

From DEPARTMENT OF LABORATORY MEDICINE
Karolinska Institutet, Stockholm, Sweden

IMMUNOTHERAPY FOR PANCREATIC CANCER

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Institutet**

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The micro-photo on the cover shows human Pancreatic cancer Tumor Tissue, PanTT26 (Panc 17 in publications) with immunostaining. I took this picture with Carlos Fernández Moro`s help.

In this picture, the brown color shows the T cell marker CD3, and the red represents the B cell marker CD20. I established the first tumor cell line in my life from PanTT26 in 2014.

T cells, B cells, fibroblasts and tumor cells can be seen in this picture, which covers almost all the subjects of my PhD studies.

The picture looks like a mature apple, so it is time to harvest the *fruits* of my PhD studies.

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Immunotherapy for Pancreatic Cancer

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献给我的母亲任素娥及离世的父亲孟宪鹏
To my father Xianpeng Meng and mother Sue Ren

To patients

ABSTRACT

Patients with pancreatic cancer have a short life expectancy, and only 20% of the patients diagnosed with pancreatic cancer can undergo surgery. Chemotherapy does not work well: Gemcitabine is the standard regimen with a 5-year survival rate of 1%–4% and a median survival time of 4-6 months; FOLFIRINOX chemotherapy regimen using four drugs was found to be more effective (median survival 11 months), but with substantial side effects. In this thesis, we aim to find new ways to treat pancreatic cancer by immunotherapy.

In **Paper I**, we established a new protocol to expand tumor-infiltrating lymphocytes (TIL) from pancreatic cancer tumor tissues. Those T cells were able to recognize and kill autologous tumor cells, and produced Th1 signature cytokines, particularly IFN- γ .

In **Paper II**, we identified the individual mutations in pancreatic cancers by whole-genome sequencing. We found that T cells from the tumor or peripheral blood could recognize the mutations, such as mutated K-ras. One CD4⁺ TIL clone recognized a novel (neo) epitope (GLLRYWRTERLF) derived from an aquaporin 1-like molecule (K7N7A8 protein product). Those T cells could be a source for T cell receptor (TCR) transfer to treat patients.

In **Paper III**, we established tumor-infiltrating B lymphocyte (TIB) cell lines from patients with pancreatic cancer and patients with glioblastoma. We found that TIB produced antibodies that could recognize EBV and/or CMV peptides. Those peptides were also recognized by TILs. One CMV peptide (CEDVPSGKLFMH) showed to be recognized by TIB, TILs, peripheral T cells and serum antibodies from one same patient, PanTT32. Antigen-specific B cell receptors (BCR) from TIBs could be a source for the construction of chimeric antigen receptor (CAR) T cells.

In **Paper IV**, we cultured peripheral blood cells from patients with pancreatic cancer with mesothelin peptides. A survival benefit was linked to IFN- γ production to peptides corresponding to the mature mesothelin and to targeted recognition of the mesothelin₆₀₁₋₆₁₅ epitope (MQEALSGTPCLLGPG; p=0.006) in the presence of IL-21. The peptide mesothelin₆₀₁₋₆₁₅ could therefore be a candidate for pancreatic cancer vaccines.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers referred to by their Roman numbers:

- I. **Qingda Meng***, Zhenjiang Liu*, Elena Rangelova*, Thomas Poiret, Aditya Ambati, Lalit Rane, Shanshan Xie, Caroline Verbeke, Ernest Doodoo, Marco Del Chiaro, Matthias Lohr, Ralf Segersvard, and Markus J. Maeurer. Expansion of Tumor-reactive T Cells from Patients with Pancreatic Cancer. *J Immunother* 2016;39:81–89
- II. **Qingda Meng**, Davide Valentini, Martin Rao, Zhenjiang Liu, Carlos Fernández Moro, Thomas Poiret, Georgia Paraschoudi, Elke Jäger, Ernest Doodoo, Elena Rangelova, Marco del Chiaro and Markus Maeurer. Neoepitopes as targets for Tumor-infiltrating Lymphocytes from Patients with Pancreatic Cancer. *Submitted manuscript*
- III. **Qingda Meng**, Davide Valentini, Martin Rao, Liu Zhenjiang, Thomas Poiret, Carlos Fernández Moro, Oscar Persson, Elena Rangelova, Marco del Chiaro, Georgia Paraschoudi, Ernest Doodoo, Markus Maeurer. Peptide microarray identifies novel CMV and EBV targets recognized by tumor-infiltrating T lymphocytes and antibodies in pancreatic cancer and glioma. *Manuscript*
- IV. **Qingda Meng**, Davide Valentini, Martin Rao, Zhenjiang Liu, Thomas Poiret, Shanshan Xie, Ann Morgell, Ernest Doodoo, Matthias Löhr, Elena Rangelova, Marco del Chiaro and Markus Maeurer. Prediction of improved survival in patients with pancreatic cancer via IL-21 enhanced detection of mesothelin epitope-reactive T-cell responses. *Accepted by Oncotarget*

* Shared first authorship

Related articles not included in the thesis:

1. Zhenjiang Liu*, **Qingda Meng***, Jiri Bartek Jr, Thomas Poiret, Oscar Persson, Lalit Rane, Elena Rangelova, Christopher Illies, Inti Harvey Peredo, Xiaohua Luo, Martin Vijayakumar Rao, Rebecca Axelsson Robertson, Ernest Dodoo and Markus Maeurer. Tumor-infiltrating lymphocytes (TILs) from patients with glioma. *OncoImmunology*, 2017, VOL. 6, NO. 2.
2. Johan Lundberg, Emma Jussing, Zhenjiang Liu, **Qingda Meng**, Martin Rao, Erik Samén, Rikard Grankvist, Peter Damberg, Ernest Dodoo, Markus Maeurer, and Staffan Holmin. Safety of Intra-Arterial Injection with Tumor-Activated T-cells to the Rabbit Brain Evaluated by MRI and SPECT/CT. *Cell Transplant*. 2017 Feb 16;26(2):283-292.
3. Qin Li, Lalit Rane, Thomas Poiret, Jiezhi Zou, Isabelle Magalhaes, Raija Ahmed, Ziming Du, Nalini Vudattu, **Qingda Meng**, Åsa Gustafsson-Jernberg, Jacek Winiarski, Olle Ringdén, Markus Maeurer, Mats Remberger and Ingemar Ernberg. Both high and low levels of cellular Epstein-Barr virus DNA in blood identify failure after hematologic stem cell transplantation in conjunction with acute GVHD and type of conditioning. *Oncotarget*, Vol. 7, No. 21.
4. Ambati, T. Poiret, B.-M. Svahn , D. Valentini , M. Khademi , I. Kockum , I. Lima , L. Arnheim-Dahlstrom ,F. Lamb , K. Fink , **Q. Meng** , A. Kumar , L. Rane , T. Olsson and M. Maeurer. Increased β -hemolytic Group A Streptococcal M6 serotype and streptodornase B-specific cellular immune responses in Swedish narcolepsy cases. *J Intern Med*. 2015, 278; 264–276.
5. Shreemanta K. Parida , Thomas Poiret, Liu Zhenjiang, **Qingda Meng**, Jan Heyckendorf, Christoph Lange, Aditya S. Ambati, Martin V. Rao, Davide Valentini, Giovanni Ferrara, Elena Rangelova, Ernest Dodoo, Alimuddin Zumla and Markus Maeurer. T-Cell Therapy: Options for Infectious Diseases. *Clinical Infectious Diseases*. Oct 2015;61(S3):S217–24.
6. Liu Zhenjiang, Martin Rao, Thomas Poiret, Silvia Nava, **Qingda Meng**, Anna von Landenberg, Jiri Bartek Jr, Shanshan Xie, Geors Sinclair, Inti Peredo, Ernest Dodoo, and Markus Maeurer. Mesothelin as a novel biomarker and immunotherapeutic target in human glioblastoma (*Oncotarget*. accepted, June, 2017)
7. Liu Zhenjiang, Martin Rao, Xiaohua Luo, Elisabeth Sandberg, Jiri Bartek, Esther Schoutrop, Anna von Landenberg, **Qingda Meng**, Davide Valentini, Poiret Thomas, Georges Sinclair, Inti-Harvey, Peredo, Ernest Dodoo and Markus Maeurer. Mesothelin-specific Immune Responses Predict Survival of Patients with Brain Metastasis. *EBioMedicine*. 2017 Sep;23:20-24.
8. Zhenjiang Liu, Thomas Poiret, Oscar Persson, **Qingda Meng**, Lalit Rane, Jiri Bartek Jr, Julia Karbach, Hans-Michael Altmannsberger, Christopher Illies, Xiaohua Luo, Inti Peredo, Elke Jäger, Ernest Dodoo, Markus Maeurer. NY-ESO-1 and survivin specific T-cell responses in the peripheral blood from glioma patients (*Cancer Immunology, Immunotherapy*. Accepted, August, 2017).

