Functional characterisation of tumour-specific T cell responses in pancreatic cancer

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I, Yuan Chen, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Abstract

Background and aims:

Pancreatic cancer (PC) has a poor prognosis and effective diagnostic tools and therapies are currently unavailable. This project has explored the role of T cell responses in PC patients as a potential therapy.

Methods:

Serum mesothelin (MSLN) levels were tested in patients with pancreatic diseases. CD4+ and CD8+ T cell responses against full-length overlapping MSLN peptide pools were identified. CEA691, together with pre-chosen MSLN547 and WT1-126 were used to stimulate T cell lines from PC patients. Antigen-specific function and phenotype were characterised in the ex-vivo expanded T cell lines after repeated Ag exposure. Further experiments used inhibitory receptor blockade to augment the function of T cells isolated from cancer patients.

Results:

Soluble MSLN was elevated in PC patients compared to normal controls, but could not distinguish the malignant from benign pancreatic disease. MSLN-specific CD4+ T cell responses were significantly increased in PC patients compared to controls, indicating an expansion of MSLN-specific CD4+ T cell in PC patients, and new epitopes were identified. It was possible to generate CEA691, WT126 and MSLN547-specific HLA-A*02 restricted T cell lines from PC patients. The ex vivo expanded CEA691-specific T cells demonstrated Ag-specific cytotoxicity and were able to recognize and kill HLA-A2+ CEA+ pancreatic cell lines in vitro. Blockade of PD-1 and TIM-3 further enhanced T cell function in vitro.

Conclusion:

We found that tumour associated antigen (TAA)-specific T cells were identifiable in the peripheral repertoire of patients with pancreatic cancer, and the function of these cells were preserved in some cases. Such antigen-specific function and cytotoxicity was more common in relatively early stages of the disease than the terminal stage, and PD-L1 blockade further improved the responses, suggesting that TAA-specific T cells together with checkpoint inhibition could be potential strategies for the treatment of pancreatic cancer.
AKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Ab: Antibody
Ag: Antigen
ALL: Acute lymphocytic leukaemia
AML: Acute myeloid leukaemia
AP-1: Activator protein-1
APC: Antigen-presenting cell
ATC: Adoptive T cell
BL: Burkitt’s lymphoma
BMI: Body mass index
CAR: Chimeric antigen receptor
CBA: Cytometric bead array
CEA: Carcinoembryonic antigen
CFSE: Carboxy fluorescein succinimidyl ester
CK: Cytokeratin
CLL: Chronic lymphocytic leukaemia
CML: Chronic myeloid leukaemia
CMV: Cytomegalovirus
CR: Complete regression
CRISPR: Clustered regularly-interspaced short palindromic repeats
CRS: Cytokine release syndrome
CT: Computerised tomography
CTL: Cytotoxic T cell
CTLA-4: Cytotoxic T-lymphocyte-associated protein 4
CXCL: Chemokine (C-X-C motif) ligand
DC: Dendritic cell
DMEM: Dulbecco's modified eagle's medium
DMSO: Dimethylsulfoxide
DNA: Deoxyribonucleic acid
EBV: Epstein Barr virus
EGF: Epidermal growth factor
ELISA: Enzyme-linked immunosorbent assay
ERCP: Endoscopic retrograde cholangiopancreatography
E:T: Effector : target
ETS: Environmental tobacco smoke
EUS: Endoscopic ultrasonography
Fab: Antigen-binding fragment
FACS: Fluorescence-activated cell sorting
FBS: Fetal bovine serum
FCS: Fetal calf serum
FNA: Fine needle aspiration
FPC: Familial pancreatic cancer
FSC: Forward scatter
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GVHD: Graft-vs-host disease
GVL: Graft vs leukaemia effect
haTCR: High affinity TCR
HLA: Human leukocyte antigen
HPV: Human papillomavirus
HSD: Honest significant difference
hTERT: Human telomerase reverse transcriptase
IBD: Inflammatory bowel disease
ICAM-1: Intercellular adhesion molecule 1
ICOS: Inducible costimulator
IFN-γ: Interferon-gamma

ITIM: Immunoreceptor tyrosine-based inhibitory motif

ITSM: Immunoreceptor tyrosine-based switch motif

KO: Knockout

LAG-3: Lymphocyte activation gene 3

LFA-3: Lymphocyte function-associated antigen 3

LMP: Latent membrane proteins

MART-1: Melanoma antigen recognized by T cells 1

MDS: Myelodysplastic Syndromes

MHC: Major histocompatibility complex

MIF: Migration inhibitory factor

MPF: Megakaryocyte-potentiating factor

MSLN: Mesothelin

NCCN: National Comprehensive Cancer Network

NF-κB: Nuclear factor-κB

NGM: Normal growth medium

NK cells: Natural killer cells

NKT cell: Natural killer T cell

NPC: Nasopharyngeal carcinoma

PBS: Phosphate-buffered saline

PC: Pancreatic cancer

PCR: Polymerase chain reaction

PD-1: Programmed death 1

PDAC: Pancreatic ductal adenocarcinoma

PD-L1: Programmed death-ligand 1

PBMC: Peripheral blood mononuclear cells

PDGF: Platelet-derived growth factor
PET: Positron emission tomography
PK: Pyruvate kinase
PMA: Phorbol myristate acetate
PR: Partial response
RCC: Renal cell carcinoma
RNA: Ribonucleic acid
ROS: Reactive oxygen species
RPMI: Roswell Park Memorial Institute
RR: Relative risk
SAGE: Serial analysis of gene expression
SPARC: Secreted protein, acidic, cysteine-rich
SSC: Sideward scatter
STAT3: Signal transducer and activator of transcription 3
TAA: Tumour associated antigen
TALENs: Transcription activator-like effector nucleases
TAMs: Tumour associated macrophages
TAP: Transporter associated with antigen processing
TCM: Central memory T cell
TCR: T cell receptor
TEM: Effector memory T cell
TGF-β: Transforming growth factor beta
Th1: Type I helper T cells
TILs: Tumour-infiltrating lymphocytes
TIM-3: T-cell/transmembrane immunoglobulin and mucin domain 3
TLR: Toll-like receptor
TLS: Tumour lysis syndrome
TNF-α: Tumour necrosis factor-alpha
TNFR: Tumour necrosis factor receptor
TNM: Tumour-node-metastasis
TRAs: Tissue restricted antigens
TRAIL: TNF-related apoptosis-inducing ligand
Tregs: Regulatory T cells
TRICOM: TRIad of COstimulatory Molecules
TSA: Tumour specific antigen
VEGF: Vascular endothelial growth factor
WT1: Wilms’ tumour antigen 1
Chapter 1

Introduction
1.1 Pancreatic Cancer

1.1.1 Pancreas: Anatomy and Histology

The pancreas is located posterior to the stomach, and lies horizontally across the posterior abdominal wall. It is divided into four parts, namely, head, neck, body, and tail of pancreas. Among them, the head rests in the concavity of the duodenum, and includes uncinate process that surrounds the superior mesenteric artery and vein. The tail of the pancreas attaches to the hilum of the spleen by the splenocolic ligament, and it is the only intraperitoneal part of pancreas. The main pancreatic duct of Wirsung courses from the tail of the pancreas to the head, where it joins the common bile duct and forms major duodenal papilla or ampulla of Vater. The sphincter of Oddi surrounds the channels of duct at the ampulla of Vater (Fig. 1.1) (1).

The head of the pancreas receives its blood supply from the anterior and posterior pancreaticoduodenal arcades, which are formed by the anastomosis of branches of celiac trunk and the superior mesenteric artery, and it has shared blood supply with the duodenum. The rest parts of the gland mainly receive blood supply from branches of the splenic artery. In addition, blood of the pancreas drains into the portal vein via the superior mesenteric vein and splenic vein (2).

![Figure 1.1 Anatomy of pancreas and related ductal system](image-url) (3). The pancreatic duct travels along from the tail to the head and joints common bile duct, finally reaching duodenum through papilla.
The pancreas consists of both endocrine and exocrine glands, composed of 80% exocrine tissue, 18% ductular system, and 2% endocrine tissue (2). The endocrine portion is a lobulated gland with secretory acinar cells, which produce enzymes such as proteases, lipases, nucleases and amylases and function in digesting proteins, lipids, nucleic acids and carbohydrates, as well as centroacinar and ductal cells, which secrete bicarbonate that can neutralizes HCl and deactivates pepsin. Enzymes and fluids are drained by interlobular ducts and main duct, and ultimately reach the duodenum. To avoid self-digestion, the draining ducts compass thick dense collagenous walls and intercellular tight junctions (2). The endocrine section contains islets of Langerhans, which are dispersed throughout the pancreas with relative concentration in the tail. The islets are made up by approximate 75%–80% of beta cells, the producer of insulin, about 15% of alpha cells, which can release glucagon, around 5% of Delta cells, taking charge in secreting somatostatin and a few PP-cells. Other secretory cells in the islets include enterochromaffin cells, which yield 5-hydroxytryptamine, and pancreatic polypeptide cells, producing pancreatic polypeptide (2).

1.1.2 General Introduction of Pancreatic Cancer

Pancreatic cancer (PC) is a worldwide health problem. Limitations in current diagnostic and therapeutic approaches confer a very poor prognosis. Despite improvements in cancer management over the last decade, the mortality of pancreatic cancer continues to increase, while the death rates of most other cancers decline (4). This section describes PC in more detail, including risk factors, and updated diagnostic and therapeutic strategies.

1.1.3 Epidemiology

Pancreatic cancer is a significant cause of tumour-related death worldwide, which attributes to 7% cancer mortality globally (5). In recent epidemiologic studies, pancreatic cancer ranked the 10th commonest cancer in men and the 11th in women. Moreover, this disease is regarded as the 4th leading cause of cancer-linked mortality in both USA (according to the data from 2004 to 2008) (6) and in Europe (records from 2007 to 2011) (7), followed by lung, colorectal and prostate (in men)/breast (in women) cancers. In the USA, among the 44,000 newly diagnosed PC patients annually, together with 37,390 estimated deaths (6), only 15-20% of patients were eligible to receive potentially curative treatment. However, even patients undergoing surgery with curative intent carried a cumulative lifetime recurrence risk up to 85% (8). There has been little improvement in overall survival over the last 30 years,
with an 5-year survival rate of 6% in 2008, compared to 2% in 1975 (6). In UK, this rate is around 5% (9). Most PC patients die within one year of diagnosis. For advanced disease, the life expectancy is 4.2 to 4.5 months for metastatic cases (10), and 6–10 months for the locally advanced ones (11).

1.1.4 Risk Factors

Not all individuals with identified risk factors will develop the disease. Advancing age is a major risk factor for pancreatic cancer, with the peak incidence in 8\textsuperscript{th} and 9\textsuperscript{th} decades, and a median age of diagnosis of 69 years to 72 years (the media ages were varied in different studies) (12, 13). Further, the black population had a higher frequency of PC, followed by the Caucasian population, then Asians (13).

1.1.4.1 External Risk Factors

Smoking is well demonstrated to be related to 20-25% of all pancreatic carcinoma cases (14). A meta-analysis of 82 published reports (between 1950 and 2007) investigated the link between smoking and pancreatic cancer, and suggested that tobacco consumers had a 75% higher risk of developing PC than non-smokers (14).

The link between chronic pancreatitis and pancreatic cancer has been studied for years. The lag period from diagnosis of chronic pancreatitis to initiation of pancreatic cancer is typically 10 to 20 years, and the chronic pancreatitis was demonstrated a significant risk factor for PC, with the relative risk (RR) ranging between 2.9 to 74.1, in different studies (15). There is also evidence to support an association between diabetes and pancreatic cancer. This risk was highest between 2-8 years after diagnosis of diabetes (16, 17).

1.1.4.2 Family History and Genetic Factors

Some pancreatic cancer patients have relevant family history, or cancer develops as a result of germ-line gene alteration, where the genetic factors may play a more important role in the initiation of pancreatic cancer than environmental factors.

1.1.4.2.1 Family History and Pancreatic Cancer

A family history of pancreatic cancer almost 2 times augments the risk of developing PC (18), with approximately 7-10% of pancreatic cancer patients having a positive family history (19). Familial pancreatic cancer (FPC) refers to families in which 2 or more first-degree
relatives have exocrine pancreatic tumours, in the absence of other tumour syndromes (e.g., Peutz-Jeghers syndrome) (20). An individual in an affected family has a 9-fold augmented risk of pancreatic cancer compared to the people in unaffected families, and this increases to 32-fold if at least 3 family members are diagnosed with pancreatic cancer (4). BRCA2, PALB2 and ATM are the most common oncogenes carried by FPC patients. Table 1.1 shows other well-studied pancreatic cancer-associated disease syndromes. This table also lists the major gene mutations and the relative risk of developing a pancreatic cancer by the age of 70 years (20).

Table 1.1 Settings of an inherited predisposition to pancreatic cancer. The table is taken from Familial pancreatic cancer – current knowledge (20).

<table>
<thead>
<tr>
<th>Setting of inherited pancreatic cancer</th>
<th>Gene</th>
<th>Risk of pancreatic cancer until 70 years (%)</th>
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<tr>
<td><strong>Hereditary tumour predisposition syndromes</strong></td>
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<tr>
<td>Peutz-Jeghers syndrome</td>
<td>LKB1</td>
<td>36</td>
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<tr>
<td>FAMMM syndrome</td>
<td>CDKN2A, CDK4</td>
<td>17</td>
</tr>
<tr>
<td>HBOC</td>
<td>BRCA1, BRCA2</td>
<td>3–8</td>
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<tr>
<td>Li-Fraumeni syndrome</td>
<td>TP53</td>
<td>&lt;5</td>
</tr>
<tr>
<td>HNPCC</td>
<td>MLH1, MSH2</td>
<td>&lt;5</td>
</tr>
<tr>
<td>FAP</td>
<td>APC</td>
<td>&lt;5</td>
</tr>
<tr>
<td><strong>Syndromes with chronic inflammation of the pancreas</strong></td>
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<td></td>
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<tr>
<td>Hereditary pancreatitis</td>
<td>PRSS1, SPINK1</td>
<td>40</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>CFTR</td>
<td>&lt;5</td>
</tr>
<tr>
<td><strong>FPC syndrome</strong></td>
<td>BRCA2, PALB2, ATM</td>
<td>2 FDR: 8–12%; &gt;3 FDR: 16–38%</td>
</tr>
</tbody>
</table>

Abbreviations: FAMMM, familial atypical mole melanoma; FAP, familial adenomatous polyposis; FDR, affected first-degree relative; FPC, familial pancreatic cancer; HBOC, hereditary breast-ovarian cancer; HNPCC, hereditary nonpolyposis colorectal carcinoma.

1.1.4.2.2 Genetic Mutations in Pancreatic Cancer

Spontaneous genetic mutations can occur in somatic cells in healthy individuals and may accumulate resulting in clonal expansion, i.e. the development of cancer (21). The well described hallmarks of malignancy are associated with the acquirement of multiple genetic mutations. These hallmarks include activation of proliferative signals (e.g. elevating RAS and MYC), inactivation of growth suppressors (e.g. loss of RB and P53 functions), resisting apoptosis (e.g. mutations in Bcl-2 families), acquisition of replicative immortality (e.g. changing functions of telomerase), angiogenesis (e.g. increasing VEGF gene expression),
invasion and metastasis (e.g., downregulation of E-cadherin expression), as well as immunosuppression and tumour-promoting inflammation (22).

Current whole-genome sequencing and copy number variation (CNV) analyses allow the systematic screening of cancer genomes and the discovery of new cancer genes. A recent paper reviewed the mutational burden of 20 different cancers, which showed that melanoma and lung cancer carried the highest frequency of mutations, whilst acute myeloid leukaemia (AML) and thyroid cancer had the lowest mutation burdens (23). It has recently been suggested that tumours with high mutational loads may generate increased numbers of neoantigens, and would be expected to have a higher likelihood of neoantigen-specific T cell reactivity, with both specificity and low toxicity that can underlie the personalized immunotherapy (23). Also, checkpoint blockade can restore the function of such neoantigen-specific T cells; therefore, cancers carrying high mutational loads, such as melanoma and lung cancer, are more likely to be sensitive to checkpoint therapies (24).

The mutational load of pancreatic cancer is in-between these two extremes, with an average about 100 coding mutations identified per tumour. Among these mutations, KRAS, TP53, SMAD4, MLL2 and CDKN2A are the most common mutations in pancreatic adenocarcinoma (21). A previous study also documented the percentage of a few genetic mutations in pancreatic cancers, which are listed in the table 1.2 below (25).

**Table 1.2. Genetic mutation in pancreatic cancer.** (cited from: Pancreatic Cancer: Epidemiology, Genetics, and Approaches to Screening (25)).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Percentage of PCs</th>
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<tr>
<td>K-ras</td>
<td>12p13</td>
<td>&gt;90%</td>
</tr>
<tr>
<td>HER2/neu</td>
<td>17q21</td>
<td>20-25%</td>
</tr>
<tr>
<td>P16INK4/CDKN2/MTSI</td>
<td>9p21</td>
<td>80%</td>
</tr>
<tr>
<td>P53</td>
<td>17p13</td>
<td>50-75%</td>
</tr>
<tr>
<td>CDPC4/SMAD4</td>
<td>18q21</td>
<td>50%</td>
</tr>
<tr>
<td>BRCA2</td>
<td>13q12</td>
<td>7%</td>
</tr>
</tbody>
</table>

Apart from these known oncogenes and tumour suppressor genes, the existence of other commonly mutated genes, such as ROBO1, ROBO2, SLIT2 and RNF43, has been reported in up to 10% PC cases, together with a long list of infrequently mutated genes (26). Due to this diversity, it may be difficult to treat PC patients with uniform and unselected strategies (27).
1.1.5 Pancreatic Cancer Stroma

The stellate cells associated with pancreatic adenocarcinoma help generate a collagens, laminin, and fibronectin-rich stroma, termed desmoplasia (28), which may interfere with drug delivery and cause hypoxia and hypoperfusion (29). In tumour microenvironments, the stellate cells also secrete VEGF, various chemokines, and SPARC (secreted protein, acidic, cysteine-rich), resulting in an immune suppressive environment favouring tumour growth and promoting angiogenesis and metastasis (30, 31). In other words, the stroma may play a part in tumour malignancy and medicine residence, contributing to poor prognosis of PC.

1.1.6 Pathology Classification of Pancreatic Cancers

Pancreatic cancer can originate from both the exocrine and endocrine sections of the pancreas. Cancers originating from endocrine pancreas are called neuroendocrine tumours, and constitute only 1.3% of all pancreatic cancers (32). Thus, the majority of the pancreatic cancers arise from the exocrine pancreas. The following table (Table 1.3) gives information about the histological classification of exocrine pancreatic cancer according to WHO published histological classification criteria, and briefly divided the cancers to solid and cystic categories. Although various types are observed, pancreatic adenocarcinoma and its variants are the dominant forms of pancreatic cancer (more than 90%) (33).

<table>
<thead>
<tr>
<th>Type</th>
<th>Frequency</th>
<th>Histological</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solid tumours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ductal adenocarcinoma and</td>
<td>90%</td>
<td>Most: Mucus-secreting columnar cells with tubular and duct-like structures</td>
</tr>
<tr>
<td>variants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acinar cell carcinoma</td>
<td>1%</td>
<td>Involving pancreatic enzyme-producing cells</td>
</tr>
<tr>
<td>Pancreatoblastoma</td>
<td>&lt;1%</td>
<td>Epithelial monomorphic cells with occasional mesenchymal substance</td>
</tr>
<tr>
<td>Endocrine tumours</td>
<td>2%</td>
<td>Origin from the cells of hormonal and nervous systems</td>
</tr>
<tr>
<td>Noepithelial tumours</td>
<td>&lt;1%</td>
<td>Epithelial original</td>
</tr>
<tr>
<td>Cystic tumours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraductal papillary</td>
<td>2%</td>
<td>Papillary proliferation of columnar mucus-secreting epithelial cells</td>
</tr>
<tr>
<td>mucinous neoplasm (IPMNs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucinous cystic neoplasm</td>
<td>1%</td>
<td>Cysts containing mucus-secreting columnar epithelial cells, occasionally with</td>
</tr>
<tr>
<td></td>
<td></td>
<td>endocrine, Paneth, or goblet cells.</td>
</tr>
<tr>
<td>Serous cystic neoplasm</td>
<td>1%</td>
<td>Flattened or cuboidal cell with round and regular nuclei and cytoplasm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PAS+ glycogen</td>
</tr>
<tr>
<td>Solid pseudopapillary</td>
<td>&lt;1%</td>
<td></td>
</tr>
<tr>
<td>neoplasm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other cystic tumours</td>
<td>1%</td>
<td></td>
</tr>
</tbody>
</table>

PAS: Periodic acid-Schiff, a special stain in the histology identification.
1.1.7 Staging of Pancreatic Cancer

According to the tumour-node-metastasis (TNM) classification, pancreatic cancer can be staged, usually based on results of computerized tomography (CT). The below tables show the definitions of different stages. This staging system can help us to evaluate the resectability and prognosis of PC, where T4 and M1 tumours are considered inoperable, while T3N0M0 and TxN1M0 tumours are recognized as being locally invasive and are potential resectable (30, 34).

Table 1.4. Clinical stages of pancreatic cancer

<table>
<thead>
<tr>
<th>T: Primary Tumour</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TX</td>
<td>Primary tumour cannot be assessed</td>
</tr>
<tr>
<td>T0</td>
<td>No evidence of primary tumour</td>
</tr>
<tr>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>T1</td>
<td>Tumour limited to the pancreas, greatest dimension ( \leq ) 2 cm</td>
</tr>
<tr>
<td>T2</td>
<td>Tumour limited to the pancreas, greatest dimension &gt; 2 cm</td>
</tr>
<tr>
<td>T3</td>
<td>Tumour extends directly into any of the following: duodenum, bile duct, peripancreatic tissues</td>
</tr>
<tr>
<td>T4</td>
<td>Tumour extends directly into any of the following: stomach, spleen, colon, adjacent large vessels</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>N: Regional Lymph Nodes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
<td>Regional lymph nodes cannot be assessed</td>
</tr>
<tr>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>N1</td>
<td>Regional lymph node metastasis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>M: Distant Metastasis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>MX</td>
<td>Distant metastasis cannot be assessed</td>
</tr>
<tr>
<td>M0</td>
<td>No distant metastasis</td>
</tr>
<tr>
<td>M1</td>
<td>Distant metastasis</td>
</tr>
</tbody>
</table>

**Staging**

<table>
<thead>
<tr>
<th>Staging</th>
<th>Tis N0 M0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Localized</td>
<td></td>
</tr>
<tr>
<td>Stage IA</td>
<td>Localized</td>
<td></td>
</tr>
<tr>
<td>Stage IB</td>
<td>Localized</td>
<td></td>
</tr>
<tr>
<td>Stage IIA</td>
<td>Locally invasive</td>
<td></td>
</tr>
<tr>
<td>Stage IIB</td>
<td>Locally invasive</td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>Locally advanced, unrespectable</td>
<td></td>
</tr>
<tr>
<td>Stage IV</td>
<td>Metastasis</td>
<td></td>
</tr>
</tbody>
</table>

1.1.8 Diagnosis of Pancreatic Cancer

Late diagnosis of pancreatic cancer contributes to the poor outcome. The early stages of pancreatic cancer are generally asymptomatic with non-specific signs, and are commonly ignored. Late symptoms of pancreatic cancer include greater than 10% weight loss,
abdominal pain, painless jaundice and changes in glucose tolerance (35, 36). Clinical features at initial presentation of PC are related to tumour location. According to previous records, 60 -70% of exocrine PC are in the head of the pancreas, compared to 20-25% are in the body or tail (37). Tumours at the head of pancreas are more likely to present with jaundice and steatorrhea (38).

As advanced PC is often unresectable by the time of diagnosis, earlier diagnosis may increase the potential for curative treatment. When considering diagnostic tools, the ability to detect pre-symptomatic tumour at early stages, and the accuracy of differentiating malignant from benign disease would likely improve therapeutic planning and interventions.

1.1.8.1 Radiologic Diagnosis

In guidelines issued by National Comprehensive Cancer Network (NCCN), triple-phase imaging and thin slice (<3mm) CT is the recommended method for suspected PC patients. CT can visualize the location of tumour and surrounding mesenteric vasculature, which assist identification of resectable cases (39-41). However, the limitations of CT include poor performance in detecting early lymph node metastases (42).

Endoscopic ultrasonography (EUS) is the most accurate technique for the measurement of tumour size and detection of early malignant lesions (43). It is, however, poor at detecting vascular infiltration and distant metastases (44). Together with the fine needle aspiration (FNA) technique, EUS can obtain diagnostic cytological specimens (45, 46).

1.1.8.2 Biomarkers of Pancreatic Cancers

The lack of an effective screening biomarker may be seen as a contributing factor for the poor prognosis of pancreatic cancer. A biomarker is defined as a measurable indicator of a certain biological condition, including normal biological conditions, pathological conditions, or therapeutic outcomes (47). Cancer biomarkers can be further classified into three categories: diagnostic, prognostic, and predictive (i.e., useful in monitoring treatment responses) (48). In pancreatic cancer, diagnostic biomarkers, which are able to distinguish the malignancy from benign pancreatic disorders at a high accuracy and are able to detect the early stage disease, are urgently needed, as they have potential to improve the survival. As it may take at least fifteen years, from the initiation of genetic mutation to the acquisition of metastatic capacity, identifying a possible window for early diagnosis of pancreatic cancer is critical (49).
CA19-9

CA19-9 has been the only FDA approved PC biomarker for clinical usage (48) and validated in the EU (50). CA19-9, a sialylated Lewis antigen, can be produced by epithelial cells of the exocrine pancreas and released into the peripheral circulation. Among the Lewis-negative population (7-10% of the general population), however, CA 19-9 concentrations are not detectable, even in advanced PC (51).

Using a cut-off value of 40U/ml, the median sensitivity and specificity of CA19-9 in the diagnosis of PC are 79% and 82%, respectively (52). Higher pre-operative CA 19-9 levels have been shown to correlate with shorter overall survival, whereas elevated pre-treatment CA19-9 predicted a poorer outcome following chemotherapy (53-55). However, CA19-9 as a biomarker has limitations: 1) it is less detectable in cases of poorly differentiated pancreatic cancer or in early stages of the disease, and 2) CA19-9 levels may also be elevated in benign diseases, such as cholangitis. In other words, the CA19-9 is not very specific (50). Thus, CA19-9 is useful in evaluating prognosis, and monitoring treatment responses and recurrences of known PC patients, but it is not recommended in universal PC screening (56).

Efforts in Detecting New Diagnosis Makers

As CA19-9 is not very useful as a diagnostic biomarker, other studies has undergone to identify potential diagnostic markers including carcinoembryonic antigen (57), and Tumour M2-pyruvate kinase (TuM2-PK) (58-60). By 2009, more than 2000 studies comprising 2500 different genes and proteins as candidate biomarkers for PC have been reviewed (61), however, none have been adopted into routinely clinical practice (48).

1.1.9 Therapeutic Strategies

1.1.9.1 Surgery

Surgery has been considered the only potential curable strategy for the treatment of PC for many years. Pancreaticoduodenectomy is offered to patients with localized pancreatic cancer. In one population-based study conducted in California, US, 10,612 patients with pancreatic adenocarcinoma were included. 22% were eligible for surgical resection and 15.8% actually underwent surgical resection. The mean survival of patients after surgery was 13.3 months compared to 3.5 months for those managed conservatively (62). In a single-institution study, at Johns Hopkins Hospital, investigators reviewed 1423 patients with pancreatic cancer who
underwent pancreaticoduodenectomy (either partial or complete) over a 36-year period (1970-2006). The post-operative median survival was 18 months, and the 1-year, 5-year, 10-year overall survival rates were 65%, 18% and 11%, respectively (63). However, 96% of the patients who underwent surgical resection had Stage I or II disease. All patients with known hepatic metastases at the time of surgery died within one year (63). Results were further complicated by the observation that 71.4% of Stage I PC patients were not offered surgery due to a combination of other medical/social reasons (64), therefore limiting interpretation.

1.1.9.2 Chemotherapy and Radiotherapy

Only a minority (20%) of patients are candidates for surgery. Nearly 50% patients have metastatic disease at diagnosis, and a further 30% have locally advanced disease (65). Gemcitabine or folfirinox-based regimens are the treatment of choice for unresectable or metastatic PC and are also used in the neoadjuvant/adjuvant setting (66-68). A phase III prospective randomised trial was completed in Europe between 1994 and 2000, involving 289 patients from 11 countries. The patients were allocated to post-operative chemotherapy or chemoradiotherapy. The study documented a survival benefit for the postoperative chemotherapy (leucovorin plus fluorouracil regimen) group, compared to surgery alone. The 5-year survival of chemotherapy group was 21%, compared to 8% in the surgery alone group (69). Treatment of locally advanced pancreatic cancer remains difficult. There is some evidence to support the use of gemcitabine, although these findings have not been consistently reproducible (70, 71). Despite this lack of evidence, gemcitabine remains the standard therapeutic option for unresectable or metastatic pancreatic cancer, despite its toxicity (66, 67).

1.1.9.3 Biological Agents

Various biological agents have been explored in pancreatic cancer, including anti-VEGF monoclonal antibodies, EGF receptor inhibitors, Src kinase inhibitors, proteasome suppression and K-ras related therapy. As single agents, results from clinical trials have shown little benefit (67). Recently, the use of monoclonal antibodies targeting inhibitory receptors on the surface of tumour infiltrating T cells has obtained promising results in patients with pancreatic cancer. These will be discussed in more detail in the tumour immunology section below.
1.2 Tumour Immunology

1.2.1 Immune Surveillance and Immunoediting

The immune surveillance hypothesis was first proposed in the 1900s and has subsequently been supported by in vitro and in vivo experimental data. According to this hypothesis, the immune system may prevent cancer development (72). Researchers found that immunodeficient mice, generated by genetic knock-out or chronic antibody stimulation, have increased susceptibility to spontaneous or chemically-triggered tumours (Fig. 1.2) (73).

In addition to animal experiments, evidence has also accumulated in human cases, where the incidence of viral-related cancers is elevated in immuno-compromised patients or patients on long-term immune suppression (74). Here, the intact immune system in healthy people or immunocompetent mice is likely to provide protection against tumour development.

Figure 1.2. Immunodeficient mice are more prone to tumours induced by chemical carcinogens (73). After being given chemical carcinogens in the same doses, more immunodeficient mice developed tumours than their wild type counterparts.

Despite this, the majority of tumours develop in individuals with normal immunity and therefore more recently the immunoediting hypothesis was postulated by Schreiber (75), who proposed that during tumour development there were three distinct phases: elimination,
equilibrium and escape (73). Evidence coming from latter experiments, where tumours formed in the absence of an intact immune system are more immunogenic than tumours arising in immunocompetent hosts (Fig. 1.3). In other words, tumours generated from immunocompetent hosts may undergo ‘editing’ and become less immunogenic (73).

Figure 1.3. Tumours in immunocompetent mice are differed from those in immunocompromising mice (73). Tumour cell lines originated from immunocompetent mice and immunodeficient mice respectively were injected into two types of recipient mice. Tumour cells from wild type (WT) donors, which had been subject to in vivo immunoediting, led to outgrowth tumours in both immunodeficient and WT recipients. On the contrary, those from immunodeficient donors caused progressive tumours only in all immunodeficient recipients, while half of WT recipients did not develop tumour.

