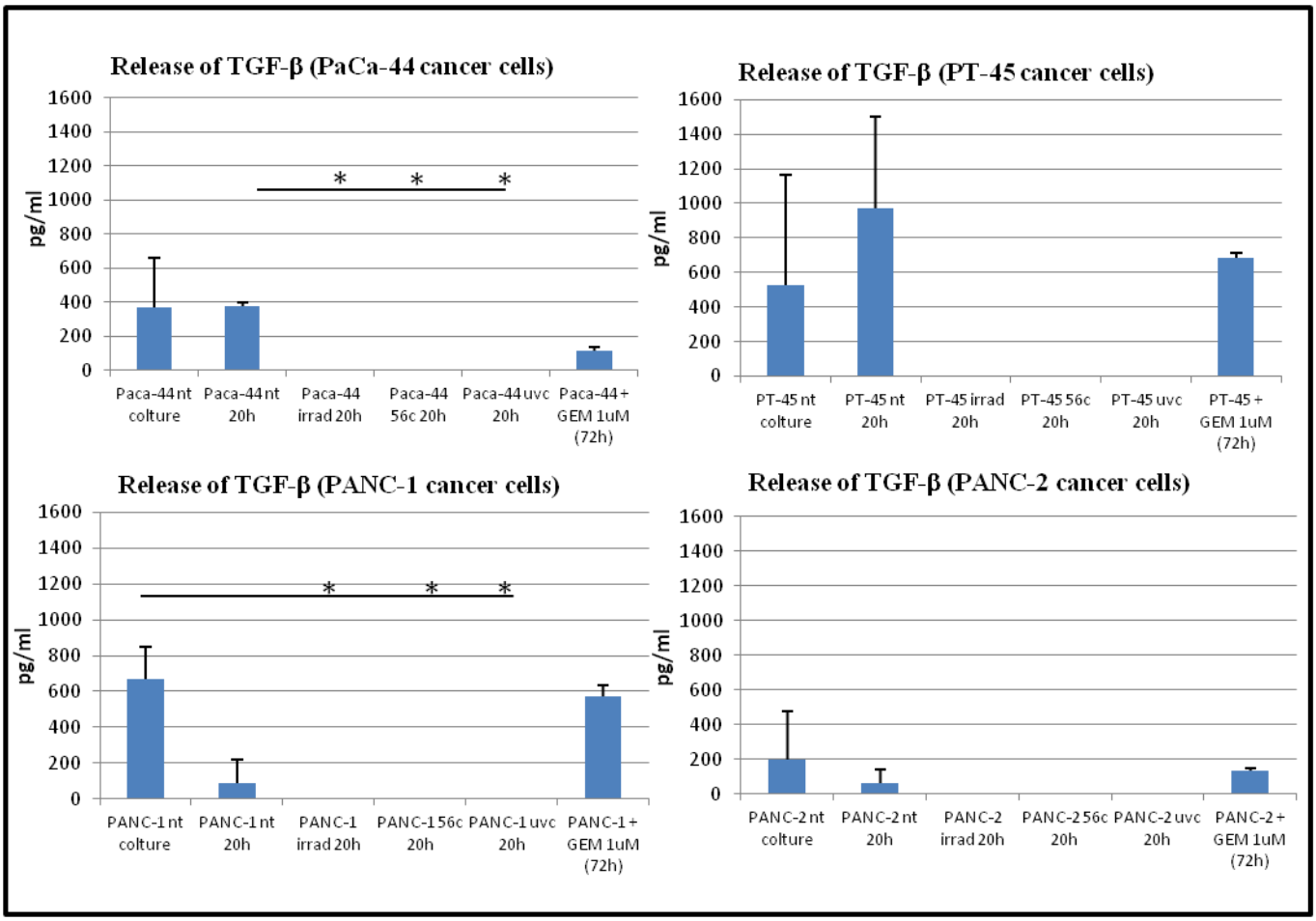


Figure 20: ELISA assay for the quantification of TGF-β protein in the supernatants of PaCa-44, PT-45, PANC-1 and PANC-2 cells. We evaluated untreated cells (NT) and cells treated at 56°C for 1 hour, with UVC for 1 hour, after γ-irradiation at 13000 cGy or treated with Gemcitabine (1μM) for 72 hours. For 56°C and UVC treatments, we analyzed the supernatants of cells incubated at 37°C for 4 or 20 hours after treatments. Each histogram indicates the mean value of two different experiments with standard deviation of the results. The statistically significant differences in TGF-β release between treated and untreated cells are indicated at the top of the histograms (*= $P < 0.05$; **=$P < 0.01$; ***=$P < 0.001$).

4.7 Hyperthermia-treated PaCa-44, PT-45, PANC-1 and PANC-2 tumor cells improved maturation of DCs compared to untreated cells

During their progression from immature to mature state, DCs up-modulate CD80 (B7-1) and MHC-II molecules and express CD83, the DCs-specific maturation marker. iDCs were incubated with PaCa-44, PT-45, PANC-1 and PANC-2 tumor cells, untreated or treated with hyperthermia, UVC or Gemcitabine as described above (see 3.4 and 3.7).

After 24 hours, they received the maturation cytokine cocktail TNF-α, IL-1β, IL-6 and PGE_2. The effect of incubating the tumor with iDCs was studied before the addition of cytokines (**Figure 21**) and after the addition of cytokines, 48 hours later (72 hours after the beginning of DCs-tumor mixed culture) (**Figure 22**).

Twenty four hours following the beginning of the experiments and before addition of maturative cytokines, DCs were stimulated with untreated and treated tumor cells.

As shown in **Figure 21**, before the addition of maturation cytokines, DCs up-modulated CD80 and CD83 molecules expression when they were stimulated with untreated and treated tumor cells, compared to iDCs .

Noteworthy, the DCs phenotype was strongly affected after 48 hours of co-culture in presence of maturation cytokines with untreated cancer cells, as indicated by the reduction of CD80, CD83 and MHC-II molecules; whereas hyperthermia-treated cells and UVC treatment were able to increase DCs maturation, although not at the same levels of mDCs ($P < 0.05$ compared to mDCs). Gemcitabine treatment affected DCs maturation 48 hours after the addition of maturation cytokines (**Figure 22**).

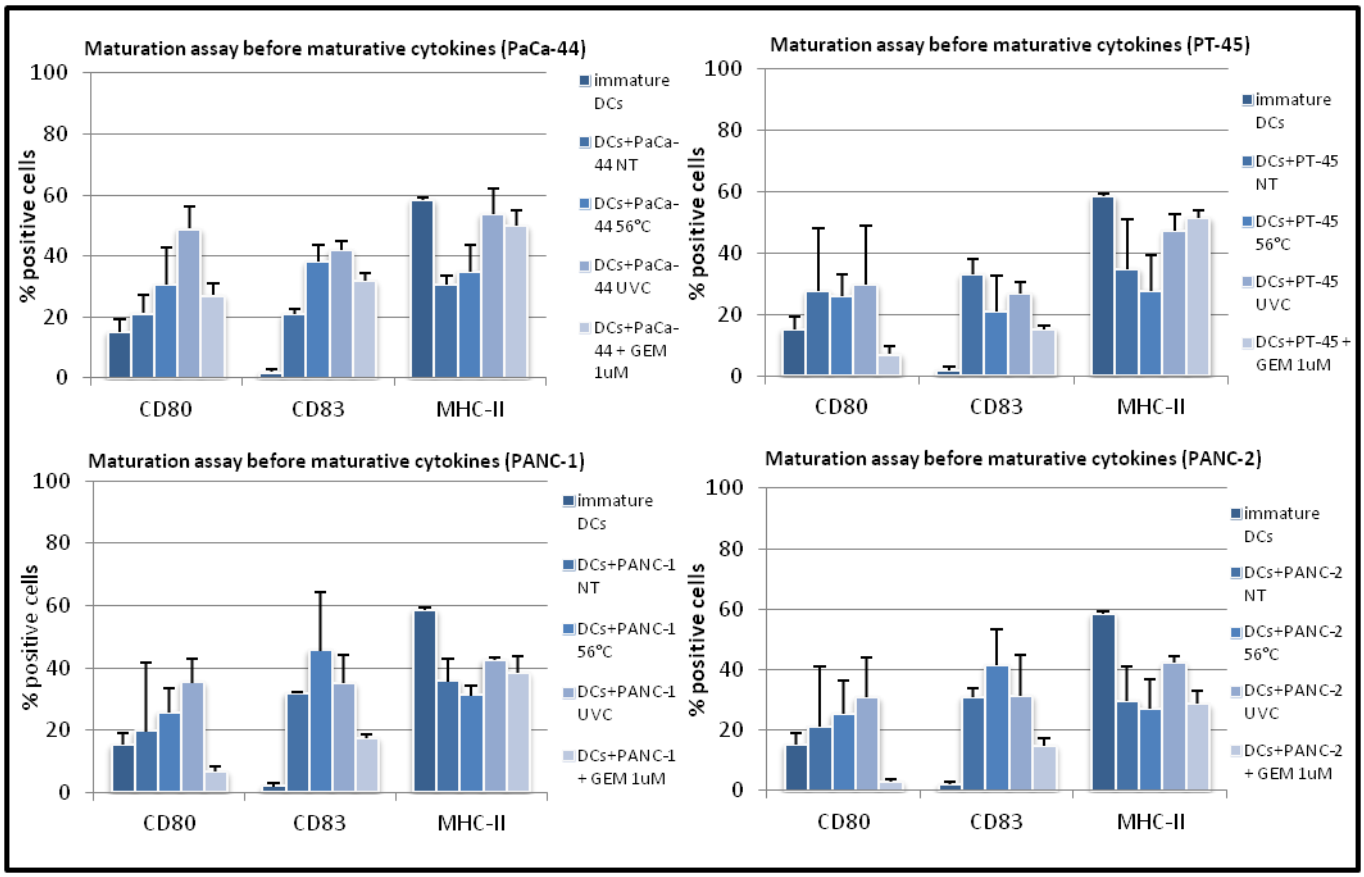


Figure 21: Hystograms with mean +/- SD percentages of CD80, CD83 and MHC-II molecules of iDCs co-cultured with PaCa-44, PT-45, PANC-1 and PANC-2 untreated cells (NT) and of the same cells

treated at 56°C for 1 hour, with UVC for 1 hour or with Gemcitabine (1µM) 24 hours after the beginning of co-cultures and before addition of maturative cytokines TNF-α, IL-1β, IL-6 and PGE_2. iDCs alone were used as control.