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LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen-presenting cell
AQP	Aquaporin
BCR	B-cell receptor
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated protein- 4
DC	Dendritic cell
DTH	Delayed-type hypersensitivity
EBNA1	Epstein–Barr virus nuclear antigen 1
EBNA3	Epstein–Barr virus nuclear antigen 3
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GVHD	Graft-versus-host disease
HLA	Human leukocyte antigen
hMSCs	Human bone marrow-derived mesenchymal stem cells
HSCT	Hematopoietic stem cell transplantation
ICS	Intracellular Cytokine Staining
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IgG	Immunoglobulin gamma

IFN- α	Interferon-alpha
IFN- γ	Interferon-gamma
IL-2	Interleukin-2
IL-15	Interleukin-15
IL-21	Interleukin-21
LAG-3	Lymphocyte-activation gene 3
LMP2	Epstein–Barr virus (EBV) latent membrane protein 2
MPF	Megakaryocyte-potentiating factor
MSCs	Mesenchymal Stem Cells
NPC	Nasopharyngeal carcinoma
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein -1
PDAC	Pancreatic ductal adenocarcinoma
TAA	Tumor-associated antigens
TCR	T-cell receptor
TGF- β	Transforming growth factor-beta
TIB	Tumor-infiltrating B lymphocytes
TIL	Tumor-infiltrating lymphocytes
TIM-3	T cell immunoglobulin-and mucin-domain-containing molecule-3
TNF- α	Tumor necrosis factor-alpha
Tregs	Regulatory T cells
WBA	Whole blood assay

1 INTRODUCTION

Pancreatic ductal adenocarcinoma, also known as pancreatic adenocarcinoma, is the most frequent form of pancreatic cancer. With a five year survival rate of 5%, it becomes one of the leading causes of cancer-related death ^[1-3]. Due to its aggressive progression, late clinical diagnosis and intense resistance to conventional chemotherapy and radiotherapy, improved and more innovative treatment modalities are urgently needed presently for patients with pancreatic cancer. Cancer immunotherapy (e.g. adoptive cellular immunotherapy) is a potential and promising strategy for cancer treatment that is expanding gradually into a broad and important subject in recent oncology ^[4,5].

This thesis will focus on tumor-infiltrating lymphocytes (TILs) (including specific T cells and B cells) expanded from the tumor tissue of patients with pancreatic cancer and identification of their targets which could be used for cellular immunotherapy, and thus benefit the development of more effective and innovative treatment options for patients with pancreatic cancer.

1.1 PANCREATIC CANCER BIOLOGY

Pancreatic cancer is subdivided into a number of different subtypes. Approximately 85% of the cases are classified as pancreatic ductal adenocarcinomas (or pancreatic adenocarcinomas) ^[3]. Generally, pancreatic cancers arise from pancreatic ductal cells, of which almost 99% derive from the exocrine component, while around 1% comes from neuroendocrine cells within the pancreas ^[3].

Pancreatic cancer is associated with advancing age. More than 50% of patients are over the age of 70, while it rarely occurs before the age of 40 ^[3,6]. Besides age, other predisposing factors such as cigarette smoking, excessive alcohol consumption, specific types of food (e.g. processed or red meat) and some medical factors (e.g. chronic pancreatitis, diabetes and obesity) are also involved ^[3,6,7].

Mutations in specific genes as K-ras, CDKN2A, TP53 and SMAD4 are associated with the tissue evolution of pancreatic adenocarcinoma. K-ras, the most common genetic alteration, is located in chromosome 12p, and is present in almost 95% of pancreatic adenocarcinomas. It encodes a member of RAS family that affects many cellular effects

including proliferation, survival and differentiation ^[8-10]. CDKN2A, the cyclin-dependent kinase inhibitor 2A, is a tumor-suppressor gene regulating the cell cycle, and resides on chromosome 9. Its mutations occur in 95% of pancreatic adenocarcinomas and are also linked to the familial atypical mole-malignant melanoma syndrome. Inherited CDKN2A mutation increases the risk of pancreatic cancer by 13 folds ^[11, 12]. In up to 85% of pancreatic cancers, there is a mutation in gene TP53, which resides on chromosome 17 and plays an important role in cell cycle, DNA repair and apoptosis; as many as 50% of all TP53 mutations could lead to loss of protein expression and null alleles ^[8]. SMAD4, located on chromosome 18, also called Mothers Against Decapentaplegic homolog 4 or Deleted in Pancreatic Cancer-4 (DPC4), is one of the Smad family proteins acting as signal transducers responding to TGF- β , regulating cell proliferation and apoptosis ^[13]. It is inactivated in 50% of advanced pancreatic cancers ^[14].

In the United States, pancreatic cancer is the fourth most frequent cause of human death ^[1, 2, 15]. And in 2015, 411,600 deaths globally were reported to be due to pancreatic cancer ^[16]. This is due to very poor prognosis. About 25% of the patients with pancreatic adenocarcinoma survive one year, whereas only 5% live at 5 years after diagnosis ^[13].

Currently, the treatment for pancreas adenocarcinoma includes surgery, chemotherapy, radiotherapy, palliative care or combinations thereof which partly depends on the cancer stage. Surgery is the only potentially curative option for those patients, however, no more than 20% are actually suitable for surgery ^[7]. Chemotherapy such as FOLFIRINOX or gemcitabine can be offered to patients with good performance status after surgery, and may also be given to patients that are not suitable for surgery to extend or improve their lives ^[17]. Gemcitabine was the first chemotherapy drug approved by FDA in 1997 for pancreatic cancer because of its improvements in life quality and a 5-weeks improved median survival duration ^[18]. More effective than the standard gemcitabine treatment, the FOLFIRINOX regimen (a combination of 4 chemicals including leucovorin, fluorouracil [5-FU], irinotecan and oxaliplatin) was found to induce the longest improvement in survival time with 11.1 months (approximately 4 months longer), but had serious side effects due to its high toxicity ^[19]. Thus, the FOLFIRINOX regimen is only a treatment option for those patients with good performance status who can tolerate the side effects ^[18].

1.2 IMMUNOTHERAPY FOR PANCREATIC CANCER

1.2.1 T CELL IMMUNOTHERAPY

Cellular immunotherapy with tumor specific T cells is a highly promising platform for treating various solid tumors, with much success observed among melanoma patients over the last 30 years [5, 20-22]. Current efforts to develop T-cell therapies for cancer focus on 3 major strategies: (i) isolation and expansion of TILs; (ii) T cells transduced with transgenic T cell receptors (TCR) specific for tumor epitopes, or T cells heterologously expressing chimeric antigen receptors (CAR) which recognize tumor cell-associated surface molecules; (iii) enrichment and expansion of tumor antigen-reactive T cells isolated from peripheral blood followed by cell selection and/or TCR cloning (**Figure 1**).

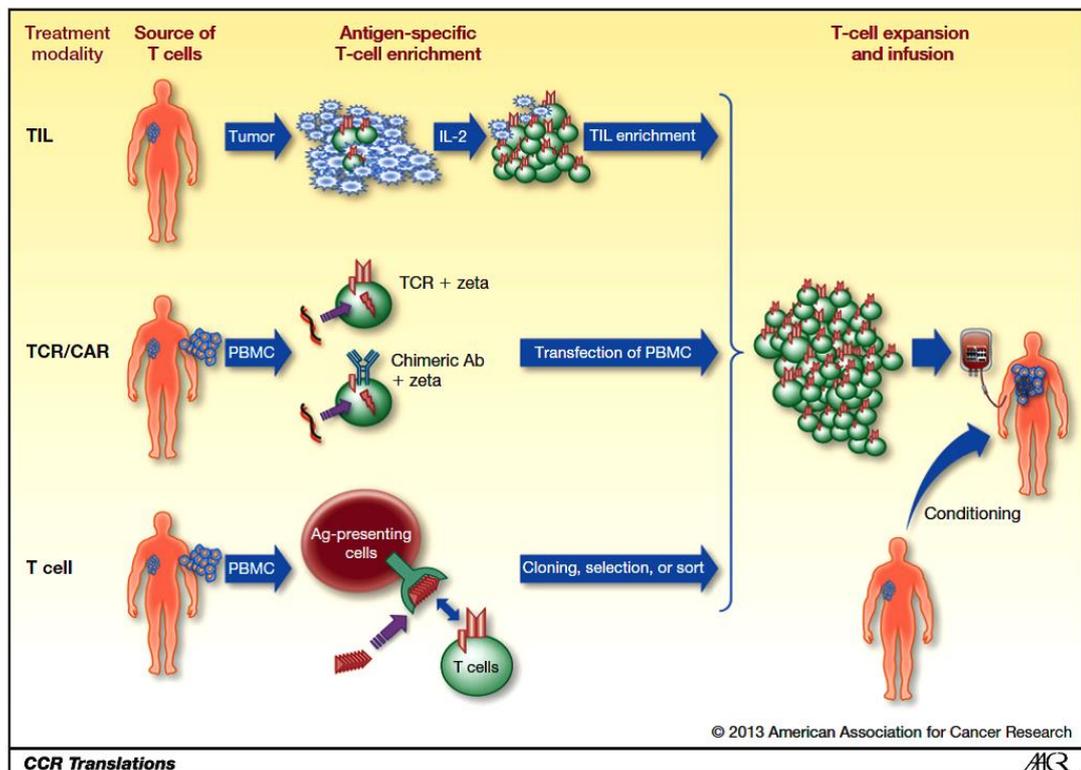


Figure 1. Adoptive cell therapy. *Source: Cassian Yee, Adoptive T-Cell Therapy for Cancer: Boutique Therapy or Treatment Modality? Clin Cancer Res, 2013.*