Scientists thought, in the elimination stage, natural killer cells, natural killer T cells and dendritic cells are activated to secrete pro-inflammatory cytokines by ‘danger signals’ (e.g. tumour debris) as a part of the initial innate immune response. The CD4+ and CD8+ T cells of the adaptive immune response are then recruited to clear cancer cells. After the initial
elimination phase, the equilibrium stage occurs when the residual tumour cells are subject to ‘pressure’ exerted by antigen-specific T cells and the immune system may limit tumour outgrowth for a variable period of time. As genetic and phenotypic mutations accumulate the cancer cells can evade immune control, causing tumour proliferation and invasion in the third stage (75). This includes the preferential growth of antigen-loss variants.

1.2.2 Adaptive Immunity and Cancer

1.2.2.1 T Cell Subsets and Their Interaction with Cancer Cells

T cells patrol the body, searching for peptide-MHC complexes able to be recognized by their T cell receptors (TCRs). T cells are normally activated by specialized antigen-presenting cells (APCs), which present peptides derived from antigens. The archetypal professional APCs are the dendritic cells (DCs) (76). T cell activation requires two signals, the antigenic signal, derived from the TCR binding to the peptide/MHC (major histocompatibility complex), and the costimulatory signal, typically through CD28 interacting with the B7 family of molecules (77). At the end of last century, Pawel Kaliński and colleagues demonstrated that DCs can provide a third signal, also termed a polarizing signal, through a number of molecules, which function to determine the differentiation of naive helper T cells into Th1 or Th2 subsets (78). More recently, IL-12 and Type I IFN have been identified as important in controlling the skewing of CD4+ T cells to Th1, Th2, or Th17, and in inducing T cell clonal expansion and differentiation (e.g., to effector or memory cells) (79).

In the T cell activation process, immunological synapses (IS) are formed between the APC and the CD4+/CD8+ T cell, which are centered on the TCR, and requires redistribution of surface receptors and remodeling of cytoskeleton (80). T cell recruitment of adhesion molecules, such as LFA-1 and ICAM-1/3 interactions is essential (81). Other surface receptors in the IS include CD40 and CD40L (which can promote IL-12 production contributing in DC maturation), and CD70 (82). There are also a group of inhibitory receptors, some of which even share the same ligands with as co-stimulatory receptors, which will be discussed in detail in Costimulatory Receptor and Coinhibitory Receptors section.

T cells play dual roles in tumour immunity. Activated tumour-infiltrating lymphocytes (TILs) have been observed in many types of tumour and they are typically associated with a better prognosis (83, 84). Endogenous peptides are loaded onto MHC class-I molecules in the endoplasmic reticulum and then transported to the cell surface. The peptide/MHC class-I
complexes are recognized by CD8+ T cells through their unique T cell receptor (TCR) (85). Activated CD8+ T cells can then migrate to the tumour microenvironment and cause lysis of tumour cells mediated by a number of different effector mechanisms including granzyme B, perforin secretion or the Fas pathway (86). MHC class II molecules (found in professional antigen-presenting cells) mainly bind exogenous peptides and these peptide/MHC class-II complexes are recognised by CD4+ T cells. Effector CD4+ T cells (‘helper T cells’) can differentiate into distinct subtypes, largely based on the pattern of cytokines they generate.

Type I helper T cells (Th1) can secrete IFN-γ and TNF-α and play an important role in anti-tumour immunity. In general, Th1 cells are critical for enhancing anti-tumour immunity through a number of mechanisms including the licensing and maturation of DCs and the generation of IL2 to support the proliferation and expansion of CD8+ cytotoxic T cells (87, 88). Besides, type II helper T cells produce IL-4, IL-5, and IL-13, on recognition of antigen and are critical for supporting B cell function and the generation of humoral immunity, and Th17 T cells are characterized by the production of IL-17, TNF-α, and IL-6, and can mediate autoimmune immunity (89). Regulatory T cells (Tregs), or CD4+ CD25+ Foxp3+ T cells, produce IL-10/TGF-β and mediate cell-to-cell dependent immune suppression, playing a part in maintaining immune tolerance (90, 91). The proportion of tumour infiltrating regulatory T cells has been correlated with a poorer prognosis in ovarian, gastric and breast cancer. However, in other types of cancer, like colon cancer and some types of lymphoma, the infiltration of Tregs may have a positive influence on the survival outcome, indicating Treg cells can play distinct roles in different tumour environments (92). It was also suggested that the expression of inhibitory receptors (such as TIM3 and LAG-3) on Treg can mediate the suppressive function of Tregs. In tumour scenarios, for example, the activation of Treg-mediated inhibition of anti-tumour immunity can contribute to tumour escaping, whilst depletion of suppressive Treg, including using Abs targeting CD25, inhibitory markers, and TGF-β pathway, may improve treatment outcomes (93).

1.2.2.2 HLA and T Cell Receptors (TCRs)

Major histocompatibility complexes (MHCs), also termed human leukocyte antigens (HLAs), permit antigen presentation and TCR recognition (94). With different loci and variable alleles, the MHC region is one of the most genetically polymorphic regions in human DNA (94). Class I MHC molecules are glycoproteins located on the surface of virtually all nucleated cells, which are encoded by 3 different MHC gene regions A, B and C on
chromosome 6, while the β2-microglobulin chain, encoded by a gene on chromosome 15 (95). Compared to Class I molecules, the expression Class II MHC molecules is usually restricted to DCs, macrophages, activated T and B cells, Langerhans and Kupffer cells (antigen presenting cells), but they are also detectable in thyroid epithelial cells and intestinal epithelial cells under inflammatory conditions (95). MHC gene regions- DP, DQ, DR and DO- encode class II HLA molecules (95). In addition to class I and II molecules, there is a third MHC region containing genes encoding immunity relating proteins, such as proteins belonging to tumour necrosis factor (TNF) family, which are involved in controlling of immune responses (94).

The structure of MHC Class I and II molecules was shown in figure 1.4. As can be seen, Class I molecule contains an α chain with 3 external domains (α1, α2, and α3) and a β2-microglobulin. A peptide-binding site exists between the α1 and α2 segments, which can bind peptides 8-10 amino acids long. A number of different epitopes can bind a single MHC class I molecule (96). HLA class II comprises one α chain and one β chain. Both chains have 2 external domains and a transmembrane segment. The peptide binding site is located between α1 and β1 segments, and binds peptides 13-18 amino acids long. A single HLA class II molecule binds a few different peptides, and stimulates CD4+ T cells via their CD4 molecule and TCR (96).

![Figure 1.4 Structure of HLA class I and II molecules](image)

**Figure 1.4 Structure of HLA class I and II molecules**

A MHC class I molecule comprise of a heavy chains, containing three polypeptide domains (α1, α2, α3), and a light chain, β-microglobulin. A peptide-binding site is formed in between α1 and α2 domains. A MHC class II molecule is a heterodimer made by an α and a β chain, at similar size. The peptide-binding cleft is located between the α chain and β chain (97).
After loading with peptides, the MHC molecule can present antigens to T cell receptors (TCRs). TCRs are heterodimeric proteins composed of either \( \alpha \) and \( \beta \) chains (95%), or \( \gamma \) and \( \delta \) chains (5%) (98). The classic model of TCR signal transduction subunits is shown in the following diagram (Fig.1.5). Here, p–MHC complex binds to both TCR and the co-receptor (CD4 or CD8) stabilizes the binding and initiates the TCR signaling cascade (99).

![Diagram of TCR/peptide–MHC complex](image)

**Figure 1.5. The classical model of T-cell receptor (TCR)/peptide–MHC complex.** The co-receptor (CD4 or CD8) help in stabilization this structure via providing the kinase LCK [which can phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs)], in the presence of the TCR/CD3 complex (99).

Phagocytosed antigens can be processed into peptides by proteasomes within the cytoplasm and then translocated to endoplasmic reticulum by transporters associated with antigen presentation (TAP1 and TAP2), which are loaded to MHC class I and cross-presented onto CD8+ T cells (100). On the other hand, exogenous antigens are normally captured by APC through pinocytosis or phagocytosis and processed by proteases. Processed peptides are subsequently loaded to MHC class II molecules and presented to CD4+ T cells (101).

MHC class I molecules can also interact with NK cells, but, in general, NK cells do not have specific receptors like T cells; instead, they contain two sets of receptors- activating (mediating NK cells to kill) and inhibitory receptors (prevent NK cells from activation) (102).
For example, the killer-cell immunoglobulin-like receptor (KIR) is a typical type of NK cell receptor, most of which are inhibitory receptors. Some KIR can recognize peptide/MHC class I complexes, reflecting the recognition of particular peptide motifs, instead of recognizing individual peptides (103). The activation status of the NK cell is determined by the balance from both types of signals. One popular hypothesis to explain the activation mechanism of NK cells is the ‘missing-self’ model, which suggests the reduction of MHC/HLA class I molecule on cells can stimulate NK to recognize them as targets and destroy related cells (102). Further studies, however, reveal that in addition to MHC molecules NK cells also impact by the signals from NK cell-activating receptors, e.g., NKG2D (102). Another hypothesis known as the ‘induced self’ theory linked the activation of NK cells to cellular stress ligands, which are involved in tumour and viral immunity. For example, under viral infection, the expression of NKG2D-activating receptors is upregulated, and NK cells therefore can be stimulated and activated (102).

1.2.2.3 T Cell Differentiation and Surface Markers

1.2.2.3.1 Naïve T Cells and Memory T Cells

Mature T cells will have undergone positive and negative selection in early life in the thymus. Cells with low affinity TCR receiving weak signal will be selected out, while T cells bearing high affinity TCR will undergo apoptosis (negative selection). Additionally, CD4+CD8- or CD4-CD8+ T cells were subjected to positive selection by binding to MHC class II or class I molecules accordingly (104).

Mature T cells in the periphery retain a naïve phenotype (CD44hiCD62LhiCCR7hi) for weeks to months, maintained by TCR signals and IL-7, in the absence of encounters with specific antigen. Before stimulation, precursor frequency of CD8+ T cells specific to certain Ag is about 1 in 10^5 T cells. After antigen stimulation, T cell precursors can expand rapidly (105). A proportion of primed T cells differentiate into memory T cells which can swiftly re-expand upon secondary encounter with the specific antigen. Memory T cells are generally characterized as CD44hi, and are further subdivided into central memory (CD62L+CCR7+) and effector memory (CD62LCCR7+) T cells (106). The proportion of memory T cells in the periphery increases with age (107). TEMs (effector memory T cells) migrate to inflamed sites from spleen and peripheral blood, while TCMs (central memory T cells) usually exist in
blood, as well as the spleen and lymph nodes (108). More recently, a new category of memory cells restricted within tissues, TRM (the tissue resident memory T cell, characterized with CD62L⁺CCR7⁻CD11a⁺) has been also identified (108). While most of the studies defining the phenotypes of memory cells have been carried out in mice, it is now known that memory cells from human and mice share similar features (108). In humans, TCMs are characterized with CD45RA⁻ CCR7⁺, while TEM are CD45RA⁻CCR7⁻. Effector T cells are known for CD45RA⁺CCR7⁻. Cells at all stages express CD27, but compared to effector cells, memory cells express lower levels of CD27 (109). The cytotoxic and proliferative capability of T cells is related to their differentiation status. (Fig.1.6) (76).

![Diagram of T cell differentiation and expression of markers](image)

**Figure 1.6. Differentiation of T cells and the expression of different markers (76).**

With the differentiation from naïve T cells to effector T cells, the cytotoxic capability of T cells increases, while the proliferative ability reduces, accompanying with upregulation in the expression of CD45RO, CD95, and CD57, and downregulation in that of CD45RA, CD62L, CCR7, CD27, and CD28.

### 1.2.2.3.2 Costimulatory Receptor and Coinhibitory Receptors

As mentioned above, T cells require two signals for Ag-specific activation. Signal 1 is via the TCR following binding to MHC I/II molecules on antigen presenting cells (APCs), which is
insufficient to activate naïve T cells. Costimulatory molecules provide additional signals (e.g., signal 2) leading to full T-cell proliferation and effector function (110, 111). Most costimulatory molecules belong to the B7 family and the tumour necrosis factor receptor (TNFR) family. Well characterised costimulatory molecules of the B7 family include CD80 (B7-1), CD86 (B7-2), PD-L1 (B7-H1), PD-L2 (B7-DC), B7-RP-1 and B7-H3. Accordingly, inhibitory-receptors for B7 members include CTLA-4 for CD80/86, programmed death 1 (PD-1) for PD-L1/ and the inducible costimulator (ICOS) for B7-H2. CD27 is a member of TNFR family (111). Co-stimulatory molecules has been shown to play a part in induction of IL-2 and effector cytokine production, promotion of T-cell survival, and mediating memory T cell development (112). The costimulatory and coinhibitory receptors on T cells and their ligands on APCs are shown on Figure 1.7.

A possible explanation for the failure of anti-cancer T cell responses is the inadequate T cell priming and insufficient duration of the effector phrase, which may be regulated by the costimulatory and coinhibitory receptors (113).

To develop potent T cell responses, it is helpful to enhance costimulatory signals. The B7 family of long recognized typical co-stimulatory molecules have been shown to participate in CD8-mediated tumour rejection (114). Also, the tumour necrosis factor (TNF) receptor family is another example of co-stimulatory molecules, including CD27, OX40, and 4-1BB, amongst others. CD27, for example, is essential in the maintenance of CD4+ and CD8+ T cell functions (115), and in a particular study, melanoma patients responding to adoptive cell transfer therapies had stable numbers of CD27+CD28+ T cells, indicating their roles in maintaining long-lasting, tumour-destructive CD8+ T cells in vivo (116). OX40 (CD134) and 4-1BB (CD137) are also considered equally important costimulatory molecules, leading to improved T cell survival and effector functions (117), and several trials had been carried out to test the efficacy of therapies targeting OX40, 4-1BB and CD40 in cancer patients (118). Interestingly, OX40 and 4-1BB are also expressed on regulatory T cells, and blocking them can result in dysfunction of Treg and reduced conversion from other type of CD4+ T cells to Treg (119, 120), while activating OX40 may also stimulate Treg expansion in the absence of IFN-γ (121).

On the other hand, suppressing the coinhibitory signals can improve anti-tumour T cell immunity. This can be achieved by blocking of ‘check point’ molecules, like PD-1 and CTLA-4. Anti-CTLA-4 (122) and anti-PD-L1/PD-1 antibodies (123, 124) have been tested in
a variety of clinical trials, achieving durable tumour remissions with an acceptable safety profile. The following section will introduce several particular costimulatory receptors (CD27 and 28) and coinhibitory receptors (PD-1, TIM3 and LAG-3) in detail.

Figure 1.7. Co-stimulation and co-inhibition of T cells at the immunological synapse (125).

A. The costimulatory receptors on T cells and their ligands on APCs. Stimulation of these receptors will lead to enhanced proliferation, memory generation, survival and cytotoxic function of T cells.

B. The coinhibitory receptors on T cells and their ligands on APCs. Activation of these signals will result in T cell tolerance and apoptosis, as well as inhibited proliferation and function.
1.2.2.3.2.1 Costimulatory Signals

CD28

CD28 is broadly and constitutively expressed on T cells, and its ligands (B7 family members) are expressed on APCs (111). To date, the CD86/80-CD28 interaction has been shown to generate the most profound costimulatory signal (derived from an APC to a T cell), and it remains the best characterized co-stimulatory pathway (126). Physiologically, CD28 costimulation has many roles in T cell mediated immunity, such as promoting T cell proliferation and IL-2 gene transcription (127), maintaining T cell responsiveness upon restimulation (128), and inhibiting T cell death (129). Although absence of CD28 does not result in complete anergy of T cells, it can lead to tolerance and impaired T cell function (130).

Loss or absence of costimulatory molecules, such as CD80 and CD86, has been frequently described in the tumour microenvironment (113). Consequently, T cells in the tumour may be rendered tolerant via chronic TCR signalling in the absence of costimulatory signals. Some studies have attempted to exploit this finding by modifying tumour cells to express CD80. When administered as a vaccine such CD80 expressing tumour cells can improve T cell priming and mediate tumour rejection (in mouse models) (113). Additionally, CD8+ T cells can also be primed via APC-mediated cross-presentation, in addition to directly from B7 family members on tumour cells (131).

To date there have been a number of clinical trials exploiting the CD86 pathway in an attempt to enhance anti-tumour immunity. For example, a new CEA-TRICOM [TRIad of COstimulatory Molecules compassing B7-1, intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function-associated antigen 3 (LFA-3)] vaccine was utilized in a phase I study which recruited 58 patients with advanced CEA-expression cancer (mainly colorectal and lung cancer). Disease responses include stable disease for a minimum of 4 months (23 patients) or more than 6 months (14 patients), which was encouraging in a cohort of patients with advanced disease (132). The CEA-MUC1-TRICOM vaccine (with additional expression of MUC-1) was tested in 25 patients with metastatic carcinoma (10 colorectal, 3 gastric, 3 ovarian and several other cancers). Prolonged clinical responses were observed in occasional patients (133). In a further phase I trial, the same CEA-MUC-1-TRICOM vaccine prolonged the survival of patients with advanced pancreatic cancer (15.1 months for treated patients vs 3.9 months for control, N=10, P=0.002) (134).
**CD27**

CD27 is a member of the tumour necrosis factor receptor (TNFR) family (135). Its expression is induced immediately after T cell activation, then gradually downregulated during repeated Ag stimulation and effector differentiation (136). It is now known that CD27 is more widely expressed on T cells subsets (including naïve, memory cells and some effector CD8+ T cells), NK cells, dendritic cells, B cells and hematopoietic progenitor cells (137-139).

CD70, the ligand for CD27, is only expressed on activated T cells, B cells and DCs (139). Its expression is upregulated by IL-1α, IL-12, TNF-α, and GM-CSF, whilst down-regulated by IL-4 and IL-10. High dose IL-2 also promotes CD70 expression, resulting in reduced CD27 expression on CD8+ T cells (140).

CD27 is required for maintenance of the T cell response and T –cell priming. CD27-deficient mice are generally healthy, viable, and able to reproduce and have a well-developed thymus and produce naive T cells. However, after viral challenge, reduced numbers of CD4+ and CD8+ effector T cells were recruited to the site of infection, and memory T cells were dramatically reduced at the time of secondary infection. These findings suggest CD27 is important in the establishment of T cell memory (115). CD27 can mediate CD127 (IL-7R) expression, suggesting a crucial role in the survival of CD8+ memory T cell (141). Further studies have demonstrated that CD27 is not required for T cell activation, but can maintain the survival of activated T cells during differentiation to effector cells (142). Moreover, evidence showed the CD27/CD70 signalling is essential in CD8+ T cell priming (143, 144).

Notably, CD27 also plays a synergistic role in anti-CD40 therapy. In 1998, modulating the expression of CD70 or CD154 (CD40 ligand) was shown to improve anti-tumour immunity (145). Later, in a B cell lymphoma mouse model, anti-CD40L MAbs were tested as adjuvants for anti-tumour vaccination, but this approach required the involvement of CD27/CD70 costimulation (146).

**Other Costimulatory Signals**

The TNF family compasses other members which are regarded as having co-stimulatory functions, such as OX40 (CD134), 4-1BB (CD137), CD40 and GITR (CD357) (118). Despite multiple functions in different types of T cells, OX40 agonistic Abs are now under testing of a few clinical trials (147). Among them, a phase I study assessing anti-OX40 demonstrated an
immune-stimulating function in the treatment of patients with end stage cancer, causing the regression of metastatic lesions in 30% patients (N=30) (148). 4-1BB MAbs typically in combination with other target drugs have also been analyzed (149). Nevertheless, all of these MAbs for costimulatory receptors are under early stages of studies in clinic setting.

1.2.2.3.2.2 Coinhibitory Signals

The increasing understanding of mechanisms that regulate cellular immune responses has resulted in the development of checkpoint-targeting antibodies. In 1994, T-lymphocyte-associated antigen 4 (CTLA-4) was reported as a negative regulator of T cell responses, by competitive binding to ligands of CD28 (150). Subsequently, scientists found anti-tumour immunity in murine models were promoted by CTLA-4 blockade (151). Another checkpoint molecule is programmed death protein 1 (PD-1), which was found to trigger immune evasion in tumour environment by mediating T cell apoptosis (152). These receptors and their ligands have been used as targets for tumour therapy, known as checkpoint therapy, as they can lead to durable long-lasting T cell responses (153).

CTLA-4 blockade had been evaluated in several phase I/II trials for multiple types of cancers, including melanoma, prostate cancer, ovarian cancer, renal cell carcinoma, and urothelial carcinoma, and had obtained encouraging results (154). The first phase III trial demonstrated that the anti-CTLA-4 treatment can restore the function of T cells and can improve the survival of melanoma patients, demonstrating the therapeutic effect of treatment targeting coinhibitory receptors (122). Encouraged by the initial success of CTLA-4 blockade, much research is now underway to identify other checkpoint blockers for cancer treatment.

The following section will focus on PD-1 (which has also been translated into the clinic), and two more recently recognized coinhibitory molecules, LAG-3 and TIM3, introducing their phenotypic and functional characteristics.

**PD-1 and PD-L1**

Programmed death 1 (PD-1) is a transmembrane protein, belonging to the immunoglobulin superfamily. The intracellular domain of PD-1 compromises a tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) (155). PD-1 is expressed on a number of immune cells. Activation induces PD-1 expression on T cells, DCs, and
monocytes. PD-1 is also expressed on NK cells and B cells (155). Notably, resting T cells do not express PD-1 (156).

To date, two ligands for PD-1 have been reported, namely, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC). The up-regulation of PD-L1 has been observed on B cells, T cells, dendritic cells, monocytes and some types of tumour cells (152, 157), or chronic viral infections (158). Interferons induce the expression of PD-L1 by microvascular endothelial cells, which may inhibit T cell development (159). In addition to PD-1, PD-L1 also binds to B7-1 (ligand for CD28, CTLA-4) (160). Another ligand, PD-L2 (B7-DC, CD273), has more restricted expressed on immune cells, like DC (161), and macrophages (162), and is induced by IL-4 (162).

PD-1/PD-L1 signalling inhibits T cell function via suppressing TCR-dependent activation of both CD4+ and CD8+ T cells, particularly, through the inhibition of their proliferation and cytokine production (including IFNγ, TNF-α, IL-2). This negative regulation can be reversed by CD28 costimulatory signalling (163). Soluble PD-1, likewise, stimulates IL-10 production via CD4+ T cells (164). PD-L1 and PD-L2 have some overlapping functions (165), but many studies suggest PD-L2 might engage in the promotion of anti-tumour T-cell function (166).

PD-1/PD-L1 signalling is important in autoimmunity and chronic viral infection. PD-1/PD-L1 pathway regulates self-tolerance in a variety of ways. Using PD-1 knock-out mice, scientists initially demonstrated that PD-1 participated in the induction and maintenance of peripheral self-tolerance (167). Further work found that PD-1/PD-L1 interactions suppress both positive and negative selection during the establishment of central tolerance (168, 169). PD-1/PD-L1 interactions are important in the pathogenesis of autoimmunity as both molecules are broadly expressed on naturally occurring and inducible Tregs (170, 171). In the presence of persistent antigenic activation, such as chronic viral infection, this pathway provides negative regulation by preventing continuous T cell activation, which could lead to tissue damage. High expression of PD-1 is observed in CD8+ T cells during HIV and HCV infection, and correlates with dysfunction and exhaustion of the antigen-specific T cells (172, 173). The inhibitory effect of PD-1 is reversible, with PD-1 blockade enhancing T cell immunity in both animal models, and more recently in clinical trials (158, 172).

Expression of PD-L1 has been reported in multiple solid cancers, including melanoma, uroepithelial cancers, ovarian, pancreatic, breast, colon, gastric and lung cancers. PD-L1 expression by the tumour cells is often associated with a poorer diagnosis or advanced
disease (174-177). PD-L1 is expressed in 80% of PC cases, of which 20% have high upregulation of PD-L1 and tend to be highly invasive and recurrent (178, 179).

The PD-1/PD-L1 blockade therapies are now being evaluated clinically. This will be discussed in later immunotherapy section.

**TIM-3**

T-cell/transmembrane immunoglobulin and mucin domain 3 (TIM-3) is a member of the TIM family (180). Initially, TIM3 expression was reported in Th1, Th17, CD8+ T cells, dendritic cells, monocytes, NK cells and restricted lymphocyte subsets (181, 182), it was shown to be negatively regulated by the transcription factor T-bet (183). Besides, a soluble version of TIM-3 was identified in mice (184).

Galectin-9 and PtdSer were identified ligands of TIM3. Galectin-9 belongs to the S-type lectins (185). The expression of galectin-9 in human endothelial cells is triggered by IFN-γ (186). Galectin-9 is expressed on many cells (182) and is broadly expressed on immune cells, including T cells, B cells, macrophages and fibroblasts (180). In addition, phosphatidylserine (PtdSer) is expressed on the surface of apoptotic cells, and is considered a ligand for TIM3 (187) and is involved in the clearance of apoptotic cells and cross-presentation (188).

TIM-3 negatively regulates T cell responses. By binding to galectin-9, TIM-3 induces Th1 cell death (189). In contrast, TIM-3 blockade could increase IFN-γ production and proliferation of Th1 (190). In chronic HIV infection, TIM-3 was expressed on 49.4 +/- SD 12.9% CD8+ T cells. TIM-3+CD8+ T cells were phenotypically exhausted, with impaired cytokine production, cytotoxicity and proliferation, which were restored by TIM-3 MAb blockade (191). In chronic HCV infection, TIM-3+ and PD-1+ co-expression was observed in many CD8+ and CD4+ T cells. Again, TIM-3+ T cells yielded less type I cytokines, and administration of TIM-3 MAb restored T-cell proliferation and IFN-γ secretion in response to HCV peptide antigen (192). In transplantation models, TIM-3 is further regarded as an inductor of peripheral tolerance (193).

Recent studies have explored the role of TIM-3 in the context of tumour immunology. TIM3 expression on endothelial cells may mediate immune evasion and enable the progression of melanoma and lymphoma (194, 195). In a murine colon cancer model, substantial co-
expression of TIM3 and PD-1 was observed in TILs, which were demonstrated impaired cytokine production and poor proliferation (196). Another experiment in melanoma patients found that TIM-3+PD-1+CD8+ T cells specific to NY-ESO-1 were more dysfunctional than TIM3- or PD-1- T cells (197). Furthermore, both experiments suggest TIM3 and PD-1 blockade could better reverse the impaired function of T cells than targeting either of them alone. However, other investigators suggested that the anti-tumour effect of TIM3 blockade requires high numbers of infiltrating IFN-γ secreting CD8+ and CD4+ T cells and that TIM3 blockade effects were observed even before TIM3+PD-1+ T cell became detectable in mouse models. Besides, they believed the therapeutic efficiency of TIM3 blockade should be improved by combining with anti-CTLA-4 and anti–PD-1, as the effect of anti-TIM3 alone was moderate (198). In a study that investigated the polymorphisms of TIM-3 in pancreatic cancer, researchers reported the +4259TG genotype were more common in cancer patients, thus concluded that polymorphisms in TIM-3 gene could be a potential risk factors for the initiation of PC (199). Less is known about TIM3 expression and its effect on peripheral T cells in pancreatic cancer.

LAG-3

Lymphocyte activation gene 3 (LAG-3, CD223) is a member of the Ig superfamily containing four extracellular Ig domains (113). MHC class II is a ligand of LAG-3, which is structurally similar to the CD4 molecules (113). LAG-3 is expressed on activated CD4+ and CD8+ T cells and NK cells. LAG-3 expression on T cells can be induced after activation and enhanced by IL-2, IL-7 and IL-12. After induction, LAG-3 plays a role in suppressing CD3/TCR-dependent immune response (200, 201). LAG-3 negatively regulates the proliferation and homeostasis of T cells (202), but amplifies Treg activity (203). Some studies revealed that co-expression of PD-1 and LAG-3 can be observed on tolerized TILs (T cell anergy due to persistent exposure to antigen), implying that these two receptors could play an important role in tumour-induced immune impairment (204).

LAG-3 blockade has been shown to relieve the immune suppression in the context of cancer. In two murine tumour models (breast cancer and prostate cancer), researchers elicited that LAG-3 Ab or gene editing can enhance the function of tumour vaccine by activating tumour specific CD8+ T cells immunity (205, 206). In a number of human studies, including melanoma, Hodgkin lymphoma and chronic viral infection, LAG-3 blockade or eradication of LAG-3+CD4+T cells can augment cytotoxic activity of antigen specific CTL in vitro (207-
Furthermore, combinatorial blockade of the PD-1 and LAG-3 pathways resulted in relieving T cell tolerance to tumour antigens and reduced tumour growth in murine models (210). Similarly, in human study (ovarian cancer), dual targeting of PD-1 and LAG-3 in the course of T-cell priming improved the proliferation and cytokine generation of NY-ESO-1–specific CD8+ T cells (211). Taken together, these studies demonstrated the role of LAG-3 as an important immune checkpoint in the tumour environment.
1.3 Tumour Immunotherapy

Tumour Immunotherapy refers to a therapeutic strategy using the immune system to target cancer, which is usually classified as passive, active or immunomodulatory therapies (212). Passive immunotherapy includes injection of immune components, such as antibodies or tumour-infiltrating lymphocytes (TILs) to enhance anti-tumour immune response, while immunomodulatory agents, including anti-CTLA-4 and PD1 antibodies, attempt to augment existing immune reactions (212). Active immunotherapy, e.g., tumour vaccination, aims to stimulate endogenous T cells to recognise TAA/TSA-expressing cancer cells (212). Recently, adoptive T cell (ATC) therapies and checkpoint inhibitors are used together to improve anti-cancer immune response. ATC transfer therapy enables the in vitro expansion of specific T cells, either antigen-stimulated TILs or T cells with genetically engineered TCRs or Chimeric Antigen Receptors (CARs), and then reinfused into the hosts to achieve an enhanced therapeutic outcome (213). The following section will focus on ATC therapy and checkpoint blockade.

1.3.1 Clinical Use of Checkpoint Blockers

As mentioned in the co-inhibitory receptor section, some receptors and ligands, such as CTLA-4 and PD-1/PD-L1, are associated with the inhibition of T cell function and may contribute to the immune suppressive microenvironment in cancer. Antibodies that target these molecules are referred to as “checkpoint inhibitors”, which function by blocking normal inhibitory regulators of the Ag-specific T cell response typically found in TILs. Their clinical benefits have recently been demonstrated in multiple cancers (214).

The first checkpoint inhibitor which entered clinical trials was Ipilimumab, a CTLA-4 blocking antibody. So far, this drug has shown efficacy in a series of phase I/II trials in patients with renal cell cancer (215), melanoma (216, 217), ovarian cancer (218), urothelial carcinoma of the bladder (219), and prostate cancer (220). These trials demonstrated a reasonable safety profile, and some led to clinical benefits in a fraction of patients. In a phase III study, Ipilimumab improved the overall survival of patients with previously treated (122) and untreated (combined with dacarbazine) (221) metastatic melanoma. Although Ipilimumab users did not show statistical differences from the placebo group in metastatic prostate cancer in a phase III study, it is still undergoing further investigation (222). More recently, a review analyzed pooled data of phase II/III studies about the effect of Ipilimumab
in advanced melanoma patients, which involved 1,861 patients and provided evidence that Ipilimumab can improve long-term survival of patients with advanced melanoma. In this study, the plateau of the survival curve started from 3 years, and some of the patients even survived after 10 years of accepting CTLA-4 blockade therapy (223). FDA had approved Ipilimumab in 2011.