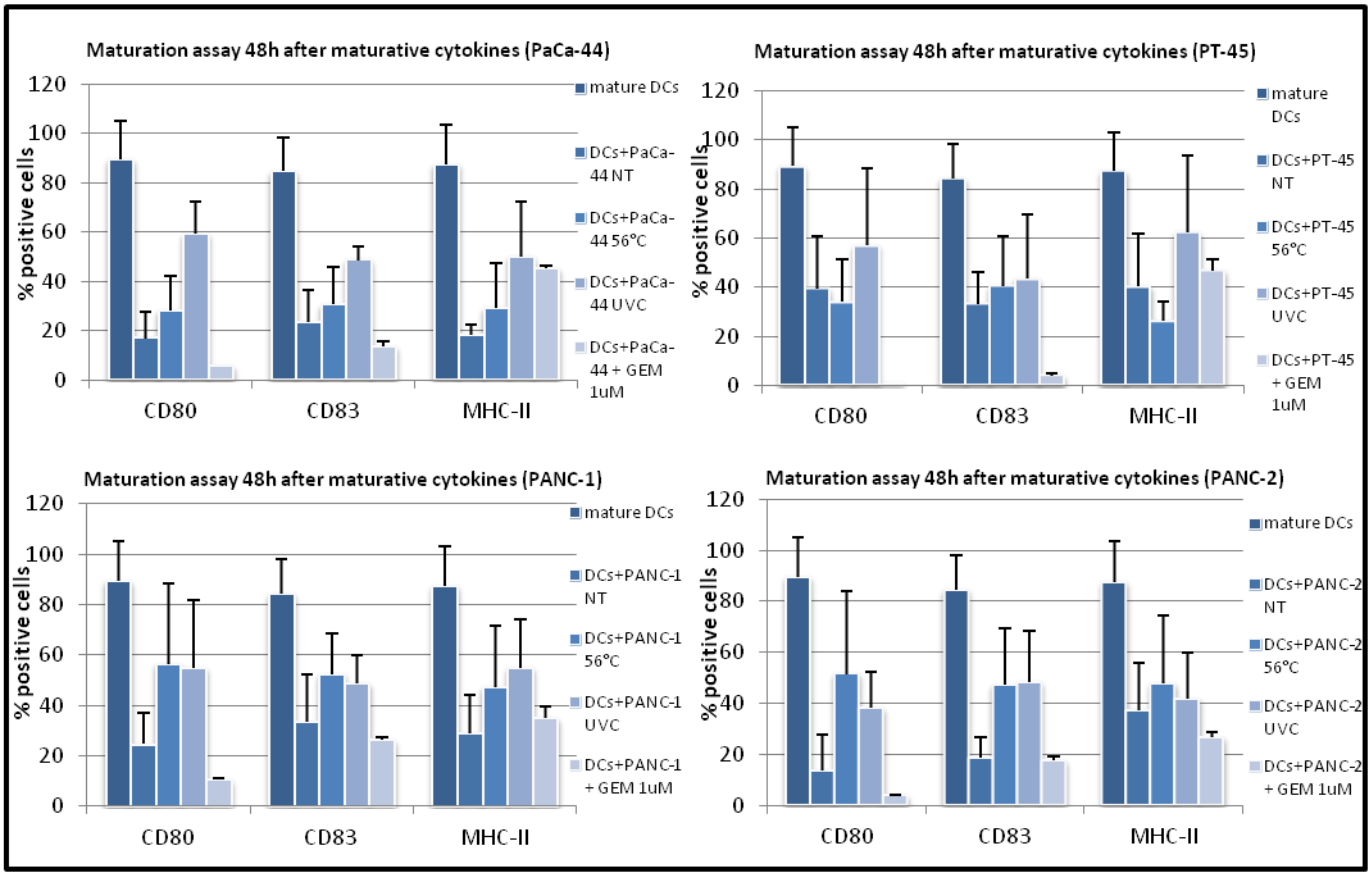


Figure 22: Hystograms with mean +/- SD percentages of CD80, CD83 and MHC-II molecules of DCs co-cultured with PaCa-44, PT-45, PANC-1 and PANC-2 untreated cells (NT) and of the same cells treated at 56°C for 1 hour, with UVC for 1 hour or with Gemcitabine (1µM) 48 hours after addition of maturative cytokines TNF-α, IL-1β, IL-6 and PGE_2. mDCs alone were used as control.

4.8 Hyperthermic treatments increased tumor phagocytosis by iDCs

PaCa-44, PT-45, PANC-1 and PANC-2 cells treated at 56°C and with UVC were then tested for their capability to induce phagocytosis by iDCs. The phagocytosis assay was performed as described in 3.8: before the treatments, pancreatic cancer cells were stained with PKH26-PE, that recognize cell membrane, and, after 24 hours of co-incubation with iDCs, the cells were stained with anti-CD80-APC, specific for DCs (**Figure 23**). DCs stained with CD1a-FITC were also analyzed by fluorescence

microscopy (**Figure 24**). As can be appreciated in **Figure 23** and **24**, 56°C and UVC treatments resulted in an increased percentage of phagocytosed PaCa-44, PT-45, PANC-1 and PANC-2 cells compared to untreated cells, even if no statistically significant effect was induced on tumor uptake by any treatments. In **Figure 23**, phagocytosis by iDCs is indicated in the upper-right quarter of each panel, as double-labelled cells on total $CD80^+$ cells.

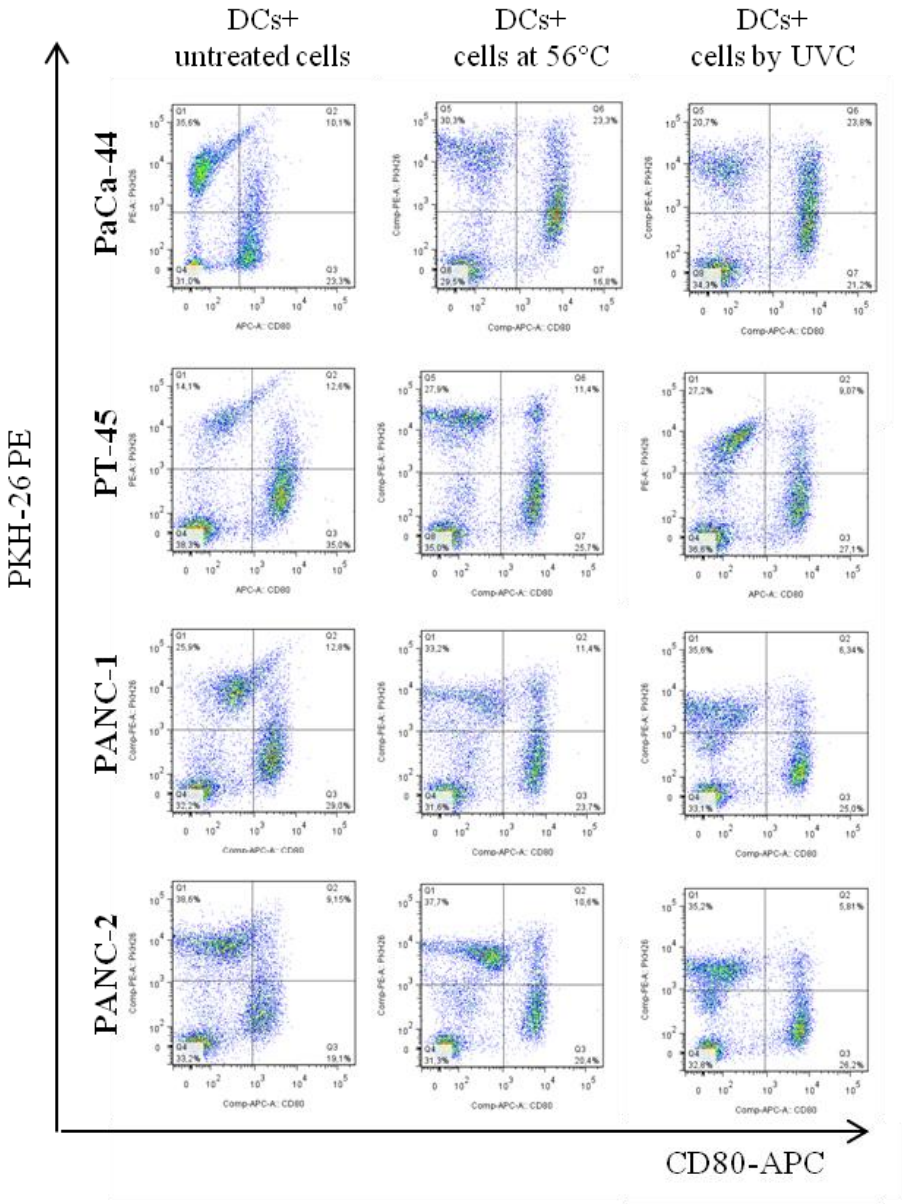


Figure 23: Uptake of dying tumor cells by iDCs. Cytofluorimetric analysis of the phagocytosis experiments performed with DCs, labelled with CD80-APC, and PaCa-44, PT-45, PANC-1 and PANC-2 cells, labelled with PKH26-PE and treated at 56°C or with UVC. The data shown are from one representative experiment out of three with similar results. Images were analyzed by FlowJo application.