TILs are generally equipped with strong and specific anti-tumor effector functions. It is however a challenging task to generate TILs in the clinical setting since T-cell isolation and expansion from tumor tissue is not always feasible due to inter-patient variabilities. Tumor-

reactive T cells from the peripheral blood of patients with melanoma have been shown to contain neoantigen-specific T cells and express the programmed cell death 1 (PD-1) on surface ^[23]. The chimeric antigen receptor (CAR) technology is being actively pursued by biopharmaceutical companies for cancer immunotherapy due to its potential for extended use. Briefly, a CAR molecule comprises a single-chain variable fragment (scFv) of a monoclonal antibody directed against a particular molecule (i.e. CD19) fused to a cytoplasmic region of a T cell activation molecules i.e. CD28, 4-1BB via a linker, and the CD3 zeta chain. Gene segments encoding these components are cloned into a lentiviral or retroviral vector for T cell transduction. CAR T cells recognizing human CD19 expressed on B cells are currently undergoing clinical testing in patients with lymphoma and B-cell leukemia. Although initial results are very encouraging, the lack of specific surface molecules exclusively expressed on cancer cells is a current limitation for an extended use. Retroviral or lentiviral vectors are also able to be applied to transfer TCRs with high affinity and avidity for tumor epitopes into autologous peripheral blood-derived T cells. The first successful clinical trial using genetically engineered autologous lymphocytes to induce tumor regression in metastatic melanoma patients was reported by Rosenberg and colleagues in 2006 ^[24]. TCRs specific for epitopes derived from NY-ESO-1, a cancer testis antigen, have also been utilized for patients with synovial cell carcinoma or melanoma ^[25].

Tumor-infiltrating lymphocytes (TILs) are a source for cancer immunotherapy. In 2016, we first reported the phenotype and function of expanded TILs from pancreatic cancer tumor tissue, and found that the TILs recognized and killed autologous tumor cells from pancreas (also shown in detail in paper I) ^[26]. Later in the same year, two other research groups also showed in their publications that TILs could be obtained from patients with pancreatic cancer ^[27,28]. Compared to them, we got more CD8⁺T cells by using IL-2, IL-15 and IL-21 cytokines in our method.

1.2.2 Regulatory T cells

Regulatory T cells (Tregs) are immunosuppressive, and can efficiently suppress or down-regulate induction and proliferation of effector T cells. Thus, Tregs exist to help regulate the strength of an inflammatory immune response and maintain tolerance to self-antigens. This, in turn establishes an equilibrium in cell-mediated immunity, and reduces the risk of developing autoimmune diseases ^[29]. Tregs are categorized as CD4 T cells which show high expression of surface CD25, along with no CD127 expression and they are positive for the

transcription factor fork head box P3 (FoxP3), also known as scurfin, and require TGF- β signal for differentiation from naïve CD4⁺ cells as well as cellular homeostasis ^[30]. It is noteworthy that surface expression of CD103 is a hallmark of tumor-infiltrating Tregs *in vitro* and *in vivo* ^[31]. Ademmer and colleagues have previously reported that among TILs isolated from pancreatic cancer tissue, approximately 8% of CD4⁺ T cells and 20% of CD8⁺ T cells are CD103⁺ ^[32]. Furthermore, there was a large variation (from 9% to 32%) observed for the distribution of CD103 expression in CD8⁺ lymphocytes, and this T cell subset was found interestingly restricted only to the fibrous interstitial tissue without periductally or intraepithelially, indicating of their roles in disease progression of pancreatic cancer ^[32].

1.2.3 Immune checkpoint inhibitors

Tumor-specific T cells express four main immune checkpoint molecules on their surface which abrogate effective anti-tumor responses within the tumor microenvironment: PD-1, CTLA-4 (cytotoxic T-lymphocyte associated protein 4), LAG-3 (lymphocyte-activation gene 3) and TIM-3 (T-cell immunoglobulin and mucin-domain containing 3). Therapeutic targeting of PD-1 and CTLA-4 in melanoma using monoclonal antibodies has been highly successful, with one anti-CTLA-4 reagent and at least two clinically approved anti-PD-1 reagents in market. Further clinical studies with newer candidates are in progress, such as anti-TIM-3 and anti-LAG-3 monoclonal antibodies. As previously mentioned, PD-1 expression on CD8⁺ T cell isolated from tumor tissues as well as peripheral blood of patients with metastatic melanoma are now known to recognize neoantigens, and can respond to them *in vitro* with IFN- γ and TNF- α production; potent cytotoxic killing of autologous tumor cells by PD-1⁺ CD8⁺ TILs has also been observed ^[33]. Blockade of PD-1 in patients with metastatic melanoma has been shown to unleash the anti-tumor activity of CD8⁺ TILs which are specific for neoantigens, leading to durable clinical responses ^[34]. Check point inhibitors such as anti-CTLA-4 and anti-PD1 antibody, improved the survival time for melanoma and lung cancer patients ^[35,36]. However, in a phase II trial, ipilimumab (fully humanized anti-CTLA4 antibody) was ineffective and did not prolong survival for 27 patients with locally or advanced metastatic pancreatic cancer ^[37].

It is now well known that tumor cells express surface molecules that inhibit the anti-tumor activity of T cells. The upregulation of surface programmed cell death 1 ligand 1 (PD-L1, also known as B7-H1) – which interacts with PD-1 on the surface of T cells to diminish their anti-tumor potential – is a major setback to activate an efficacious immune responses to

kill tumor cells ^[38]. Anti-PD-L1 monoclonal antibodies are also employed in clinical trials for various cancers. Pre-screening patients for PD-L1 expression on the tumor cells prior to initiating therapy may provide an early indication for the chances of treatment success.

PDL1 and indoleamine-pyrrole 2, 3-dioxygenase (IDO) are expressed by tumor and infiltrating immune cells and could be upregulated by IFN- γ . This forms a feedback loop inducing a PD-1 signal that holds immunosuppression dominantly. The immunosuppressive tumor microenvironment is the principle reason why many immunotherapies aiming to directly trigger the antitumor immune response failed ^[39].

1.2.4 B cells

B cells function as antigen-presenting cells (APCs), which are well-known for production of antibodies against broad antigens and can also produce cytokines (Table 1) ^[40]. We found that EBV-B cells also could produce IL-21 which is important for memory T cells (unpublished data). A Japan research group in 2015 found that the tumor infiltrating B lymphocytes (TIB) represent a good positive prognostic factor for pancreatic cancer. TIBs were antigen-experienced and secreted antibodies which can bind to allogeneic tumor cell lines ^[41]. These antibodies may also have reactivity to neoantigens and could potentially be developed further to therapeutic agents. Identification of the tumor specific antibodies from TIBs to be used to engineer CAR T cells could be a novel way to treat cancer.

Table 1. Cytokine production from B lymphocytes and EBV transformed B cells

Cytokines*	Fresh B cells	LCLs*
IL-1, 2, 3, 4, 6, 10, 13	+	+
INF- γ	+	+
IL-7	-	+
LT- β	+	-
GM-CSF	+	-
G-CSF, M-CSF	+	+

* LCL, lymphoblastoid cell line; IL, interleukin; IFN, interferon; LT, lymphotoxin; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; M-CSF, macrophage colony-stimulating factor.