Antibodies blocking the PD-1/PD-L1 pathway are another important group of checkpoint inhibitors. Recently, PD-1/PD-L1 antibodies have also shown efficacy in clinical trials. Anti-PD-1 antibodies, (such as Nivolumab and Pembrolizumab) and Anti-PD-L1 antibodies (Pidilizumab), have been tested in a variety of phase I clinical trials. Nivolumab has achieved durable tumour remissions with an acceptable safety profile in non-small-cell lung cancer, melanoma, and renal-cell cancer (224), and long-term (2 years) survival benefits and safety has also been reported in melanoma patients (123). Anti-PD-L1 antibodies also showed similar effects in the abovementioned types of cancers (124) and urothelial bladder cancer (225). Another PD-1 blocker, named Pembrolizumab or lambrolizumab, had a response rate at 37-38% in advanced melanoma (226). In ipilimumab-refractory advanced melanoma, the Nivolumab (227) and Pembrolizumab (228) can still induce prolonged survival benefits, and less adverse effects related to Ipilimumab was seen in studied PD-1-inhibitor users. More recently, a phase III study had demonstrated a significant progression-free survival in advanced melanoma patients receiving Nivolumab (overall survival rate at 72.9%) compared to dacarbazine therapy (229). Lately, phase III studies using Nivolumab to treat advanced squamous-cell non–small-cell lung cancer gained better overall survival and response rate than docetaxel, regardless of the PD-L1 expression degree (230, 231). Lambrolizumab and Nivolumab have got FDA-approval in 2014 and 2015 respectively.

The efficacy of checkpoint blockers has also been checked in pancreatic cancer. Although these inhibitors showed therapeutic efficacy in many experiments based on murine models (232, 233), they lack support from large clinical trials (234). In a phase I study using a PD-L1 antibody -atezolizumab- to treat Japanese patients with advanced solid tumors, the only pancreatic patient in this study had a more than 12 months’ progression-free survival, and the drug was well tolerated in these patients (235).

To improve clinical responses, scientists have also attempted to identify predictive biomarkers to select patients most likely to respond to checkpoint antibodies. Some clinical studies revealed that the number of genetic mutations and generation of neoantigens was
positively associated with rates of CTLA-4 responsiveness. In other words, where there are high mutational loads resulting in more neoepitopes that can potentially be recognized by immune system and in particular tumour infiltrating T cells (TILs), the overall anti-tumour response is more likely to be augmented by checkpoint blockers (236). Interestingly, the expression of PD-L1 on tumour tissues correlated with the response rates to anti-PD-1 antibodies, but it does not correlate with survival rates. Yet, in Hodgkin lymphoma, which includes cells constitutively expressing PD-L1 and PD-L2, the level of PD-L1/2 expression can predict the outcome of treatment (237).

### 1.3.2 Early Development of Adoptive Immunotherapy

In the 1960s, the efficacy of adoptive immunotherapy was first described in patients with haematological malignancies, who underwent allogeneic stem cell transplantation, where the immune components from the donors acted against the malignant cells from the host (238). This phenomenon is now known as graft-vs-host disease (GVHD) which is associated with the graft vs leukaemia (GVL) effect, and clinical evidence has shown it can prevent leukemia relapse (239). The role of adoptively transferred T cells has also unfolded. T cell depletion performed with the aim of reducing GVHD was shown to increase recurrence rates, especially in chronic myeloid leukemia (CML) (240). Some fifty years later the main challenge in allogeneic hematopoietic stem cell transplantation (HSCT) is how to limit GVHD, whilst retaining the graft vs leukaemia (GVL) effect. For the benefit, the T cell–depletion was now routinely given after allogeneic HSCT. (241). Another limitation of allogeneic transplantation is the requirement for post-transplant immune suppression, which impairs antiviral immunity. EBV-associated lymphoproliferative disorders can be seen in patients with post-transplant status, caused by clonal expansion of B cells (in 95% of cases) or T cells (in 5% of cases) (242). Other major challenges are CMV and adenovirus reactivation (243).

Also, the adoptive transfer of ex vivo expanded tumour infiltrating lymphocytes (TIL) has been an effective approach in the treatment of melanoma (244, 245), where the infusion of autologous TILs (CD4+T cells and CD8+T cells) after lympho-depletion resulted in regression of metastatic cancer in up to 50% of patients. However, the adoptive T cell therapy was only generally used in melanoma.
1.3.3 Tumour Specific Antigens (TSA), Tumour Associated Antigens (TAA) and Immunological Tolerance

Successful tumour specific T cells responses rely on recognition of tumour-associated antigens (TAAs) or tumour-specific antigens (TSAs) by T cells (246), and the ability to discriminate these antigens from self (247). Antigenic discontinuity can determine the immunogenicity rather than “self and non-self”, meaning that an epitope can be immunogenic if it is ‘unusual’, but not essentially new (248). Thus, the genetic and epigenetic alternations and abnormal expression of proteins in cancer cells may also lead to the processing of immunogenic antigens and triggering relevant immune responses (248).

Tumour antigens include TSAs and TAAs. TSAs are only expressed in tumour cells, not in normal cells, whilst TAA are usually overexpressed in tumour cells but low level expression can be found in normal tissues (249). TSAs are considered ideal targets for anti-tumour vaccination, since TSA-specific T cells are not subjected to pre-existing immunological tolerance, and are not likely to target normal tissues. Generally, TSAs can be divided into three classes: exogenous antigens from transforming viruses (e.g. EBV derived peptide epitopes), unique TSAs (such as mutated RAS) and idiootypic TSAs (found in B cell malignancies where the malignant clone expresses rearranged genes encoding a specific immunoglobin receptor) (249). However, TSA are not always efficiently processed and presented by MHC class I molecule (250).

The majority T cells in the autologous repertoire specific for TAAs are of low avidity. According to central tolerance mechanisms, T cell precursors with high affinity TCR binding to MHC/self-peptide are deleted during early development in thymus (251). As a result, peripheral T cells usually express low-affinity TCR towards self-antigens. These T cells typically exert ineffective or transient anti-tumour effects. In addition, mature T cells which have low avidity for MHC/self-peptide complexes may be controlled by peripheral tolerance mechanisms, such as the physical barrier between naïve T cells and parenchymal cells presenting tissue restricted antigens (TRAs). Furthermore, autoreactive T cells usually undergo apoptosis after recognition of self-peptide pulsed MHC molecules (activation induced cell death/deletion) (252). Other peripheral tolerance mechanisms include ignorance, anergy, TCR downregulation or suppression by Treg (249). However, both central and peripheral tolerances mechanisms are leaky, allowing existence of a small number of self-reacting T cells, which escape thymic deletion, or, the effects of peripheral tolerance.
The generation of high avidity T cells specific for TAA may therefore overcome peripheral tolerance mechanisms. It is important, however, that T cells “ignore” low level (physiological) TAAs expressions on normal cells, to avoid autoimmune response directed against normal tissues (253).

1.3.4 Anti-tumour Vaccination

Vaccination can use TAA or TSA, either in the form of inactivated whole tumour cells or component of tumour cells (such as protein, cell lysates or DNA) following administration are taken up by antigen presenting cells (APCs) and induce CD4+ and/or CD8+ T cell responses against the tumour bearing the relevant antigen (254).

Viral antigens are the most commonly targeted TSA in oncology. For example, human papillomavirus (HPV) is known to play an essential role in the carcinogenesis of human cervical cancer (255), and HPV vaccines have been widely examined. An HPV-16/18 bivalent vaccine demonstrated a 90% reduction in morbidity and persistent HPV infection of the cervix, together with a reduction of cytological abnormalities linked to chronic HPV infection. Another quadrivalent HPV vaccine (directed against 4 oncogenic types of HPV-HPV-6,-11, -16 and -18) can prevent 70% of cervical cancers, and also 90% of genital warts (256). Such vaccines are now being used as part of the UK-wide national immunization programme and can effectively prevent the initiation of up to 70% of cervical cancers (257). The latent herpes virus EBV can drive tumourigenesis in some epithelial cell tumours, particularly, nasopharyngeal carcinoma (NPC), and Burkitt’s lymphoma (BL) (258).

Transferring vectors encoding CTL peptide epitopes derived from the latent membrane proteins (LMP1 and LMP2) of EBV have been used to stimulate T cell responses against EBV-associated cancers. The efficacy of similar EBV-vaccine approaches have been demonstrated in HLA A2/Kb transgenic mice models (259). Subsequently, in a phase II clinical trial, Toh’s group has used LMP1-LMP2 transduced DC to vaccinate 16 advanced NPC patients. The trial confirmed the safety of this approach, while efficacy required more testing (3 patients achieved partial responses and two secured stable disease) (260). Heslop and Rooney developed another approach using allogeneic cell transplantation (from EBV-seropositive donors) to obtain EBV- specific CTL, which cause relief of EBV-driven lymphoproliferative disorders and tumours both in vitro and in vivo (261). The first trial using this method performed in 1995 and validated the efficacy of this method in controlling EBV-driven lymphoproliferation (262).
Despite encouraging results from TAA-based vaccine in animal models, clinical responses are rarely achieved. The majority of vaccination trials demonstrating some clinical responses have been in patients with melanoma or ovarian cancer. For example, a DC-based vaccine expressing five HLA-A24 restricted melanoma-associated peptides (gp100, tyrosinase, MAGE-A2, MAGE-A3 and MART-1) was tested in a phase II clinical trial. Vaccinated stage III/IV patients had a significantly better overall survival (263). Another trial performed in 25 melanoma and 22 ovarian cancer patients using an NY-ESO-1 vaccine, resulted in 14% complete or partial responses, and 52% disease stabilization (264).

In patients with established or progressive tumours, immune evasion strategies have been established to escape or resist T cell responses, including down-regulation of TAAs and/or MHC molecules, up-regulation of PD-1 and CTLA-4, as well as the local production of immunosuppressive cytokines (250, 265).

This increasing evidence that TAA specific T cells developed suppressive phenotype after repeated antigen exposure, such as vaccination or repeated interaction with tumour cells in the tumour microenvironment. For example increased in expression of the co-inhibitory receptors, PD-1 and LAG-3 have been observed (211). Thus, T cells based therapy together with co-inhibitory receptor blockade may improve the outcome of related immunotherapy.

1.3.5. Adoptive Immunotherapy Using T Cell Engineering

For success cellular immunotherapy, it is important to expand T cells with high avidity to appropriate tumour antigens. Optimal T cells should have high functional avidity in response to the antigen, which is in part determined by the specific TCR affinity for peptide/MHC and the numbers of TCR expressed on T cell surface. Affinity refers to the binding energy between an antibody/TCR and a univalent epitope, whilst avidity measures the total binding energy of an antibody/TCR to multivalent (multiple affinities) of antigen. Functional avidity is typically used to describe the polyfunctional response of a T cells to a given antigen. T cells can be fully activated when a defined threshold of TCR/MHC signaling is reached, which also relies on the extent and strength of co-stimulatory signals received at the same time (266). Currently, there are three T cell engineering methods used to redirect Ag specificity under testing, including TCR, haTCR and CAR T cells. The comparisons of these strategies and TIL are listed in the below table (Table 1.5).
Table 1.5. General comparison of tumour-infiltrating lymphocytes (TIL), TCR engineered T cells (TCR T cells), high affinity TCR engineered T cells (haTCR T cells), and chimeric antigen receptor engineered T cells (CAR T cells). [Content in this table mainly cited from (267)]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TIL</th>
<th>TCR T cells</th>
<th>haTCR T cells</th>
<th>CAR T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue of origin</td>
<td>Tumour</td>
<td>PBMC</td>
<td>PBMC</td>
<td>PBMC</td>
</tr>
<tr>
<td>Efficiency</td>
<td>Moderate</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td><em>Ex vivo</em> manipulation time</td>
<td>17–30 days</td>
<td>8–12 days</td>
<td>8–12 days</td>
<td>8–12 days</td>
</tr>
<tr>
<td>Genetic modification</td>
<td>Not necessary</td>
<td>Required</td>
<td>Required</td>
<td>Required</td>
</tr>
<tr>
<td>Mispairing</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Antigens targeted</td>
<td>All</td>
<td>All</td>
<td>All</td>
<td>Mainly surface</td>
</tr>
<tr>
<td>Antigenic specificity</td>
<td>Undefined</td>
<td>Defined</td>
<td>Defined</td>
<td>Defined</td>
</tr>
<tr>
<td>HLA restriction</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Co-signalling</td>
<td>Physiological</td>
<td>Physiological</td>
<td>Physiological</td>
<td>Engineered</td>
</tr>
<tr>
<td>Effector functionality</td>
<td>Undefined</td>
<td>Low-Moderate</td>
<td>High</td>
<td>Very high</td>
</tr>
<tr>
<td>Potential for on target toxicity</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Potential for off target toxicity</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
</tbody>
</table>

1.3.5.1 TCR Gene-Modified T Cells

TCR gene transfer provides a potential solution to re-direct the specificity of patient T cells by introducing an exogenous (tumour-specific) TCR using viral vector encoding the genes for the TCR alpha and beta chains (254). To date, a number of different TCR have been used in TCR gene transfer to target HLA-A2 presented TAAs, including MDM2, MART1, WT1 and mutated p53 (254).

Due to the HLA-restriction of TCR, the TCR gene transfer approach is necessarily restricted by HLA type and may also be influenced by the downregulation of HLA molecule or variability of antigen presentation in tumour cells. HLA-A2 is the highest frequency class I HLA allele in Caucasian populations (268). Previous studies have found that HLA-A*0202 and HLA-A*0201 are structurally similar and some antigen specific CTL can cross-recognize certain peptides presented by both HLA-A*0201 and A*0202 (269).

Since the TCR are heterodimeric receptors consisting of one α and one β chain, following expression of viral vector encoded exogenous TCR chains, it is possible that the introduced chains will mispair with one endogenous chains resulting in cell surface expression of an αβ
TCR with unknown, and potentially self-reactive specificity. In addition, this TCR will compete with the desired, introduced correct αβ TCR by forming complexes with the endogenous CD3. Such TCR mispairing can reduce ability of the TCR gene modified T cell to respond to target antigen, and also introduce the risk of autoimmunity against self-antigens (Fig. 1.8) (270). A variety of methods have been developed to reduce the mispairing, including the establishment of single-chain TCR constructs (271), editing TCR structure by adding additional disulphide bonds within the constant region to facilitate appropriate pairing (272), or downregulation of endogenous TCR expression using zinc-figure nucleases (273), siRNAs (274), transcription activator-like effector nucleases (TALENs) (275) and CRISPR/Cas technologies (276).

Figure 1.8. Mispairing of TCR chains in TCR-transduced T cells (277). The introduced α chain (or β chain) can form new TCR with endogenous β chain (or α chain).

Enhancing the avidity of TCR-transduced T cell can be achieved by increasing the affinity of TCR itself or increasing the number of specific TCR on T cell surface. It is expected that such T cells will recognize target cells expressing lower concentrations of the target antigen.
Diverse strategies have been developed to isolate high-affinity antigen-specific T cells clones (278, 279). These include the isolation of allo-restricted TCRs (280) and affinity maturation of TCRs applying libraries of mutant peptides to direct antigen binding regions of TCR (270, 281). However, high-affinity TCR (able to recognize target peptide concentrations of less than 1nM) may impair the function of T cells, as the expression of haTCR need lower peptide/HLA complex concentration, and haTCR has an elevated risk of cross-reactivity (282).

Genetically engineered MART-1-specific TCRs have been tested in clinical trials recruiting melanoma patients and demonstrated anti-tumour efficacy in end stage patients (283). Subsequently, genetically modified NY-ESO-1 specific TCR-transduced T cells were infused into 11 patients with metastatic melanoma or synovial cell sarcoma, resulting in 2 complete regression (CR) and 1 partial response (PR) persisting up to 18 months (284). Some typical ongoing TCR trials are listed in table 1.6 (285).

**Table 1.6. Examples of recent clinical trials involving genetically redirected T cells.** Table was cited from *Adoptive Immunotherapy for Cancer or Viruses* (285). More information added from clinicaltrial.org.

<table>
<thead>
<tr>
<th>Target antigens</th>
<th>Cancers</th>
<th>Receptor</th>
<th>Combinatorial/engineering strategies (biologicals, drugs)</th>
<th>Phase; ID</th>
<th>Sponsor</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1</td>
<td>AML; CML</td>
<td>TCR</td>
<td>IL-2</td>
<td>I/II; NCT01621724</td>
<td>University College, London</td>
</tr>
<tr>
<td>WT1</td>
<td>MDS; AML</td>
<td>TCR</td>
<td>IL-2</td>
<td>I/II; NCT02550535</td>
<td>Cell Therapy Catapult</td>
</tr>
<tr>
<td>WT1</td>
<td>AML, MDS, or CML</td>
<td>TCR</td>
<td>Aldesleukin; virus-specific CD8+ T cells</td>
<td>I/II; NCT01640301</td>
<td>Fred Hutchinson Cancer Research Center/University of Washington Cancer Consortium</td>
</tr>
<tr>
<td>NY-ESO-1</td>
<td>Melanoma</td>
<td>TCR</td>
<td>None</td>
<td>I/II; NCT01350401</td>
<td>Adaptimmune</td>
</tr>
<tr>
<td>NY-ESO-1/LAGE-1</td>
<td>Multiple myeloma</td>
<td>TCR</td>
<td>None</td>
<td>I/II; NCT01892293</td>
<td>Adaptimmune</td>
</tr>
<tr>
<td>NY-ESO-1/MAGE-A3/6</td>
<td>Multiple myeloma</td>
<td>TCR</td>
<td>None</td>
<td>I/II; NCT01352286</td>
<td>Adaptimmune</td>
</tr>
<tr>
<td>NY-ESO-1</td>
<td>Oesophageal Cancer</td>
<td>TCR</td>
<td>IL-2</td>
<td>II; NCT01795976</td>
<td>Christie Hospital</td>
</tr>
<tr>
<td>NY-ESO-1</td>
<td>Synovial Sarcoma</td>
<td>TCR</td>
<td>None</td>
<td>I; NCT01343043</td>
<td>Adaptimmune</td>
</tr>
<tr>
<td>CEA</td>
<td>Metastatic cancers</td>
<td>IgCD28 TCR</td>
<td>None</td>
<td>II; NCT01723306</td>
<td>Roger Williams Medical Center</td>
</tr>
<tr>
<td>MART-1</td>
<td>Metastatic melanoma</td>
<td>TCR</td>
<td>Administration of MART-126-35-pulsed dendritic cells and IL-2</td>
<td>II; NCT00910650</td>
<td>Jonsson Comprehensive Cancer Center</td>
</tr>
</tbody>
</table>

*Abbreviations: AML, acute myelogenous leukemia; CEA, carcinoembryonic antigen; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndromes; TCR, T cell receptor.*
1.3.5.2 Chimeric Antigen Receptor Gene-Modified T Cells (CAR T Cells)

Another way to improve T cell avidity and re-directing specificity is to use artificial antigen recognition receptors, termed chimeric antigen receptors (CARs). The CAR structure is modelled on the antigen-binding fragment (Fab/Fv) of an antibody. As an antibody-based antigen receptor, CARs directly bind antigen and are therefore not restricted by HLA or affected by the downregulation of HLA molecules (286). Despite these advantages, CARs cannot recognize intracellular antigens presented in the context of MHC. Work is currently underway to generate CARs able to target HLA-presented intracellular antigens (287).

First generation CARs contained only CD3ζ or FcR-γ signalling domains, and lacked co-stimulatory signaling at the time of antigen recognition. As predicted, these CAR T cells failed to generate significant clinical responses (288, 289). However, much progress has been made and second generation constructs have been designed which include fusion domains containing various combinations of B7 family members or TNFR members, such as CD28, CD137, or OX-40 to enhance the proliferation and persistence of CAR T cells can enhance persistence and survival (290). As with other antigen-specific T cells (naturally occurring or gene-modified) the addition of low dose IL-2 can enhance persistence and survival (291). It has also been demonstrated that for TCR or CAR modified T cells, the introduction of the receptor into virus-specific T cells can also increase the persistence of the gene modified cells through viral antigen-mediated stimulation (292). Noticeably, some studies demonstrated that using of exogenous IL-2 may result in toxicity (293).

Many pre-clinical models have demonstrated that IL-7, IL15 and IL-21 can improve persistence of CTL responses (294-296). Clinical trials using second-generation CAR-T cells have generated some excellent clinical responses, which has brought much attention to this field. Numerous CD19 specific CAR-T cells have been tested (or are being tested) against various B cell malignancies, such as ALL (acute lymphocytic leukaemia), CLL, small cell lymphoma, follicular lymphoma and diffuse large B cell lymphoma (297-301). With regards to the relative efficacy of CD28 and CD137 (4-1BB) co-stimulatory molecules, the results remain variable (302, 303).

More recently, the third generation CARs utilize fusion domains such as CD3ζ, CD28 and 4-BB generating improved cytokine production and longer survival in vivo (286). The enhanced efficacy of the updated CARs was demonstrated in vitro (304), and in xenogenic
mouse models (305). However, whether the third-generation CARs are superior to the second generation CARs in clinical practice is still under debate. In a study, researchers utilized ERBB2-specific CARs, which was transduced with CD28/4-1BBCD3ζ, to treat a patient with colon cancer. This CAR failed to generate protective response, but lead to patient’s death after cytokine storm-related multi-organ failure (306). So far, CARs have not displayed clinical benefit in solid cancer. Related clinical trials are described above (285).

1.3.6 Toxicity and Safety of Immunotherapies

The use of gene-engineered T cell therapies can be limited by toxicity. On-target and off-target toxicities are more common when using T cells modified to express haTCR and CAR. Off-tissue toxicity, or on-target toxicity, occurs when CTLs target normal tissues expressing low levels of the target antigen, while off-target toxicity can cause damage of tissues not presenting the tumour antigens, which are usually unexpected (267).

An example of on-target/off-tumour toxicity was observed in a melanoma trial using MART-1 specific TCR transduced T cells, where patients achieving improved anti-tumour responses also developed skin rash and uveitis (307). Also, in a clinical trial using a CEA-specific TCR, generated from an HLA-A2 transgenic mouse model, CEA-TCR transduced autologous T cells were used to treat colon cancer patients, and severe colitis developed in 2 out of 3 patients (308). To prevent on-target toxicity, it is important to select suitable targets, which are ideally absent in normal tissue. An alternative is to identify the expression level of target protein in normal tissues and understand the threshold which can induce this kind of toxicity (309). On the other hand, the risks of off-target toxicity may be increased by elevating TCR affinity beyond its normal range. In that case, artificial TCR may attack un-specified antigens (282).

As most targets of CAR T cells have also been expressed on normal tissues, the on-target/off-tumour toxicity becomes an expected side effect (310). Besides, the majority of CAR T cells use domains originally derived from murine antibodies, and therefore, may be recognized as foreign agents and induce acute anaphylaxis (310). One strategy to improve safety is the addition of the inducible caspase 9 suicide switch into therapeutic T cells (311). Other potential ways include using pharmacological immunosuppression, such as MAbs directed against the IL-6R and corticosteroids to prevent the over-response of CAR T cells and introduce an ‘on-switch’ design, e.g., dual antigen binding (310).
When CAR T cells recognize target cells, further toxicity can be caused such as cytokine release syndrome (CRS) and neurotoxicity. These can be severe or fatal and associated with supra physiological levels of circulating cytokines (including IL-6, and IFN-γ) (312). For example, it occurs when using CD19 CAR treated patients with advanced B-cell malignancies, where inflammatory cytokine levels were elevated (313). Tumour lysis syndrome (TLS) is common in haematologic malignances, when the disease burden is high at the time of T cell infusion. TLS follows the release of tumour contents into the circulation, resulting in severe hyperkalemia and hypocalcemia, which may cause cardiac arrhythmias and multi-organ failure (314).

Checkpoint blockers also have systemic adverse effects such as fatigue, headache, and fever, dermatologic adverse effects (e.g., vitiligo and rash), digestive system adverse effects (e.g., colitis and diarrhea), pulmonary adverse effects (e.g., pneumonia and dyspnoea), endocrine adverse effects (e.g., hypothyroidism) and neurological adverse effects and autoimmunity (315). However, compared to chemotherapy and targeted therapy, checkpoint blockade is complicated by fewer overall and life-threatening toxic side effects (315). In general, more adverse events of CTLA-4 blockade therapy are reported than anti-PD1/PD-L1 therapy (316). Most side effects resolve when the treatment is stopped. Thus, early detection and intervention are essential in the management of these adverse events (317). Also, it is suggested that predictive biomarkers can be used to predict the efficacy and toxicities of checkpoint inhibitors (318). For example, increasing numbers of TILs and high diversity of T cell repertoire are associated with high response rates in patients treated with anti-CTLA-4 or anti-PD-1 antibodies, while increased circulating IL-7 and neutrophil infiltration in the colon are observed when checkpoint blockers cause colitis (318).

### 1.3.7 Immunotherapeutic Targets for the Treatment of Pancreatic Cancer

#### 1.3.7.1 Tumour Associated Antigen in PC

Potential immunotherapeutic targets in pancreatic cancer, which can stimulate T cell responses, are over-expressed pancreatic TAA’s. As previously stated, ideal targets are over-expressed in cancer cells, not or minimally expressed in normal tissue, and critical for maintaining the malignant phenotype. A number of previously characterized TAA’s in pancreatic cancer are detailed in table 1.7.
As Mesothelin and CEA are the most frequently expressed TAAs in PC, they will be described in more detail in the following sections.

Table 1.7. Tumour antigens expressed on pancreatic cancer cells.

<table>
<thead>
<tr>
<th>Tumour associated antigen (TAA)</th>
<th>Frequency of expression in PC</th>
<th>Normal physiology</th>
<th>Putative role in pathogenesis of PC</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesothelin</td>
<td>&gt; 90%</td>
<td>Not clear</td>
<td>Contribution to metastasis</td>
<td>(319)</td>
</tr>
<tr>
<td>Carcinoembryonic antigen (CEA)</td>
<td>&gt;90%</td>
<td>Produced in fetus, with unclear roles.</td>
<td>L-selectin and E-selectin ligands, function in metastasis</td>
<td>(320, 321)</td>
</tr>
<tr>
<td>Human telomerase reverse transcriptase (hTERT)</td>
<td>88%</td>
<td>Function in the de novo synthesis of telomeric DNA</td>
<td>Stabilize telomeres and DNA, causing cell immortality</td>
<td>(322)</td>
</tr>
<tr>
<td>Mucin proteins</td>
<td>&gt;85%</td>
<td>Protection: secreted mucins form chemical barriers and also involved in cellular signalling</td>
<td>Involved in tumour progression, invasion and metastasis; possible receptor of tyrosine kinase signalling pathways; antiadhersion</td>
<td>(323)</td>
</tr>
<tr>
<td>Survivin</td>
<td>77%</td>
<td>Regulates apoptosis and mitosis; expression during fetal development</td>
<td>Prevent apoptosis.</td>
<td>(324, 325)</td>
</tr>
<tr>
<td>Wilms’ tumour antigen 1 (WT1)</td>
<td>75%</td>
<td>Tumour-suppressor gene</td>
<td>Contributes to tumourigenesis</td>
<td>(326)</td>
</tr>
<tr>
<td>Mutated K-RAS</td>
<td>&gt;90%</td>
<td>Guanine NBP in growth pathway signal transduction</td>
<td>Enhance tumour cell proliferation and survival, mutated at early stage of pancreatic cancer</td>
<td>(27)</td>
</tr>
<tr>
<td>p53</td>
<td>&gt;60%</td>
<td>Tumour suppressor gene</td>
<td>Cells expressing mutated p53 resist apoptosis, promote angiogenesis and proliferation</td>
<td>(327)</td>
</tr>
<tr>
<td>HER-2/neu</td>
<td>61.2%</td>
<td>Encode transmembrane tyrosine kinase receptor related to growth factors</td>
<td>Associated with tumour progression in breast, lung and gastric carcinomas</td>
<td>(328)</td>
</tr>
</tbody>
</table>

NBP: nucleotide-binding protein
1.3.7.2 Mesothelin

The mesothelin (MSLN) gene contains an 1884bp open reading frame which encodes a 69 kDa precursor, which is processed into a 40 kDa membrane-bound form together with a 31 kDa megakaryocyte-potentiating factor (MPF). The 40 kDa mesothelin protein product is a GPI-anchored glycoprotein expressed on the cell surface (Fig.1.9) (329).

![Figure 1.9. Mesothelin protein synthesis.](image)

**Figure 1.9. Mesothelin protein synthesis.** Precursor protein which contains a potential signal peptide (SP) at NH2 terminus, the glycosylphosphatidylinositol anchor signal sequence (GASS) at COOH terminus, and a furin cleavage site (RR). The precursor gives rise to the membrane located mesothelin and a secretory protein called megakaryocyte-potentiating factor (MPF) following cleavage at the furin site (319).

1.3.7.2.1 Mesothelin as a Diagnostic Biomarker

Tissue localisation and expression of mesothelin has been studied by serial analysis of gene expression (SAGE) tag analysis (http://www.ncbi.nlm.nih.gov/projects/SAGE/) and staining with the monoclonal antibodies Mab K1 and 5B2. The Mab K1 anti-mesothelin antibody was generated by immunizing mice with the ovarian carcinoma cell line OVCAR-3, whereas Mab 5B2 was generated following immunisation with recombinant mesothelin related peptides. Using these antibodies, the tissue distribution of mesothelin was determined by immunohistochemistry. Mesothelin has limited expression in normal mesothelial cells (a single cell layer) located on pleura, peritoneum, and pericardium (330). However, as TAA, it
is overexpressed in many cancers, including mesothelioma, ovarian, pancreatic/biliary and lung cancers (319). A large number of studies have assessed immunohistochemical methods to detect mesothelin over-expression in tumour biopsies (331-333).

Elevated concentrations of soluble mesothelin in the serum of patients with mesothelioma and ovarian cancer have been described with a significant reduction after surgical resection (334). Also, higher serum mesothelin levels in ovarian cancer patients were associated with a poorer prognosis (335). This published data supports the role for mesothelin as a tool for diagnosis and monitoring of these cancers. Prior to starting this project there was little published data on the role of circulating mesothelin in pancreatic cancer, with one study describing elevation of soluble mesothelin in 99% of investigated pancreatic cancer cases (336).

1.3.7.2.2 Biology and Pathological Function

The physiological function of mesothelin is not clearly understood. In a mesothelin knockout (KO) mouse model, no phenotypic changes, functional abnormality or adverse influence on reproduction were observed (337), indicating that mesothelin was not an essential protein for survival.

There is increasing evidence to support the role of mesothelin in the initiation and development of cancer. A study has shown that mesothelin and tuberous sclerosis-2 (Tsc-2, plays a role in renal carcinogenesis) KO mice are more susceptible to renal cell carcinoma than mice that have Tsc-2 knocked out only. Thus, mesothelin may play a role in renal tumourigenesis (338). Researchers also found that the peptides derived from mesothelin can bind to ovarian cancer antigens CA125 and MUC16, which promote metastasis of CA125/MUC16 positive tumour cells metastases into mesothelin-expressing tissues, such as the peritoneal cavity (339). Further studies have identified mesothelin 296-359, the N-terminal portion (residues 296-359) present on the cell surface as a binding site for CA125 (340). These results indicate that the expression of mesothelin may play a role in the metastasis of ovarian cancers, which express MUC16 or CA125.

1.3.5.2.3 Function in Pancreatic Cancer

Over-expressions of mesothelin and CA125 have been described in pancreatic cancer. Mesothelin expression correlated positively with the degree of vascular permeation and high co-expression of mesothelin and CA125 was associated with poorer overall survival and
relapse-free survival (341). Another study has shown that mesothelin can promote pancreatic cancer tumour development and also migration/metastases in mice. In contrast, cell lines with disrupted mesothelin expression (through siRNA) gave rise to smaller, more localized tumours (342). Others have shown mesothelin to activate the Akt/NF-κB pathway in pancreatic cancer cells, increasing IL-6 expression and generating resistance to TNF-alpha induced apoptosis (343, 344).