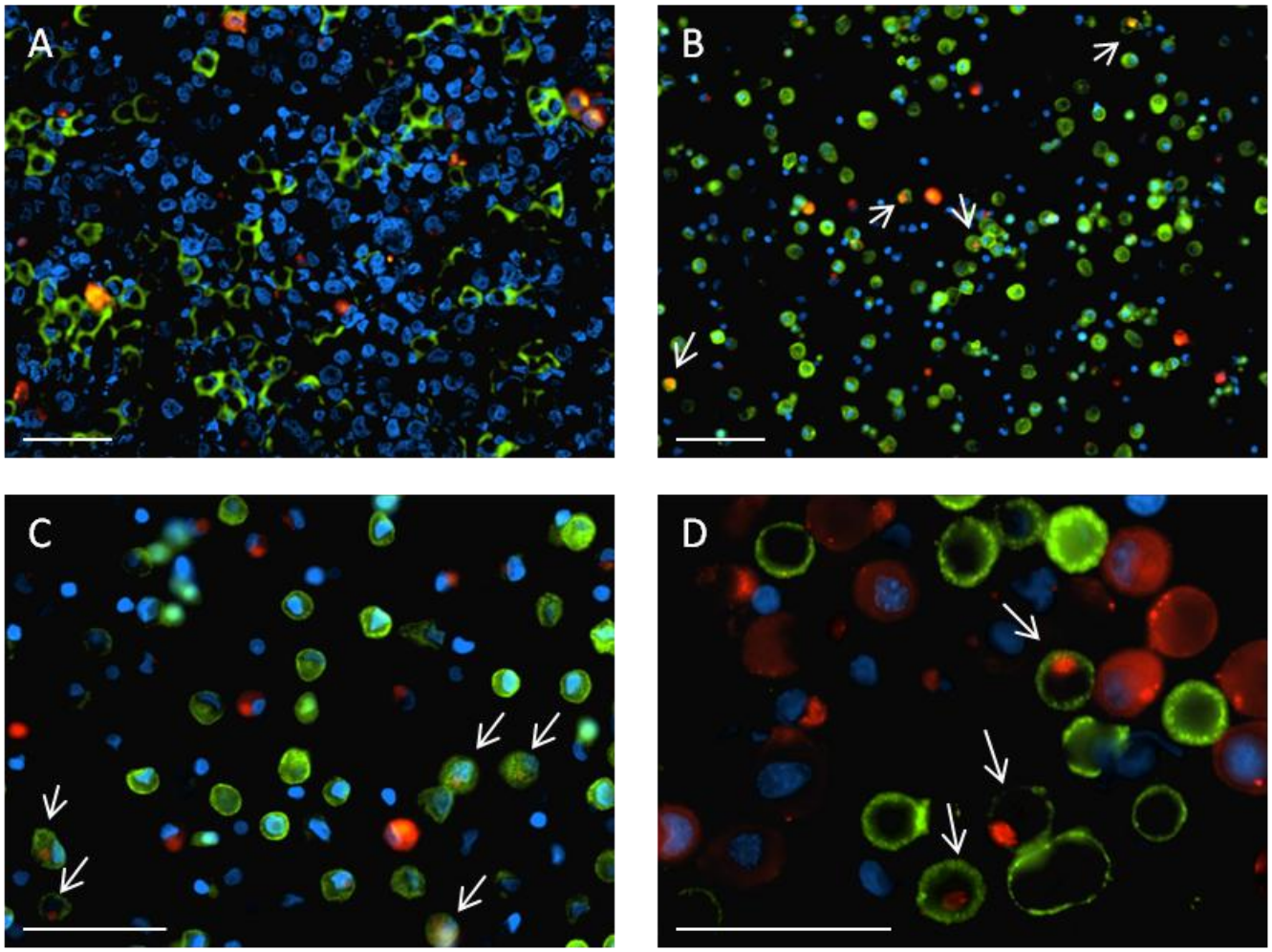


Figure 24: Uptake of dying tumor cells by iDCs. Fluorescence microscopy on co-cultures of DCs, labelled with CD1a-FITC, and PaCa-44, labelled with PKH26-PE, not-treated (A; 200× magnification) or treated by UVC (B, 200× magnification; C, 400× magnification; D, 600× magnification). Nuclear staining was obtained by DAPI (4',6-diamidino-2-phenylindole). Data obtained with cells treated by UVC were confirmed also for 56°C and all cell lines used. Bars represent 50 μm. The data shown are from one representative experiment out of two with similar results.

4.9 Generation of CTLs was increased by loading of iDCs with hyperthermic treated tumor cells

Four hours after hyperthermic treatments, PaCa-44, PT-45, PANC-1 and PANC-2 cells were incubated with iDCs. Twenty hours later, iDCs were exposed to the maturation cytokine cocktail to optimize their antigen-presenting activity and after 48 hours they were used as stimulators for autologous lymphocytes. Stimulation was repeated twice. Cytofluorimetric analysis of co-cultures showed an increased percentage of $CD8^+$ and $CD4^+$ T cells when lymphocytes were co-cultured with DCs loaded with necrotic cells (obtained by both 56°C incubation or UVC treatment), while DCs loaded with untreated cells did not stimulate efficiently lymphocytes and the number of $CD8^+$ and $CD4^+$ T cells decreased rapidly after repeated stimulations (**Figure 25**).

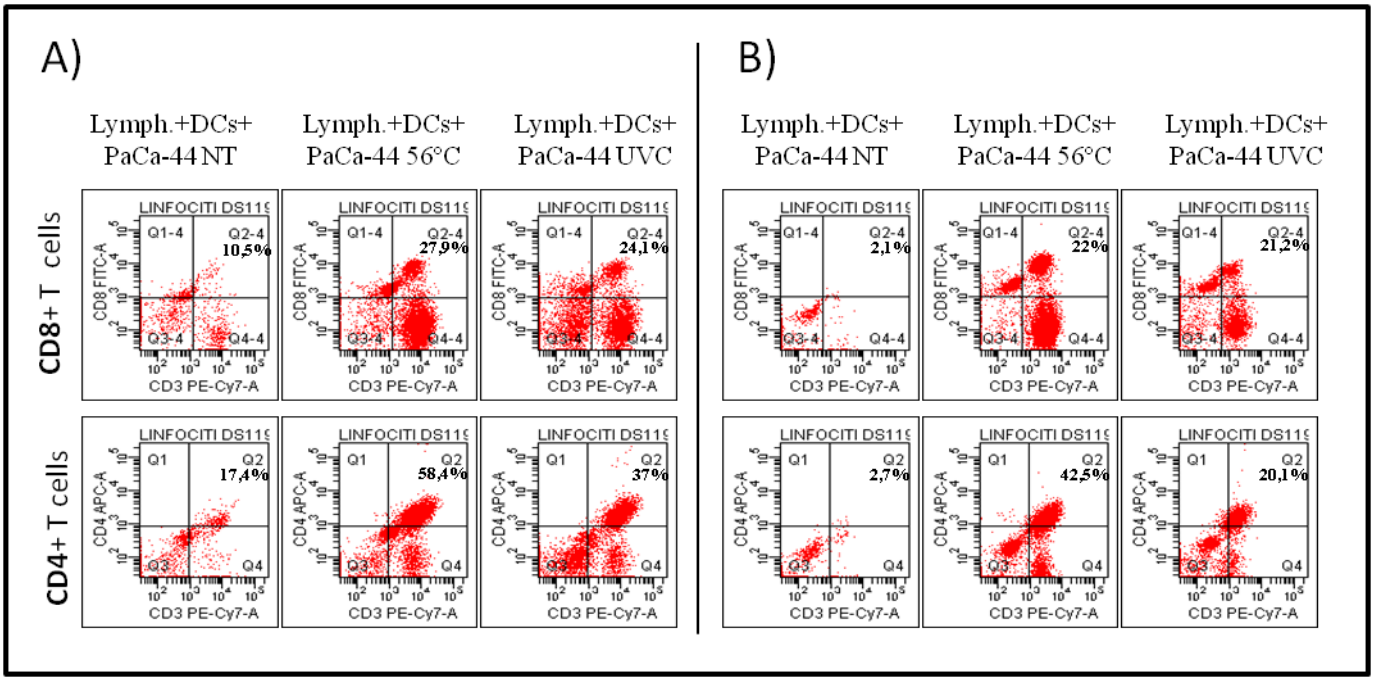


Figure 25: Cytofluorimetric analysis of the co-cultures of lymphocytes with DCs loaded with PaCa-44 not-treated (NT) or treated at 56°C or by UVC, six days after the first stimulation (A) or six days after the second stimulation (B). Similar results were obtained for PT-45, PANC-1 and PANC-2 cancer cells. The results shown are from one representative experiment out of four with similar outcome.