By immunostaining, we found that there were both TILs and TIBs in pancreatic cancer tumor tissue (Figure 2). I have established the TIB cell lines from tissues of both pancreatic cancer and brain tumors. They produced high levels of IgG and or IgA antibodies (Unpublished data). In 1996, Williams S. found that the TIB from lung cancer could produce tumor-specific IgG detected by Western Blotting ^[42]. Staoru Imahayashi showed similar results in the year of 2000 ^[43]. B cells may also express Fas ligand with the potential to kill autologous tumor cells ^[44].

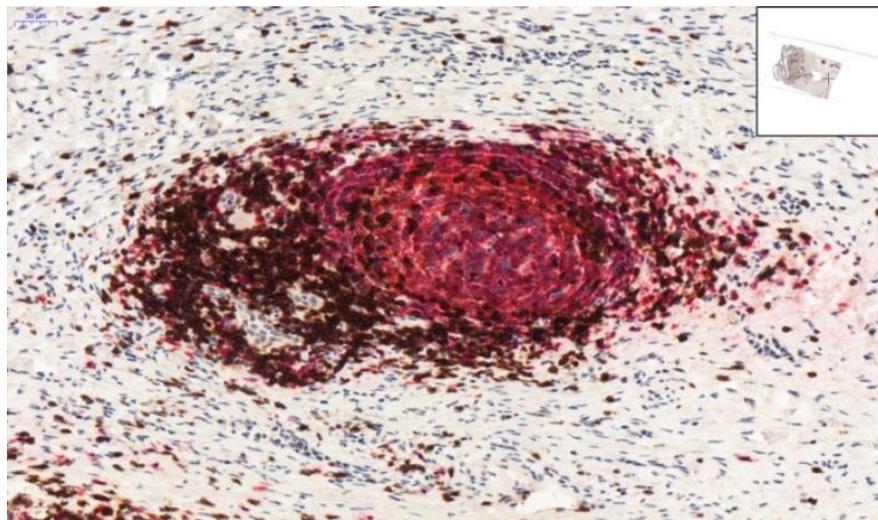


Figure 2. Microphoto of pancreas cancer tumor tissue with immunostaining CD3 (brown) & CD20 (Red); taken by Carlos Fernández Moro & Qingda Meng

Recent studies have shown that certain B cells especially B1 B cells, which produce high levels of IgM, can promote the growth of pancreatic cancer or metastasis ^[45-47]. TIB not only can be a source for B cell receptor (BCR) to get individual CAR T cells, but also a target for pancreatic cancer therapy.

1.2.5 Dendritic cells (DCs) and vaccine

Dendritic cells form an essential connection between the innate and adaptive immune responses. It has been previously reported that DCs requires the type I interferon for tumor rejection ^[48, 49]. DC-based cancer vaccines represent a promising area of investigation for cancer immunotherapy.

MUC1 is overexpressed in pancreatic cancer and it can be recognized by cytotoxic T lymphocytes and antibodies ^[50]. MUC1 is used for DC-based cancer vaccine against pancreatic cancer ^[51]. A phase I/II trial of a DC vaccine loaded with MUC1 peptide for patients with pancreatic and biliary cancer, showed that four of the twelve patients are alive at four years after vaccination without recurrence ^[52].

In an additional report, three of 14 patients were observed to have a more than 25 months of disease-free survival time in a trial for a GM-CSF-secreting pancreatic tumor vaccine and this vaccine could induce delayed-type hypersensitivity (DTH) responses against the autologous tumor ^[53]. Subsequent studies by the same group showed that the mesothelin specific CD8⁺ T cells were induced by this vaccine for those three patients ^[54]. In a phase I/II trial for a K-ras vaccine with 48 patients with pancreatic cancer enrolled, one individual single mutant K-ras peptide has been reported to be derived from the patient tumor mutations or a mixed mutant K-ras including G12D, G12C, G12V and G12R combined with GM-CSF; this K-ras peptide vaccine could significantly increase the responder survival time (148 days) as compared to non-responders (61 days) ^[55].

In this thesis, we found that there were a quite high percentage of mesothelin specific CD8⁺ T cells among TILs. We identified one mesothelin peptide, mesothelin₆₀₁₋₆₁₅ (MQEALSGTPCLLPG) to be dominant in this response. The immune response (IFN- γ production) for this particular peptide was linked to a survival benefit. Our data suggested that mesothelin₆₀₁₋₆₁₅ could be a new peptide epitope to be used for a pancreatic cancer vaccine. We have established various TIL cell lines from patients with pancreatic cancer. Among them, a TIL cell line and tumor cell line were from patient Panc17, and the TILs showed a very high IFN- γ response (453 pg/mL) to mutated K-ras, after three times stimulation with the autologous tumor cell line.

1.2.6 Cytokine immunotherapy

IFN- α is the first cytokine shown to provide anti-tumor effects for patients with progressive melanoma. In phase I/II trials of IFN α , almost 20% of patients with metastatic disease were observed to get objective tumor regression ^[56,57]. Early in the year of 1995, recombinant IFN- α -2b was approved by FDA in the US as the first adjuvant immunotherapy to treat patients with stage IIB/III melanoma.

Interleukin (IL)-2 plays an essential role in regulating the immune system and T cell proliferation. It was shown in clinical trials that recombinant IL-2 had significant anti-tumor activity for patients with progressive metastatic melanoma ^[58-61].

IL-15, as a T-cell growth factor, is secreted by phagocytes such as dendritic cells and macrophages, having effects on both T cells and natural killer cells. IL-15b can inhibit the antigen-induced cell death (AICD) of T cells, reverse T cell anergy and activate natural killer cells ^[62-64].

IL-21, which is mainly produced by activated CD4⁺ memory T cells, is a member of the γ -chain family of cytokines ^[65,66]. When acting with IL-7 or 15 together, it is able to stimulate naïve and memory CD8⁺ T cell to expand, and raise the affinity to tumor-reactive antigen-specific CTL clones ^[65,67]. IL-21 also inhibits the CD80 and CD86 expression on immature DCs, and suppresses DC activation and cytokine production ^[68,69]. It has been demonstrated that IL-21 could promote tumor rejection and immune memory, and avoid metastases ^[68,69]. IL-21 can be critical when in vitro generating and expanding tumor antigen-specific CTL lines/clones from PBMCs or TILs used for adoptive immunotherapy.

1.2.7 Mesenchymal Stem Cells (MSCs) engineered for Cancer Therapy

Mesenchymal Stem Cells (MSCs) are able to be recruited into solid organs when reacting to tissue stress. Because of its tropism for brain tumors, human bone marrow-derived mesenchymal stem cells (hMSCs) can possibly be applied as potent delivery vehicles to treat gliomas. It was reported in 2005 that after isolating and fluorescently labelling hMSCs, Akira and coworkers injected the cells to mice bearing human glioma xenografts and observed that these cells were only restricted in the brain tumors, whereas widespread distribution of delivered cells was induced by fibroblasts or U87 glioma cells without tumor specificity ^[70].

HMSC-IFN- β is MSCs that has been engineered to release IFN- β , which was reported to be able to treat human glioma xenografts in vivo and notably prolong the survival of animals as compared to control group ^[70]. Similar to HMSC-IFN- β , transplanted MSCs that produce IL-18 was related to improved T cell infiltration and prolonged anti-tumor effects in mice with gliomas ^[71]. Oncolytic adenoviruses loaded MSC was found capable of suppressing the growth of glioma and markedly improving animal survival to eliminate tumors when compared with the control group ^[72]. MSC can also delivery other cytokines

including IL-2, 12 and IFN- γ , as well as drugs. Figure 3 is the summary of the use of MSC therapy in cancer.

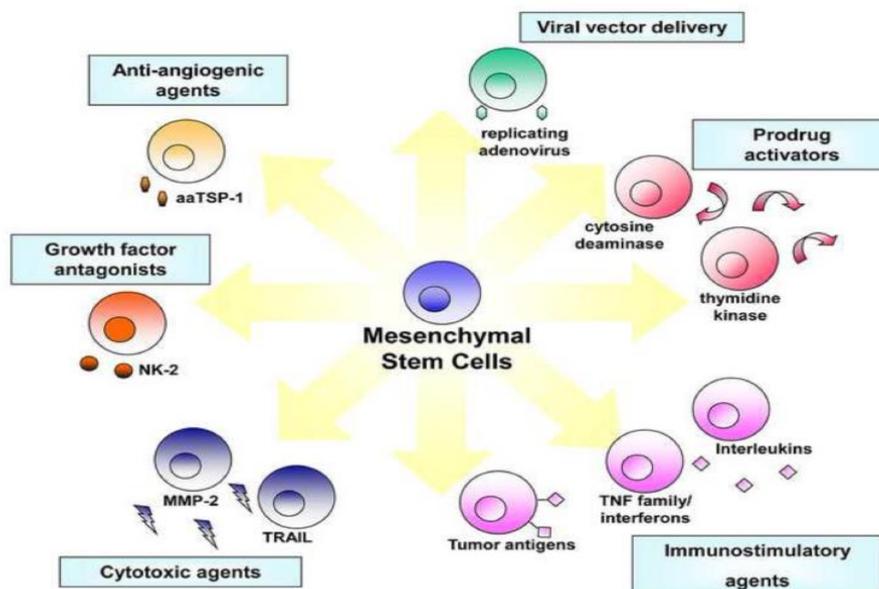


Figure 3. Transgene strategies potentiating MSC for tumor. *Source: Khalid Shah, Adv Drug Deliv Rev, 2012.*

MSCs have advantages of easy availability and handling in vitro, non-immunogenic nature, and an ability of preferentially migrating towards local inflamed tissue, which make them very promising candidates for cell based treatment options [73].