1.3.7.2.4 Mesothelin and Cellular Immunity

As discussed earlier in this chapter, cellular immunity is important in antitumour immune responses. The number of tumour infiltrating CD4+ T cells, CD8+ T cells and DC can be correlated with the prognosis of pancreatic cancer patients (345), i.e., the more infiltrating immune cells, the better the prognosis. The induction of effective anti-tumour immune responses to TAA-related vaccines require professional APCs (especially DCs) to enroll low affinity or tolerant T cells (346), stimulate potent CD8+ T cell responses and recruit CD4+ helper T cells (90).

Mesothelin-specific T cell responses in 14 pancreatic cancer patients immunized with a GM-CSF–secreting pancreatic cancer lines vaccine were assessed for IFN-γ secretion and cytotoxicity pre and post vaccination (346). In this study, MSLN-specific T cell responses were induced. The mesothelin-specific CD8+ T cells killed a mesothelin positive pancreatic cancer cell line in vitro. Moreover, in a pilot study, the safety and immunogenicity of a GM-CSF-secreting vaccine has been evaluated in advanced pancreatic adenocarcinoma patients. This vaccine was able to enhance HLA-A1, A2 and A3 restricted CD8+ T cell responses against several mesothelin-derived peptides:

- **HLA-A1**: mesothelin310-318 EIDESLIFY and 429-437 TLDTLTAFY;
- **HLA-A2**: mesothelin20-28 SLLFLFSL and 531-539 VLPLTVAEV;
- **HLA-A3**: mesothelin225-234.

In detail, an increased frequency of IFN-γ producing CD8+ T cells was detected after vaccination, and a correlation between the numbers of post-vaccination MSLN531-539 tetramer binding cells and overall survival rate was observed (347). In a subsequent phase II study, 60 resected pancreatic cancer patients were recruited and the presence of cytokine-
secreting mesothelin-specific CD8+ T cells was correlated with disease-free survival in HLA-0101 and HLA-0201 patients (348).

A DNA vaccine targeting human mesothelin induced CD8+ T cell anti-tumour responses against ovarian cancer cells in an HLA-A2 transgenic mouse model (349), with the same group demonstrating regression of ovarian cancer following adoptive transfer of mesothelin-specific CD8+ T cells (350). Compared to using peptide-pulsed DC for vaccination, the potential advantage of DNA-based vaccines is the ability to stimulate CTLs able to recognize multiple and unknown mesothelin-derived epitopes (351).

Others have attempted to expand antigen-specific T cells from mesothelin-pulsed PBMC isolated from pancreatic cancer patients. After 1 or 2 rounds of peptide stimulation, IFN-γ secretion was identified by ELISA and flow cytometry-based intracellular cytokine staining assays. The frequency of both IFN-γ secreting CD4+ and CD8+ T cells in the total T cell population were higher in PBMC isolated from patients compared to normal controls, after mesothelin stimulation. Interestingly, mesothelin-specific T cell responses were also seen in approximately 50% of subjects tested with benign pancreatic disorders (336).

It is known that peptide mutagenesis can increase the potency of anti-tumour vaccines. For this purpose, Tsang’s group modified the mesothelin 547-566 peptide (which was identified by computer algorithm and binding assays) to increase the affinity of HLA-A2 binding. The PBMCs from healthy donors and cancer patients which were stimulated with modified peptides exhibited enhanced antigen-specific function (cytokine secretion and cytotoxicity of relevant cell lines (352).

More recently, mesothelin-specific CAR T cells were tested in a phase I study for patients with pleural malignancies (mesothelioma), which induced potent and persistent anti-tumour responses (353).

1.3.7.3 Carcinoembryonic Antigen (CEA)

1.3.7.3.1 Structure of CEA

Carcinoembryonic antigen (CEA, CEACAM5; CD66e) is an 180 kDa glycosylated membrane protein belong to the immunoglobulin supergene family, and has been studied as an attractive target for immunotherapy (321). In addition to CEA itself, the CEA family includes a series of proteins, including CEA cell adhesion molecule 1 and 6, meconium
antigen, and Tex. CEA family members consists of variable combinations of a 34 amino acid leader sequence, a 108 amino acid N-terminal IgV-like region, three 178 amino acid C2 Ig-like regions and a 27 amino acid C-terminus (containing a glycoprophatidylinositol structure) (Fig. 1.10) (354).

Figure 1.10. The structure of CEA and subgroup molecules. [cited from The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues (354).]
1.3.7.3.2 Expression and Function of CEA

During early embryogenesis development no CEA is detected, but between 9 and 12 weeks gestation CEA expression is observed on mucosal surfaces, including the lower oesophagus, the pyloric antrum, the gastro-esophageal junction and the epithelium of tongue. CEA expression then reduces in the 3rd trimester. Post-natally, CEA expression is limited to the squamous epithelium of tongue, the lower esophagus, the stomach cardia and pyloric antrum, the appendix and the colon at low levels (355). However, CEA is the most commonly over-expressed TAA in tumours originating from the gastrointestinal tract (Table 1.8):

Table 1.8. The expression of CEA in different cancers.

<table>
<thead>
<tr>
<th>Type of tumour</th>
<th>Frequency of CEA expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal carcinoma</td>
<td>94.5%</td>
<td>(356)</td>
</tr>
<tr>
<td>Pancreatic carcinoma</td>
<td>&gt;90%</td>
<td>(320)</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td>92%</td>
<td>(357)</td>
</tr>
<tr>
<td>female reproductive tract carcinoma</td>
<td>47-75%</td>
<td>(358)</td>
</tr>
<tr>
<td>Gall bladder carcinoma</td>
<td>63%</td>
<td>(359)</td>
</tr>
<tr>
<td>Non-small cell lung carcinoma</td>
<td>70%</td>
<td>(321, 360)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>50%</td>
<td>(321, 360)</td>
</tr>
</tbody>
</table>

CEA can be released by tumour cells, thus supporting its use as a serological biomarker for CEA-expressing tumours. In pancreatic cancer, a meta-study analysed the data from 1900 patients. In this cohort, the detection of elevated serum CEA had a diagnostic sensitivity of 44.2% and specificity of 82.8% with respect to identifying patients with pancreatic cancer (361). Currently, its routine use in PC screening is not yet approved (362-364). Besides, CEA is also associated with intercellular adhesions and may assist metastatic tumour spread (365).

To sum up, CEA is considered a valuable target for anti-tumour immunotherapy based on the following facts: 1) There is a differential over-expression of CEA on tumour tissue compared to normal tissue; 2) CEA is expressed in multiple cancers; 3) CEA is involved in the pathogenesis of tumour development and metastasis; 4) CEA peptides are naturally processed and presented on the surface of tumour cells (366); and 5) As a TAA, CEA-specific T cell responses are subject to immunological tolerance mechanisms, but there is evidence that tolerance is incomplete (366, 367).
1.3.7.3.3 HLA-A2 Epitopes for CEA and Clinical Usage

A number of CTL epitopes for CEA have been identified and were discussed in a recent review (321). Most studies focused on HLA-A2 restricted epitopes, due to its frequency in Caucasian populations (268). These data demonstrated that T cells isolated from healthy donors could recognize relevant CEA epitopes, after in vitro stimulation of PBMCs with peptide-pulsed APCs (321). Other strategies have been used for TAA epitope prediction including high performance liquid chromatography mass spectrometry. This method has identified CEA605, CEA691, CEA694 and other HLA-A2 binding CEA-derived epitopes (368).

The best studied epitope derived from CEA is CEA 605-631 /Cap1. Initially, using a vaccinia virus expressing CEA, functional T cell lines specific for CAP1 were generated from patients with metastatic CEA positive cancer. These T cell lines could kill colorectal cell lines in vitro (369). A number of different approaches have been used to generate CAP1 specific T cells responses, using avipox-derived vectors (370), HLA-A2 transgenic mice (371), and peptide mutagenesis (372).

Table 1.9 lists the active immunotherapy clinical trials as of August 2015 (http://www.clinicaltrials.gov) targeting CEA. Of the total of 18 clinical trials targeting CEA, 11 were vaccination-related with the majority using the modified Cap1-6D target epitope. Generally speaking, the Cap-1 or Cap-6D vaccines were safe and able to generate antigen-specific CTL responses in some of the treated patients, with occasional clinical responses (132, 373-376). Other studies were unable to trigger protective CTL responses (377). It is clear that vaccination strategies used alone are typically inadequate at inducing durable and potent antitumour responses (308). CEA694-702 is another HLA-A2 binding peptide epitope. It is thought to be of low immunogenicity and simulated low-avidity CTL (378).

CEA691 specific CD8+ T cell lines were first stimulated from PBMCs of normal donors (10 out of 48 cultures had CEA specific responses) and one T cell line was able to kill CEA-expressing colon cancer cell lines (367). A later study suggested a series of CEA691 analogs, which changed one amino acid of the CEA691-699 epitope (M3 and H5), could induce more sensitive peptide specific IFN-γ production. In this study, only 2% of tested healthy donors had wild type CEA specific response (379). No cytokines were used in the T cell cultures, which may have contributed to low responses. The modified CEA epitopes have not been used in other studies.
An HLA-A2 transgenic mouse model has been used to stimulate CEA691 specific T cells in an attempt to overcome immunological tolerance (380) and isolate high-affinity TCR for gene transfer (278). The anti-tumour efficacy of the CEA-TCR transduced T cells were evaluated in a phase I clinical trial. Three patients with metastatic colorectal carcinoma were treated. In one cancer regression was observed, but severe transient colitis was also observed resulting in early termination of the trial (308).

Two further clinical trials are assessing the safety and efficacy of CEA-targeting CAR modified T cells (381).
Table 1.9. Active clinical trials targeting CEA [correct at time of writing, July 2015]*

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Cancers</th>
<th>Phase;ID</th>
<th>Sponsor</th>
<th>Status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus vectors encoding CEA protein</td>
<td>Colon, lung, and breast cancer</td>
<td>I/II; NCT01147965</td>
<td>Etubics Corporation, Duke University</td>
<td>Completed</td>
<td>(376)</td>
</tr>
<tr>
<td>Alphavirus replicon (VRP) encoding the CEA(6D)</td>
<td>Colorectal, lung, breast and pancreatic cancer</td>
<td>I/II; NCT00529984</td>
<td>AlphaVax, Inc.; Duke University</td>
<td>Completed</td>
<td></td>
</tr>
<tr>
<td>CEA(6D) VRP Vaccine</td>
<td>Stage III Colon Cancer</td>
<td>I; NCT01890213</td>
<td>Duke University; AlphaVax, Inc.</td>
<td>Recruiting</td>
<td></td>
</tr>
<tr>
<td>Recombinant Fowlpox-CEA(6D)/TRICOM Vaccine+IFN-γ-2b, GM-CSF</td>
<td>Metastatic CEA-positive cancer</td>
<td>I; NCT00217373</td>
<td>National Cancer Institute (NCI)</td>
<td>Completed</td>
<td>(132)</td>
</tr>
<tr>
<td>Modified CEA peptide CAP 1-6D</td>
<td>Pancreatic Adenocarcinoma</td>
<td>I/II; NCT0203892</td>
<td>University of Chicago</td>
<td>Completed</td>
<td>(382)</td>
</tr>
<tr>
<td>rF-CEA(6D)-TRICOM loaded DC</td>
<td>CEA-positive cancer</td>
<td>I; NCT00027534</td>
<td>Michael Morse, MD; NCI</td>
<td>Completed</td>
<td></td>
</tr>
<tr>
<td>Vaccinia-CEA(6D)-TRICOM and Fowlpox-CEA(6D)-TRICOM + GM-CSF + Docetaxel</td>
<td>Metastatic lung cancer and colorectal cancer</td>
<td>I; NCT00088933</td>
<td>NCI</td>
<td>Terminated</td>
<td></td>
</tr>
<tr>
<td>Vaccinia-CEA(6D)-TRICOM Fowlpox-CEA(6D)-TRICOM + GM-CSF</td>
<td>Unspecified Adult Solid Tumour; Protocol Specific</td>
<td>I; NCT0009958</td>
<td>NCI</td>
<td>Terminated</td>
<td></td>
</tr>
<tr>
<td>Fowlpox-CEA(6D)/TRICOM</td>
<td>Advanced CEA positive adenocarcinoma</td>
<td>I; NCT00028496</td>
<td>NCI</td>
<td>Completed</td>
<td></td>
</tr>
<tr>
<td>CEA pulsed DCs + IL-2</td>
<td>Colorectal cancer</td>
<td>I/II; NCT00154713</td>
<td>National Taiwan University Hospital</td>
<td>N.A.</td>
<td>(374)</td>
</tr>
<tr>
<td>CEA (either peptide or mRNA) pulsed DCs</td>
<td>Colorectal cancer, liver metastases</td>
<td>I/II; NCT00228189</td>
<td>Radboud University</td>
<td>Completed</td>
<td>(375)</td>
</tr>
</tbody>
</table>

**Cell therapy**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Cancers</th>
<th>Phase;ID</th>
<th>Sponsor</th>
<th>Status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA Specific CTL stimulated by DC (infected by Adenovirus encoding CEA gene)</td>
<td>stage IV gastric cancer</td>
<td>I; NCT02496273</td>
<td>The First People's Hospital of Changzhou</td>
<td>Not yet recruiting</td>
<td></td>
</tr>
<tr>
<td>CEA691 specific haTCR CTL</td>
<td>Colon cancer</td>
<td>I; NCT00923806</td>
<td>NCI</td>
<td>Terminated</td>
<td>(308)</td>
</tr>
<tr>
<td>CAR-T (2nd generation)</td>
<td>Liver Metastases</td>
<td>I; NCT01373047</td>
<td>Roger Williams Medical Centre</td>
<td>Completed</td>
<td>(381)</td>
</tr>
<tr>
<td>CAR-T</td>
<td>Liver Metastases</td>
<td>I; NCT02416466</td>
<td>Roger Williams Medical Centre</td>
<td>Recruiting</td>
<td></td>
</tr>
<tr>
<td>CAR-T</td>
<td>Colorectal, lung, breast, gastric and pancreatic cancers</td>
<td>I; NCT02349724</td>
<td>Southwest Hospital, China</td>
<td>Recruiting</td>
<td></td>
</tr>
</tbody>
</table>

**mAb therapy**

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Cancers</th>
<th>Phase;ID</th>
<th>Sponsor</th>
<th>Status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CEA mAb M5M</td>
<td>CEA-positive cancer</td>
<td>NP; NCT02293954</td>
<td>City of Hope Medical Center; NCI</td>
<td>Recruiting</td>
<td></td>
</tr>
<tr>
<td>Anti-CEA mAb M5M</td>
<td>Unspecified Adult Solid Tumour, Protocol Specific</td>
<td>I; NCT00645060</td>
<td>City of Hope Medical Center; NCI</td>
<td>Not yet recruiting</td>
<td></td>
</tr>
</tbody>
</table>

*Note: Some trials are under recruitment, whilst others are to date unpublished. Thus, some of the trials are missing references.*
1.3.7.4 WT1 (Wilms’ Tumour Antigen 1)

The wilms’ tumour antigen 1 (WT1) gene, which encodes at least 24 proteins, functioning in regulating target gene in tissue development, differentiation and apoptosis, is essential in the normal development of several organs, including kidney, cardiovascular system, haematopoietic system, spleen, liver and lung (383). While WT1 is widely expressed in developmental stages of embryo, its expression is downregulated in many adult tissues, except some sites including adult intestine, lung mesothelium, sertoli & granulosa cells, uterus, and haematopoietic progenitor cells. The WT1 protein is also abnormally overexpressed in a variety of cancer cells, including acute myeloid leukemias (384), renal cell carcinoma (385), pancreatic cancer (326), and many other cancers initiated from breast, lung, colon, etc (386). It has been utilized as a target in developing vaccine-based immunotherapy to treat AML (387, 388), CML and myelodysplastic syndrome (389, 390), gynecological cancers (391), pancreatic cancer (392) and many other solid cancers (393). In cancer originating from the pancreatobiliary system, 65% to 75% of cases overexpressed WT1 (326, 386). After treatment with gemcitabine in vitro, the expression of WT1 was further elevated on pancreatic cancer cell lines (394).

Originally, WT1 was reported as a zinc finger transcription factor, localized on human chromosome 11 at the Wilms’ tumour locus, which functioned as a tumour suppressor gene in Wilms’ tumour - a paediatric renal cancer (395). Furthermore, WT1 was shown to be critical for cancer progression and maintenance of the malignant phenotype in leukaemia and breast cancer (396, 397). Accordingly, the degrees of WT1 over-expression in haematological malignancies and breast cancer have been correlated with poorer prognoses (384, 398). WT1 can also inhibit some growth factors that are believed to contribute in cancer development, including transcription of platelet-derived growth factor (PDGF) A chain (399), insulin-like growth factor II (400) and promoter activity of colony-stimulating factor-1 (401).

WT1 is an endogenous antigen, which can be routinely processed and presented by HLA class I molecules. Two WT1 epitopes have been carefully studied in cellular immunotherapy, namely, the HLA-A*0201 restricted pWT126 peptide (WT1 126-134) and the HLA-A*2402 restricted pWT235 peptide (WT1 235–243) (402, 403). Naturally occurring WT1-126 specific T cells have been identified in in patients with leukaemia (404), breast cancer (405) and prostate cancer (406) and also in some healthy donors, suggesting incomplete tolerance to the self-antigen. WT1-126 peptide based vaccines have been evaluated in patients with
AML and with advanced pancreatic or biliary tract cancer (392, 407). HLA-A24 restricted WT1 peptide-pulsed DC vaccination has also attracted broad interest and has been tested in preclinical studies and phase I clinical trials in combination with gemcitabine for patients with pancreatic cancer (408, 409). The results of these studies confirmed the safety of WT1-specific vaccines. However, the efficacy of the approach was limited.

Clinical grade WT1-TCR transduced T cells have been developed in our laboratory. High avidity pWT126 specific CTL were isolated using the allo-restricted approach (280). The TCR genes from this haCTL were sequenced and cloned into viral vectors. These vectors have been used to redirect the specificity of T cells, isolated from either healthy donors or leukaemia patients, using retroviral transduction methods. Antigen-specific cytotoxicity and cytokine secretion of the redirected T cells was demonstrated in vitro and in vivo (402, 410). These gene-modified T cells are currently being evaluated in phase I/II clinical trials in myelodysplasia and leukaemia patients (285, 410).
1.4 Aims and Hypothesis

This study aims to explore new strategies to improve the diagnosis and therapy of pancreatic cancers.

Initially, we investigated the role of soluble mesothelin as a potential biomarker in pancreatic cancer patients. We hypothesized that an elevation of serum mesothelin concentration would be present in patients with pancreatic cancer. We aimed to determine if circulating mesothelin levels were able to distinguish PC from pancreatic benign disease.

Next, we aimed to identify TAA-specific T cells responses in the peripheral blood of patients with pancreatic cancer. We anticipated that TAA-specific T cells were not fully tolerated and can be isolated from the circulation of PC patients. Thus, we chose mesothelin as target, and detected if CD4+ and CD8+ T cells isolated from peripheral blood of PC patients could specifically respond to peptides derived from mesothelin.

We also planned to determine the immunogenicity of TAA’s and explore their potential as targets for T cell mediated immunotherapy for PC. For this purpose, we hypothesized that CD8+ T cells specific to some epitopes from particular TAA may be more readily detectable in the peripheral blood of PC patients, and set out to identify the most immunogenic epitope in pancreatic cancer patients and generate T cell lines specific for this.

Over-expression of an antigen can stimulate T cell expansion and cytotoxicity, but can also lead to tolerance. Thus, we wanted to understand if functional T cells lines could be generated, or if they were subject to tolerance and deletion. Hence, we hypothesized that the functional T cell responses may be observable in fractions of PC patients, but in patients with terminal stages of PC, poor T cell responses may be observed. The availability of serial samples from individual patients at different stages would have been more helpful to detect the changes of T cell responses with clinical progression.

Finally, we attempted to identify if PC patients-derived TAA specific T cells, which express exhaustion makers after chronic antigen exposure in vitro, could have improved ex vivo function following PD-L1 blockade. We expected that PD-L1 blockade would enhance the TAA-specific T cell responses of pancreatic cancer patients in vitro.
Chapter 2

Materials and Methods
2.1 Patients and Samples

This study was approved by the Central London REC 3 Research Ethics Committee, and all patients gave written, informed consent. The trial was run in accordance with the Declaration of Helsinki. Following written informed consent, blood samples were obtained from patients at University College London Hospital NHS Foundation Trust, the Royal Free London NHS Foundation Trust, and Charing Cross Hospital, Imperial College London Healthcare NHS Foundation Trust. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples of patients with pancreatic cancer, benign pancreatic disease and healthy donors. Pancreatic cancer (PC) diagnosis was confirmed by standard cytopathology or histopathology after biopsy. The clinical staging of patients with pancreatic carcinoma was determined using the WHO histological classification of tumours of the exocrine pancreas.

In more detail, peripheral blood mononuclear cells were isolated from whole blood samples of patients with pancreatic cancer using Ficoll density gradient centrifugation (Lymphoprep-Apogent Discoveries, Wilmslow, United Kingdom). The cells were stored in liquid nitrogen (-196 °C) after dilution in freezing solution (10% DMSO, 50% FCS and 40% RPMI). Lymph node samples were collected from surgical patients with PC, where possible during surgical resection of the pancreatic tumour. Single cell suspensions were made by dividing the tissues thoroughly using sterile scissors. In addition, plasma samples were prepared within 20 mins after venesection. Plasma was separated from peripheral blood after spinning at 1500rpm for 5 mins, prior to storage at -20 °C in the freezer, without adding any further media.

2.2 HLA-A2 Tying

Fluorescence-activated cell sorting (FACS) analysis was used to detect HLA-A2 positive PBMCs, after staining with PE-labeled -anti-HLA-A2 antibodies (BB7.2 clone; BD Science).

2.3 Mesothelin ELISA

Plasma mesothelin (MSLN) concentration was measured by Quantikine (Catalog Number DMSLN0, R&D Systems Europe, Ltd, Abingdon, UK) - a validated double determinant sandwich ELISA. In brief, 5µl plasma (1 in 10 diluted in assay diluent) was incubated in MSLN antibody pre-coated microplates at room temperature for 2 hours. After washing unbound elements, a MSLN-specific enzyme-linked monoclonal antibody was added and incubated for a further 2 hours, prior to adding substrate for 30 minute incubation. The MSLN antibody was conjugated to horseradish peroxidase with preservatives, and the
substrate solution contained stabilized hydrogen peroxide and tetramethylbenzidine. A microplate reader was used to determine optical density after stopping the development reaction with sulfuric acid.

2.4 Cytometric Bead Array (CBA)

Concentrations of IL-1β, IL-6, IL-8, IL-10, IL-12, and TNF-α in plasma samples were determined using the BD cytometric bead array human inflammatory cytokines kit (BD Biosciences, CA, US). Manufacturer’s instructions were followed. In brief, beads coated with antibodies binding the above-mentioned cytokines were incubated with plasma for 3 hours in order to ‘capture’ soluble cytokines. The samples were analyzed by FACSCanto II flow cytometer (BD Biosciences) in the FL-3 channel using FCAP Array Software v3.0 (BD Biosciences). The cytokine kit also provided standards for each cytokine, which could also be read out by FACS machine and analyzed by FCAP Array. By making duplicate dilutions, the standards were used to generate a standard curve.

2.5 Peptide Library

Overlapping peptides corresponding to the full length amino acid sequence of Mesothelin and 18 PC-related HLA-A2 restricted peptides were synthesized by Mimotopes Pty Ltd. (Clayton Victoria, Australia). For MSLN, fifteen amino acid long peptides, overlapping by five amino acids, were pooled into 7 distinct peptide pools. Details of amino acid sequences are given in related chapters. All peptides were dissolved first in DMSO and then PBS at a stock concentration of 2 mmol/L and stored at -20 °C.

2.6 Flow Cytometry

The antibodies used in our experiment were: PE-Cy7 CD3 (Clone: SK7), Horizon v450 CD8 (RPA-T8), Horizon v500 CD4 (RPA-T4), FITC IFN-γ (B27), PerCP-Cy5.5 CD27 (M-T271), PE PD-1 (MIH4), APC CD28 (CD28.2), BV650 CD45RO (UCHL1) (all abovementioned were purchased from BD Biosciences Oxford, United Kingdom), FITC LAG-3 (Clone: #87450)1, AF700 TIM3 (#344823) (R&D Systems, Abingdon, United Kingdom), and APC-Cy7 CD62L (Clone: DREG56) (eBioscience, Hatfield, United Kingdom). Surface staining was performed using relevant antibodies in aliquots of 1×10^6 cells. One microliter of a 1:50 dilution of the antibody was added for 30 minutes at 4 °C (on ice). Cells were washed twice with PBS/1% FCS and then resuspended in 200 μL PBS/1% FCS for data acquisition.
Propidium iodide was added to discriminate dead cells from viable cells. All data acquisition was obtained using FACSLSR-II and analysed using FlowJo software.

2.7 Tetramer Staining of Peripheral T Cells

APC-labeled tetramers consisting of CEA691 peptide bound to HLA-A*0201 molecules were used to detect CEA-specific T cells (TCMetrix, Zurich, Switzerland). The tetramers were stored at 4 °C. Cells were spun down and the supernatant was discarded, before they were resuspended in 1 μl tetramer together with 50 μl FACS buffers. After 30 mins’ incubation at room temperature (avoiding light), the cells were washed twice in FACS buffer prior to surface staining and FACS analysis.

2.8 Short Term in Vitro T Cell Expansion

Short-term (9-10 days) culture of T cells was performed to identify the presence T cells responding to individual peptides or peptide pools. In brief, PBMCs were resuspended at a concentration of 1.5 × 10^6/mL in in normal growth medium (NGM) containing RPMI 1640 (Invitrogen, Paisley, United Kingdom) supplemented with 2mmol/L glutamine, 1% penicillin plus streptomycin (Sigma-Aldrich, St. Louis, MO), and 10% heat-inactivated FCS (BioWest, Ringmer, United Kingdom). PBMCs were stimulated with different peptides or MSLN peptide pools at a final concentration of 2μmol/L (for each individual peptide), in the presence of recombinant IL-2 (20UL/mL). The cells were cultured at 37 °C, 0.5%CO2, and harvested after up to 10 days in culture.

T cell lines were re-stimulated with the same peptide pools for a further 5 hours in the presence of Brefeldin A. Cells were surface stained with anti-CD4 and anti-CD8 antibodies (BD PharMingen, Cowley, United Kingdom), then permeabilized and fixed using formaldehyde and saponin. After fixation, the cells were stained for intracellular cytokines with FITC-conjugated anti IFN-γ (Clone: #25718), PE-conjugated anti-IL-2 (#5334), and APC-conjugated anti-TNFα (#6402) (R&D Systems, Abingdon, United Kingdom), prior to FACs analysis.

2.9 Individual MSLN-Peptide Stimulation Experiments

As stated above, the MSLN overlapping peptide -pools were used to scan for peptide specific T cell responses in patients and controls. In some patients where MSLN-specific CD4+ T cell responses were observed and surplus PBMC were available for further testing, additional
experiments were performed to identify the specific immunogenic cognate peptides. PBMCs were cultured with relevant stimulating peptide pool in the presence of IL-2 containing NGM (as before) for up to 10 days. On day 9 or 10, these cells were further cultured for 5 hours in the presence of individual peptides from the relevant peptide pool, or with an irrelevant control peptide. The T cells were then stained with anti-CD4, anti-CD8 and IFN-γ antibodies prior to analysis by flow cytometry.

2.10 Generation Long Term T Cell Line

PBMCs were further expanded by 4 rounds of in vitro peptide-specific stimulation. Initially, PBMCs were resuspended in NGM at a density of 3×10⁶ cells per 2 mL medium in a 24-well plate. The cytokines IL-2 (Roche, Basel, Switzerland), IL-7 (R&D Systems, Abingdon, Oxfordshire, UK), IL-15 (R&D Systems) and IL-21 (R&D Systems) were added to the medium on the first day of each stimulation. The final concentration of cytokines used was as follows: 20 U/ml of human recombinant IL-2, 2 ng/ml human recombinant IL-7, 5 ng/ml human recombinant IL-15 and 0.5 ng/ml mouse recombinant IL-21. Peptide-specific stimulation was done by adding CEA691, MSLN 547, WT1 126 or CMV p65 directly to the medium at a concentration of 10μmol/L. After a culture period of 7-9 days an aliquot of cells were tested for intracellular cytokine secretion prior to replating for subsequent restimulation. Cells were seeded at 5×10⁵ cells per well (24-well plate) in 2 ml NGM with cytokines as above. T cells were stimulated with irradiated 2×10⁵ (70 Gy) T2 cells pulsed with 10μmol/L relevant peptide. Irradiated (35 Gy) autologous PBMCs (2×10⁶) or PBMC from healthy HLA-A2 positive donors obtained from the National Blood Service (Colindale, UK) were used as feeder cells.

2.11 Intracellular Cytokine Staining

Antigen-specific intracellular cytokine secretion (IFN-γ, TNF-α and IL-2) was detected using FACs analysis. 1×10⁶ T cells were stimulated with 1×10⁶ T2 cells loaded with 10μmol/L of relevant peptide or irrelevant peptide for 5 hours. Typical irrelevant control peptides were HLA-A2 restricted but derived from unrelated tumour associated antigens. Brefeldin A (Sigma) was added at a concentration of 10μg/mL to block cytokine secretion. After 5 hours incubation at 37 °C, cells were washed prior to staining with CD3 (PE-Cy7), CD4 (Horizon v500) and CD8 (Horizon v450) antibodies for 20 minutes in dark at 4 °C. Following this, cells were fixed with FACS fix/perm solution A (Invitrogen, Paisley, United Kingdom). After
20 minutes incubation at 4°C and two washes with PBS/1% FCS, cells were stained with IFN-γ (FITC), TNF-α (APC) and IL-2 (PE) antibodies in fix/perm solution B for 30 minutes at 4 °C. Finally, samples were resuspended in 200 μL of PBS/1% FCS prior to data acquisition. An antigen-specific immune response was defined as a ≥ two-fold increase in the frequency of cytokine-producing cells above the response generated to the control/irrelevant peptide.

2.12 Tumour Cell Lines

Six pancreatic cancer cell lines (Panc-1, MiaPaca2, PK-1, Bx-Pc-3 KLM-1 and PK-45H) were obtained from PIKEN BioResource centre (PIKEN BRC, Tsukuba, Japan). Among them, Panc-1, MiaPaca2, PK-1, and Bx-Pc-3 were used in cytotoxicity assays. KLM-1 and PK-45H were not for further cytotoxic test. Apart from MiaPaca2, all other cell lines were maintained in normal growth medium, described above. MiaPaca2 cells, were cultured in DMEM (Invitrogen, Paisley, United Kingdom) with 1% penicillin plus streptomycin and 10% heat-inactivated FCS. The HLA-A2-positive T2 cell line is deficient in the transporter associated with antigen processing (TAP) and can be efficiently loaded with exogenous peptides (411). T2 cells were maintained in NGM.

2.13 Cytotoxic Assay

A carboxy fluorescein succinimidyl ester (CFSE) cytotoxicity assay was used in our study to determine the antigen-specific cytotoxicity of expanded T cell lines. CEA691 peptide-loaded T2 cells or HLA-A2 positive pancreatic cancer cell lines (known to be CEA+) were used as specific target cells. T2 cells pulsed with irrelevant peptides and HLA-A2 negative pancreatic cancer cell lines were used as control target cells.