4.10 Lymphocytes stimulated by DCs loaded with hyperthermic-treated tumor cells produced a specific anti-tumor response

Five days after the last stimulation, the induction of a specific anti-tumor response was assessed by IFN-γ ELISA assays on supernatants of co-cultures (**Figure 26**) and by standard cytotoxicity assays with stimulated lymphocytes against untreated cancer cells as targets (**Figure 27**).

Human IFN-γ ELISA assays showed an increase of IFN-γ release when lymphocytes were stimulated with DCs loaded with treated cancer cells, in particular when DCs were loaded with UVC-treated necrotic cells ($P < 0.01$ for PaCa-44 and PT-45 *versus* not-stimulated autologous lymphocytes). In the case of treatment at 56°C, only lymphocytes stimulated with DCs loaded with PT-45 released a statistically significant level of IFN-γ *versus* not-stimulated autologous lymphocytes ($P < 0.01$). Interestingly, also untreated PT-45 cells seemed to stimulate an effective immune response even if it was not statistically significant. Lymphocytes stimulated with UVC or 56°C-treated PANC-1 and PANC-2 cancer cells showed an increase of IFN-γ release compared to lymphocytes alone or lymphocytes stimulated with DCs plus not-treated cancer cells, although it was not statistically significant. Nevertheless, the variability of the response was donor-dependent (**Figure 26**).

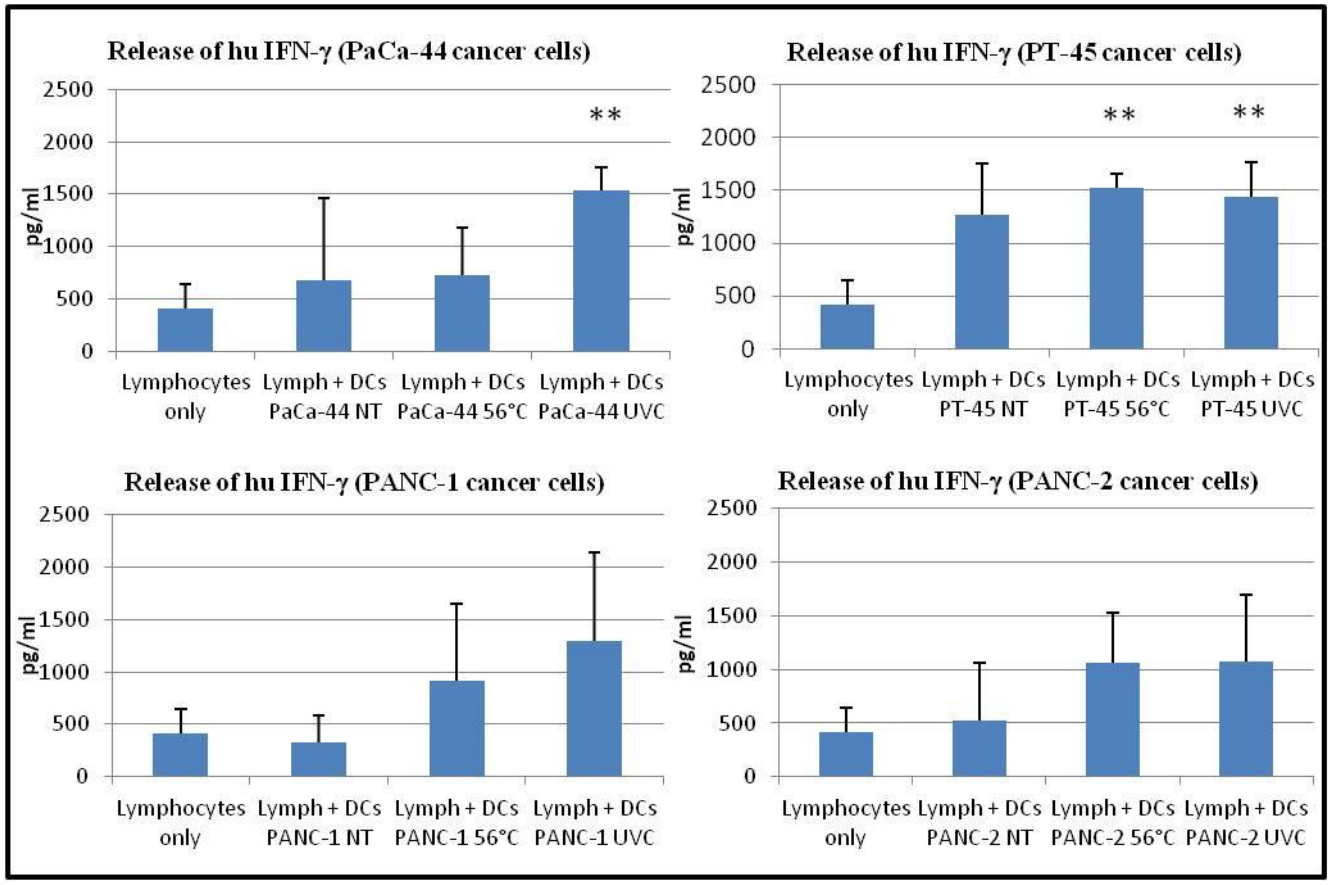


Figure 26: Human IFN-γ levels in the supernatants of cross-priming co-cultures for PaCa-44, PT-45, PANC-1 and PANC-2 cells, 5 days after the last stimulation. Each histogram indicates the mean value with standard deviation of the results relative to three different experiments. The statistically significant differences in IFN-γ release between treated and untreated cells are indicated at the top of the histograms (**=$P < 0.01$).

Cytotoxicity assays revealed the induction of low levels of cytotoxic activity against PaCa-44 and PT-45 cancer cell lines, when lymphocytes were stimulated with DCs loaded with UVC-treated cells, but not against PANC-1 and PANC-2 cell lines. Lymphocytes stimulated with DCs loaded with cells treated at 56°C did not show appreciable target-specific cytotoxic activity (**Figure 27**).

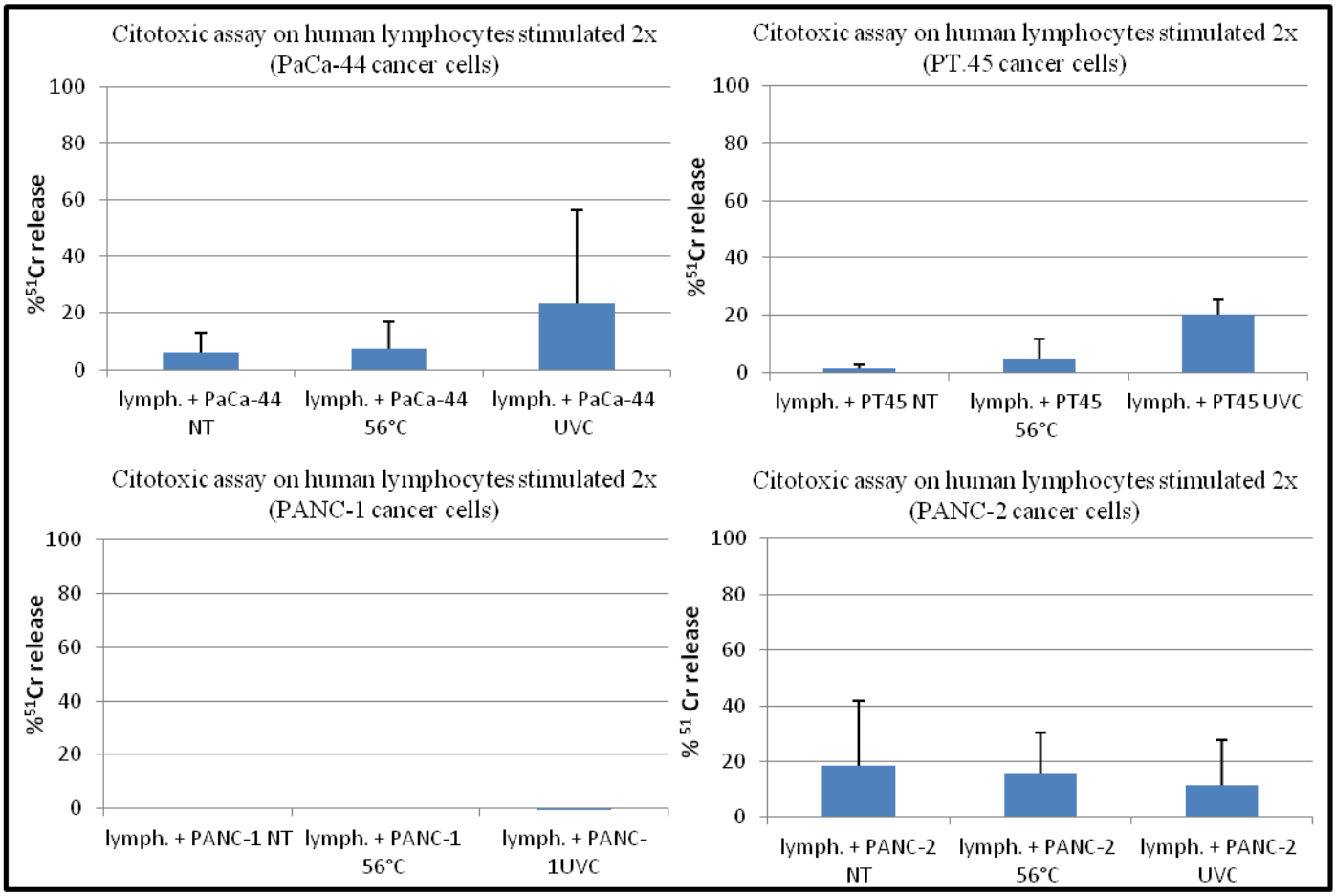


Figure 27: Cytotoxic response against PaCa-44, PT-45, PANC-1 and PANC-2 cancer cells of lymphocytes stimulated twice with DCs loaded with not treated or treated tumor cells at 56°C or with UVC. The percentage of ^{51}Cr release is on the ordinate. All experiments were performed with a 50:1 effector:target ratio. Each histogram indicates the mean value with standard deviation of the results relative to two different experiments.