I hypothesize that MSCs after engineered with IL-2/15/21 may deliver these cytokines to the tumor tissue. Thus, transfer of MSCs together with tumor specific TILs to patients could be a more powerful therapy for cancer.

1.2.8 Natural killer (NK) cell therapy

In contrast to cytotoxic T lymphocytes (CTLs), the anti-tumor effects of NK cells are antigen-independent. Especially, it is possible to use both autologous NK cells and allogeneic ones. IgG Fc receptors (Fc γ RIII, CD16) are highly expressed on the CD56^{dim} NK cell subset, which can regulate ADCC, the antibody-dependent cellular cytotoxicity [74]. NK cell activity is dependent of NKG2D and NCRs (natural cytotoxicity receptors) for receptor activations, and

relies on inhibitory receptors such as KIRs (killer immunoglobulin-like receptors) for receptor suppression. KIRs interact with human leukocyte antigen (HLA) class I molecules. Besides, inhibitory receptor NKG2A is also able to bind to non-classical HLA class I, HLA-E ^[75].

In 2005, Miller and coworkers first established and published the safety and efficacy report about adoptive transfer of haploidentical NK cells, whereas nineteen patients with acute myeloid leukemia (AML) received the cell infusions and IL-2, and complete remission was observed in all of the five patients ^[76]. NK cells are appealing to CAR expression because of its cytotoxic function and not causing graft-versus-host disease (GVHD) that is different from T cells ^[77]. However, in pancreatic cancer, there are very few NK cells detected to infiltrate into the tumor tissue ^[32].

1.2.9 Hematopoietic Stem Cell Transplantation (HSCT)

Allogeneic HSCT is primarily used for patients with hematologic malignancies ^[78]. Studies showed that HSCT had also effects for solid tumors, e.g. renal cell carcinoma ^[79-81], colorectal cancer ^[82, 83] and ovarian cancer ^[84].

In 2009, Yasunobu Abe reported a study using HSCT for five patients with pancreatic cancer, and found that 2 patients showed tumor reduction (one with tumor disappearance, whereas the other approximately 20% reduction) ^[85]. Additionally in 2016, Brigitta Omazic and Olle Ringdén from our division (Therapeutic Immunology, Karolinska Institutet), reported in a long-term follow-up study of HSCT for solid cancer (pancreatic cancer included) that three patients with pancreatic cancer were treated with HSCT, 2 of which were alive and cancer-free 8 years after HSCT ^[86]. A subsequent study in the year of 2017 showed that two patients developed immune responses against INO80E and UCLH3, two tumor-associated antigens after HSCT ^[87].

2 AIMS

The general aims of this thesis were:

- 1) To expand tumor specific T cells from pancreatic cancer tumor tissue, characterize their phenotype, function and specificity, and also identify their targets, which could potentially be used for cellular immunotherapy.
- 2) To identify targets of tumor-infiltrating B lymphocytes (TIBs), as BCR from the TIBs could then be used for CAR T cells, and identified epitope could be used as a tumor vaccine.
- 3) To understand the relation between the peripheral blood immune reactions against mesothelin and overall survival, and to identify if the mesothelin epitopes were associated with overall survival of the patients.

Specific aims:

- To expand tumor specific T cells from pancreatic cancer tumor tissue with IL-2, IL-15 and IL-21, and test the T cell phenotype, function, specificity and clonality (Paper I).
- To identify mutations in tumors by whole-genome sequencing, using this information to synthesize neoepitopes, and to identify T cell targets by culturing TILs with neoepitope peptides (Paper II).
- To identify EBV and CMV epitopes recognized by tumor-infiltrating B lymphocytes and/or tumor-infiltrating T lymphocytes. Map the response to these epitopes among peripheral T cells and serum antibodies from patients with pancreatic cancer or glioma (Paper III).
- To explore the relationship between immune reactions against a tumor associated antigen (mesothelin) and patients overall survival, and to identify specific mesothelin epitopes (Paper IV).

3 MATERIALS AND METHODS

3.1 PERIPHERAL BLOOD AND TUMOR TISSUE

Peripheral blood and tumor tissue samples from patients with pancreatic cancer or brain tumors were obtained from Karolinska hospital, Sweden after informed consent. The study was approved by the regional ethical review board at Karolinska Institutet, Stockholm, Sweden (Dnr: 2013/977-31/1, Dnr: 2013/1332-31/3 and Dnr: 2013/576-31).

3.2 CELL LINE ESTABLISHMENT

In these studies, we have established tumor-infiltrating T lymphocytes cell lines, tumor-infiltrating B lymphocytes cell lines and pancreatic cancer tumor cell lines. Tumor tissues from clinical biopsies or surgery were cut with surgical scissors and a scalpel. Individual single tumor fragments (1–2 mm³) were placed in each well of a 24-well tissue culture plate.

Tumor-infiltrating T lymphocytes were cultured with Cellgro GMP Serum-free medium (Cell-Genix, Freiburg, Germany) with 10% human AB serum (Innovative Research, MI) supplemented with IL-2 (1000 IU/mL), IL-15 (10 ng/mL) and IL-21 (10 ng/mL). (Paper I, II and III)

Tumor-infiltrating B lymphocytes were cultured with 70% Cellgro GMP-grade serum-free medium (CellGenix, Freiburg, Germany), 20% B95-8 supernatant containing EBV virus, 10% FBS (Life Technologies). (Paper III)

Pancreatic cancer cell lines were cultured with RPMI 1640 with 10% FBS (Life Technologies), epidermal growth factor (EGF, 20 ng/mL; ImmunoTools, Friesoythe, Germany). When tumor cell lines were established, the cells were cultured and passaged without EGF. (Paper I and II)

3.3 WHOLE-BLOOD ASSAY (WBA)

Peripheral blood was diluted 5x with RPMI 1640 (1 blood: 4 RPMI 1640), with or without recombinant human IL-21 (10 ng/ml; Prospec, Rehovot, Israel) in 96-well plates (200µl diluted blood/well). The diluted blood was stimulated with different antigens. Anti-human

CD3 antibody (OKT3) was used as positive control and cells without stimulation (medium only) as negative control. Supernatants were harvested after 7 days incubation at 37°C with 5% CO₂.

The supernatants were used for IFN- γ ELISA test. (Paper IV)

3.4 ELISA

IFN- γ was detected by sandwich ELISA (Mabtech, Stockholm, Sweden) after T cells or diluted peripheral blood cells were co-cultured with peptides, OKT3 or tumor cells. (Paper I, II, III and IV)

3.5 FLOW CYTOMETRY

We used a 12 color flow cytometer (FACS Aria) to measure the TIL phenotypes, exhaustion level and TCR V β repertoire, as well as for testing T cell function by CD107a assay and intracellular cytokine staining (ICS). (Paper I and II)

In paper I, antibodies for phenotype characterization: CD3, CD4, CD8, CD45RA, CCR7, CD107a, C-kit and CD127; Antibodies for activation/exhaustion: CD3, CD4, CD8, CD45RA, CCR7, 4-1BB, CD127, LAG-3, CD25, TIM3, PD-1 and CTLA-4; Antibodies used for ICS panel were: CD3, CD4, CD8, IL-2, IL-17, IFN- γ and TNF- α ; Antibodies used for TCR V β panel were: CD3, CD4, CD8 and TCR V β Repertoire Kit (Beckman Coulter) which measures 24 TCR V β with antibodies in 8 tubes.

In paper II, antibodies for phenotyping: CD3, CD4, CD8 and CXCR3; Antibodies for CD107a assay: CD3, CD4, CD8 and CD107a.

3.6 CR⁵¹ RELEASE ASSAY

Chromium 51 (Cr⁵¹) release assay was performed in paper I to detect cytotoxic T-cell responses.