In detail, we set up two wells which included only the specific and control target cells (both types of cells at the same number) as standards. Thus, after gating on the T2 cells/PC cells, singlet, and stained cells, two nearly equal peaks (cell numbers) should have been observed. The mean of the two wells were calculated and used as the standard, meaning in other wells, the original proportion of the specific and control target should be similar to this number. In the wells with both target cells and effector cells, effector cells can kill a fraction of specific target cells. Thus, more debris can be seen in the FSC-SSC figure, and a group of unstained cells were gated out before we read the proportions of the two CFSE stained peaks (Fig. 5.1).
1 x 10^6 target cells were suspended in PBS/1%FCS at a concentration of 10^6/mL and then stained with CFSE. For sensitive targets, 0.5μl of CFSE stock solution (5mM) was added to 1 ml of cell suspension, while for control targets, 0.5μl of diluted CFSE at 500μM was used. After 4 minutes’ incubation at room temperature, 9mL of PBS/1%FCS was added to stop the reaction. The cells were washed with PBS then re-suspended at 5x10^4 cells/mL prior to setting up co-cultures with the effector cells. T cells (effectors) were added to round-bottomed 96-well plates to obtain a total volume of 200 mL/well (412). The E:T ratio (effector : target) was titrated at 100:1, 50:1, 20:1, 10:1, 5:1, 2.5:1, 1.25:1 and 0.625:1 respectively. Assay plates were incubated for 4 hours at 37 °C, 5% CO2. Cells were washed in PBS prior to FACS analysis (FACSCalibur). For peptide titration assays, CFSE-stained T2 cells were loaded with variable concentration of peptides, at 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}, 10^{-12} M, respectively (413).

2.14 PD-L1 and TIM-3 blockade

PBMCs isolated from PC patients were cultured in NGM in the presence of CEA691 peptide and cytokines as described above, at a concentration of 2x10^5/mL in 200mL media. 10 μg/mL anti-PD-L1 MAb (Clone: MIH1), and 10μg/mL anti-TIM-3 mAb (Clone: F38-2E2) (both mouse MAb from eBioscience) were added to the media separately or in combination on day 1, and after 7 days’ incubation at 37C, the cells were harvested for intracellular cytokine secretion assays and tetramer staining.

2.15 IL-10 Blockade

In selected cases (where sufficient cells were available), peptide stimulation of PBMC was performed in the presence of IL-10 blocking antibodies. MSLN-specific T cell lines were generated (as described above) in the presence of anti-IL10 (Clone: JES3-9D7, 5 μg/mL) and anti-IL-10R (Clone:1B1.3A,10 μg/mL) antibodies (eBioscience). On day 4, 150μl supernatant was removed, and replenished with 150μl of fresh medium with additional anti-IL-10 (5 μg/ml), IL-10Ra (10 μg/ml) antibodies, and the cytokine IL-2 (25UL/mL). The re-stimulation and intracellular cytokine staining at day 9-10 was performed as described above.

2.16 Reverse Transcription, Ligation, Sequencing and High-Throughput Sequencing

1 μg of RNA was treated with RQ1 DNase (Promega) to remove any genomic DNA. Two primers (αRC2 and βRC2) specific for the constant region of the TcR alpha and beta chain were used to reverse transcribe the RNA into cDNA with Superscript III reverse transcriptase
(all Life Technologies). The cDNA was purified via MinElute PCR (polymerase chain reaction) purification columns (Qiagen) using manufacturer’s recommendations and eluted in 10 μl water.

A ssDNA oligo was ligated to the 3’ end of the cDNA. The ssDNA oligo contained an Illumina Sequencing Primer 2 (SP2) sequence as well as one or two random hexamers to uniquely label each cDNA molecule with a barcode.

TcR chains were then amplified using two consecutive PCR reactions. In the first PCR TcRs that had been ligated to the ssDNA oligo were amplified. The PCR product was purified using AMPure beads at a ratio of 1:1 beads to sample and eluted in 30μl water. During the second PCR two 6 basepair indices were added to each end of the product in order to multiplex several different samples on one sequencing run. Additionally, an Illumina Sequencing Primer 1 (SP1) complementary sequence was introduced directly followed by a random hexamer to increase the diversity of the bases used at the beginning of the sequencing run. The Illumina adapters P5 and P7 were introduced either end of the product to enable the DNA to bind to the flow cell of an Illumina sequencing machine. The sample was split in two to amplify the alpha and beta chain of the TcRs separately. The PCR was performed as a Cybergreen qPCR (ABI) in order to stop the PCR reaction once enough products had been formed (Ct threshold was set to 0.01). PCR 2 products were bead purified and eluted in 30 μl water. The concentration of the final product was measured using a high-sensitivity dsDNA kit on a qu-bit spectrophotometer and the size of the product was analysed using a TapeStation or Bioanalyzer.

A 4 nM pool of 10-12 samples was sequenced on a MiSeq machine (Illumina) according to manufacturer’s instructions.

2.17 Statistical Analysis

Statistical analysis was performed using SPSS 17.0 for windows (for Chapter 3). The Mann-Whitney U test (for 2 groups) and Kruskal-Wallis test (for 3 groups) analyses were applied to compare the plasma levels of MSLN and the 6 cytokines in PC patients compared to control subjects. The Chi-squared test was used to determine statistical significance of MSLN-specific CD4+ and CD8+ T cell response rates in PC and control groups. The Spearman rank coefficient was utilized to check the correlation between plasma MSLN levels and T cell
response levels. Kruskal-Wallis test was employed to analyse the levels of T cell response between PC, benign pancreatic disease patients and healthy volunteers. A significant interval was defined at 95%, or two tailed p-value ≤ 0.05.

Due to the updating of software, SPSS 21 software was used for statistical analysis in Chapter 4 and 5. Data were tested for normal distribution (using Skewness–Kurtosis test) and homogeneity of variance (homogeneity of variance test) to evaluate whether they were parametric. If the data were parametric, the T test was used to determine the statistical significance. For nonparametric data, the Mann–Whitney U test was applied. When comparing the dataset for more than two groups, the one-way ANOVA test (for parametric data) or Kruskal–Wallis test (for nonparametric data) was used. When the overall P values were statistically significant, posthoc pairwise comparison with the Tukey Honest Significant Difference (HSD) method was performed. P < 0.05 was considered to be statistical significant. Grouped data was analyzed by paired t test for parametric data or Wilcoxon matched-pairs signed rank test for nonparametric data.
Chapter 3

Anti-Mesothelin T Cell Responses and Identification of Novel Class II-Restricted Peptides in Patients with Pancreatic Cancer
3.1 Introduction

Cell-based anti-tumour immunotherapy is a promising strategy for the treatment of pancreatic cancer (86). Th1 type helper CD4+ T cells with the ability to secrete IFN-γ and TNF-α can act as effectors in anti-tumour immunity and provide ‘help’ for the development of potent anti-tumour CTLs (90, 91). Activated tumour-infiltrating lymphocytes have also been observed in many types of tumours and can confer a better prognosis (83, 84).

Successful anti-tumour T cells responses rely on the recognition of tumour-associated antigens (TAAs) by T cells (414). Mesothelin is a 40k-Da GPI-anchored glycoprotein expressed on the cell surface, and can also be released by Phospholipase C (329), which is expressed in normal cells at low level (330), but overexpressed in many kinds of cancers, including pancreatic cancer (319). Studies have shown that mesothelin can be identified in 91%-100% pancreatic cancer tissue (331-333). In addition, mesothelin was reported to be associated with poor prognosis (341) and promoted metastasis of tumour cells (342). A pilot study further illustrated that an allogeneic GM-CSF secreting pancreatic cancer cell vaccine could stimulate MSLN specific T cells in gemcitabine-resistant advanced pancreatic cancer patients (346). Thus, MSLN is a possible biomarker for pancreatic cancer and a target for T-cell based immunotherapy.

The profile of circulating cytokines, e.g., IL-10, may also change in PC patients. IL-10 is generally regarded as an anti-inflammatory and immunosuppressive cytokine. This cytokine is secreted by regulatory T cells, macrophages, Th1 cells and DCs. During infection, it inhibits the activity of Th1 cells, NK cells, and macrophages, resulting in balance of pathogen clearance and tissue damage (415). Other studies have shown that IL-10 secretion reduces the expression of MHC class I, which makes tumour cells less readily recognized by CTLs, whereas susceptible to NK cell–mediated tumour cell lysis (416).

However, less is known about the diagnostic value of circulating mesothelin levels in PC patients, and there is very little information on naturally occurring MSLN-specific T cell responses in cancer patients. This study aimed to identify and functionally characterise MSLN-specific T cell responses and establish whether the elevation of immune-regulatory cytokines such as IL-10 can modulate this response.
To detect the T cell responses to mesothelin peptides and discover possible new epitopes, we generated a mesothelin derived peptide library. The Mesothelin derived overlapping peptide library contained peptides of 15 mer amino acids and was generated by Minotopes Ltd (Wirral, UK). The sequence information of individual peptides contained within the library is shown in Table 3.1. This approach cannot cover all the potential peptides derived from mesothelin (possible with a 14AA overlapping library, in which each peptide has only one AA in common), but it can be used to quickly scan unknown epitopes and shorten the list of potential peptide epitopes (417). Due to the limitation of patient samples, stimulating T cells with every peptide was also not possible. We therefore pooled peptides into 7 pools as shown in the Table. The pools are generated according to the orders of peptides. Only after having observed T cells responses to any certain pool, we further stimulated the fresh PBMCs again using different peptides in that pool.

Table 3.1. Mesothelin-devided peptides and pools

<table>
<thead>
<tr>
<th>Amino acid start</th>
<th>pool</th>
<th>sequence</th>
<th>Amino acid start</th>
<th>pool</th>
<th>sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>MALPTARPLGSCGT</td>
<td>321</td>
<td>4</td>
<td>WELEACVDAAALLATQ</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>GSCGTPLGALLSFLF</td>
<td>331</td>
<td>4</td>
<td>LLATQRMDRVAIPIFT</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>LFLFSLGQWQPSR</td>
<td>341</td>
<td>4</td>
<td>AIPDTYEQLDVKHLK</td>
</tr>
<tr>
<td>31</td>
<td>1</td>
<td>VQPSRLAGETGQA</td>
<td>351</td>
<td>4</td>
<td>VLPKHLDELYPGPGY</td>
</tr>
<tr>
<td>41</td>
<td>1</td>
<td>TGGEAAPLDGVLANP</td>
<td>361</td>
<td>4</td>
<td>PQQYESVICQHGLY</td>
</tr>
<tr>
<td>51</td>
<td>1</td>
<td>VLAPNPNSSLSQPR</td>
<td>371</td>
<td>5</td>
<td>HLGYLFLKMSGPDIR</td>
</tr>
<tr>
<td>61</td>
<td>1</td>
<td>LSPRQLLGFPCEAVS</td>
<td>381</td>
<td>4</td>
<td>PEDIKRVNVTSELT</td>
</tr>
<tr>
<td>71</td>
<td>1</td>
<td>GAEVSLSTKERVREL</td>
<td>391</td>
<td>4</td>
<td>SLETKLALAVNQKH</td>
</tr>
<tr>
<td>81</td>
<td>1</td>
<td>RVLRELAVALAQNKV</td>
<td>401</td>
<td>5</td>
<td>VNKGHEMSQAPPRRP</td>
</tr>
<tr>
<td>91</td>
<td>1</td>
<td>QKNVSLQSTKRCAL</td>
<td>411</td>
<td>5</td>
<td>APRRLPQVATLDR</td>
</tr>
<tr>
<td>101</td>
<td>2</td>
<td>LRLCLAHLSEPPDLDL</td>
<td>421</td>
<td>5</td>
<td>QLIDRQVNFGRQGDK</td>
</tr>
<tr>
<td>111</td>
<td>2</td>
<td>PPDLLDALPLDLLL</td>
<td>431</td>
<td>5</td>
<td>LQLDDKQTLTDLAFY</td>
</tr>
<tr>
<td>121</td>
<td>2</td>
<td>DLLFLNPDAFSGPGQ</td>
<td>441</td>
<td>5</td>
<td>LTAQTYPGYLCILSPE</td>
</tr>
<tr>
<td>131</td>
<td>2</td>
<td>FSGPOACTRFFSRIT</td>
<td>451</td>
<td>5</td>
<td>SLSPEELSSVPSSS</td>
</tr>
<tr>
<td>141</td>
<td>2</td>
<td>FSRTKANVQDLPRLG</td>
<td>461</td>
<td>5</td>
<td>PPSWNAVQRPQDLD</td>
</tr>
<tr>
<td>151</td>
<td>2</td>
<td>LPRPQAGERPLLPA</td>
<td>471</td>
<td>5</td>
<td>QQDLTDCTQPLQDLDVY</td>
</tr>
<tr>
<td>161</td>
<td>2</td>
<td>RLLLAACLVWGRGDS</td>
<td>481</td>
<td>5</td>
<td>LVLYLPKARLAFQNM</td>
</tr>
<tr>
<td>171</td>
<td>2</td>
<td>GVRRSSSLSEYVRA</td>
<td>491</td>
<td>5</td>
<td>AFQCNMNGSEYFKVIQ</td>
</tr>
<tr>
<td>181</td>
<td>2</td>
<td>DVRALGGLACDLPG</td>
<td>501</td>
<td>6</td>
<td>FVQKIQSLGFGAPTED</td>
</tr>
<tr>
<td>191</td>
<td>2</td>
<td>DLPGRFVAEAVEL</td>
<td>511</td>
<td>6</td>
<td>APTEVKALSQLQVS</td>
</tr>
<tr>
<td>201</td>
<td>2</td>
<td>AEVLPRLVSCPQPGL</td>
<td>521</td>
<td>6</td>
<td>QCNQSMDLATFMKLR</td>
</tr>
<tr>
<td>211</td>
<td>3</td>
<td>CPGPQLDQOQEAAAA</td>
<td>531</td>
<td>6</td>
<td>FKLRTQAVLVPLTVA</td>
</tr>
<tr>
<td>221</td>
<td>3</td>
<td>EARAALQGGGPGPQG</td>
<td>541</td>
<td>6</td>
<td>PLTVAEVQKLGGPPH</td>
</tr>
<tr>
<td>231</td>
<td>3</td>
<td>GPPYGGPSTWKSVM</td>
<td>551</td>
<td>6</td>
<td>LGPHVEGKAEHRH</td>
</tr>
<tr>
<td>241</td>
<td>3</td>
<td>SYSTMADLRRLLPV</td>
<td>561</td>
<td>6</td>
<td>EERHPDVRLWORQR</td>
</tr>
<tr>
<td>251</td>
<td>3</td>
<td>LLPVGLQPIRSIQQ</td>
<td>571</td>
<td>7</td>
<td>ILRQRQDDLDLTLGG</td>
</tr>
<tr>
<td>261</td>
<td>3</td>
<td>RISPQGIIIAYWRQIA</td>
<td>581</td>
<td>7</td>
<td>TLGILLGGGIPNGY</td>
</tr>
<tr>
<td>271</td>
<td>3</td>
<td>WQRJRSRDSVPWRQPOE</td>
<td>591</td>
<td>7</td>
<td>PNYGILDSMQLQAE</td>
</tr>
<tr>
<td>281</td>
<td>3</td>
<td>WIRQPIERTLPRFPRRF</td>
<td>601</td>
<td>7</td>
<td>MPEALSSTGCLLPPG</td>
</tr>
<tr>
<td>291</td>
<td>3</td>
<td>PFRFREVEKTAPSG</td>
<td>611</td>
<td>7</td>
<td>LLGPGFLVVTAYLL</td>
</tr>
<tr>
<td>301</td>
<td>4</td>
<td>ACPSGKKAREIDSL</td>
<td>616</td>
<td>7</td>
<td>PVLYVLALLLASTLA</td>
</tr>
<tr>
<td>311</td>
<td>4</td>
<td>IDESLFYYKVWELEA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.4. Results

Analysis of peripheral blood and PBMC samples

In total, peripheral blood samples of 34 patients with pancreatic cancer (Table 3.2), 15 patients with benign pancreatic disease (Table 3.3), and 16 healthy donors (Table 3.4) had been collected for our experiment in this stage. All patients were reviewed at University College London Hospitals, NHS Foundation Trust, Royal Free London Hospital, NHS Foundation Trust, and Charing Cross Hospital (IC, London, UK). Recruited patients gave informed consent and the study was approved by the National Research Ethics Service (study No 06/Q0512/106). 15-25ml blood samples were obtained using heparin containing Vacutainers (BD, NJ) from patients with pancreatic cancer or benign pancreatic disease. PC diagnosis was confirmed by standard cytopathology or histopathology after biopsy as routine clinical service.

Peripheral blood mononuclear cells (PBMCs) were isolated from 25 PC patients, 15 benign pancreatic disease patients and 16 healthy controls to stimulate and expand short term T cell lines in the presence of mesothelin peptides pools prior to analysis for IFN-γ production using intracellular cytokine assays. The individual peptides which stimulated IFN-γ production within the relevant responding peptide pools were further identified. 9 out of 34 PC patients (PC26-34) who we did not collect enough amounts of peripheral blood were not used in T cell stimulation cultures. However, we still isolated the plasma from these samples for analysis.
Table 3.2. Patients demographic information (PC patients only, n=34)

<table>
<thead>
<tr>
<th>ID of PC patients</th>
<th>Age (year)</th>
<th>Gender</th>
<th>Histological diagnosis</th>
<th>Stage</th>
<th>Plasma mesothelin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC01</td>
<td>63</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>IV</td>
<td>21.8</td>
</tr>
<tr>
<td>PC02</td>
<td>77</td>
<td>M</td>
<td>Adenocarcinoma with squamous differentiation</td>
<td>IV</td>
<td>21.6</td>
</tr>
<tr>
<td>PC03</td>
<td>47</td>
<td>M</td>
<td>PDAC</td>
<td>IIB</td>
<td>74.0</td>
</tr>
<tr>
<td>PC04</td>
<td>80</td>
<td>M</td>
<td>Epithelial neoplasia</td>
<td>n.a.</td>
<td>53.4</td>
</tr>
<tr>
<td>PC05</td>
<td>66</td>
<td>F</td>
<td>PDAC</td>
<td>n.a.</td>
<td>13.8</td>
</tr>
<tr>
<td>PC06</td>
<td>80</td>
<td>F</td>
<td>PDAC</td>
<td>n.a.</td>
<td>20.1</td>
</tr>
<tr>
<td>PC07</td>
<td>72</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>n.a.</td>
<td>13.4</td>
</tr>
<tr>
<td>PC08</td>
<td>54</td>
<td>F</td>
<td>Poorly differentiated adenocarcinoma</td>
<td>IIB</td>
<td>n.a.</td>
</tr>
<tr>
<td>PC09</td>
<td>41</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>n.a.</td>
<td>13.3</td>
</tr>
<tr>
<td>PC10</td>
<td>71</td>
<td>F</td>
<td>Morderately differentiated AD</td>
<td>IIA</td>
<td>34.9</td>
</tr>
<tr>
<td>PC11</td>
<td>86</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>III</td>
<td>n.a.</td>
</tr>
<tr>
<td>PC12</td>
<td>83</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>IIA</td>
<td>27.3</td>
</tr>
<tr>
<td>PC13</td>
<td>n.a.</td>
<td>F</td>
<td>Moderately differentiated AD</td>
<td>n.a.</td>
<td>42.3</td>
</tr>
<tr>
<td>PC14</td>
<td>60</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>IIB</td>
<td>26.4</td>
</tr>
<tr>
<td>PC15</td>
<td>80</td>
<td>F</td>
<td>Morderately differentiated AD</td>
<td>IIB</td>
<td>52.3</td>
</tr>
<tr>
<td>PC16</td>
<td>74</td>
<td>M</td>
<td>PDAC</td>
<td>IV</td>
<td>75.78</td>
</tr>
<tr>
<td>PC17</td>
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<td>M</td>
<td>PDAC</td>
<td>IV</td>
<td>27.034</td>
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<tr>
<td>PC18</td>
<td>67</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>Post-operative</td>
<td>18.17</td>
</tr>
<tr>
<td>PC19</td>
<td>94</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>IV</td>
<td>40.1</td>
</tr>
<tr>
<td>PC20</td>
<td>76</td>
<td>M</td>
<td>PDAC</td>
<td>III</td>
<td>38.9</td>
</tr>
<tr>
<td>PC21</td>
<td>69</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>IV</td>
<td>27.6</td>
</tr>
<tr>
<td>PC22</td>
<td>65</td>
<td>M</td>
<td>Recurrent adenocarcinoma</td>
<td>III</td>
<td>100.956</td>
</tr>
<tr>
<td>PC23</td>
<td>61</td>
<td>M</td>
<td>Recurrent adenocarcinoma</td>
<td>III</td>
<td>30.7</td>
</tr>
<tr>
<td>PC24</td>
<td>41</td>
<td>M</td>
<td>Poorly differentiated ampullarycancer</td>
<td>IV</td>
<td>18.2</td>
</tr>
<tr>
<td>PC25</td>
<td>69</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>IIB</td>
<td>32.7</td>
</tr>
<tr>
<td>PC26</td>
<td>49</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>n.a.</td>
<td>14.1</td>
</tr>
<tr>
<td>PC27</td>
<td>63</td>
<td>F</td>
<td>Morderately differentiated AD</td>
<td>IIB</td>
<td>61.1</td>
</tr>
<tr>
<td>PC28</td>
<td>77</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>IV</td>
<td>43.4</td>
</tr>
<tr>
<td>PC29</td>
<td>79</td>
<td>F</td>
<td>n.a. (malignant cells were seen)</td>
<td>n.a.</td>
<td>23.6</td>
</tr>
<tr>
<td>PC30</td>
<td>70</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>n.a.</td>
<td>7.3</td>
</tr>
<tr>
<td>PC31</td>
<td>61</td>
<td>M</td>
<td>Ampullary cancer</td>
<td>IV</td>
<td>40.6</td>
</tr>
<tr>
<td>PC32</td>
<td>67</td>
<td>F</td>
<td>adenocarcinoma</td>
<td>IIB</td>
<td>46.7</td>
</tr>
<tr>
<td>PC33</td>
<td>58</td>
<td>M</td>
<td>PDAC</td>
<td>n.a.</td>
<td>11.5</td>
</tr>
<tr>
<td>PC34</td>
<td>74</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>IV</td>
<td>53.2</td>
</tr>
</tbody>
</table>

PDAC: pancreatic ductal adenocarcinoma. AD: adenocarcinoma. n.a.: not available.
Table 3.3 Patients demographic information (Benign disease patients, n=15)

<table>
<thead>
<tr>
<th>ID of control patients</th>
<th>Age (year)</th>
<th>Gender</th>
<th>Diagnosis</th>
<th>Plasma mesothelin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON01</td>
<td>40</td>
<td>M</td>
<td>Chronic pancreatitis</td>
<td>56.7</td>
</tr>
<tr>
<td>CON02</td>
<td>50</td>
<td>F</td>
<td>Acute pancreatitis, cyst</td>
<td>31.6</td>
</tr>
<tr>
<td>CON03</td>
<td>77</td>
<td>F</td>
<td>Mucinous cystic lesion</td>
<td>11.5</td>
</tr>
<tr>
<td>CON04</td>
<td>54</td>
<td>F</td>
<td>Benign lesion in pancreas</td>
<td>17.1</td>
</tr>
<tr>
<td>CON05</td>
<td>32</td>
<td>F</td>
<td>Recurrent pancreatitis</td>
<td>n.a.</td>
</tr>
<tr>
<td>CON06</td>
<td>50</td>
<td>F</td>
<td>Acute pancreatitis secondary</td>
<td>n.a.</td>
</tr>
<tr>
<td>CON07</td>
<td>63</td>
<td>M</td>
<td>Cystic disease</td>
<td>33.9</td>
</tr>
<tr>
<td>CON08</td>
<td>41</td>
<td>M</td>
<td>Chronic pancreatitis</td>
<td>62.5</td>
</tr>
<tr>
<td>CON09</td>
<td>82</td>
<td>M</td>
<td>Cystic disease</td>
<td>18.2</td>
</tr>
<tr>
<td>CON10</td>
<td>n.a.</td>
<td>F</td>
<td>Chronic pancreatitis</td>
<td>7.6</td>
</tr>
<tr>
<td>CON11</td>
<td>46</td>
<td>F</td>
<td>Chronic pancreatitis</td>
<td>11.3</td>
</tr>
<tr>
<td>CON12</td>
<td>24</td>
<td>M</td>
<td>Cystic disease, acute pancreatitis</td>
<td>0</td>
</tr>
<tr>
<td>CON13</td>
<td>59</td>
<td>F</td>
<td>Recurrent pancreatitis</td>
<td>31.0</td>
</tr>
<tr>
<td>CON14</td>
<td>50</td>
<td>M</td>
<td>Necrotic pancreatitis</td>
<td>19.1</td>
</tr>
<tr>
<td>CON15</td>
<td>53</td>
<td>F</td>
<td>Cyst in body of pancreas</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

The healthy volunteers were collected from staff in the Division of Hepatology UCL, and at Charing Cross Hospital. The healthy donors must have no known clinical diagnosis of acute or chronic disease and are asymptomatic at the time of donation and are not on regular medication. None of the healthy volunteers are known to have developed cancer to date. The information of these samples was given in the following tables:

Table 3.4 Demographic information for healthy controls (n=16)

<table>
<thead>
<tr>
<th>ID of healthy control</th>
<th>Age (year)</th>
<th>Gender</th>
<th>Plasma mesothelin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H01</td>
<td>22</td>
<td>M</td>
<td>32.20</td>
</tr>
<tr>
<td>H02</td>
<td>n.a.</td>
<td>F</td>
<td>n.a.</td>
</tr>
<tr>
<td>H03</td>
<td>28</td>
<td>M</td>
<td>22.60</td>
</tr>
<tr>
<td>H04</td>
<td>45</td>
<td>M</td>
<td>4.13</td>
</tr>
<tr>
<td>H05</td>
<td>42</td>
<td>F</td>
<td>12.31</td>
</tr>
<tr>
<td>H06</td>
<td>27</td>
<td>F</td>
<td>9.19</td>
</tr>
<tr>
<td>H07</td>
<td>47</td>
<td>M</td>
<td>9.06</td>
</tr>
<tr>
<td>H08</td>
<td>29</td>
<td>M</td>
<td>0</td>
</tr>
<tr>
<td>H09</td>
<td>23</td>
<td>F</td>
<td>n.a.</td>
</tr>
<tr>
<td>H10</td>
<td>27</td>
<td>F</td>
<td>16.77</td>
</tr>
<tr>
<td>H11</td>
<td>34</td>
<td>M</td>
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</tr>
<tr>
<td>H12</td>
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<td>9.55</td>
</tr>
<tr>
<td>H13</td>
<td>n.a.</td>
<td>M</td>
<td>n.a.</td>
</tr>
<tr>
<td>H14</td>
<td>n.a.</td>
<td>M</td>
<td>0</td>
</tr>
<tr>
<td>H15</td>
<td>38</td>
<td>F</td>
<td>13.00</td>
</tr>
<tr>
<td>H16</td>
<td>37</td>
<td>F</td>
<td>17.20</td>
</tr>
</tbody>
</table>

It is noticeable that the median age of PC patients was 69 years (range 41 to 94 years), 50 years (range 24 to 82 years) for benign control and 31.5 years (range 24 to 47 years) for healthy donors.
Plasma IL-6, IL-8 and IL-10 Levels Are Increased In Pancreatic Cancer Patients

The inflammatory state in cancer patients promote cancer progression, and worsen the prognosis of cancer (418). For example, animal studies shown that IL-6 can activate mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/SRC kinase) signals, which are required for carcinogenesis of pancreatic cancer (419). Elevated serum IL-6 concentration in pancreatic cancer patients was reported long ago (420). Here, we tested a series of inflammatory cytokines, including IL-1β, IL6, IL8, IL-10, IL-12 and TNF-α, in plasma samples of patients with cancer or benign pancreatic disease, as well as healthy donors. We aimed to identify a relationship between pancreatic disease and circulating cytokine concentration. For this purpose, we detected concentrations of 6 cytokines by a cytometric bead array assay as mentioned in Section 2.

As shown in figure 3.1, plasma IL-6 concentrations were significantly higher in patients with benign pancreatic disease compared to normal controls (p<0.01), while plasma IL-6 concentrations in patients with malignant disease were even higher than that observed in the benign group (p=0.03). Similarly, plasma IL-10 levels were significantly elevated in pancreatic cancer patients compared to both benign disease patients (p=0.047) and normal controls (p<0.001). However, no significant differences were observed in IL-10 concentrations between healthy controls and benign disease controls (p=0.14). Significant differences were observed in the plasma IL-8 concentrations between healthy controls and patients with either benign (p=0.004) or malignant (p<0.001) pancreatic disease, but not between non-cancer and cancer patients. Statistically significant differences of plasma TNF-α levels were only detected between PC patients and healthy subjects (p=0.007). There were no significant differences in IL-1β and IL-12p70 levels between different groups. Table 3.5 shows the mean± standard variation of plasma cytokine levels observed in the different disease groups (normal, benign disease and PC). Statistical comparisons were made using the Kruskal-Wallis test to determine any differences within the 3 subject groups, while Mann-Whitney test was used to compare the difference between each individual group (Fig 3.1).

Due to limited numbers, we pooled all the patients with benign pancreatic disease together as ‘benign pancreatic disease’ for analysis. However, it is expected that inflammation may influence the levels of these cytokines in patients with pancreatitis more than the cystic disease. Hence, we divided patients in the benign groups further to 3 categories: the acute pancreatitis group (including recurrent pancreatitis with acute reaction, n=5), chronic
pancreatitis group (n=4) and other disorders (including cystic disease and benign lesions, n=6). After analysis of the subdivided data using one-way ANOVA, we found that except for IL-8 (P=0.049), no differences were observed in the plasma levels of IL-6 (P=0.56), IL-10 (P=0.97), TNF-α (P=0.95), IL-1β (P=0.66) and IL-12 (P=0.15). Plasma IL-8 levels in acute pancreatitis patients were higher than those in cystic disease group (P=0.035).

### Table 3.5. Plasma cytokine levels according to disease groups

<table>
<thead>
<tr>
<th>cytokine</th>
<th>Healthy (n=13)</th>
<th>Benign pancreatic disease (n=15)</th>
<th>PC (n=34)</th>
<th>Kruskal-Wallis (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6(pg/ml)</td>
<td>0.92 ± 0.27</td>
<td>4.00 ± 0.83</td>
<td>8.92 ± 2.30</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>IL-10(pg/ml)</td>
<td>1.02 ± 0.27</td>
<td>1.73 ± 0.34</td>
<td>3.25 ± 0.45</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>IL-8(pg/ml)</td>
<td>55.71 ± 34.89</td>
<td>63.33 ± 11.54</td>
<td>331.80 ± 127.10</td>
<td>0.001**</td>
</tr>
<tr>
<td>TNF-α(pg/ml)</td>
<td>0.93 ± 0.66</td>
<td>1.69 ± 0.46</td>
<td>1.96 ± 0.30</td>
<td>0.032*</td>
</tr>
<tr>
<td>IL-1β(pg/ml)</td>
<td>0.88 ± 0.49</td>
<td>2.23 ± 0.52</td>
<td>1.93 ± 0.44</td>
<td>0.058</td>
</tr>
<tr>
<td>IL-12(pg/ml)</td>
<td>0.58 ± 0.41</td>
<td>1.31 ± 0.33</td>
<td>1.33 ± 0.42</td>
<td>0.407</td>
</tr>
</tbody>
</table>

a. PC, pancreatic cancer  
b. Statistical analysis was performed through Kruskal-Wallis test. P <0.05 (2-tailed) was considered to be statistically significant.  
**P<0.01
Figure 3.1. Plasma cytokine levels, including IL-10, IL-6, IL-1β, IL-8, TNF-α and IL-12, according to disease groups. Cytokines were tested by Cytometric Bead Array. Each dot represents the plasma cytokine concentration of one individual in PC group (n=34), patients with benign pancreatic disorders group (n=15), or healthy controls (n = 13). The horizontal line displays the median value for each group. Mann-Whitey was used to test the statistical differences between each two groups, and P<0.05 was considered as statistically significant. Data is from one experiment.