4.11 Adoptive transfer of human T-lymphocytes stimulated *in vitro* with DCs loaded with UVC-treated PaCa-44 and PT-45 tumor cells did not inhibit tumor growth, but increased overall survival

The results of the *in vitro* cytotoxicity assays described above indicated that PaCa-44 and PT-45 cells might be better targets for CTLs than PANC-1 and PANC-2. Therefore they were chosen to test the *in vivo* efficacy of a protocol of curative immunotherapy. $Rag2^{-/-}$ γ-$chain^{-/-}$ immunodeficient mice (n=4/ group) were injected s.c. with PaCa-44 or PT-45 tumor cells ($2.5x10^6$ cells/ mouse), and, when s.c. tumor was palpable, they were adoptively transferred with T-lymphocytes ($5x10^6$cells/mouse) stimulated twice *in vitro*

by DCs from same donor, loaded with necrotic tumor cells. When tumor diameter was 1-1,5 cm, mice were sacrified and peripheral blood, tumor and organs were collected for FACS analysis and immunohistochemistry.
All mice developed palpable tumors during the experiment, that did not decrease after immunotherapeutic treatments.

4.11.1 PaCa-44

Overall survival (OS) for animals inoculated with PaCa-44 was between 12-22 days after tumor inoculation (median OS: day +17) in control animals (n=4) and it was comparable to OS of animals treated by ACT performed either i.v. or i.p. with T cells stimulated with DCs loaded with 56°C-treated and i.v. with UVC-treated PaCa-44 cells (median OS: day + 17, day +18 and day + 18 respectively). We observed an increase, although not statistically significant, in median OS only when mice received an i.p. injection of T-lymphocytes stimulated with DCs loaded with UVC-treated PaCa-44 cells (17-36 days; median OS: day +27) (**Figure 28**).

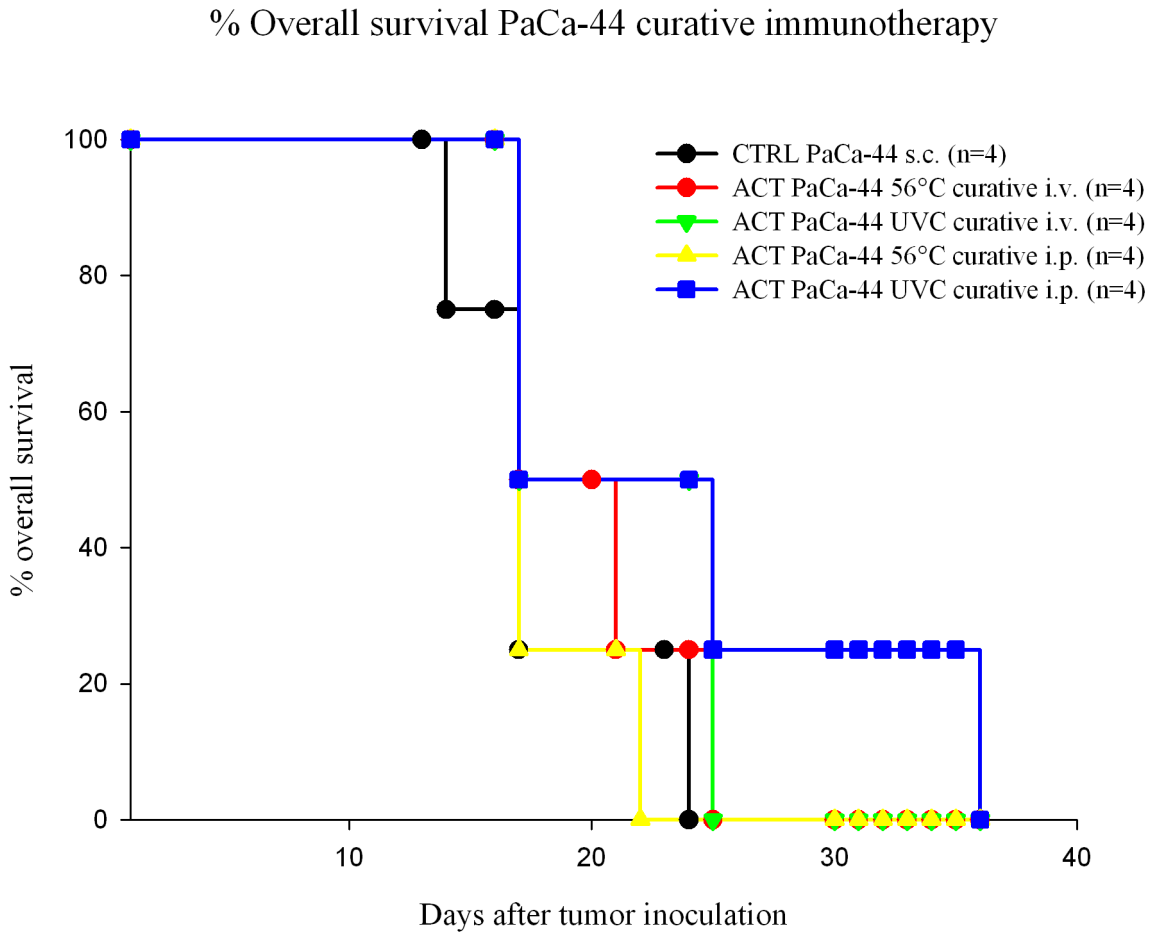


Figure 28: OS in PaCa-44 curative immunotherapy. OS of Rag2$^{-/-}$ γ-chain$^{-/-}$ mice injected s.c. with 2.5x10^{6} PaCa-44 tumor cells, followed, when tumor s.c. was palpable, by i.v. or i.p. adoptive transfer of 5x10^{6} T-lymphocytes stimulated twice *in vitro* by DCs loaded with necrotic tumor cells by 56°C incubation or UVC exposure, as indicated.

We observed a trend towards slower tumor growth in the groups of mice that received immunotherapeutic approach with T-lymphocytes stimulated *in vitro* with DCs loaded with 56°C or UVC-treated PaCa-44 cells with respect to control mice injected with w.t. tumor cells only. In particular, i.v. ACT with T-lymphocytes stimulated with UVC-treated PaCa-44 cells and i.p. ACT with T-lymphocytes stimulated with 56°C-treated PaCa-44 cancer cells seemed to work better in term of decrease of tumor growth rate, although these differencies were not statistically significant. PaCa-44 mean volume at day +10 was 0.48±0.17 cm^3 (n=4) for i.v. ACT with UVC-treated PaCa-44 cells, and 0.48±0.05 cm^3 (n=4) for i.p. ACT with 56°C-treated PaCa-44 cells. By comparison, mean volume at the same time point was 0.67 ±0.15 cm^3 (n=4) in the case of control

mice (PaCa-44 injected alone). All other immunotherapeutic approaches resulted in mean tumor volumes comparable with control mice (**Figure 29**)