The tumor target cells were labeled with Na²CrO₄ at 37°C for 1 hour. After washing with PBS, the Cr⁵¹ labeled tumor cells were co-cultured with TILs in a 96-well plate at

10,000 cells/well for 4 hours at 37 °C, 5% CO₂. After 4 hours of incubation, the Cr⁵¹ radioactivity from each well was measured with 1450 MicroBeta Trilux scintillation counter. The percentage of specific lysis was calculated with the negative control and positive control.

3.7 PEPTIDE MICROARRAY

In Paper III, peptide microarray chips embedded with 12-mer peptides were used from CMV and EBV proteins: CMVpp65, EBNA1, EBNA3, LMP2 and BMLF1. The 12-mer peptides with a 11-amino acid overlap were selected to cover the five protein sequences entirely. These five proteins were spanned by a total number of 3067 peptides, with seven technical replicates for each peptide on the chip. Goat Anti-Human IgG, Fcγ Fragment Specific was used as detection antibodies. After incubation, the microarrays were scanned with a NimbleGen MS200 scanner and the signal was extracted with internal Roche software called SlideViewer.

3.8 WHOLE-GENOME SEQUENCING

Genomic DNA from patient samples (tumor tissues and TILs) was fragmented to construct an Illumina DNA library. DNA exons were captured and analyzed. Tumor mutations were determined by comparing to both the reference human genome sequence and non-malignant cell sequence from the same patient.

3.9 IMMUNOSTAINING

In Paper II, pancreatic cancer tumor tissues were performed with immunohistochemistry and stained with CD3-CD20 or CD4-CD8 antibodies. CD3 and CD4 antibodies were labeled with 3, 3'-diaminobenzidine (DAB) (brown), while CD20 and CD8 with alkaline phosphatase (AP) (red). The tissues for staining were selected by Dr. Carlos Fernández Moro, a specialist pancreatic pathologist.

3.10 STATISTICS

In Paper IV, survival probability was evaluated using Kaplan-Meier survival analysis and log-rank test. The cut-off median values of antigen-specific IFN- γ production were determined based on the intensity of the response to each antigenic stimulus tested.

4 RESULTS AND DISCUSSION

4.1 PAPER I

“Expansion of Tumor-reactive T Cells from Patients with Pancreatic Cancer”

In PAPER I, we generated TILs from surgical or biopsy specimens from 17/17 patients with pancreas cancer using the IL-2/IL-15/IL-21 cytokine cocktail. Our data showed that the combination of IL-2, IL-15 along with IL-21 is capable of expanding TILs with a preference for central ($CD45RA^-CCR7^+$) and effector ($CD45RA^+CCR7^-$) memory subsets (Figure 4). It has been reported that central-memory T cells exhibit stronger proliferative potential and are perhaps the best candidates to provide long-term antitumor reactivity ^[88, 89].

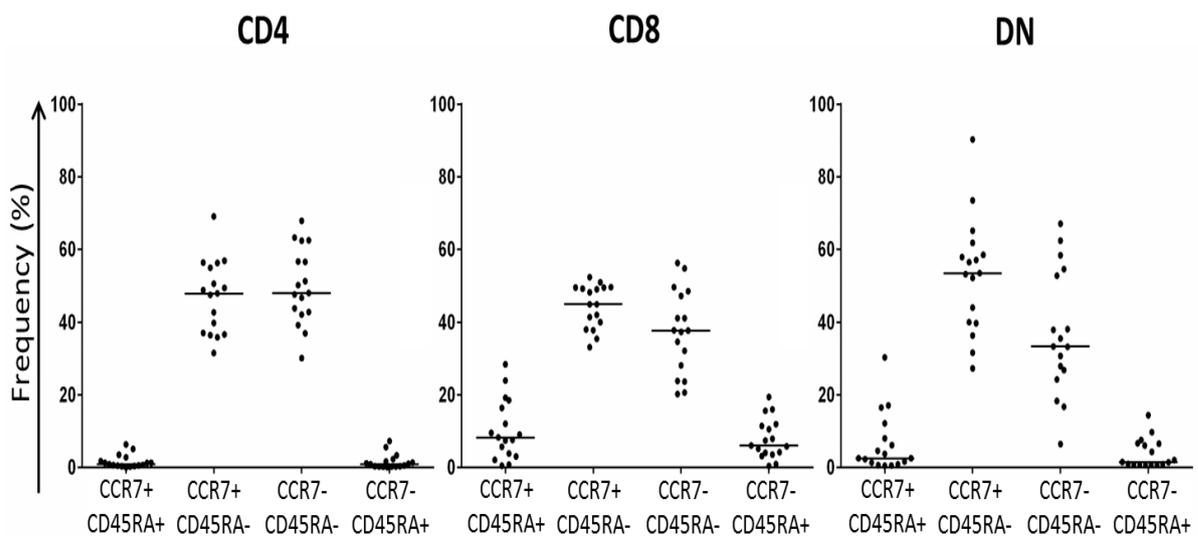


Figure 4. TIL Immunophenotype

TILs were observed to have a low expression of exhaustion marks such as CTLA-4 and TIM3. However, the PD-1 expression was high in $CD8^+$ T cells (median 36%). LAG-3⁺ cells were frequent in $CD8^+$ T cells with 96% as compared to almost 2% in $CD4^+$ T cells (median).

By analyzing the TCR V β families in TILs with flow cytometry, we found that twelve of 17 TILs expressed preferential expansion of certain TCR V β families (e.g. 99.2%

V β 13.2 in CD8⁺ T cell from Panc9 and 77% V β 1 in CD8⁺ T cell from Panc1).

Mesothelin, survivin and NY-ESO-1, the commonly shared tumor-associated antigens (TAAs), were also found to be recognized by TILs (Figure 5). NY-ESO-1 acts as one of the cancer-testis antigens ^[90], and could induce specific HLA class I- and class II-restricted T cell responses ^[91, 92]. We observed strong IFN- γ and TNF- α induction of TILs upon NY-ESO-1 exposure, e.g. up to 8.2% and 12.0% in CD8⁺ TILs from patient Panc6 (Figure 5).

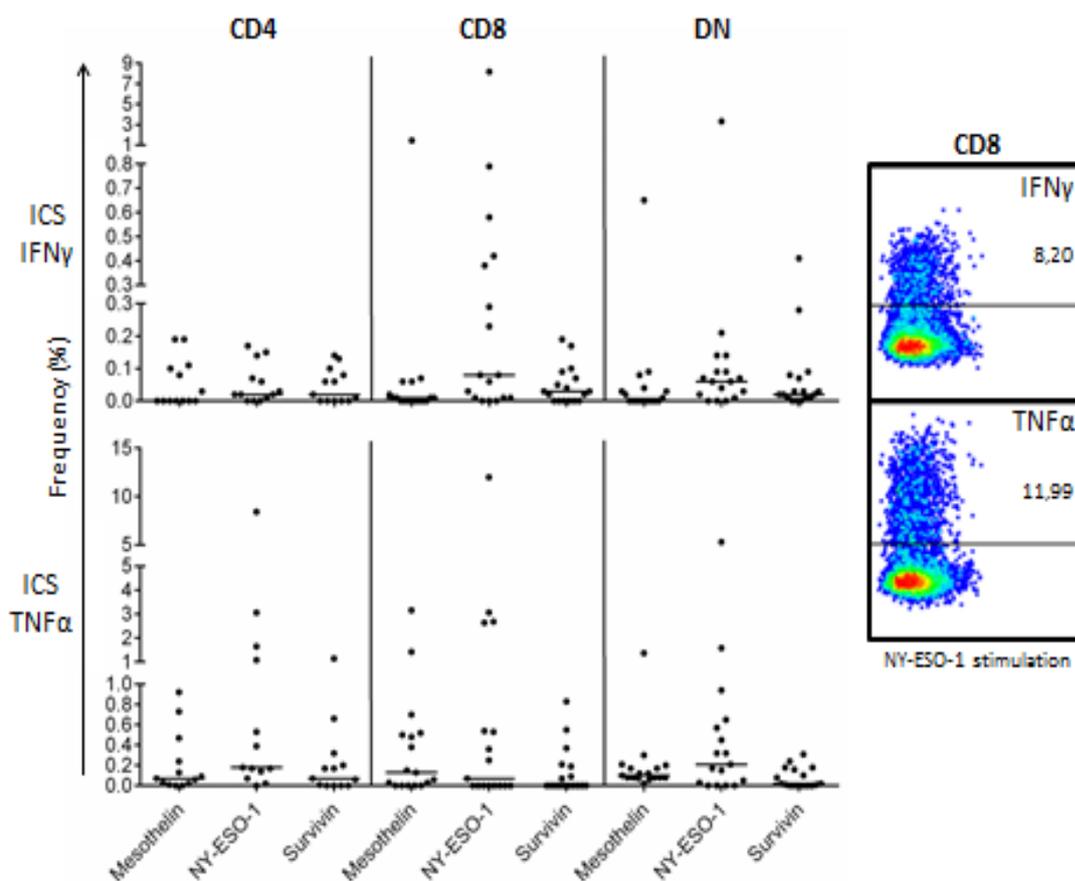


Figure 5. Frequency of TAA-reactive T cells defined by IFN- γ and TNF- α intracellular cytokine staining in TILs from patients with pancreatic cancer. Right panel: ICS analysis of IFN- γ and TNF- α production after NY-ESO-1 stimulation in CD8⁺ and T cells from Panc 6 TILs.