Plasma Mesothelin Levels Are Increased in Pancreatic Cancer Patients

Based on our hypothesis, with disease progresses, mesothelin can be released from cell surface of malignant cells, resulting in increased courses of circulating soluble MSLN. Thus, we used an ELISA method to detect circulating mesothelin levels. The method is described in detail in the Material and Methods section.

Of a total 13 healthy controls, 11 (85%) had mesothelin levels less than 20 ng/ml, with the median value at 12.0 ng/ml (ranged from 0ng/ml to 32.2ng/ml). Compared to healthy controls, the plasma mesothelin levels were significantly elevated in patients with benign pancreatic disease (N=13, P<0.05), where the median concentration was 19.1 ng/ml (range: 0 to 62.5 ng/ml). The median plasma mesothelin concentration in 32 pancreatic cancer patients (PC8 and PC11 were not used in this test, due to insufficient plasma after cytokine analysis) was 27.4 ng/ml, with the minimum of 3.7 ng/ml and maximum of 101.0 ng/ml. Additionally, statistically significant difference was observed between soluble mesothelin levels of PC patients and that of healthy volunteers (P<0.001), but no significant difference between the
plasma mesothelin concentration in the cancer group and that of the non-cancer group (P=0.17, Fig 3.2A). No significant difference in soluble mesothelin concentration was observed between patients with metastatic pancreatic cancer (stage IV) compared to other PC patients (P=0.48, Fig. 3.2B). These results suggest that circulating mesothelin levels are significantly increased in patients with pancreatic disease, particularly, with pancreatic cancer, but that in our study, admittedly limited by relatively low numbers, no differences were observed as stage increased.

Figure 3.2. Plasma mesothelin concentrations.

A. This figure shows plasma mesothelin concentrations in pancreatic cancer patients (n=32), patients with benign pancreatic disease (n=13), or healthy controls (n = 13). The horizontal line displays the median value for each group. The median mesothelin concentrations in the plasma of pancreatic cancer patients was 27.4 ng/mL (n=32), which was significantly higher than healthy donors (P < 0.001). There was also a significant difference between benign pancreatic disease (median plasma mesothelin level at 19.1 ng/mL, n=13), and normal volunteers (median plasma mesothelin level at 12 ng/mL, n=13), P=0.045. However, no statistically significant difference was seen between patients with benign pancreatic disease and those with pancreatic carcinoma (P=0.17).

B. This figure shows the plasma mesothelin concentrations in pancreatic cancer patients at different stages. Due to missing data with respect to the stage information, only 22 patients were included here. No statistically significant difference was observed between the median of mesothelin concentrations of patients in different stages (P=0.48), using Kruskal–Wallis test. Data is from one experiment.

Detection of Mesothelin-Specific T Cells in PBMCs Isolated From Pancreatic Cancer Patients and Non-Cancer Controls
PBMCs were isolated from 25 patients with pancreatic cancer (PC01-PC25), 15 patients with benign pancreatic disease (CON01-CON15), and 16 healthy controls (H01-H16). Patients’ demographics are described in Table 3.2 and 3.3. The clinical stage of the pancreatic cancer was known in 19 (out of 25 patients, PC01-PC25), and 89% (17 out of 19) had advanced stage disease (stage III/IV). T cells were stimulated in the presence of IL-2 with mesothelin peptide pool (Table 3.3) pulsed autologous PBMCs over 9-10 days. Then, a 5 hour stimulation was performed using specific (relevant), or control (irrelevant) peptides pools. For example, if Pool 1 was used to culture the PBMC, the Pool 1 was defined as the relevant pool when performing 5-hour re-stimulation, whilst peptide Pool 2 was used as an irrelevant peptide pool. MSLN specific T cell responses were measured using intracellular cytokine staining for IFN-γ. PMA/Ionomycin was used to test the viability of cells after 9-10 days incubation. An example of the gating strategy is shown in Figure 3.3A, and the PMA stimulated/non-stimulated control is illustrated in Figure 3.3B. No IFN-γ secretion was observed without in vitro expansion in the presence of MSLN derived peptides.

**Figure 3.3. The gating strategy and PMA stimulation control.**

A. The cells were stained for CD4, CD8 and IFN-γ. PBMCs were firstly gated on lymphocytes and then gated on CD4+ or CD8+ T cells for analyzing.

B. PMA/Ionomycin was used to test the viability of the cells after 9-10 days’ stimulation. In cells stimulated with PMA/Ionomycin, viable CD4+ and CD8+ T cells secreted IFN-γ.
Representative FACS plots of CD4+ and CD8+ T cells are shown (Fig. 3.4A). A positive response was defined as more than two-fold increase in the percentage of IFN-γ secreting cells within the CD4 or CD8 compartment. If a tested individual demonstrated positive responses to one or more of the 7 pools, they were defined as a ‘responder’. Mesothelin-specific CD4+ T cell responses were observed in 84% of pancreatic cancer patients (21 out of 25), compared to 66.7% of benign controls (10 out of 16) and 43.7% of healthy donors (7 out of 15). The percentage of responders within the PC and healthy groups were significantly different (p=0.014, $\chi^2$ test). Anti-mesothelin CD8+ T cell responses were detected in 36% of PC patients (9 out of 25), in 20% benign pancreatic disease patients (3 out of 15) and in only 6.3% healthy controls (1 out of 16, Fig 3.4B). Where CD8+ T cell responses were observed, so were CD4+ T cell responses. However, different subjects responded to different numbers of peptide pools, as shown in Figure 3.4C. For a number of patients analyzed, it was not possible to identify additional data at the time of PBMC isolation, such as FBC (full blood count), lymphocyte counts, plasma CEA and CA19-9 levels. These would be useful for the interpretation of T cell responses.
Figure 3.4 CD4+ and CD8+ T cell responses following in vitro stimulation by mesothelin peptide pools.

A. The identification of mesothelin (MSLN) peptide-specific CD4+ T cells (top) and CD8+ T cells (bottom). Data shown are representative of two individual experiments using PBMC isolated from the peripheral blood of a patient with Ca Pancreas (PC25). PBMC were stimulated with different MSLN peptide pools.
peptide pools for 10 days prior to an overnight restimulation with the same peptides prior to intracellular cytokine staining. The production of mesothelin-specific IFNγ by CD4+ T and CD8+ T cells was assessed using intracellular cytokine assay, and compared to unstimulated controls (left). The percentage of cytokine-producing cells of the total viable CD4+ or CD8+ T cells is shown.

**B.** The percentage of patients/healthy volunteers with identifiable MSLN-specific CD4+ (top) and CD8+ (bottom) T cell responses amongst the total subjects tested are shown. MSLN-specific responses were more frequent in PBMC isolated from patients with pancreatic cancer (PC group) than in patients with benign pancreatic disease (Benign group) or healthy volunteers (Normal/Healthy group).

**C.** PBMC isolated from each subject (PC, CON or H groups) were stimulated with 7 different pools of overlapping MSLN-derived peptides, as listed in table 3.1. Positive responses were defined as ≥2 times the frequency of IFNγ+ T cells which are stimulated with MSLN than that of unstimulated control. The numbers of peptide pools stimulating IFNγ-secreting T cells are shown. The mean number of peptide pools able to stimulate functional CD4+ T cell responses in PBMC from the healthy control, benign pancreatic disease and pancreatic cancer groups were 1, 1.5, and 2, respectively. The difference between the numbers of peptide pools recognized by different groups is statistically significant (p<0.001, one-way ANOVA).

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**Assessment of Anti-Mesothelin CD4+ T Cells Response in Pancreatic Cancer Patients and Controls**

The CD4+ T cell responses stimulated by each peptide pool are further shown in Figure 3.5A. In brief, T cells isolated from pancreatic cancer patients responded to more peptide pools and the frequency of anti-mesothelin specific CD4+ T cells identified in the blood of cancer patients were significantly higher than those observed in the blood of benign disease patients (p=0.0053) and normal controls (Mann Whitney, p=0.0004, Fig. 3.5B).
Following 10 day in vitro stimulation with MSLN peptide pools, T cell lines were re-stimulated overnight with the same peptide pools prior to intracellular cytokine staining for IFNγ production. Summary results are shown for the 25 patients with pancreatic cancer (PC01 to PC25), 15 patients with benign pancreatic disease (CON01 to CON15) and 16 healthy donors (h01 to h16). Positive responses were defined as ≥2 times the frequency of IFNγ+ T cells which are stimulated with MSLN than that of unstimulated control.

Note: Pool 1=peptide 1-100, Pool 2=peptide 101-200, Pool 3=peptide 201-300, Pool 4=peptide 301-400, Pool 5=peptide 401-500, Pool 6=peptide 501-570, Pool 7=peptide 571-630.
B. Each symbol represents the percentage of MSLN specific IFN\(\gamma\)-producing CD4+ T cells of viable CD4+ T cells in normal control, non-cancer pancreatic disease patients and pancreatic cancer patients. The PC group had significantly higher percentage of IFN\(\gamma\) producing CD4+ T cells than both control groups, Benign and Normal (Mann-Whitney U Test, p <0.01). Data is from one experiment.

**Identification of Immunogenic Mesothelin Peptides**

All peptide pools were recognized by CD4+ T cell lines from PC patients, while Pool 1-specific responses were more frequently detected and the percentages of IFN-producing T cells within the CD4 compartment were more than those stimulated with other pools (Fig 3.6A). No CD4+ T cell responses were observed to Pool 1 in healthy controls.

To further identify the specific peptides within the peptide pools recognized by the T cells, we assessed IFN-\(\gamma\) production by CD4+ and/or CD8+ T cells after short term stimulation of T cell lines with individual peptides from various peptide pools. Among the responders, PBMCs from 10 pancreatic cancer (PC01, PC02, PC13, PC17, PC18, PC19, PC12, PC23, PC24, PC25), 6 benign pancreatic disease patients (CON01, CON02, CON04, CON09, CON10, CON11), and 1 healthy volunteer (H02) were available for further studies. The specific peptide epitope which induced CD4+ or CD8+ T cell responses are shown Table 3.6. Among them, CD4+ T cell responses to peptide 3 were detected in 6 out of 14 examined patients. Using online MHC class-II binding peptide prediction engine MHC2Pred (http://www.imtech.res.in/raghava/mhc2pred/), we found this peptide could bind to a series of HLA class II alleles, including HLA- DR9, DR11, DQ4, DQB1*0301, DRB1*0404, DRB1*0405.

Some peptides were subsequently analyzed for their ability to stimulate intracellular IL-2 and TNF-\(\alpha\) production. The FACS plots shown (Figure 3.6B) indicate that the CD4+ T cells were functional, antigen-specific able to generate both IL-2 and TNF-\(\alpha\).
Table 3.6. Immunogenic peptides within MSLN peptide pools.

<table>
<thead>
<tr>
<th>Peptide pool</th>
<th>Peptide No</th>
<th>sequence</th>
<th>Response type and positive rate</th>
<th>Sample ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1</td>
<td>Peptide 3</td>
<td>MSLN&lt;sub&gt;21-35&lt;/sub&gt; LLFLFFSLGWVGPSR</td>
<td>CD4 response, positive 6 in 14 (42.9%)</td>
<td>PC01, PC13, PC17, PC18, CON 02, CON04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CD8 response, positive 1 in 14 (7.2%)</td>
<td>PC17</td>
</tr>
<tr>
<td>Pool 5</td>
<td>Peptide 10</td>
<td>MSLN&lt;sub&gt;91-105&lt;/sub&gt; QKNVKLSTEQLRCLA</td>
<td>CD4 &amp; CD8, positive 1 time</td>
<td>CON01</td>
</tr>
<tr>
<td></td>
<td>Peptide 41&amp;42</td>
<td>MSLN&lt;sub&gt;401-415&lt;/sub&gt; VNKGHEMSPQAPRRP</td>
<td>CD4 response 1 in 2</td>
<td>PC25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MSLN&lt;sub&gt;411-425&lt;/sub&gt; APRRPLPQVATUHDR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool 6</td>
<td>Peptide 43</td>
<td>MSLN&lt;sub&gt;421-435&lt;/sub&gt; TUDRFVKGRQLDK</td>
<td>CD4 response 1 in 2</td>
<td>PC 22</td>
</tr>
<tr>
<td></td>
<td>Peptide 54</td>
<td>MSLN&lt;sub&gt;533-545&lt;/sub&gt; FMKLRTDAVLPLTVA</td>
<td>CD4 response, positive 2 in 5</td>
<td>PC23, PC24</td>
</tr>
<tr>
<td>Pool 7</td>
<td>Peptide 60&amp;61</td>
<td>MSLN&lt;sub&gt;591-605&lt;/sub&gt; PNGYLVLDLSMQEAL</td>
<td>CD4 response 1 in 4</td>
<td>H02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MSLN&lt;sub&gt;601-615&lt;/sub&gt; MQEALSHTPCCLGPG</td>
<td>CD4 response 1 in 4</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.6. In vitro IFNγ, TNFα and IL2 production by mesothelin-specific CD4+ T cells.

**A.** In vitro stimulation with MSLN peptide pool 1 generated higher frequencies of IFNγ producing CD4+ T cells compared to stimulation with peptide pools 2-7 (p=0.04, Kruskal-Wallis test). Data is from one experiment.

**B.** CD4+ T cell lines secreting IFNγ in response to MSLN peptide pool 1, were re-stimulated with individual MSLN-derived peptides prior to intracellular cytokine staining, as before. Secretion of IFNγ, TNFα and IL-2 were analysed by flow cytometry. A representative example (PC25) demonstrating IFNγ, TNFα and IL-2 secretion by CD4+ T cells after re-stimulation with peptide 41 (MSLN 401-415).
IL-10 Blockade Enhances MSLN-Specific IFN-γ Production by CD4+ and CD8+ T Cells

In the first section (Fig. 3.1), we found that the plasma levels of IL-6, IL-8 and IL-10 were increased in patients with PC compared to healthy volunteers, with the increases in IL-6 and IL-10 concentration being statistically significant. We therefore decided to study IL-10-blockade, as IL-10 is an important immunosuppressive cytokine, which has been shown to impair T cell function. IL-10-blockade in pancreatic cancer has been less studied than IL-6.

PBMCs from 7 patients with malignant disease and 2 patients with benign disease were restimulated in the presence of a concentration of anti-IL-10 and anti-IL-10 receptor blocking antibodies (added to cultures of day1 and day 4 of incubation). The T cell lines were restimulated with irrelevant or relevant peptide pools prior to intracellular cytokine staining for IFN-γ secretion. Next, we compared the percentage of IFN-γ-producing CD4+/CD8+ T cells with IL-10 blockade (plus peptide stimulation) and that with peptide stimulation only. The detectable limit was set as the % of IFN-producing T cells without specific peptide stimulation.

After stimulation in the presence of IL-10 blockade, the frequency of MSLN-specific CD8+ T cells increased in PBMC from 2 out of 7 patients with pancreatic cancer (Fig. 3.7A). Similarly, PBMC from 4 out of 9 patients demonstrated increased frequency of MSLN-specific IFN-γ producing CD4+ T cells after IL-10 blockade (Fig. 3.7B). Taken together, the frequencies of IFN-γ production CD4+ T cells were significantly increased in IL-10 blockade group compared to non-blockade group (p=0.04). These results suggest that the blockade of IL-10 may restore or promote IFN-γ production in mesothelin specific T cells isolated from patients with pancreatic cancer.
Figure 3.7. IL-10 blockade enhanced MSLN-specific IFNγ production by CD8+ and CD4+ T cells. Representative FACs plots of IFN-γ producing CD8+ (A) and CD4+ (B) T cells in the presence of both anti-IL-10 and anti-IL10Ra MAbs. Intracellular cytokine staining was performed as before. Paired summary data for T cells isolated from patients with either malignant or benign pancreatic disease are shown demonstrating the percentage of IFN-γ producing CD4+ (C) and CD8+ (D) T cells in the presence or absence of anti-IL10 blockade. Data is from one experiment. These data were paired, and was analyzed by paired t test for CD4 and Wilcoxon matched-pairs signed rank test for CD8. Different statistical tests were used to compare CD4 and CD8 data as the distributions of data were different. CD8 data are non-parametric data because the division variations of two groups are not equal.
Plasma IL-10/MSLN levels and the MSLN-specific CD4+ T cell responses in PC patients

According to the presence and frequency of MSLN-specific CD4+T cell responses, we divided the cancer patients into strong-responders and weak-responders. The weak-responders were defined as secreting IFN-γ in response to 2 or fewer different MSLN peptide pools and with less than or equal to 0.5% of total CD4+ T cells responding to any MSLN peptide pool. Strong-responders were defined as patients whose CD4+ T cells responded to more than 2 MSLN peptide pools with a percentage of IFN-γ-producing CD4+ T cells more than 0.5%. In total, there were 11 weak-responders and 14 strong responders in the PC samples. We then compared the plasma IL-10 levels and MSLN levels in these two groups (Fig. 3.8 A&B). However, no statistical significant difference was observed between the circulating IL-10 concentration in MSLN weak or strong responders (p=0.36), or between plasma MSLN levels and MSLN responding patients (p=0.29).

![Figure 3.8](image)

**Figure 3.8** The levels of plasma MSLN and IL-10 in PC patients with different degrees of MSLN-specific CD4 responses. The plasma MSLN concentrations in the strong-responder group were not significantly different with those in the weak-responder group (p=0.36, A), and no statistically significant differences were observed with IL-10 levels (p=0.29, B).
3.3 Discussion

There is little information on the status of naturally occurring MSLN-specific CD4+ and CD8+ T cell responses in PC patients. The experiments described in this chapter attempted to identify the role of plasma mesothelin, naturally occurring mesothelin-specific T cell responses and serum cytokines as biomarkers in pancreatic carcinoma patients, and aimed to assess whether there was any association between MSLN-T cell response and the levels of plasma MSLN or cytokines.

Elsewhere, elevated serum cytokines and/or chemokines have been observed in cancer patients (e.g., TNF-α and IL-6), whilst others, like IL-12, are often downregulated (421), and as such tumour-related cytokines may be effective biomarkers for cancer diagnosis. To identify the relationship between cytokines and pancreatic cancer, we measured the concentrations of 6 cytokines in plasma of 34 PC patients, 15 patients with benign pancreatic disease and 13 healthy donors. IL-6 and IL-8 are tumour promoting cytokines, with antiapoptotic and angiogenic function (422, 423), which have been shown to be significantly elevated in pancreatic disease, especially, pancreatic cancer. The concentration of IL-10, an important immune suppressive cytokine, was also elevated in PC patients (statistically significant compared to patients with benign disease). Plasma levels of TNF-α were significantly higher in the pancreatic cancer group compared to healthy subjects. However, IL-12, which facilitates Th1 differentiation (424), and IL-1β, an important cytokine in inflammation and infection, were unchanged between the healthy control and patients with pancreatic disease. These results suggest that the plasma levels of IL-6, IL-8 and IL-10 may undergo quantitative change during the development of pancreatic cancer in certain patients.

Theoretically, in benign pancreatic disease, an increase of inflammatory related cytokines is usually seen in acute pancreatitis, rather than the chronic and cystic forms of the disease. After stratification of our benign diseases group, we found that, apart from IL-8, no difference was observed in any circulating cytokine concentrations between three benign groups (acute, chronic and cystic disease groups). However, interpretation is limited as biopsies were not available to determine the degree of inflammation at the time of blood sampling. Other limitations include the fact that the sensitivity of our method had not been optimized. Undetectable cytokine concentrations may have reflected the detection limits of the assay. In addition, too many “0” (or undetectable) data points can impact the accuracy of statistical analysis, particularly when the overall sample size is small. Furthermore, samples
were taken at a single time point, with no serial data available from the same patients. Finally, the patients in the cancer group were older than the other groups (benign disease and healthy controls), which may result in a potential bias. Thus, if we had been able to recruit more patients and increase the number of samples collected, including at serial time points, the results would have been more reliable. Nevertheless, our results from an unselected group of ‘real’ patient samples suggest that certain cytokines, including IL-6, and IL-10 deserve more attention in larger studies.

Mesothelin has been reported to be expressed on more than 90% of pancreatic cancer tissue (331-333). To our knowledge, only one study (336) to date have reported the elevation of circulating MSLN in pancreatic cancer patients compared to healthy controls. Compared to their performance, our study increased the number of healthy and benign pancreatic disease controls, which were considered as risk factors for pancreatic cancer. Meanwhile, majority of the malignant subjects were recruited when the biopsy specimen for diagnosis was taken, which means these patients have not been firmly diagnosed as pancreatic cancer and not received related treatment when we collected the blood samples. In total, we detected soluble mesothelin in the plasma of 13 healthy volunteers, 13 patients with non-malignant pancreatic disease and 32 pancreatic cancer patients. The concentration of mesothelin identified in the cancer group was significantly higher than in the healthy control group, but no statistically significant difference was observed between that the cancer group and the benign disease group. Based on our preliminary results, it is difficult to confirm the potential use of MSLN, IL-8, IL-6, and IL-10 as candidate biomarkers for further clinical studies. It will be necessary to collect additional samples from patients at different stages of disease progression, including ‘treatment naïve’ early stage PC patients and late stage patients. Through stratifying patients or combining biomarkers it may be possible to determine the diagnostic value of these factors.

We could not establish any association between the levels of elevated plasma IL-10 and the presence or intensity of MSLN-specific T cell responses. As we only collected the plasma samples on a single time point, we are not able to describe the change of plasma levels of mesothelin and IL-10 during the progression of pancreatic cancer based on our data. However, our data suggested that circulating IL-10 and MSLN levels may undergo quantitative change when malignancy had developed compared to normal status.
Furthermore, evidence from other studies has shown that using whole mesothelin protein can stimulate in vitro T cell responses in pancreatic cancer patients (336, 352), which suggests that mesothelin over-expression may stimulate a clinically meaningful T cell response. Most studies to date have focused on CD8+ T cell responses. However, CD4+ T cell responses are known to be critical for activating and promoting anti-tumour CD8+ T cell memory. We next tried to identify mesothelin peptide pool-specific CD8+ and CD4+ T cell responses and subsequently individual CD4+ T cell recognised peptide epitopes using 15mer amino acid-long overlapping mesothelin peptides. Anti-mesothelin CD4+ T cell responses were detected in 83% PC patients compared to 66.7% of benign controls and 43.7% of healthy subjects. Anti-mesothelin CD8+ T cell responses were observed in 36% PC patients. The results have confirmed that CD4+ T cell responses could be generated in pancreatic cancer patients, and that these patients had an increased frequency of IFN-γ secreting CD4+ T cells, compared to controls. CD8+ T cell responses were observed less frequently in our patients than CD4+ T cell responses. A possible explanation for this is that we used 15mer AA peptides rather than 8-10mer AA peptides in this experiment. HLA class I molecules can bind 8-10mer AA peptides and present to the class I restricted TCR on CD8+ T cells, while HLA class II molecules bind to 13-15mer AA peptide and present to class II restricted CD4+ T cells. The 15mer AA peptides need to be processed in APC before smaller peptide epitopes are loaded onto the HLA class I molecule. This additional processing may impair T cell responses due to impaired Ag presentation. Directly using 8-10 AA peptides may improve HLA class I loading and CD8 stimulation, but due to cost limitations and cell numbers, we only used the 15 AA peptides to screen for possible CD8+ T cell epitopes. We also found that there was no difference in circulating plasma mesothelin levels in patients with MSLN-stimulated CD4+ T cell responses and those with or without weak responses. This may infer that mesothelin-specific immune tolerance was not established even in the presence of high circulating soluble mesothelin.

Moreover, several potential T cell epitopes were identified (Table 3.6). Among them, MSLN21-35 LLFLLLFLGWVPSR was recognized by CD4+ T cells and stimulation with the peptide generated IFN-γ production in 6 out of 14 patients with pancreatic cancer or benign pancreatic disease, and one of them also have CD8 responses to this peptide. MSLN401-415 VNKGHEMSQAPRRP, MSLN531-545 FMKLRTDAVLPLTVA, could also stimulate secretion of IL-2 and TNF-α. Interestingly, MSLN20-18 and MSLN531-539 were reported as HLA-A2 binding epitopes, which were included in MSLN21-35 and MSLN531-545, but here
we provided the first evidence that these peptides were able to generate functional CD4+ T cell responses. Also, other novel epitopes able to stimulate T cell responses have been reported by us (Table 3.6). However, due to the limitation of cell numbers (PBMCs isolated from the PC patients), we were unable to test the HLA class II subtype of these patients. MSLN HLA class II epitopes were also less studied than the class I epitopes.

We observed significantly increased circulating IL-10 levels in pancreatic cancer patients, compared to controls. As a result, we assessed the influence of IL-10 blockade on MSLN-specific T cell responses in 9 pancreatic disease patients. The anti-IL10 blockade increased the frequency of CD8+ T cell responses in 2 patients and CD4+ T cell responses in 4 patients.

In conclusion, we evaluated the potential role of mesothelin and IL-6, IL-8, IL-10 in the diagnosis of pancreatic cancer, demonstrated that mesothelin was immunogenic, reported some epitopes of mesothelin required for the induction of specific T cell responses and described an in vitro method to promote these T cell responses, which may contribute relevant information to the development of mesothelin related immunotherapy against pancreatic cancer.
Chapter 4

Identification of TAA-Specific CD8+ T Cell Responses in Patients with Pancreatic Cancer
4.1 Introduction

In order to characterize further Ag-specific T cell responses in PC patients we isolated PBMC from HLA-A2+ patients and examined responses to 18 previously described HLA-A2 restricted peptide epitopes. The main TAA of pancreatic cancer was previously shown in table 1.7.

In this study, we tested 18 known cancer associated HLA-A2 restricted peptides to characterize their immunogenicity in pancreatic cancer patients. We selected one peptide (CEA, which appeared to be the most immunogenic in tested patients), together with WT1 and MSLN derived HLA class I restricted peptides, for long term cultures, with the aim of generating Ag specific T cell lines to functionally characterize in vitro.

CEA is reported to be over-expressed in approximately 90% of pancreatic cancers (320), and is naturally processed and presented on tumour cells (366). MSLN is also expressed on 91%-100% of PC tissues (331-333). The peptides, MSLN547-552, chosen here, had been reported as HLA-A2 binding epitopes (352). WT1 is inducibly overexpressed on PC cells after Gemcitabine (394). Our laboratory has investigated this epitope in other malignancies and has adequate reagents to test its potential as a target antigen for immunotherapy. Collectively, these three antigens may be good targets for T cell mediated immunotherapy in PC patients.

Finally, we examined expression of the co-stimulatory and co-inhibitory receptors on T cells isolated from PC patients’ PBMCs, as part of further in vitro functional and phenotypic characterization.
4.2. Results

Functional CEA691 and Mucin12 Specific T Cells Were Isolated In Pancreatic Cancer Patients

From 2011, my colleagues Dr Sanju Matthew and Shyam Masrani had tested various TAAs (listed in Table 4.1) to determine which were the most immunogenic and able to stimulate T cells derived from PC patients. They passed their data and samples to me in 2012 when our supervisor Dr Behboudi left UCL. Overall, 8 experiments based on PC patient samples (PC1, PC2, PC3, PC5, PC8, PC10, PC11, and PC12 in Table 3.2) were performed by Shyam and Sanju, while I performed 5 experiments, including PC14, PC 16, PC17, PC20, and PC24. Also, they did 5 experiments using healthy controls, which are H1, H3, H5, H10 (Table 3.4), and H12, whilst I conducted 5 experiments in H11, H15 and three buffy coat samples. PBMCs from all the above 13 patients with pancreatic cancer and 10 healthy controls were stimulated with 18 peptides (Table 4.1) derived from different PC related TAAs.

Table 4.1 Amino Acid Sequences of HLA-A2 restricted peptides derived from PC related TAAs

<table>
<thead>
<tr>
<th>No</th>
<th>antigen</th>
<th>Sequence No</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cadherin 3/P-cadherin CDH3</td>
<td>655-663</td>
<td>FILPVLGAV</td>
</tr>
<tr>
<td>2</td>
<td>Cadherin 3/P-cadherin CDH3</td>
<td>757-765</td>
<td>FIIENLKAA</td>
</tr>
<tr>
<td>3</td>
<td>CEA</td>
<td>605-613</td>
<td>YLSGANLN</td>
</tr>
<tr>
<td>4</td>
<td>CEA</td>
<td>691-699</td>
<td>IMIGLVGV</td>
</tr>
<tr>
<td>5</td>
<td>CEA</td>
<td>694-702</td>
<td>GVLVGVALI</td>
</tr>
<tr>
<td>6</td>
<td>Her2/Neu GP2</td>
<td>654-662</td>
<td>IISAVVGIL</td>
</tr>
<tr>
<td>7</td>
<td>Her2/Neu GP2</td>
<td>369–377</td>
<td>KIFGSLAFL</td>
</tr>
<tr>
<td>8</td>
<td>Indoleamine 2,3-dioxygenase IDO</td>
<td>273-281</td>
<td>VLHAFDEFL</td>
</tr>
<tr>
<td>9</td>
<td>Indoleamine 2,3-dioxygenase IDO</td>
<td>386-394</td>
<td>AVMSFLKSV</td>
</tr>
<tr>
<td>10</td>
<td>Mesothelin</td>
<td>547-552</td>
<td>KLLGPHVLGV</td>
</tr>
<tr>
<td>11</td>
<td>Mucin-1</td>
<td>12-20</td>
<td>LLLLTVLTV</td>
</tr>
<tr>
<td>12</td>
<td>Mutated Ras(Val12)</td>
<td>5-14</td>
<td>KLVVVGAAGV</td>
</tr>
<tr>
<td>13</td>
<td>Mutated Ras(Asp12)</td>
<td>6-14</td>
<td>LVVVGADGV</td>
</tr>
<tr>
<td>14</td>
<td>Mutated Ras(Arg12)</td>
<td>6-14</td>
<td>LVVVGARGV</td>
</tr>
<tr>
<td>15</td>
<td>Survivin</td>
<td>96-104</td>
<td>LMLGFLKL</td>
</tr>
<tr>
<td>16</td>
<td>Telomerase</td>
<td>540-548</td>
<td>ILAKFLHWL</td>
</tr>
<tr>
<td>17</td>
<td>WT1</td>
<td>126-134</td>
<td>RMPFNAPYL</td>
</tr>
<tr>
<td>21</td>
<td>Mucin-1</td>
<td>950-958</td>
<td>STAPPVHNV</td>
</tr>
</tbody>
</table>
CD8+ T cell responses were identified using intracellular cytokine staining assays for IFN-γ. Representative dot plots of CEA691-specific CD8+T cell responses and Mucin12-specific CD8+ T cell responses are shown (Figure 4.1A). Anti-CEA691 CD8+ T cell responses were detected in 53.8% (7 out of 13) of patients with pancreatic carcinoma, and 20% (2 out of 10) of healthy controls. In addition, 46.1% of cancer patients (6 out of 13) and 30% of normal donors (3 out of 10) had mucin12 specific CD8+ T cells in their PBMCs (Fig. 4.1B). No CD8+ T cell response to other peptides was identified in healthy donors, whilst Her2-specific responses were observed in 2 PC patients and anti-CDH3 655, CEA694 and Mucin950 responses were each observed in one PC patient respectively (Figure 4.1B). Furthermore, combined CEA and Mucin responses were detected in 4 cancer patients (50% of responders, Fig4.1C), indicating a certain number of individuals may have within their peripheral repertoires, T cells specific to more than one HLA-A2 restricted TAA. With regards to the frequency of the antigen-specific responses in different people, Figure 4.1D displayed the percentage of IFN-γ producing CD8+ T cells against CEA691 and Mucin 12 in PC and normal individuals. CTLs specific for other peptides were moderately expanded (less than 0.5% in total CD8+ T cells, Fig. 4.1E). These data suggested CEA691 and Mucin12-specific CTL were the most commonly identified CD8+ T cell responses generated in vitro in PBMCs of pancreatic cancer patients and healthy controls.