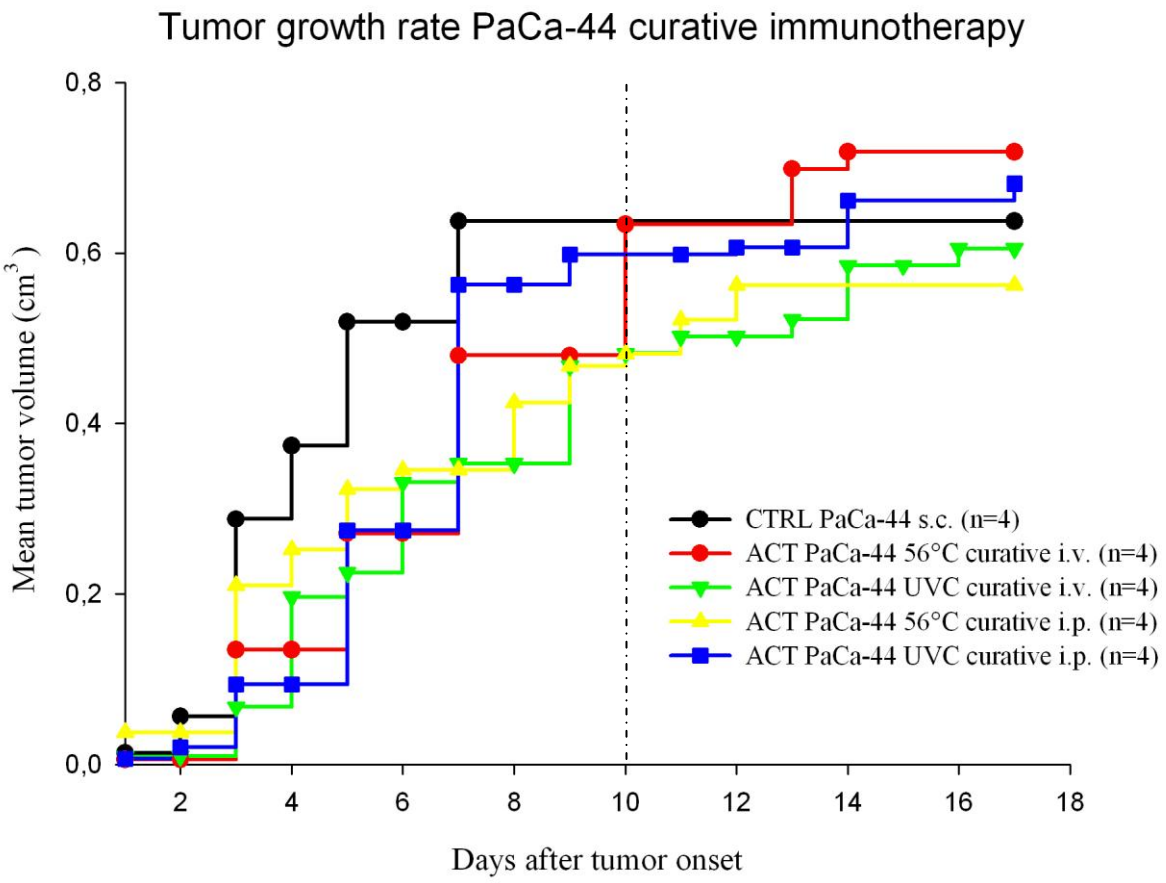


Figure 29: Tumor growth rate in PaCa-44 curative immunotherapy. Tumor growth rate after tumor onset (mean volumes) of $Rag2^{-/-}$ γ-$chain^{-/-}$ mice injected s.c. with $2.5x10^6$ PaCa-44 tumor cells, followed by i.v. or i.p. adoptive transfer of $5x10^6$ T-lymphocytes stimulated twice *in vitro* by DCs loaded with necrotic tumor cells.

Treated mice did not show any human $CD45^+$ cells detectable in peripheral blood (data not shown). Immunohistochemistry showed no human $CD45^+$ cells in their spleens, except for one mouse among those injected with lymphocytes stimulated with DCs loaded with UVC-treated PACA-44 cells (n=1/4), showing low CD45+ human cells. No human $CD45^+$ cells were found in tumor masses of all mice, either ACT-treated and ACT-untreated control mice. We also observed inflammation and tumor metastases in lungs of some ACT-untreated control and ACT-treated mice, using an anti-cytokeratin-

8 antibody, specific for epithelial cells, but it seemed to decrease after immunotherapeutic approach. (**Figure 30**).

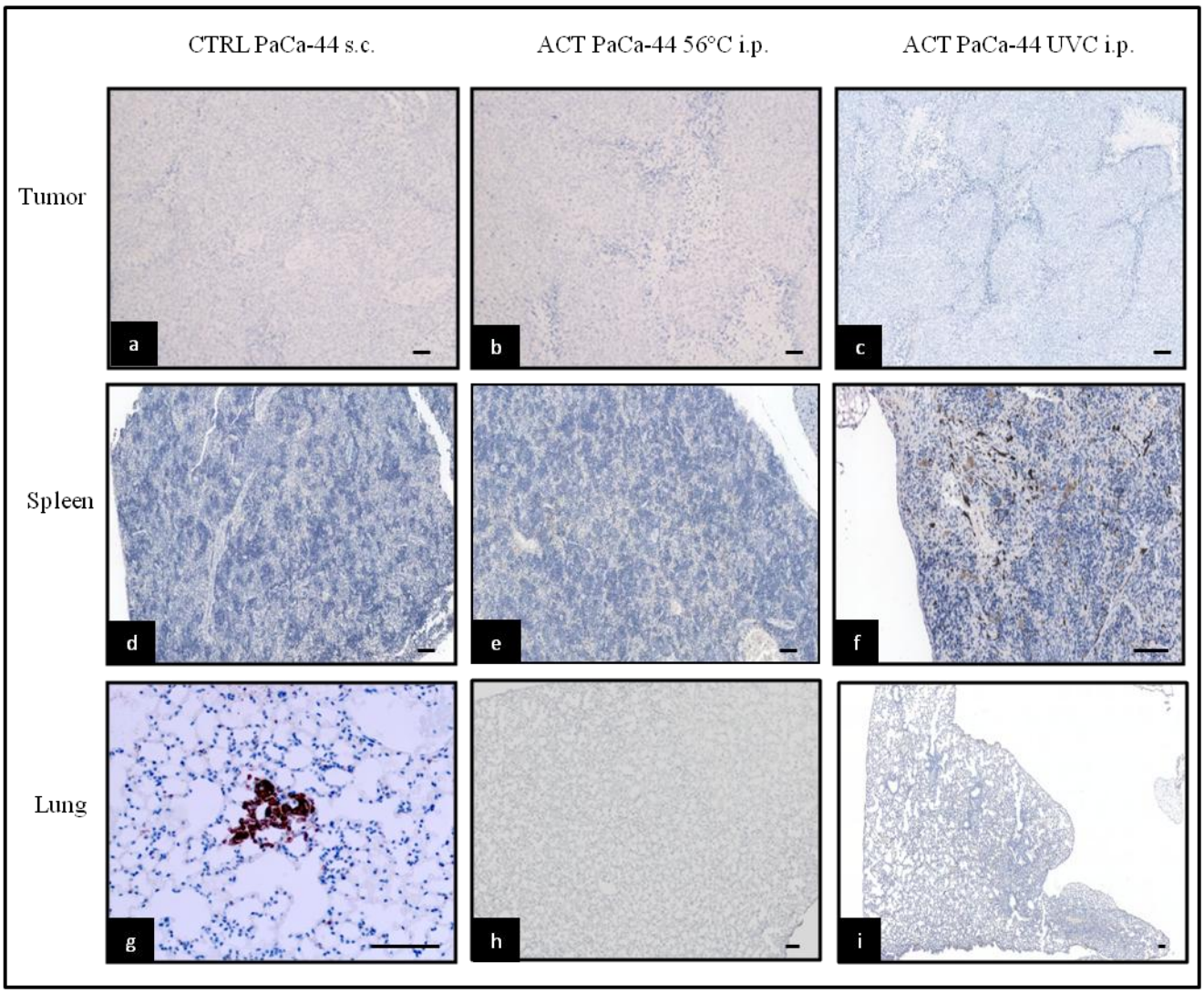


Figure 30: Immunohistochemical analysis of tumor mass (a-c) and of spleen (d-f), with human CD45 marker, and of lung (g-i) with cytokeratin-8 (CK-8) of: a control mouse injected s.c. with w.t. PaCa-44 tumor (mass: a; 100× magnification; spleen: d; 100× magnification;¸lung: g; 400× magnification), a mouse injected with w.t. tumor followed by i.p.ACT with lymphocytes stimulated twice *in vitro* by DCs loaded with 56°C-treated tumor cells (mass: b; 100× magnification; spleen: e; 100× magnification;¸lung: h; 40× magnification) and mouse injected with w.t. tumor followed by i.p. ACT with lymphocytes stimulated twice *in vitro* by DCs loaded with UVC- treated tumor cells (mass: c; 100× magnification; spleen: f; 200× magnification;¸lung: i; 20× magnification) No differencies were observed between i.p. or i.v. injected mice. Bars represent 250 µm for 100×, 200× and 400× magnifications and 500 µm for 20x and 40× magnifications. The figure shows a representative case of different treated $Rag2^{-/-}$ γ-$chain^{-/-}$ mice.

4.11.2 PT-45

OS for animals inoculated with PT-45 was between 43-78 days after tumor inoculation (median OS: day +61) in control animals (n=4). We observed an increase in median OS when mice received immunotherapeutic approaches, in particular i.v. ACT with T cells stimulated with DCs loaded with 56°C-treated PT-45 cells (43-99 days; median OS: day +71) and i.p. ACT with T-lymphocytes stimulated with UVC-treated PT-45 cells (60-98 days; median OS: day +79). This OS increase was not statistically significant. (**Figure 31**).