TILs could recognize and kill autologous tumor cells. The Panc9 TILs (a V β 13.2 CD8⁺ T cell line) prompted IFN- γ production against autologous tumor cells (Figure 6A), which were able to be blocked by anti-MHC-I antibody, whereas not by MHC-II. Panc17 TILs were found to be able to kill autologous tumor cell line through a standard chromium 51 release assay (Figure 6B).

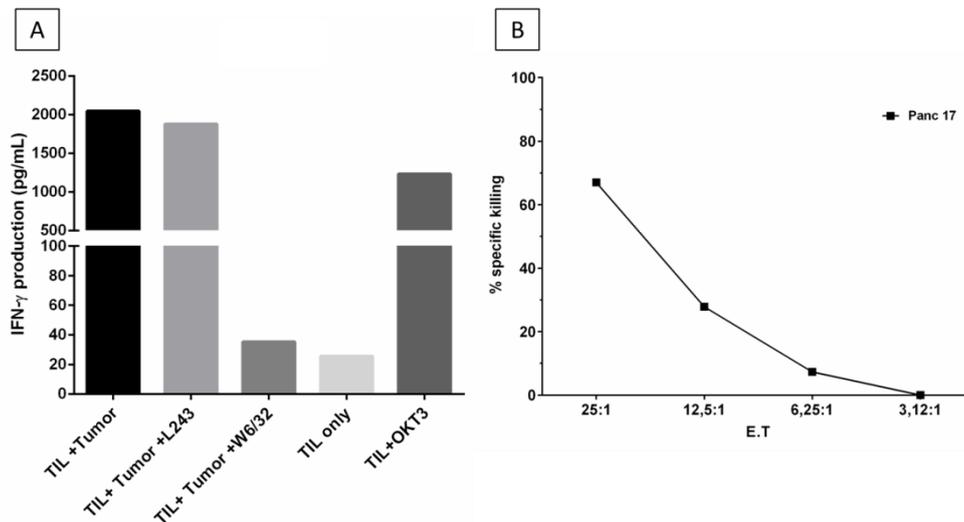


Figure 6. (A) IFN- γ production in TILs after co-culture with autologous tumor cells (Panc 9); (B) Recognition of autologous tumor cell line in Panc17 (PanTT26) TILs by a chromium 51 release assay.

Taken together, our results presented in PAPER I show that: Pancreatic cancer TILs with a Th1 profile can be reliably expanded; Individual TIL cell lines can recognize commonly shared TAAs and response to autologous tumor cells; Preferential expansion of TCR V β families in TILs has been associated with antigen-driven expansion^[93]. Taken together this all suggests that IL-2/IL-15/IL-21-driven expansion of pancreatic cancer TILs indicates a feasible source for cells to be used for cancer immunotherapy.

4.2 PAPER II

“Neoepitopes as targets for Tumor-infiltrating Lymphocytes from Patients with Pancreatic Cancer”

Neoantigens are host proteins harboring point mutations that are disease-associated i.e. cancer, from which peptide sequences containing specific mutated amino acids, namely neoepitopes can be derived. These structures reflect ‘private mutations’ that are unique from patient to patient, thus representing an individually tailored and highly specific antigen signature ^[94]. As such, neoantigen/neoepitope-directed approaches are clinically relevant for refining T cell immunotherapies and cancer vaccines. Studies have previously shown that extraordinary clinical responses were induced by specialized T cell-based therapies selecting private mutations of patients with metastatic cancers as targets ^[95-97]. The identification of neoepitopes described in our studies employed a highly sophisticated method, which involves whole exome sequencing using genomic DNA from patients’ tumor material, followed by *in silico* processing of data and application of bioinformatic prediction algorithms. In PAPER I, we successfully isolated and expanded TILs from human pancreatic cancer tumor with a cocktail of the gamma chain cytokines IL-2, IL-15 and IL-21. In addition, we demonstrated that these TILs could recognize and kill autologous pancreatic cancer tumor cells. Thus, analysis of the immune response of TILs to patient-specific neoepitopes potentially expressed by autologous tumor cells – based on cytokine (IFN- γ and/or TNF- α) response and/or cytotoxic activity (CD107a induction) – would enable a thorough screening platform to identify legitimate immunogenic targets in human pancreatic cancer, an idea which led to the studies reported in PAPER II.

We have established TILs and tumor cell lines from patient Panc17. Since IFN- γ production by TILs was negatively reversed by adding the W6/32 (anti-HLA class I) antibody to the TIL:tumor cell co-culture, the anti-tumor effect of Panc17 TILs thus reflected a dominant HLA class I-restricted, CD8⁺ T cell-mediated activity. Accordingly, we observed that the composition of TILs was approximately 59% of the CD8⁺ T cells and 22% CD4⁺ T cells – the number of CD8⁺ T cells almost 3 times that of the CD4⁺ T cells. We also observed that stimulating the TILs with the autologous tumor cells enriched for CD8⁺ T cells (almost 100%), which displayed strong immune responses (IFN- γ production) directed against mutated host peptides, including one neoepitope derived from the K-RAS protein (KLVVVGAVGVGKSAL). Thus, we concluded that it is highly likely that TILs from patient Panc17 contained several neoepitope-specific T cells that can be expanded and

activated with the IL-2, -15 and -21 cytokine cocktail as well as autologous tumor cells.

From patient Panc39, we were able to isolate a CD4⁺ TIL clone recognizing a neoepitope (GLLRYWRTERLF) derived from an aquaporin (AQP)-like molecule (K7N7A8 protein product). In fact, the AQP2 gene mutations, which result in aberrantly expressed protein products, have been related to the onset of autosomal nephrogenic diabetes insipidus ^[98]. Even though weak binding of potentially pathogenic IgG molecules (pertinent to the neurodegenerative disease neuromyelitis optica) has been demonstrated to be induced by site-specific AQP4 mutations ^[99], there is still no report about clinically relevant immune responses to mutant AQP proteins. As far as we know, the study presented in PAPER II is the first to describe the productive anti-tumor responses directed against a mutant epitope derived from an AQP-like molecule, and furthermore mediated by CD4⁺ TILs. Importantly, we also noticed that the CD4⁺ TIL clone reactivity to the GLLRYWRTERLF neoepitope was dose-dependent, and was amenable to blockade by the L243 antibody (anti-HLA-DR), but not by the W6/32 antibody (anti-HLA-A/B/C), further affirming that GLLRYWRTERLF involved a nominal HLA class II neoepitope.

TILs and PBMCs derived from patient Panc77 were compared for T-cell recognition pattern of mutant targets, defined by IFN- γ production. Six mutant epitopes recognized by TILs and PBMCs, while other neoepitopes showed exclusive recognition by either TILs (nine mutated peptides) or PBMCs (five mutated peptides). An interesting TIL-specific neoepitope target was derived from PPP1R15B (Protein Phosphatase 1 Regulatory Subunit 15B), as part of an enzyme that dephosphorylates the eukaryotic translation initiation factor 2A when cells are under stress and has been reported to exhibit pro-oncogenic characteristics in breast cancer ^[100].

Identifying and characterizing neoepitope-specific cellular immune responses presents a revolutionary therapy platform for treating patients with advanced cancers. Our aim therefore is to make the findings presented in this thesis (thus, scientific articles) a clinical reality – developing the next generation T-cell based immunotherapies for patients with pancreatic cancer, even including individuals ineligible for surgery.

4.3 PAPER III

“Peptide microarray identifies novel CMV and EBV targets recognized by tumor-infiltrating T lymphocytes and antibodies in pancreatic cancer and glioma”

As previously mentioned, TILs perform a key role in regulating immune responses in the tumor microenvironment, conversely influencing the clinical outcome in patients with solid cancers e.g. melanoma, pancreatic cancer and glioma ^[101-105]. Even though T cells are the major component of TILs, a substantial and noteworthy proportion of these cells also comprise tumor-infiltrating B cells (TIB). In essence, TIBs, together with their T-cell counterparts, actively shape and participate in mediating intratumoral immune responses ^[41, 106]. Besides of processing and presenting antigens to organize local cellular immune response, TIBs are capable of producing effector cytokines such as IFN- γ , TNF- α and IL-10 ^[41]. It is also important that TIBs – which are differentiated into plasma cells – secrete antibodies against TAAs or persistent viral antigens e.g. those derived from cytomegalovirus (CMV) and Epstein-Barr virus (EBV).