As mentioned in the introduction, T cell responses to self antigens can be subject to immunological tolerance through either central or peripheral mechanisms. Thus, T cells with high affinity to TAAs listed here (most of those we examined are overexpressed self proteins without mutations), may fail to respond or be subject to activation induced cell death, rather than activation. However, it has been shown by others that tolerance mechanisms are ‘leaky’, and self-reactive T cells can be detected in the peripheral repertoire of some people. Our findings here are in agreement with this, suggesting immune tolerance to particular self-peptides (CEA and Mucin) may be incomplete, and that functional CD8+ T cells specific to these two TAAs are detectable in patients with PC. Interestingly, whilst responses to additional peptides were elicited in PC patients, the same was not observed for healthy individuals, suggesting the lack of sufficient priming and/or better preserved immune tolerance in the latter.
Figure 4.1. T cell responses to 18 different HLA-A2 restricted peptides.

A. Example FACS plot of IFN-γ production by CD8+ T cells (from PC8 for CEA691 and PC20 for Mucin12) responding to relevant peptide or control peptide after 9 days’ stimulation with CEA691 peptide or Mucin12 peptide, respectively. After 9 days’ stimulation with 18 HLA-A2 restricted peptides, T cells were restimulated with relevant peptide or irrelevant peptide for 5 hours before intracellular cytokine staining to determine IFN-γ production specific to certain peptides.

B. The percentage of individuals responding to certain HLA-A2 peptide in pancreatic cancer patients (N=13) or healthy controls (N=10). Individuals who had more than 2-fold higher percentage of IFN-γ CD8+ T cells after re-stimulation with relevant peptide compared to control peptide were regarded as responders. A number of these experiments were performed by colleagues Shyam and Shanju. I performed 5 individual experiments.

C. The number of pancreatic cancer patients who generated CEA691 and/or Mucin12 responses.
D. The percentage IFN-γ producing CD8+ T cells specific to CEA691 or Mucin12 peptides. Each symbol represents one subject, and the percentage of certain peptide-induced IFN-γ producing CD8+ T cells in healthy controls or patients with pancreatic carcinoma (after gating on total CD8+ T cells) was shown. The Mann-Whitney test was used to test statistical differences.

E. The percentage IFN-γ producing CD8+ T cells specific to other TAA peptides. Each symbol represents one subject, and the percentage of certain peptide-induced IFN-γ producing CD8+ T cells in healthy controls or patients with pancreatic carcinoma was shown. Data is from one experiment.

Prolonged Antigen Stimulation Leads to identification of Additional MSLN547- And WT1-126-Specific CTL Responses in PC

In the last chapter, we demonstrated that mesothelin peptide pool-specific CD8+ T cells could be expanded from PC patients’ PBMCs (425), and it is known that mesothelin-specific CD8+ T cells have also provided important information on priming mechanisms during vaccination of PC patients (346). Moreover, WT1-specific CTLs have been recently described as being of therapeutic relevance in the context of PC (394, 408, 426). Although we studied these two TAAs, no mesothelin-, nor WT1-specific CTL responses were observed after short term stimulation of PC patients’ PBMC. In our experience, prolonged antigen exposure was required to reveal additional WT1-specific CTLs in the peripheral blood of patients with breast cancer (405). Thus, in order to investigate WT1 and MSLN-specific CTL responses, longer stimulation assays were performed (Fig. 4.2), which consisted of four rounds of cell expansion, after which antigen-specific responses were again measured in terms of IFN-γ production after re-antigen stimulation.

Finally, in the last section we demonstrated that CEA691 and Mucin12 specific CTL were detectable in PC patients. Hence, we also hypothesised that CTL specific for these two peptides may be further expanded by long-term stimulation. Due to the limitation of available PBMCs, we only chose CEA691 to stimulate T cells (CEA691 responses were detected in 7 out of 13 PC patients after one round of peptide stimulation).
18 new pancreatic cancer patients were used in the prolonged stimulation. Additional demographic information regarding these patients is shown in the below table (Table 4.2). These patients had not previously been analysed for MSLN specific responses in the previous chapter.

**Table 4.2 Patients demographic information (PBMC samples, N=18)**

<table>
<thead>
<tr>
<th>ID of PC patients</th>
<th>Age (year)</th>
<th>Gender</th>
<th>Histological diagnosis</th>
<th>Stage</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA01</td>
<td>51</td>
<td>M</td>
<td>AC</td>
<td>IV</td>
<td>Untreated</td>
</tr>
<tr>
<td>CA02</td>
<td>37</td>
<td>M</td>
<td>PDAC</td>
<td>IV</td>
<td>Untreated</td>
</tr>
<tr>
<td>CA03</td>
<td>86</td>
<td>F</td>
<td>AC</td>
<td>III</td>
<td>Untreated</td>
</tr>
<tr>
<td>CA04</td>
<td>56</td>
<td>F</td>
<td>Poorly differentiated AC</td>
<td>IV</td>
<td>Resected + FOLFOX</td>
</tr>
<tr>
<td>CA05</td>
<td>67</td>
<td>M</td>
<td>AC</td>
<td>IV</td>
<td>GemCap</td>
</tr>
<tr>
<td>CA06</td>
<td>66</td>
<td>M</td>
<td>Moderately differentiated PDAC</td>
<td>IIB</td>
<td>Resected, Pre-chem</td>
</tr>
<tr>
<td>CA07</td>
<td>60</td>
<td>M</td>
<td>Ductal AC</td>
<td>IIA</td>
<td>Pre-chem</td>
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<tr>
<td>CA08</td>
<td>73</td>
<td>F</td>
<td>Mucinous carcinoma</td>
<td>IIA</td>
<td>Gemcitabine</td>
</tr>
<tr>
<td>CA09</td>
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<td>AC</td>
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<td>FOLFIRINOX</td>
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<tr>
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<td>AC</td>
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<tr>
<td>CA11</td>
<td>68</td>
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<td>Moderately differentiated AC</td>
<td>IIB</td>
<td>Resected + GemCap</td>
</tr>
<tr>
<td>CA12</td>
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<td>Adenocarcinoma</td>
<td>IIB</td>
<td>Resected + GemCap</td>
</tr>
<tr>
<td>CA13</td>
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<td>IIB</td>
<td>Post-operation</td>
</tr>
<tr>
<td>CA14</td>
<td>46</td>
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<tr>
<td>CA15</td>
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<td>AC</td>
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</tr>
<tr>
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<td>AC</td>
<td>IV</td>
<td>Resected + GemCap</td>
</tr>
<tr>
<td>CA17</td>
<td>45</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>IIB</td>
<td>FOLFIRINOX</td>
</tr>
<tr>
<td>CA18</td>
<td>55</td>
<td>M</td>
<td>Moderately differentiated AC</td>
<td>III</td>
<td>Resected, pre-chem</td>
</tr>
</tbody>
</table>

We used intracellular cytokine staining to test the frequency of IFN-γ producing CD8+ T cells after 4 rounds of stimulation with relevant peptides. After 4 rounds of expansion, the CEA691 specific CD8+ T cells were re-stimulated with CEA691, WT1-126, or MSLN547 pulsed-T2 cells, while Telomerase540 peptide (irrelevant peptide) loaded T2 cells were used as negative control to stimulate T cells from the same well. The following figure 4.3 showed the results of CEA691 specific T cells from 18 HLA-A2 positive pancreatic cancer patients.

Figure 4.3A shows a typical expansion profile of IFN-γ producing CD8+ T cells from a pancreatic cancer patient (CA11). At the end of the 4th round, IFN-γ producing CD8+ T cells were detectable in 10 out of 18 patients (Fig. 4.3B shows 8 of them), suggesting that these cells specifically recognized CEA691 loaded APC. Additionally, CTLs producing both TNF-α and IFN-γ were observed in 5 patients. Importantly, more than 30% of CD8+ T cells from CA07, CA11 and CA18 specifically responded to CEA691 when compared to control peptide. Between 20 and 70% of these CD8+ T cells produced both IFN-γ and TNF-α in response to specific peptide (Fig. 4.3B), indicating these T cell lines were polyfunctional. (N.B.: when using anti-TNF-α antibodies for intracellular staining, a higher background was observed in the control peptide stimulated cells, therefore we only used the frequency of IFN-γ producing cells to define the positive responses. If the frequency of IFN-γ producing CD8+ T cells induced by CEA691 doubled that stimulated by control peptide, we defined the response as a positive response.)
Figure 4.3. CEA691 specific T cells isolated from pancreatic cancer patients produce type 1 cytokines.

A. A typical example of T cells expanded during stimulation with CEA691 loaded T2 cells. Between the second (2R) and fourth round (4R) of stimulation, the percentage of IFN-γ producing CD8+ T cells gradually increased. By the end of the 4th round stimulation, 77.9% of CD8+ T cells were IFN-γ secreting, compared to 2% after 2 rounds.

B. Percentage of IFN-γ and TNF-α producing CD8+ T cells stimulated by CEA691 pulsed T2 cells and control peptide loaded T2 cells after 4 rounds of stimulation (Gated on CD8+ T cells). PBMCs of 18 pancreatic cancer patients were stimulated with CEA691, and 10 of them showed response after 4 rounds expansion. Gated on CD8+ T cells, the IFN-γ and TNF-α production is presented in this figure. Single cytokine (IFN-γ) producing CD8+ T cells were observed in e patients (upper row to CA14), whilst dual secreting (IFN-γ and TNF-α) CD8+ T cells were seen in the latter 5 patients (CA02 and lower row).

Here, PBMCs from 15 healthy controls were also used, which were labeled as HC01 to HC15. The PBMCs of healthy controls were treated in the same way as those isolated from PC patients, and CEA specific responses were observed in 5 out of 15 donors. FACS data of IFN-γ/TNF-α producing T cells from four out of five healthy controls with responses were shown in figure 4.4.

Figure 4.4. FACs plots demonstrating the percentage of IFN-γ and TNF-α producing CD8+ T cells stimulated by CEA691 pulsed T2 cells and control peptide loaded T2 cells after 4 rounds of stimulation (gated on CD8+ T cells). PBMCs of 15 healthy controls were stimulated with CEA691, and 5 of them showed responses after 4 rounds expansion. Four representative plots are shown here.

In addition to the generation of the expected specific response against CEA691 (Fig. 4.5A), prolonged antigen stimulation elicited the expansion of MSLN547- (Fig. 4.5B) and WT1-specific (Fig. 4.5C) CTLs in PC. The frequency of responders to the CEA691 peptide was the same in both short- (7 out of 13, about 53.8%) and long-term cultures (10 out of 18,
approximately 55.6%), but MSLN and WT1 specific T cell responses were only observed after long-term stimulation.

Of note, although the observed percentage of CEA691 responding T cells was much higher in PC patients than in healthy controls (Fig. 4.5A), the same was not observed for MSLN547, where the levels of IFN-γ produced by PC in response to MSLN547 peptide stimulation were in the same range as those obtained from healthy controls (Fig. 4.5B). On the other hand, our previous studies on WT1 specific T cell responses also showed a higher percentage of WT1-specific CD8+ T-cells (in term of WT1-specific IFN-γ mRNA expression) in patients with CML and AML than in healthy donors (404, 427), which was similar to the results observed after stimulation with CEA691.

Finally, after four rounds stimulation, IFN-γ producing CEA691-specific T cell lines were generated in 55.6% of pancreatic cancers (one responder, CA11, had 73% of CEA691 specific T cells at the end of four-round stimulation) and 33.3% healthy controls (the responder with highest percentage of IFN-gamma-producing CD8+ T cells have 4.1% CEA691 specific IFN-γ+ CD8+ T cells after stimulating for 4 rounds) (Fig 4.5D). MSLN547-specific CTL response was observed in 16.7% of cancer patients (3 out of 18) and 13.3% of normal controls (2 out of 15) (Fig 4.5D). However, in pancreatic cancer patients, only two WT1-126 responders were detected (11.2% of patients), with 1.81% of WT1-126 specific IFN-γ+ CD8+ T cells after 4 rounds of stimulation observed in the responder with highest percentage of IFN-γ-producing CD8+ T cells (Fig 4.5C & D). This stimulation was not performed in healthy controls (as it has been performed and published previously). In conclusion, in vitro CTL responses to CEA691, MSLN547, and WT1-126 were detectable in peripheral T cells from PC patients and the control group. CEA691 specific CD8+ T cell lines were more readily generated from PC patients than T cell lines specific for other two peptides, in agreement with the results from short term cultures.
Figure 4.5. Long-term ex vivo expansion of T cells stimulated by CEA691, MSLN547, and WT1-126, in 18 HLA-A2 positive patients with pancreatic cancer and 15 healthy controls.

A. FACs plots demonstrating the frequencies of IFN-γ secreting CD8+ T cells after 4 rounds of stimulation with CEA691 loaded T2 cells, in a PC patient (CA11) and a healthy control (HC08).

B. FACs plots demonstrating the frequencies of IFN-γ secreting CD8+ T cells after 4 rounds of stimulation with MSLN547 loaded T2 cells, in a PC patient (PC03) and a healthy control (HC04).

C. FACs plots demonstrating the frequencies of IFN-γ secreting CD8+ T cells after 4 rounds of stimulation with WT1-126 loaded T2 cells, in PC patient.

D. The percentages of PC patients or healthy controls with CD8+ T cells reacting to CEA691, MSLN547 or WT1-126. N.B. Responders were defined as those having a two-fold increase in the frequency of IFN-γ producing CD8+ T cells above control peptide, and the percentage of specific T cells was calculated as the percentage of IFN-γ+ CD8+ T cells stimulated by relevant peptides minus that stimulated by control peptide.
PC Disease Progression Is Associated with Impaired Mounting of Antigen-Specific CTL Responses

Levels of CEA691 expression have been previously shown to positively correlate with the degree of PC tumour differentiation (428). Hence, we further investigated whether PC patients at different stages of disease were able to mount distinct CEA691-specific CTL responses.

Of the 18 patients whose cells were used for the long-term cultures, 6 were at stage IV PC (with metastases being observed), whilst the rest were at stages III (N=3) and II (N=9) PC (Fig. 4.6A). As shown, even though 75% (9 out of 12) of stage II/III patients were able to elicit CEA691-specific responses, this capacity progressively decreased with disease progression, with only 17% (1 out of 6) of patients at stage IV being able to produce IFN-γ upon antigen-specific stimulation (Fig. 4.6B). Relative frequencies of CEA691-specific CD8+ T cells were also observed to be significantly reduced in stage IV PC responders, in comparison to their stage II-III counterparts (Fig. 4.6B). Most importantly, PC patients with inoperable tumours (Fig. 4.6D) or who had gone through chemotherapy (Fig. 4.6C) showed lower frequencies of CEA691-specific CTLs compared to the surgical and non-chemo patients respectively, with a finding that reached statistical significance between the surgery and inoperable group (P=0.013). Patients treated with chemotherapy are typically rendered lymphopenic and would therefore not be expected to elicit significant or measurable T cell responses. It may be also assumed that patients with inoperable disease have late stage or metastatic disease, and therefore a longer duration of exposure of endogenous T cells to tumour associated antigens.

Taken together, our results suggest in the context of PC disease progression the ability to generate efficient anti-tumour responses may be compromised. Continuous cell activation (chronic antigentic exposure) may give way to exhaustion of immune responses instead of contributing to enhance antigen-specific responses. Also, certain medical interventions can impact on the T cell responses.

In order to adequately prove this hypothesis, more data would be required, including a serial samples collected from individual patients during the process of disease, as well as before & after a treatment is given. Other factors that may have influenced the results include the total lymphocyte count at the time of PBMC analysis, percentage of Tregs in the peripheral blood and the tumour microenvironment /draining lymphnodes and the concentration of suppressive cytokines.
Figure 4.6. CEA691-specific CD8+ T-cell responses in different PC patients.

A. Pie charts illustrate the frequency of pancreatic cancer patients at different disease stages (II-IV).

Frequencies of IFN-γ+ cells within the CD8+ T-cell population are shown for patients stratified according to B. disease stage, C. prior to administration of chemotherapy, and D. submission to surgery. Each symbol represents one individual and horizontal bars represent median. P values <0.5 were considered statistically significant and Mann-Whitney test was used for statistical test. Data is from one experiment.

Differentiation and Exhaustion Status of CD8+ T Cells in PC Patients and Healthy Controls

The phenotype of untreated T cells from 18 PC patients and 13 healthy controls, were analysed. The expression of co-inhibitory (PD-1, TIM3 and LAG-3) and co-stimulatory receptors (CD27 and CD28) was analyzed directly ex vivo. The gating strategy is shown in Figure 4.7A & B.
Patients who had detectable CEA691 specific CD8+ T cell responses after four rounds of antigen-specific stimulation were defined as “responders” (as previously described), and were analysed for the expression of exhaustion and differentiation markers PD-1, TIM3, and LAG3, and co-stimulatory makers CD27 and CD28. As shown, the frequency of PD-1+CD4+ T cells and PD-1+CD8+ T cells (Fig 4.8A) was significantly higher in PC patients who had not generated CEA-specific T cells during 4 rounds stimulation compared to CEA-specific PC responders (t test, P=0.048) and healthy volunteers (t test, P=0.005). Moreover, significant differences were also detected in the frequencies of CD4+ and CD8+ T cells expressing LAG-3 or TIM3 between PC groups (responder or non-responders) and normal controls (a higher percentage of LAG-3+ and TIM-3+ T cells in cancer patients were...
observed, Mann-Whitney, P<0.01), but not between PC responders and non-responders (Fig 4.8A). Significantly higher percentages of CD27+CD4+T cells were detected in CEA responders, compared to non-responders (P<0.001) and healthy controls (Mann-Whitney, P=0.016, Fig 4.8B). These data suggested a decrease in the frequency of T cells expressing co-inhibitory receptors and an increase in frequency of T cells expressing co-stimulatory receptors in PC patients able to generate a CEA691 specific response.

A possible confounder is T cell differentiation status. Differentiation status influences the expression of some markers, such as PD-1 and CD27. Here, we identified naïve, central memory (TCM), effector memory (TEM), end-stage/effector stages based on CD62L and CD45RO expression (i.e., TCM was defined as CD62L+CD45RO+ T cells, TEM is CD62L−CD45RO+T cells, Naïve T cells are CD62L+CD45RO−, and end-stage/effector T cells are CD62L−CD45RO−). As shown in figure 4.8C, there was no statistically significant difference in the percentage of gated CD4+ or CD8+ T cells at various differentiation stages between PC responders and non-responders (Statistical comparisons were made using Mann-Whitney U test or t test based on the states of the data).
Figure 4.8 Ex vivo phenotype of fresh T cells from pancreatic cancer patients and healthy controls.

A. The expression of co-inhibitory receptors, namely PD-1, TIM3, LAG-3, in healthy controls, PC responders and PC non-responders on gated viable CD4+ or CD8+ T cells.

B. The expression of co-stimulatory receptors, CD27 and CD28, in healthy controls, PC responders and PC non-responders.

C. The percentage of Naïve, central memory, effector memory and end-stage/effector CD4+ and CD8+ T cells in PC responders or non-responders. Data is from one experiment.
Phenotypic Characterization of T Cells from Peripheral Blood or Draining Lymph Node of Pancreatic Cancer Patients

CD8+ T-cell priming and activation take place in draining lymph nodes where, upon interaction with antigen-presenting cells, naïve cells become fully-activated and differentiate into fully-functional antigen-specific CTLs. To further explore the mechanisms underlying negative regulatory molecule modulation in PC, PD-1, TIM-3 and LAG-3 levels were evaluated in matched peripheral- and lymph node-derived CD8+ cells obtained from three PC patients, whose information were shown in table 4.3, and the FACS results are shown below (Fig. 4.9 A, B and C).

Table 4.3 Patients demographic information (LN samples, n=3)

<table>
<thead>
<tr>
<th>ID of PC patients</th>
<th>Age (year)</th>
<th>Gender</th>
<th>Histological diagnosis</th>
<th>Grade</th>
<th>HLA-A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA13</td>
<td>60</td>
<td>M</td>
<td>Adenocarcinoma</td>
<td>IIB</td>
<td>+</td>
</tr>
<tr>
<td>CA19</td>
<td>52</td>
<td>M</td>
<td>Moderately differentiated AC</td>
<td>IIB</td>
<td>-</td>
</tr>
<tr>
<td>CA20</td>
<td>51</td>
<td>F</td>
<td>Adenocarcinoma</td>
<td>IIB</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 4.9 A
Figure 4.9 B
Figure 4.9 Phenotypic characterisation of T cells derived from PBMCs and LNs of 3 PC patients. Peripheral blood and lymph node samples were collected from each PC patient at the same time. PBMCs were isolated from whole blood and single cell suspensions were made from LNs immediately after the samples were obtained. The cells were stained for CD3, CD4, CD8, TIM3, LAG-3, PD-1, CD27 and CD28. The proportions of the CD8+/CD4+ cells which expressed relating markers can be read out in FACS figures.

As can be seen in Figure 4.9, lymph node-derived CD8+ T cells expressed higher levels of PD-1 and TIM-3, but not LAG-3, both in terms of relative expression (Fig. 4.10A), and number of molecules per cell, as indicated by mean fluorescence intensity, MFI (Fig. 4.10B), an observation that reached statistical significance for both PD1 and TIM3.

In order to further address the relative contribution of negative regulatory pathways in the generation of antigen-specific responses in PC, PBMCs obtained from peripheral blood and lymph nodes were cultured with the CEA691 peptide in the presence or absence of anti-PD-L1 and/or anti-TIM-3 blocking antibodies. PD-L1 is the ligand for PD-1. PD-1 has two known ligands, PD-L1 and PD-L2. As PD-L1 is widely expressed in various types of tissue and cells, including some tumour cells, and its functions are better elucidated than PD-L2, we
attempted to test if blocking signals from PD-L1 in co-cultures could enhance the in vitro T cell responses. It is important to note that after PD-L1 blockade, PD-1 may also receive signal from PD-L2. Thus our study focused on PD-L1/PD-1 signal pathway only. Blockade of the PD-L1/PD-1 pathway, but not of the TIM-3 pathway, led to an increase in IFN-γ production by both circulating and lymph node-derived CTLs, suggesting a role for the PD-1/PD-L1 axis in the modulation of IFN-γ responses in PC (Fig. 4.10C). Of note, PD-1 and TIM-3 expression was higher in lymph node-derived CD8+ T cells. These cells also recovered the ability to produce IFN-γ upon inhibitory pathway disruption (Fig. 4.10C&D).

These observations suggest that the expression of negative regulatory molecules in circulating and lymph node-derived CTLs may have distinct origins. Whilst high levels of PD-1 and TIM-3 expression in lymph node-derived CTLs is likely to be associated with antigen exposure and cell activation, their expression in circulating CTLs of PC patients may result from a state of terminal exhaustion, with cell function no longer being able to be fully restored by blockade of the inhibitory pathways. This is in agreement with previously published reports suggesting immune exhaustion to be progressive and hierarchical process, with proliferation and CTL function being lost first, followed by the loss of IL-2, TNF-α and IFN-γ (429, 430). Unfortunately, we were unable to test antigen specific proliferation due to low number of T cells.
Figure 4.10 Different phenotype of T cells from PBMCs and LNs of PC.

A. The percentage of co-inhibitory receptors (left) and co-stimulatory receptors (right) expressed on CD8+ or CD4+ T cells from PBMCs or lymph nodes of PC (data compared by t test or Mann-Whitney test).

B. Median fluorescence intensity of co-inhibitory receptors (left) and co-stimulatory receptors (right) expressed on CD8+ or CD4+ T cells from PBMCs or lymph nodes of PC. Data is from one experiment.

C,D. The FACs dot plot and summary figure of the CEA-specific IFN-γ production after stimulation with CEA peptides, in the presence of PD-L1, TIM-3 antibodies, alone or in combination. The two experiments were duplicated and data are pooled from two independent experiments.

PD-L1/PD-1 and TIM3 Pathway Blockade Promotes the Expansion of CEA691 Specific T Cells and Restores Antigen-specific Function in vitro

Stimulation of T cells with CEA691 in the presence of PD-L1 and/or TIM3 blockade was performed to assess their effect of T cell expansion and Ag-specific function. 11 samples of PBMCs from pancreatic cancer patients and T cells from one draining lymph node were stimulated by directly adding CEA691 peptide or control peptide, in the presence of IL-2 and blocking antibodies (PD-L1 and/or Tim3) for 7 days. On day 7, CEA691 tetramer staining and surface staining was performed to evaluate the phenotype of the CEA691-specific T cells. Intracellular cytokine secretion assays were performed as described previously. An example FACs plot was presented in figure 4.11A. The percentages of IFN-γ producing cells within the CD8+ T cells significantly increased in the presence of anti-PD-L1 and/or TIM3 blocking antibodies, compared to cells treated with CEA691 alone (Fig. 4.11B). In addition to the observation of a significant increase in the percentage of CEA specific CTL in the presence of PD-L1 antibody (paired t test, P = 0.023), TIM-3 antibody (P=0.038), or adding both antibodies (P=0.022), as compared with CEA stimulation alone (Fig. 4.11D), a significant increase in frequency of CEA691 tetramer binding CD8+ T cells was also observed in T cells treated with PD-L1 antibody (P=0.030) or both antibodies (P=0.045) as compared with pCEA691 alone (Fig. 4.11C, E), suggesting that anti-PD-L1 treatment enhances the expansion of CEA-specific CTL. We have shown that in the presence of anti PD-L1 the expansion of functional, tetramer-binding CEA691 specific T cells was enhanced. The addition of TIM3 blockade did not significantly augment the effect.
Figure 4.11 Ex vivo PD-L1 and TIM3 blockade promoted the expansion of CEA691 specific T cells and restored their antigen-specific function.

A. Representative FACs plot of IFN-γ production and CEA691 tetramer-binding T cells after TIM3/PD-L1 blockade. The cells (from CA10) were stimulated with CEA691 peptides for 7 days, in the presence of specific blocking antibodies alone or in combination, and then re-stimulated once with the same peptide or control peptides.

B. TIM3 or PD-L1 blockade alone can increase the frequency of IFN-γ producing T cells in vitro (*=P<0.05, using multiple comparison).

C. PD-L1 blockade can increase the frequency of CEA691-tetramer- binding T cells in vitro (*=P<0.05, using multiple comparison). As the T cells had been cultured with CEA691 and blocking antibodies for 7 days, the tetramer-binding CD8+ T cells reflected the expansion of CEA691 specific T cells within this period.

D. Effect of TIM3 or PD-L1 blockade alone or in combination in the frequency of IFN-γ producing T cells in vitro (using paired t test or Wilcoxon matched-pairs signed rank test).

E. Effect of TIM3 or PD-L1 blockade alone or in combination in the frequency of CEA691-tetramer-binding CTLs in vitro (using paired t test or Wilcoxon matched-pairs signed rank test). Data are pooled from two independent experiments.
4.3 Discussion

We have shown that CEA691 and Mucin-12 were the most immunogenic peptides of the 18 PC-related TAA derived peptides tested (Table 4.1). Stimulation of T cells from PBMCs of pancreatic cancer patients and healthy controls indicated the existence of Ag-specific T cells within the peripheral repertoire. In the PBMCs isolated from 13 PC patients that had been analysed after short-term stimulation, 61.5% responded to either CEA or MUC1, while 50% of the responders had specific CD8+ T cells responding to both peptides. Although both healthy control and PC patients could generate CEA or MUC1 specific T cell responses, the frequency of IFN-γ secreting T cells was higher in PBMC isolated from PC patients.

Three peptides were selected for prolonged in vitro culture: CEA691, MSLN547 and WT1-126. WT1-126 has been targeted in our lab for immunotherapy of hematological malignancies. CEA 691 was the most immunogenic peptide in short term cultures (one-round stimulation). It is also the most widely expressed TAA in PC (more than 90% tumour cells express CEA) (320). CEA is also the ligand for L-selectin and E-selectin, which may contribute to the metastasis of cancer cells as it may increase cancer cell adhesion (365). Together, these findings suggest CEA can be an attractive target for cancer immunotherapy. However, CEA is also over-expressed in tumours originating from the GI tract, and is expressed at low level in normal colon (354, 358). To date, most research into CEA-specific CTL responses have been conducted in colorectal cancer. In fact genetically engineered CEA691 specific T cells have been studied in patients with metastatic colorectal carcinoma (308). The third peptide studied was from mesothelin (also studied in Chapter3). Mesothelin is also expressed on more than 90% of pancreatic tumour cells (331-333), and can enhance tumour metastasis (341). MSLN has been studied previously in ovarian cancers rather than pancreatic cancer. MSLN547-spesific T cell lines isolated from healthy volunteers have been shown to kill pancreatic cancer cell lines in vitro (352), but they have not to date been generated from PC patients. The WT1-126 peptide has been studied by others who investigated a WT1-126 peptide vaccine in patients with advanced pancreatic or biliary tract cancer, with concurrent gemcitabine chemotherapy (392). The vaccine was well tolerated. Previously, our lab has developed the use of WT1-TCR transduced T cells and vaccination to boost WT1-126 specific CTL immunity in different types of cancer (388, 405, 406). Mucin1 was not included in long-term stimulation cultures, due to the limited numbers of PBMCs
isolated from patients. Another aim of the long-term cultures were to identify high avidity TAA-specific T cells which could be cloned for the purpose of T cell receptor gene therapy.

After 4 rounds of antigen-specific T cell expansion, CD8+ T cell lines specific for CEA691, MSLN547, and WT1-126 were generated. Again, CEA691 specific responses were more readily detectable in pancreatic cancer patients than responses against the other two HLA-A2 restricted peptides tested. Of the 18 patients we tested, 10 had T cells with CEA691 specific IFN-γ production, and 5 produced both IFN-γ and TNF-α in response to CEA691. After stratification of patients according to disease stage, we found that T cells responding to CEA691 were more readily detectable in PC patients at earlier stages of disease (Stage II-III). In addition, CEA specific CTL were more frequently detected after surgical resection of tumours (which can be considered a surrogate marker of early stage disease) compared to patients with inoperable disease. One possible explanation for this is that the CEA691-specific CTL expanded in patients during the initial immune response to the tumour, but following chronic exposure to high levels of TAA, or their chronic exposure to the immunosuppressive tumour microenvironment, tumour reactive T cell numbers reduced. This is consistent with the immune editing theory. Also, different types of treatment that patients received may also impact on the TAA-specific immune response in PC. The influence of medical interventions on T cell response has observed in our previous study, in which embolization improved alpha fetoprotein-specific CD4+ T cell responses in patients with hepatocellular carcinoma (431).

Our data also demonstrated that CEA691 specific T cells were present in the repertoire of healthy individuals in addition to PC patients. It was noticeable that functional CEA691 T cells were less readily detectable in the peripheral repertoire of patients with advanced disease. For example, CEA691 specific T cells from CA11 reduced from 70% to 20% of CD8+ T cells after disease progression. Most current vaccine based immunotherapies are tested in patients with metastatic disease (132, 374, 376). However, our data suggest that these patients may be less able to mount an effective self-restricted T cell response to TAAs such as CEA691. Similar findings were observed in a study testing a CEA605-(6D) vaccine, where the only complete response was observed in a patient with locally advanced disease but not in patients with metastatic disease (382).

Unfortunately, we were unable to collect PBMCs at different points over the course of tumour progression from individual patients. Interestingly, the CA11 patient with impressive
CEA691-specific responses had had stable disease for 15 months, after the blood sample was collected until eventual progression. Advanced PC patients may have other factors impairing their ability to mount an effective TAA specific immune response, such as aging, cachexia, chemotherapy and lymphopenia.