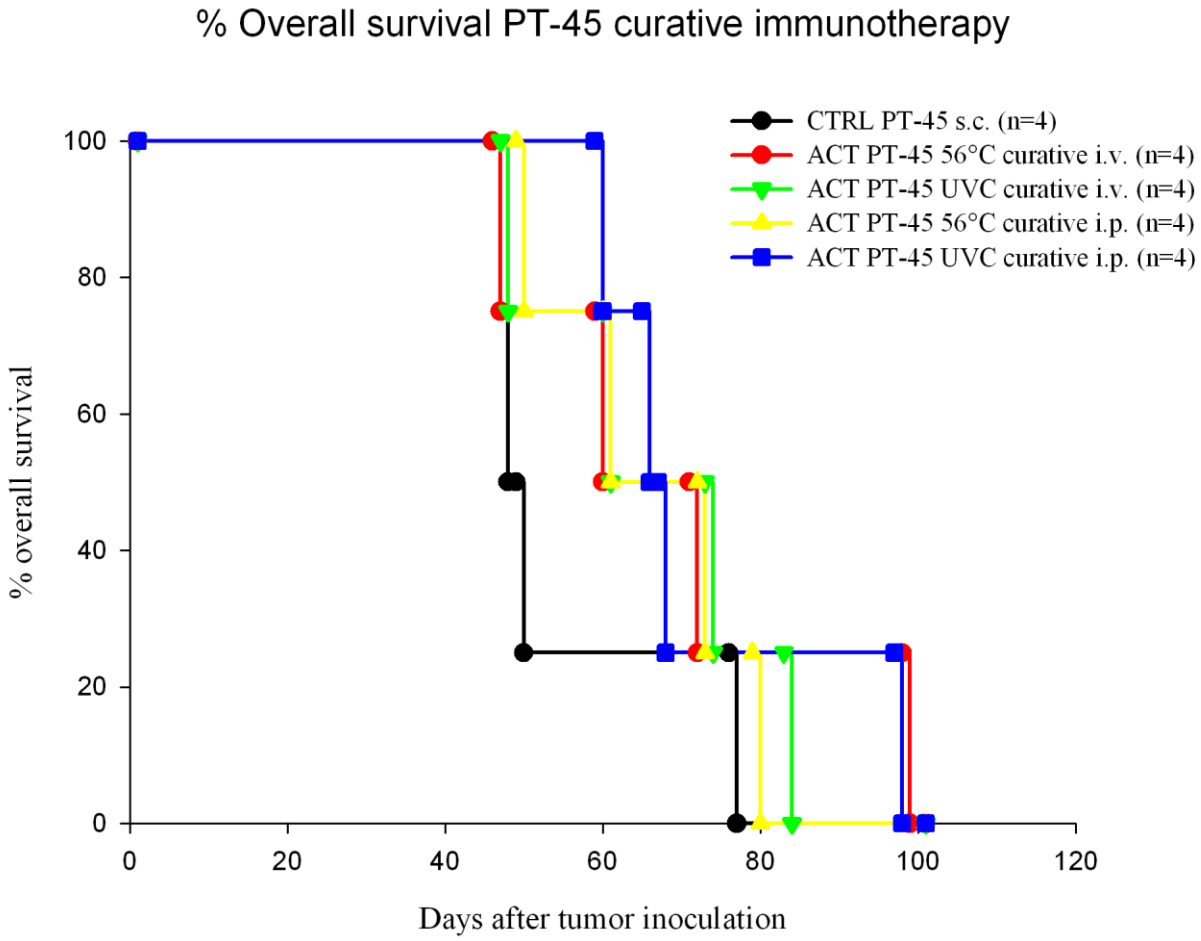


Figure 31: OS in PT-45 curative immunotherapy. OS of Rag2$^{-/-}$ γ-chain$^{-/-}$ mice injected s.c. with 2.5x10^{6} PT-45 tumor cells, followed, when tumor s.c. was palpable, by i.v. or i.p. adoptive transfer of 5x10^{6} T-lymphocytes stimulated twice *in vitro* by DCs loaded with necrotic tumor cells by 56°C incubation or UVC exposure, as indicated.

.

We observed a statistically significant decrease in tumor growth rate in the groups of mice that received ACT with T-lymphocytes stimulated *in vitro* with UVC-treated PT-45 cells (either i.v or i.p; $P < 0.01$ and $P < 0.001$ at day +20, respectively), compared to control mice injected only with w.t. tumor cells. PT-45 mean volume at day +20 was 0.22±0.2 cm^3 (n=4) for i.v. ACT with T-lymphocytes stimulated *in vitro* with UVC-treated PT-45, and 0.13±0.11 cm^3 (n=4) for i.p. ACT with T-lymphocytes stimulated *in vitro* with UVC-treated PT-45. By comparison, mean volume at the same time point was 0.59 ±0.2 cm^3 (n=4) in the case of control mice injected with PT-45 without ACT. ACT with T-lymphocytes stimulated *in vitro* with PT-45 incubated at 56°C did not reduce tumor growth respect to control mice and, when T-lymphocytes were injected i.p., tumor growth rate increased more rapidly than in control mice (**Figure 32**).

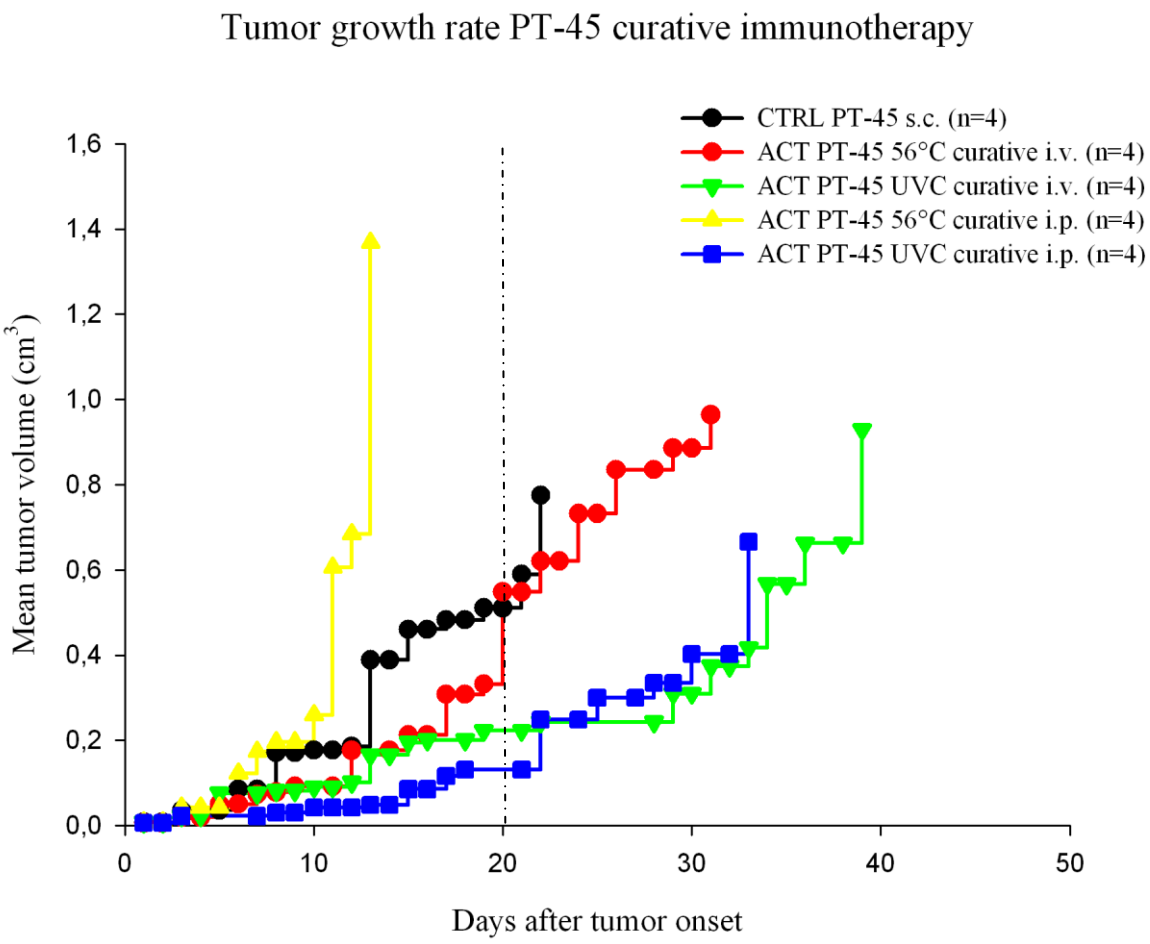


Figure 32: Tumor growth rate in PT-45-specific curative immunotherapy Tumor growth rate after tumor onset (mean volumes) of $Rag2^{-/-}$ γ-$chain^{-/-}$ mice injected s.c. with $2.5x10^6$ PT-45 tumor cells, followed by

i.v. or i.p. adoptive transfer of $5x10^6$ T-lymphocytes stimulated twice *in vitro* by DCs loaded with necrotic tumor cells.

ACT-treated mice did not show any human $CD45^+$ cells in peripheral blood, spleen and tumor masses (data not shown).

We also observed PT-45 tumor metastases and, in some cases, inflammation in lungs of control and treated mice, but they seemed to decrease after immunotherapeutic approaches (data not shown).

V. Discussion

This study is focused on the establishment of a preclinical model of immunodeficient mouse to study pancreatic cancer immunotherapy. Mouse models harbouring human cells or tissues (also called "humanized mouse models") have been very helpful to investigate underlying immunological mechanisms in human biology, immune-mediated diseases, transplantation and cross-talk between cancer and immune system. Although the "humanized mouse" is a difficult field of research, it still remains a relevant model for preclinical studies.

The original project was to develop patients-derived "humanized mouse" bearing human immune system from pancreas adenocarcinoma patients starting from bone marrow-derived CD34+ immune-precursor cells, engraft the autologous tumor and study the interactions between tumor and immune system and eventually try to develop systems to enhance tumor-specific immune responses and inhibit tumor-mediated immunosuppressive mechanisms. So far we could not obtain bone marrow neither from these patients or from healthy donors, therefore we focused this study to analyze:

1. the possibility of engraftment of human PBMCs and purified T-lymphocytes in newborn or adult immunodeficient mice and
2. the development of an immunotherapeutic approach for pancreatic cancer in immunodeficient mice bearing growing human pancreas tumors.