Intact immune responses against CMV and EBV, and the ability to effectively contain infection without causing disease manifestation serve as a crucial indication of the immunophysiological fitness of an individual ^[107-109]. Targeted immune responses to these pathogens seem to link with positive clinical outcomes in patients with cancer. Even there already have large details about human T-cell reactivity to CMV and EBV proteins, the IgG responses therein still have not been thoroughly investigated for solid cancers, thus as the background for the study presented in PAPER III.

In this paper, we showed for the first time that anti-CMV and anti-EBV humoral immune responses exist in the tumor microenvironment, defined by TIB-derived IgG reactivity to viral epitopes *in vitro*. This was performed on a high content peptide microarray chip embedded 12-mer peptides with 11-mer overlapping sequences spanning entire proteins from CMV (pp65) and EBV (EBNA-1a, EBNA-3, BMLF1, LMP2) – which are major antigens from these pathogens. We also confirmed that TIB-IgG responses were more diverse than serum IgG response, whereas serum IgG response was usually more restricted hence focused. TIB-derived IgG molecules can broadly recognize viral targets, which may therefore represent a unique imprint of humoral immune responses in the tumor microenvironment.

There might also be a role for virus (CMV/EBV)-specific T cells in human cancer. A report earlier this year showed in mouse model of melanoma that CMV-specific T cells had capacity of infiltrating tumor lesions without loss of functionality, in spite of PD-1 expression ^[110]. T-cell epitopes are possibly recognized also by antibodies and provide a valuable platform for diagnosis, e.g. formerly demonstrated in the context of NY-ESO-1-mediated immune responses in patients with cancer ^[90, 111, 112]. In line with this, we also found that the TILs from the same patients showed reactivity (IFN- γ production) to CMV and EBV peptides that were at first recognized by the TIB-derived IgG. Interestingly, one particular CMV peptide (CEDVPSGKLFMH) was recognized by TIB-IgG, TIL, PBMCs and serum-IgG from a patient with pancreatic cancer (PanTT32).

Antigen-specific T-cell responses and therapy thereof can induce tumor regression and may improve the survival of patients with advanced cancer ^[23, 33, 113]. In accord, it is highly plausible that virus-specific adaptive immune cells presenting in cancer lesions i.e. TIL and TIB play a biologically relevant role in mediating anti-tumor responses. Our data showed that the immune responses directed against CMV and EBV antigens in cancer are heterogeneous, because of their both patient- and disease-specific. This antiviral immune response could potentially be exploited as yet another novel immunotherapeutic strategy to treat advanced cancers. Indeed, administration of a CMV pp65-based cellular vaccine to patients with glioblastoma was recently shown to contribute to increased survival ^[114].

4.4 PAPER IV

“Prediction of improved survival in patients with pancreatic cancer via IL-21 enhanced detection of mesothelin epitope-reactive T-cell responses”

Novel immunogenic targets as well as biomarkers which can regulate biologically and clinically relevant immune responses in while enabling better diagnosis of cancer are now urgently needed. Mesothelin is a cell surface-bound TAA and expressed by mesothelial cells lining various internal organs, such as the pericardium, peritoneum, pancreas, and pleura ^[115]. Mesothelin has been identified to be overexpressed in a number of cancers including mesothelioma, ovarian cancer, colorectal cancer, lung cancer and pancreatic cancer, and usually in its native form ^[115-119]. Furthermore, the expression of mesothelin has been reported in almost all pancreatic ductal adenocarcinomas ^[120-122]. Structurally, mesothelin consists of: i) the megakaryocyte-potentiating factor (MPF), which is cleaved by furin after being translocated to the cell surface and shed into systemic circulation, and ii) the cell surface-bound GPI-anchored mature mesothelin component with overexpression in tumor tissues ^[115]. We therefore asked the question whether mature mesothelin possess a prognostic role and how this might be related to immune responses and survival in pancreatic cancer.

PAPER IV is the first report to show that IL-21 conditioning promotes mesothelin-specific T cell responses, based on IFN- γ production in PBMCs. Furthermore, this enhanced cellular immune response correlates with the increased survival of patients with pancreatic cancer who were also surgically treated. A crucial point presented in this study is that only the mature mesothelin component but not the shed MPF component of full-length mesothelin is associated with survival of the patients with pancreatic cancer. In particular, immune responses to the immunodominant MQEALSGTPCLLGPG peptide (mature mesothelin, aa 601-615) is a single target that is independently linked to better patient survival profile. We therefore concluded that mesothelin-directed IFN- γ responses provide a clinically relevant immunological readout associated with improved clinical outcome in patients with pancreatic cancer.

IL-21, which belongs to the family of gamma chain cytokines, can maintain the function and survival of memory CD8+ T cells, as well as aiding their proliferation *in vitro* after isolation from clinical samples sourced by patients with melanoma or pancreatic cancer ^[123]. The IL-21/IL-21 receptor pathway in T cells also facilitates better immunological control of viral and bacterial infection ^[124]. It was shown that IL-21

inhibited the expression of forkhead box P3 (FoxP3), the cardinal transcription factor linked with Tregs in conjunction after encountering cancer cells – just like TGF- β blockade ^[125]. Our previous report demonstrated that the combination of IL-2, IL-15 and IL-21 increases the population of central memory TILs from pancreatic cancer and glioma, following *ex vivo* expansion ^{[26] [125]}.

Mesothelin epitopes have been previously described to be recognized by CD8+ T cells ^[54, 126, 127]. In PAPER IV, we established a previously unknown link between the mesothelin₆₀₁₋₆₁₅ epitope (MQEALSGTPCLLGPG) and improved survival of patients with pancreatic cancer but in the presence of IL-21 conditioning. This peptide may therefore have implications for rational vaccine design, T-cell expansions as well as a novel cancer biomarker.

5 CONCLUSION AND FUTURE POSSIBILITIES

The work of this thesis showed a new protocol to culture tumor-infiltrating lymphocytes using IL-2, IL15 and IL-21 cytokines. Cells presented low exhaustion markers after expansion and could recognize and kill autologous tumor cells. We identified targets of T cells and TIBs. We also detected a specific immune response to a single peptide p41 mesothelin₆₀₁₋₆₁₅ epitope (MQEALSGTPCLLGPG), which is associated with the improved patient survival.

The future work following the study reported in Paper I and II would be to obtain the TCR for tumor mutation epitopes, such as the TCR for aquaporin 1-like protein (K7N7A8) in adoptive immunotherapy. We found that V β 2 and V β 9 in PanTT39 could recognize autologous tumor cells; therefore, the next step would be to identify the location of antigen-specific T cells in the tissue by TCR V β immunostaining.

In paper III, we found that TIB and TIL recognize certain EBV and CMV peptides, which are different from the peptides recognized by serum antibodies. In the future, we may choose to sequence BCR from the TIB and TCR from the TIL as well to identify EBV and CMV specific BCR/TCR for engineering EBV and/or CMV specific CAR T cells for cancer therapy. The identified peptides that TIBs and TILs recognize can be used as candidates for anti-cancer vaccines.

In Paper IV, we identified the peptide 41 (P41), mesothelin₆₀₁₋₆₁₅ epitope (MQEALSGTPCLLGPG), which is associated with improved patient survival. We therefore would like to measure the serum antibody against the peptide and analyze the relationship between antibody levels and patient survival, and use this peptide for pancreatic cancer vaccine. By culturing PBMCs with p41, we could obtain specific T cells for p41, selecting those T cells by flow cytometry, and sequence the TCR in order to engineer p41 specific T cells which could be used for targeting mesothelin-positive cancers.

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In the last few years, we received and processed tumor tissues and/or blood from approximately 300 patients. The median survival time of patients with pancreatic cancer is very short. One patient passed away, just 3 days after donating blood. I would like to start this section by thanking all the patients who have donated samples. Thank you for your great contribution for this study, and without you, my project would never have started. I hope and believe this project will help the patients with pancreatic cancer, even with other cancers.

When I arrived in Sweden alone, I spoke very poor English and knew little about cancer biology and immunology. **Prof. Markus Maeurer**, my main supervisor, paid a lot of patience and great help for my English, and gave me the courage to study and work in this group. With your support, I have cultured a lot of samples and many different types of cells. You are so active a person; so many times we are discussing about science after 1am in the middle of the night.

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