In our mouse facilities we developed a colony of C57BL/6-Rag2$^{-/-}$ γ chain$^{-/-}$ mice. These animals lack T and B cells, they have a compromised innate immunity and, even if a small number of NK cells remains, they do not function correctly, and according to current literature, should offer technical advantages over SCID recipients, because they appear to accept more readily human PBMCs, show improved reconstitution rates and reject more reliably human transplants (Abele-Ohl, 2010).

Newborn immunodeficient mice were γ-irradiated after birth and injected intrahepatically with PBMCs. As already described (see Results 4.1), C57BL/6-Rag2$^{-/-}$ γ chain$^{-/-}$ mice displayed high mortality at newborn age: 67% (22/33) animals died between 15-20 days after intrahepatically injection. In addition only 45% (5/11) of live treated animals showed human CD45+, CD3+/CD8+ or CD3+/CD4+ cells in peripheral

blood 4 weeks after injection. B-lymphocytes were always absent in the blood of these mice, according to literature, as well as cells of myelo-monocyte origin (Mosier et al., 1988; van Rijn et al.,2009; Abele-Ohl et al., 2010). These mice showed also the presence of T and B cells in the spleen and in the lungs (**Figure 9 and 10** of Results-section I). Interestingly, B cells were absent in peripheral blood, but they were detected in spleen.

In adult mice, γ-irradiated or not, receiving an intraperitoneal injection of PBMCs or T-lymphocytes, engrafment of human cells appeared independent from γ-irradiation in both cases. These mice did not display any mortality following PBMCs injection, but peripheral blood of all treated mice showed very low levels of human $CD45^+$ cells (3.75±0.3% of total blood cells, vs 27.1±0.5% found in mice treated at newborn age). Nevertheless immunohistochemical analysis detected positive staining for human T and B cells in the spleen of these mice injected at adult age (**Figure 12 and 13** of Results-section I), similarly to mice injected at newborn age, suggesting that delivery of PBMCs at adult age allows T and B cells colonization of secondary lymphoid organs but it is poorly permissive for blood circulation of these cells.

Therefore engrafment of human cells from PBMCs resulted more effective when human PBMCs were inoculated in newborn animals, even if mortality of newborns following PBMCs inoculation was close to 70%.

The proliferation capacity of engrafted human T and B cells was analyzed and confirmed by the expression of the Ki67 marker. Ki-67 is an excellent marker to determine the growth fraction of a given cell population, because it is a nuclear protein associated with and maybe necessary for cellular proliferation (Scholzen T et al., 2000). Surprisingly, while other organs were normal and similar to those of untreated mice, lungs appeared highly repopulated by human T and B cells, with characteristics close to those T and B cells found in the spleen. These results were reproducible for all treated newborn and adult mice. Moreover, mice did not present any symptoms of pain, even if there was an acute state of pulmonary inflammation. The presence of human cells T and B in lungs is possible because engrafted cells can reach the different organs through blood and lungs are the largest filter of peripheral blood. The reasons for the accumulation of human lymphocytes in the lungs are still unclear, since these mice were not injected intravenously with human PBMCs, but intrahepathically or

intraperitoneally. It may depend on the activation of a mechanism like that of the CD11b+CD103+ DCs secreting Th2-stimulating chemokines, where these DCs, resident in both spleen and lung, are able to act across inter-species barriers and recruit and stimulate Th2 cells (del Rio ML et al., 2010). This observation, even if it needs to be better analyzed may be extremely interesting and helpful for pre-clinical models of pulmonary inflammation and related diseases. The results described are referred to C57BL/6 $Rag2^{-/-}$ γ $chain^{-/-}$ mice and they cannot be generalized to other immunocompromised mouse strains. However, for further studies, it could be very interesting to perform the same experiments with other immunocompromised mouse strains.

The second part of the work presented was focused on the development of an immunotherapeutic approach for pancreatic cancer.

The aim of antigen-specific active antitumor immunotherapy is to break tolerance against tumor-associated antigens (TAAs) by presenting them in the context of co-stimulatory signals on antigen presenting cells, as dendritic cells (DCs).

The immunogenic potential of dying tumor cells received great attention for both its importance in enhancing T cell-directed immunotherapy and its indication of the best immunogenic source for *ex vivo* TAA DCs loading (Lake et al., 2005; Nowak et al., 2006). Apoptosis is generally regarded as tolerance-inducing cell death (Voll et al., 1997 Steinman et al., 2000) but it was recently reported to be immunogenic *in vitro* and *in vivo* (D'Hooghe et al., 2007); moreover, necrotic death has long been considered highly immunogenic because of the danger signals it conveys (Gallucci et al., 1999; Sauter B et al., 2000).

DCs capture necrotic tumor cells, process them and present the relevant T-cell antigen epitope in the context of both class I and class II MHC, thus allowing the cross-priming of $CD8^+$ T lymphocytes (Banchereau et al., 1998).

We focused our attention on the activation of a pancreas adenocarcinoma-protective immune response using the human PaCa-44, PT-45, PANC-1 and PANC-2 pancreatic tumor cell lines made necrotic by means of hyperthermia treatment at 56°C or UVC exposure, because they could represent a vaccination approach translatable to clinical protocols.

The use of 4 different cell lines is important to point out different responses that could be obtained with different human pancreatic tumor clones.

According to Brusa *et al.* (Brusa et al., 2009), treatment of PaCa-44, PT-45, PANC-1 and PANC-2 cells at the temperature of 56°C caused secondary necrosis in about 90% of cell population already after a 15 minutes incubation, while UVC treatment needed longer exposure times (1 hour). Necrotic cell death induced by 56°C heating or by UVC treatment produced better results in terms of percentage of necrotic cells than γ-irradiation or treatment with Gemcitabine, actually the gold standard treatment for pancreatic cancer.

Cell death by hyperthermia or UVC generates necrotic material producing factors that induce DCs activity of antigen processing and cross-priming of CTLs (Shi et al., 2007). Necrotic cell death induced by 56°C heating or by UVC exposure determines a remarkable release outside the cell of molecules named "danger associated molecular pattern" (DAMPs) HSP70 and HMGB-1. When released outside tumor cells, these molecules promote increased expression of MHC-peptide complexes, reduction of iDCs-maturation time, reversion of the suppressive action of tumor cells on CTL cross-priming and increased expression of the chemokine receptor CCR7, responsible of DCs migration to lymph nodes (Brusa et al., 2008 and 2009).

In addition, necrotic cell death induced by 56°C heating or by UVC treatment reduces the release of TGF-β molecule, involved in the immunosuppressive mechanisms of tumor escape. Not only necrotic PaCa-44, PT-45, PANC-1 and PANC-2 pancreatic cancer cells result more palatable to iDCs, but also mDCs loaded with necrotic tumor cells are able to activate an immune response when they are co-cultured with autologous T-lymphocytes.

UVC treatment resulted more effective than 56°C heating in the induction of tumor-specific immune response, and the best results *in vitro* were obtained with PaCa-44 and PT-45 tumor cells, while we were not able to obtain a specific cytotoxic T response for PANC-1 and PANC-2, even if high levels of IFN-γ were detectable in co-culture supernatants. The different results obtained with any single cell line need to be further investigated comparing the different expression profiles of immune- and cancer-related molecules of each cell line, in order to unveil the possible mechanisms that make one pancreatic cancer more sensitive to immunotherapeutic approach with respect to another one.

The *in vitro* results led us to investigate this immunotherapeutic approach also *in vivo*, using protocols of curative immunotherapy. Curative immunotherapy with PT45 and PaCa-44 produced some encouraging results.

$Rag2^{-/-}$ γ-$chain^{-/-}$ mice were injected s.c. with tumor cells, followed by i.v. or i.p. adoptive transfer of T-lymphocytes stimulated twice *in vitro* by DCs loaded with necrotic tumor cells. All treated animals developed tumors, but mice treated with curative immunotherapy showed a higher OS and a lower tumor growth rate compared to animals that did not receive the immunotherapeutic approach, but were injected with w.t. tumors. The effect was more evident when UVC-treated tumor cells were used. The difference was statistically significant only for tumor growth rate using UVC-treated PT45 cells *versus* control mice.

The critical points of this model are: 1) the low number of T-lymphocytes that can be injected in mice, obtained by *in vitro* restimulation; 2) the low number of mice for each groups (n=4) due to the low number of activated lymphocytes obtained; 3) the donor–dependent variability of the T-lymphocytes response against pancreatic tumors. In these experiments, PT-45 cancer cells resulted more immunogenic than PaCa-44 cells, expecially when treated with UVC. The route of injection (i.v. or i.p.) of human lymphocytes does not seem to compromise the efficacy of the immunotherapeutic approach. Interestingly, we did not find human lymphocytes in the blood and tumors of ACT-treated mice, while we found CD45+ human cells in the spleen of one ACT-treated mouse where lymphocytes were stimulated with DCs loaded with UVC-treated PACA-44 cells (n=1/4). Tumor metastasis and inflammation were present in lungs of some control and treated mice but they were more evident in control mice compared to treated mice. These data suggest that the ACT treatment may be able to impair metastatization, but more experiments are needed on a more extended number of animals to validate this hypothesis.

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