Interestingly, PD1 expression was increased on T cells in PBMC from patients failing to respond to CEA-specific ex vivo expansion. Upregulation of co-inhibitory receptors has been observed in NYESO-1 specific T cells in patients with ovarian cancer and melanoma after vaccination with related peptides (197, 211). In PC, PD-1/PD-L1 pathway has been described to be associated with poor prognosis (432). No difference in differentiation status was observed between CEA- ‘responders’ and ‘non-responders’. In addition, the percentages of TIM-3 and LAG-3 positive T cells were higher in PC patients compared to healthy controls.

To our knowledge this is the first study examining TIM-3 and LAG-3 expression on PBMCs in pancreatic cancer. These results suggest that T cells in pancreatic cancer patients may have an inhibitory phenotype in terms of TIM-3 and LAG-3 expression, whilst PD-1 upregulation may be associated with the CEA-specific response. This data supports the use of checkpoint blockade to enhance the T cell function. In vitro PD-1/PD-L1 pathway and TIM-3 blockade was performed in 11 PC patients. Both IFN-γ production and tetramer binding T cells were increased after treating with anti-PD-L1 antibodies. Noticeably, the PD-1 can also receive signals from PD-L2, but our results suggested that the blocking PD-L1 alone was sufficient to promote the cytokine-production and proliferation of CEA specific T cells in PC patients.

Further, we were able to examine T cells from draining LNs which may better reflect the characteristics of T cells at the tumour site, as the local T cells migrate directly to these LNs. In LN samples, PD-1+CD4+ T cells and TIM3+CD8+ T cells were significantly increased in comparison to the paired PBMC samples, suggesting that T cells located at the tumour site may express higher levels of inhibitory receptors. Actually, about 80% of PC cases express PD-L1, of which 20% have upregulated expression of PD-L1 and tend to be highly invasive and recurrent (178, 179). Further blockade experiments in a HLA-A2 PC patient supported that the PD-1/PD-L1 pathway contributed to the functional dysfunction of peripheral T cells.

Overall, our results provided evidence of potential epitopes for vaccination and/or TCR gene therapy against pancreatic cancer. Anti-CEA691 and MUC1-12 CTL responses were more likely to be detected in PC patients, whereas CEA691 specific CTL responses were more impaired in patients suffering with advanced or inoperable disease. The results presented in
this chapter also provided evidence for the role of co-inhibitory checkpoint blockade to improve anti-tumour performance of TAA-specific T cells in pancreatic cancer patients.

However, to demonstrate the efficacy of anti-PD-L1 and anti-TIM3 treatment in PC patients, further investigations will be required. Firstly, we should determine the relative expression of PD-L1 in immune and pancreatic cancer cells, and document that changes in the expression levels of PD-L1 correlate to the improved T cell function observed. Secondly, we should identify individuals whose T cells express high TIM3 to determine if TIM3 blockade can have more profound effects on reversing T cell exhaustion. Finally, all experiments should be repeated in vivo to confirm the translational potential. Establishing PC in vivo models by injecting PC cells, including those expressing PD-L1, into mice model could help test the anti-tumour effects of CEA691 specific T cells and the function anti-inhibitory receptors or their related ligands.
Chapter 5

Functional Characterization of CEA691 Specific T Cells Isolated from Peripheral Blood of HLA-A2 Positive Pancreatic Cancer Patients
5.1 Introduction

CEA is overexpressed in tumours originated from gastrointestinal system (354, 358) and also expressed in normal tissues, such as the pyloric antrum and colon, at low levels (355), and has been considered as a target in the immunotherapy for patients with different types of cancers, particularly in colorectal carcinoma (321, 433). Some HLA-A2 restricted CEA epitopes have been reported (369), and three of them (CAP1-6D, CEA691 and CEA694) have been assessed in cancer patients (308, 378, 382). Our data in last section also indicated that CEA691 specific T cells were more frequently expanded in patients with pancreatic cancer than other two epitopes tested (MSLN and WT1).

As a self-antigen, CEA-specific T cells may be subjected to immunological tolerance. Although both others’ and our studies demonstrated that tolerance to CEA691 is incomplete (366), little is known about the function of CEA691 specific CTL in pancreatic cancer patients. Previously, T cells genetically engineered to express CEA691 specific TCR were administered to 3 patients with metastatic colorectal carcinoma, causing regression of the cancer, but also severe transient colitis (308).

We therefore continued to attempt to identify and characterize a CEA691 specific T cell clone isolated from pancreatic cancer patients. We hypothesized that it was possible that T cells isolated from a cancer patient may demonstrate anti-tumour function, without generating severe adverse effects, if the TCR bound only target cells expressing very high concentrations of CEA691. Typically, TCR recognising TAA are of low-moderate avidity and may therefore be less likely to cause off-tissue, on-target toxicity if there is a significant differential in Ag expression between normal tissue and tumour tissue.

Since these T cell lines we generated are likely to contain a number of dominant T cell clones, including moderate-high avidity T cell clones able to kill tumour cells overexpressing CEA, we isolated the total RNAs from the T cell lines and sequenced the TCRs, aiming to find out the dominant clone in some functional CTL lines.
5.2 Results

CEA691 Specific CTL Lines Demonstrated Killing Activity against Peptide Loaded T2 Cells

The T cell lines were generated by expanding the PBMCs of PC patients using CEA691 loaded T2 cells for 4 rounds as described in chapter 4. We observed that more than 30% of CD8+ T cells from CA07, CA11 and CA18 specifically responded to CEA691 at the end of the 4\textsuperscript{th} round of stimulation (Fig. 4.3). In other words, in the T cell lines we generated from these three patients at the end of 4\textsuperscript{th} round stimulation, at least 30% of the CD8+ T cells produced both IFN-\(\gamma\) and TNF-\(\alpha\) in response to CEA691. Hence, CFSE killing assays were performed with the CEA691 stimulated T cell lines, which contained the highest frequencies of IFN-\(\gamma\) & TNF-\(\alpha\) producing CD8+ T cells (isolated from CA07, CA 11 and CA18).

CEA691 peptide-loaded T2 cells were used as specific target cells (CFSEhi), whereas T2 cells pulsed with irrelevant peptides were used as control target cells (CFSElo). Figure 5.1 shows the gating strategy. The method used for setting negative controls was described in the Materials and Methods section.

![Figure 5.1. Gating strategy for CFSE killing assays.](image)

Specific and control target cells were co-cultured in the absence of effector cells. After 4 hours incubation, the reaction was stopped and gated on the T2 cells, singlet, and stained cells in that order. Two peaks represented specific target cells (CFSEhi, specific T2) and control target cells (CFSElo, control T2), and the proportion of cells counted was used as standard (upper row). The lower row shows target cells together with effector cells. Thus, three groups were seen, including effector cells.
(unstained), specific target cells (CFSEhi) and control target cells (CFSElo). If a fraction of specific target cells were killed, the proportion of specific target cells would be expected to be less than the control cells.

Figure 5.2A shows the reduction in T2 cells loaded with 1µM (10^{-6}M) CEA691 peptide (CFSEhi) compared to irrelevant peptide loaded T2 cells (CFSElo), after co-culture with CTL from CA11 at different E:T ratios from 100:1 to 0.6:1. Unstimulated HLA-A2+ PBMCs were used as control effector cells, and no cytotoxicity was observed (Fig 5.2B). Figure 5.2B also shows the cytotoxicity of CEA691 stimulated T cell lines from CA07, 11, 18, as well as control PBMCs. Peptide titration assays were performed to determine functional avidity. T cell lines were stimulated with T2 cells loaded with 10^{-5} to 10^{-12}M (10µM to 1pM) CEA691 peptide for 4 h (E:T=20:1). Figure 5.2C showed that T cells from CA11 were able to kill T2 cells loaded with 10^{-9}M CEA691 (1nM), at a E:T ratio = 20:1. Summary data with the PBMC control is shown in figure 5.2D.
Figure 5.2. CEA691-specific cytotoxic activity of CTL lines isolated from PC patients.

A. T cell line from CA11 were measured for their capability to recognise and kill T2 cells coated with 1mM (10^{-6} M) of CEA691, compared to control peptide, in 4-h CFSE cytotoxicity assay.

B. The percentage specific cytotoxicity of CEA 691 loaded T2 cells (1mM peptide) at various E:T ratio, using CTL lines from 3 PC patients (CA07, 11, and 18). Data is from one experiment.

C. The cytotoxicity of T cells from CA11 against T2 cells coated with 10^{-5} to 10^{-12} M CEA691 peptide and control peptide, at E:T ratio 20:1 (left). The cytotoxicity curve was summarised on the bottom (D). Data is from one experiment.

CEA691-Specific CTL Lines from 3 Pancreatic Cancer Patients Recognized and Killed Pancreatic Cancer Cell Lines in vitro

We next investigated whether the in vitro expanded T cell lines could recognize and kill pancreatic cancer cell lines, and the gating strategy was shown in figure 5.3. Six pancreatic cancer cell lines, namely MiaPaca-2, PK-45, Panc-1, KLM-1, Bx-Pc-3 and PK-1, were stained for CEA and HLA-A2 and analysed using flow cytometry (Fig. 5.4A).

Figure 5.3. Examples of Gating strategy for HLA-A2+, CEA+ cells. Isotype stained cells were used as controls. Isotype control staining profiles were used to set the gate for each type of cells. Here, the example shown is the PC cell line PK-1.

MiaPaca-2 was negative for both CEA and HLA-A2. Similarly, PK-45 was HLA-A2 negative with very low level CEA expression. The Panc-1 cell line was HLA-A2 positive, but expressed low level of CEA. Bx-Pc-3 and PK-1 were HLA-A2 positive cell lines with high expression of CEA. KLM-1 had high expression of CEA, however, only half of them showed HLA-A2 positive, possibly due to the down-regulation of class I HLA molecules during cell
Accordingly, MiaPaca-2 was used as the negative control and PK-1, Bx-Bc-3 (HLA-A2 + and CEA +), together with Panc-1 (HLA-A2+ and CEA low) were used as specific targets. KLM-1 was not included since the cells were not homogeneously HLA-A2 positive. Subsequently, we labelled MiaPaca-2 (HLA-A2- and CEA-) with low concentration of CFSE, while PK-1, Bx-Pc-3 and Panc-1 were labelled with high concentration. Figure 5.4B shows the relative reduction in PK-1 cells after co-culture with the CA11 T cell line and MiaPaca-2 cells, at different E:T ratios. A summary of relative killing of specific target compared to control target by T cell lines from CA07 and CA11 is shown in figure 5.4C. T cell lines isolated from these two different patients can kill PK-1 and Bx-Pc-3 cancer cell lines, but not Panc-1, compared to negative control (MiaPaca-2), demonstrating their CEA691-specific cytotoxicity. Figure 5.5 shows the unstimulated PBMCs could not kill pancreatic cancer lines (Fig. 5.5A), and specific cytotoxicity was not observed using CEA-specific T cells isolated from patient CA18 (Fig. 5.5B).
Figure 5.4. Cytotoxic activity of CTL lines against pancreatic cancer cell lines.
A. CEA and HLA-A2 expression of 6 pancreatic cancer cell lines. The results were analysed by FACS and presented in histogram (upper). Isotype antibodies were used to determine the background. Percentage and MFI of HLA-A2/CEA expressions by different PC cell lines was also shown (bottom). N.B. The unstained cells were gated out. Gating strategy was described in Figure 5.1.

B. FACS analysis of CA11 T cells killing activity in response to recognise and kill PK-1 cell line (HLA-A2+, CEA+, labelled with high dose CFSE), and MiaPaca-2 cell line (HLA-A2-, CEA-, labelled with low dose CFSE), at different E:T ratios.

C. The percentage of relative killing of PK-1, Panc-1 and Bx-Pc-3 by CTLs from CA07 or CA11, compared to MiaPaca-2, at different E:T ratios. All the experiments were repeated twice and the mean of the results was shown in the figure.

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**Figure 5.5. Cytotoxic activity of T cells against pancreatic cancer cell lines (negative example).**

A. Unstimulated HLA-A2+ PBMCs did not show IFN-γ production in intracellular cytokine assays following overnight stimulation with CEA691 peptides were co-cultured in a cytotoxicity assay with PK-1 cells. As can be seen these unstimulated PBMCs could not kill PK-1, even at an E:T ratio of 100:1.

B. T cell lines from CA18 were added to pancreatic cancer cell line PK-1 (specific target) and MiaPaca-2 (control target) at different ratio from 100:1 to 1:1. No cytotoxic effect was observed.
Figure 5.6 shows anti-tumour specific cytokine secretion after stimulation by the same pancreatic cancer cell lines, as assessed by intracellular cytokine staining (E:T ratio = 1:1). Example FACS plots of IFN-γ and TNF-α production by CD8+ T cells expanded from CA07 after being stimulated with four different cancer cell lines are shown in figure 5.6A. Similar results were observed with the T cell line from patient CA11 (Fig. 5.6B), but not CA18 (Fig. 5.6C). In summary, T cell lines from CA07 and CA11 displayed anti-tumour function against HLA-A2+ pancreatic cancer cells with high CEA expression.

Figure 5.6 Representative FACS plots data of IFN-γ and TNF-α by T cell line from CA07 (A), CA11(B) and CA18 (C), after 5h-stimulation with different cancer cell lines (Gated on CD8+ T cells).
Sequencing of TCR α and β Chains Isolated from Functional T Cell Clones from Patient CA11

The CEA specific T cells line generated from CA11 had demonstrated cytotoxicity against PC cell lines. We used TCR sequencing strategies to identify expansion of one or more immunedominant T cell clone. RNA was isolated from the CA11 T cell lines and sequencing was performed as described in the Materials and Methods section. Figure 5.7 displays the sequencing results of T cells from CA11. Figure 5.7A shows the expansion of cytokine producing CEA691 specific CA11 T cells between the 3rd and 5th round. A number of TCR chains were identified. Figure 5.7B presented the percentages of identified sequences of α and β chain at the end of 5th round stimulation respectively, the top 10 most commonly detected chains are labelled in colours (which consist more than 60% of all detected chains). Among them, three α chain sequences and three β chain sequences were presented throughout and expanded in all three tested rounds and remained the dominant sequences at the end of 5th round. They were named as sequence 1, 2 and 3 (Fig 5.7C). For example, TCR α chain sequence 3 and β chain sequence 3 were the most frequently identified sequences at the end of round 5. Moreover, the percentages of the two chains (of all α or β chains) were similar to one another and gradually expanded from 3R to 5R (Fig. 5.7C). These data may suggest that the sequence3 α chain and β chain may pair to form a functional TCR. The only way to prove this is to clone these TCR sequences into retroviral vector and to perform TCR gene transfer experiments and test the Ag-specificity and function of the transduced T cells. Similar results were seen with sequence 1, which was the second most frequent sequence, and with sequence 2.
Figure 5.7. Identification of dominate TCR sequences during CEA691-specific CD8+ T cell expansions.

A. The expansion of functional CEA691 specific T cells of CA11 from 3rd to 5th round stimulation.

B. The percentage of identified TCR α and β chain sequences at the end of 5th round. The top 10 most commonly detected chains were labelled in colors. The other sequences were merged and labelled in white. Three dominant sequences of α chain and β chain were labelled as sequence 1, 2 and 3.

C. TCR sequence 1, 2 and 3 were presented and expanded stably from 3rd round to 5th round. The red line represents the percentage of β chain of interest in all β chains and blue line represents the percentage of curtain α chain in all α chains. The expansion trends of α chain and β chain with the
same label were generally matched to one another. For example, when 5% of α chains at 3R were sequence 3, about 5% of the β chains were also sequence 3. The percentages of α sequence 3 and β sequence 3 both increased to just above 15% at the end of 4R, and to about 36% vs 43% at the end of 5R. N.B. the sequencing was performed in Professor Chain’s lab.

Fitness and Phenotype of CD8+ T cell Lines after CEA691-Specific Stimulation

To identify the phenotypic characteristics of T cells lines generated after CEA691 specific stimulation, we examined the change in proportions of CTL expressing the following surface markers: CD62L, CD45RO, CD27, CD28, PD-1, TIM3 and LAG-3, after each round of stimulation. Figure 5.8A show the phenotype of CD8+ T cells from CA11, between the 2nd and 6th round of stimulation, and the percentage of CEA-specific IFNγ-producing CTLs (gated on CD8+ T cells). When more than 70% of the T cells produced IFN-γ, less CD8+ T cells expressed PD-1, TIM-3 and LAG-3 than in other rounds, and most of the CD8+ T cells expressed a memory phenotype. At the 6R, when the T cells lost function, the percentage T cells expressing these co-inhibitory markers increased (Fig. 5.8B). The percentages of T cells expressing co-stimulatory markers, CD27 and CD28 were reduced along with stimulation (Fig 5.8B).

The results of functional tests differed between T cells from different patients; the change in phenotype also demonstrated various trends. However, in the three T cell lines with the most impressive antigen-specific function (from CA07, CA11, and CA18), the CD8+ T cells all presented reduction in percentage of PD-1+ CD8+ T cells, when the T cell responses (i.e., most T cells having CEA-specific IFN-γ production) were observed. Figure 5.9 compared the co-stimulatory markers and co-inhibitory markers between T cells from CA07, CA11 and CA18 cultured with CEA691 (thus, have 30-70% of T cells responded to CEA691) at the end of 4th round stimulation, and T cells from the same patients, but cultured with MSLN547 (where none or less than 1% of T cells responded to MSLN547 were observed) after 4 rounds of stimulation. Compared to MSLN-culturing T cells, less CEA-culturing T cells with memory phenotype expressed PD-1 (paired t test, N=3, P=0.011 for TCM, and 0.022 for TEM).
Figure 5.8. Phenotypic characteristics of CD8+ T cells from CA11 during CEA-specific expansion.

A. Dot plot of CD8+ T cells from CA11 producing IFN-γ after CEA-stimulation, and expressing PD-1, LAG-3, TIM3, CD27, CD28, CD62L and CD45RO, between 2\textsuperscript{nd} and 6\textsuperscript{th} round expansion.
B. The trend of expression of co-inhibitory makers, co-stimulatory markers and memory differentiation by CD8+ T cells from CA11 during ex vivo expansion.

Figure 5.9. Phenotypic difference of CTL lines cultured with CEA691 and MSLN547 after four-round antigen specific stimulation.

CD8+ T cells from CA07, CA11 and CA18 were grouped to naïve, TCM, TEM and End-stage/effector, according to the expressions of CD62L and CD45RO. At this point, 30-70% of CEA-culturing T cells can produce IFN-γ after stimulated with CEA691. But MSLN-culturing T cells hardly had functional responses to MSLN. This chart showed the different percentages of naïve or memory CD8+ T cells expressing co-stimulatory or co-inhibitory markers between CEA-culturing T cells and MSLN-culturing T cells. Data is from one experiment.
5.3 Discussion

CEA is a candidate target for cellular immunotherapy of pancreatic cancer. Firstly, CEA is overexpressed on more than 90% of pancreatic cancer tissues (320), but expressed on normal tissues at a much lower density. Secondly, CEA was expressed on various types of tumours, including >90% colorectal cancer (356), 92% gastric carcinoma (357), 70% lung cancer (360) and 63% gall bladder cancer (359), hence, anti-CEA strategies may be used as a universal approach in different cancers. Thirdly, CEA may contribute to progression of tumours as it is important for intercellular adhesion and influences mechanisms of metastasis (365). Additionally, CEA peptides are naturally processed and presented on the surface of tumour cells (366). Finally, evidence has demonstrated that immune tolerance of CEA is incomplete (366, 367). Three HLA-A2 restricted CEA epitopes were previously reported to be presented and processed by tumour cells, namely, CEA605, CEA691 and CEA694 (308, 378, 382), but we chose CEA691 to generate Ag-specific CTL lines, based on our previous results indicating that CEA691 specific CTL were more frequently detected in pancreatic cancer patients than CTL specific for other two epitopes in Chapter 4. Besides, previous data also showed the CEA691 may have a better HLA-A2 binding affinity than CEA605 (366).

The majority of studies to date relating to CEA691 epitopes have focused on colorectal cancer. To our knowledge, CEA691 specific T cell lines have not previously been generated from pancreatic cancer patients, or their ability to kill pancreatic cancer cell lines tested. In the past study, CEA691 specific T cells were detected from PBMCs of normal donors after stimulation (366, 367), but these studies did not provide the functional details of the cell lines. A well-studied CEA691 specific T-cell line has been generated by immunizing HLA-A2 transgenic mice (380). The subsequently isolated humanized TCR was high affinity and when introduced into TCR engineered human CTL, they were able to lyse colon cancer cell lines in vitro (278). The engineered TCR was subsequently used in a phase I clinical trial, leading to one (out of three) tumour regression, but all three patients developed severe transient colitis (308). As CEA was also expressed on multiple normal tissues (355), there is a risk of on-target toxicity outside the tumour. The observed autoimmune response indicated this high-affinity TCR-CTL could recognize CEA expression on normal tissues. This may have been influenced by the high affinity of the haTCR-CTL, or by an increase in the expression of CEA in normal colon tissues in these patients. In this study, we concentrated on the immunogenicity and function of natural-occurring CEA691 specific CTL.
Of the three T cell lines with the most impressive cytokine production, only T cells from CA11 could kill 80% of the 1μM peptide loaded T2 cells at a E:T ratio 20:1. At an E:T ratio of 20:1, the CA11 CTL line recognized and killed peptide-loaded T2 cells at nano-molar level, which are comparable to the concentration of TAA presenting on the surface of tumour cells (434), indicating high-avidity of this T cell lines. However, such in vitro results are likely to overestimate the functional cytotoxicity of Ag-specific T cells compared to in vivo function in the tumour microenviroment. It is known that the tumour may down-regulate the expression of CEA or MHC molecules (250, 265). Also, to reach equivalent E:T ratios as observed in the in vitro experiment, T cells need to accumulate in the tumour site, after successful trafficking to the tumour site with effector phenotype (76). Nevertheless, the results demonstrated in some PC patients where CEA was overexpressed, high-avidity CEA691 specific T cells are still observable in PBMCs.

In our experiments, the effector T cells were most potent at the end of four rounds of stimulation. In patients with ‘strong’ responses (i.e., large number of CEA691 specific IFN-γ secreting T cells), most T cells displayed a memory phenotype at that time point. This was associated with a reduction in PD-1 expression. CD28+ and CD27+ T cells were also consistently reduced following Ag-stimulation.

Thus, we have identified self-restricted CEA691-specific CTL, with tumour-killing potential, which can be expanded from the peripheral T cell repertoire of patients with pancreatic cancer. These results may provide support for the use of CEA-based vaccine in PC patient. We also sequenced the functional T cells aiming to help in generating TCR constructs for TCR-based cancer immunotherapy. To generate ‘safe’ T cell clones avoiding off-target toxicity, the TCR should be able to induce cytotoxic responses to CEA-presenting tumour cells but not recognize normal cells, which express CEA at low levels. As patient CA11 who had CEA specific T cell responses survived longer than the average survival time of general PC patients, these responding T cells may have contributed to the prolonged survival of the patient and did not knowingly cause any off-target effects (e.g., colitis).

However, the use of high concentrations of CEA peptide may have favoured the selection of low avidity T cells rather than high avidity T cells which can recognize tumour cells presenting relatively lower levels of peptide/HLA-A2 molecules than in the experimental environment. Alternatively, using CEA691 tetramer to bind CEA691 specific T cells has been shown to be a useful way of selecting out high affinity TCRs. One approach is using the
CEA691 tetramer to select out high avidity T cells from PBMCs, and then expand the cells using CEA691 peptides. This would be expected to promote the selective expansion of high avidity T cells, but it would require a large number of PBMCs, as the proportion of CEA691 T cells in the starting PBMC cultures are likely to be minimal. Also, the CEA691 tetramer can be used when the CEA691 specific T cells have been expanded after a few rounds of stimulation. This strategy may avoidably expand multiple clones of CEA691 specific T cells, and the expansion of one clone may compete with other clones during in vitro stimulation, possibly resulting in poor expansion and low numbers of some high-avidity clones. But the advantage of this method would be that it is feasible even when the numbers of collected PBMCs are relatively low (as in our study when we were only entitled to collect 30ml of peripheral blood from each patient). Unfortunately our tetramer expired at the end of the project and was no longer effective at binding CEA691-specific T cells.
Chapter 6

Summary and Discussion
The prognosis for patients with pancreatic cancer remains extremely poor and novel treatments such as T cell based immunotherapies are required to improve the survival rate. Our study attempted to provide rationale for the development of new strategies to improve the immunotherapy for pancreatic cancer, especially, T cell-based immunotherapy. Basically, such new immunotherapies require the identification of suitable target antigens, and a mechanism to reverse the immune tolerance within the tumour environment.

**Possible biomarkers for PC**

An important contributing factor for the poor prognosis of pancreatic cancer is late diagnosis. It is possible the survival of PC patients could be improved with earlier diagnosis leading to more patients being eligible for surgical approaches. In our study, we aimed to determine if the concentration of certain biomarkers (mesothelin and some inflammatory cytokines) in the peripheral blood were different between PC patients and non-cancer individuals.

We chose mesothelin as it is expressed on more than 90% of pancreatic cancer tissues, and the protein contains a secreted portion (319). Thus, we hypothesized that an increase in mesothelin expression in malignant pancreatic tissue could lead to an increase in circulating soluble mesothelin protein, and therefore potentially predict the development or progression of pancreatic cancer. After testing plasma samples from 32 PC patients, 13 patients with non-cancer pancreatic disease and 13 healthy volunteers, we observed that the mean concentration of mesothelin identified in the PC patients was statistically significantly higher than in the healthy control group, but this difference did not reach statistical significance when compared to the mesothelin levels observed in the benign disease group. The result suggests that the plasma mesothelin concentration could increase significantly in association with pancreatic cancer, but it may have limited efficacy in distinguishing the benign pancreatic diseases from malignant disease.

Other candidate biomarkers we analysed were 6 cytokines associated with inflammation, namely IL-10, IL-6, IL-1β, IL-8, TNF-α and IL-12, based on the fact that elevated or reduced serum cytokines have been detected in some cancer patients (421). After investigating the concentration of given cytokines in plasma samples from 34 PC patients, 15 patients with benign pancreatic disease and 13 healthy donors, we found that the concentration of IL-10 was significantly elevated in PC patients compared to both control groups.
Our outcomes suggested that the concentration of some cytokines and mesothelin in serum may be abnormally elevated in some PC patient patients, and should be evaluated further in larger studies.

One of the major limitations of our study is that the sample size was small. For example, the benign controls in this study were a mixture of patients with different clinical conditions, such as acute and chronic pancreatitis, which can have different effects on cytokines levels. It would be necessary to recruit more samples, especially patients with chronic pancreatitis, in order to gain a more accurate insight into the relationship between soluble cytokines/mesothelin and PC. Besides, other factors such as age, gender, and race, may also impact the levels of mesothelin and cytokines, and in an ideal study case matched controls would be included, with paired age and gender. As with other studies, it remains a challenge to include patients in the early stages of PC due to delayed onset of presenting symptoms. In summary, we cannot prove that the predictive utility of these biomarkers in the diagnosis of pancreatic cancer is better than existing diagnostic methodology. It may be possible to address this by initiating a cohort study to recruit non-cancer patients with a high risk of PC and prospectively follow changes in circulating mesothelin and cytokines regularly. Results from such a study may demonstrate whether such biomarkers are likely to be useful as early diagnostic indicators in the clinical setting.

**TAA-specific T cells responses from PC patients**

In this study, we put significant emphasis on detecting antigen-specific T cells responses in pancreatic cancer patients. To identify potential targets, the first TAA we tested was mesothelin. As discussed above, mesothelin is overexpressed in more than 90% of pancreatic cancer cases and is involved in the biological mechanism of metastasis. In our opinion, it therefore has potential as a target for T cell based therapies directed against pancreatic tumour cells. To date, there has been little published information on the status of naturally occurring MSLN-specific CD4+ and CD8+ T cell responses in cancer patients.

As shown in chapter 3, we investigated T cell (CD4+ and CD8+) responses stimulated by overlapping mesothelin peptides (15AA) covering the whole length of the mesothelin protein, and demonstrated that the breadth and magnitude of MSLN-specific CD4+ T cell responses (defined by the percentage of IFNγ producing CD4+ T cells after 9-10 days antigen specific stimulation) was significantly higher in patients with PC, compared to patients with benign pancreatic disease and healthy controls. CD4+ T cell responses are known to be critical for
activating and promoting anti-tumour CD8$^+$ T cell memory (90) and therefore these results suggest MSLN over-expression may stimulate clinically meaningful self-restricted T cell responses. Meanwhile, we identified new MSLN epitopes which were recognized by CD4+ T cells from PC patients.

Nevertheless, it was noticeable that in using overlapping peptides with 5 different AAs, some epitopes not included in our pools would not be screened. Also, as the first screen was performed using peptide pools, the high affinity peptides may have competed with each other, resulting in T cells with TCR of relatively weaker affinity to not expand. Ideally, the sequences of peptides that had been selected out should be modified and tested to confirm the exact epitopes with highest affinity, and the relevant HLA class II molecules should be determined as well.

Prior to performing the experiments, we also expected to identify dominant mesothelin epitopes in pancreatic cancer patients. However, our results showed that the epitopes which stimulated CD4/CD8 responses were distributed in different segments of the MSLN protein and were predicted to bind a wide range of HLA types. Here, the peptide 3 stimulated relatively more samples to respond than other peptides, but the percentage of patients who responded to this peptide in all PC patients in our study was relatively low. Unlike HLA-class I restricted epitopes, which usually have stronger binding affinity to one class I allele, the HLA-class II epitopes can bind to several class-II alleles. The peptide 3, for example, was shown to be able to binding to HLA- DR9, DR11, DQ4, DQB1*0301, DRB1*0404, DRB1*0405, based on online epitope prediction engines. Due to the limited number of T cells we were able to collect, we did not perform HLA-typing. If we had done that, we could have matched the HLA type to particular epitopes. Such an approach predicts the design of vaccines for PC patients with certain HLA class II types.

In addition to CD4+T cell responses, we were keen to investigate CD8+ T cell responses, as they have been considered more important in anti-tumour immunity. However, the number of MSLN-specific CD8$^+$ T cells identified (after in vitro stimulation with peptide pools) in PC patients was not significantly different to that observed in patients with benign pancreatic disease or healthy controls. We used 15mer AAs peptides to stimulate the cells, which may favor the detection of CD4 responses rather than CD8 responses, because the TCRs in CD8+ cells bind to peptide/HLA class I complexes which usually contain peptides at a length of 8-10 mer AAs, and the peptides in our studies had to be processed prior to loading onto class I
molecules. This may partly explain why we detected more CD4+ T cell responses than CD8+. To screen potential CD8 restricted epitopes, the 8-10 mer AAs overlapping peptides may be more efficient. Besides, PBMCs in our study were only stimulated ex vivo for a total of 9-10 days and, according to our experience (405), it is possible that the expansion of responsive CD8+ T cells was not sufficient for detection by flow cytometry, which is not as sensitive in detecting cytokine production as ELISA. However, we did not adopt ELISA, as the limitation of ELISA is that it is unable to trace whether individual CD4+ or CD8+ T cells generated the relevant cytokines.

Meanwhile, although a number of TAAs have been identified as over-expressed in PC (320, 326, 333), very little is known about which particular CD8 restricted epitopes are sufficiently immunogenic to elicit a potent T cell response and could therefore be useful for the development of novel immunotherapeutic approaches (435). In order to answer this question, we sought to characterise further the antigen-specific CTL responses generated upon short-term culture of PC patient derived PBMC with 18 different HLA-A2 restricted peptides listed in Chapter 4. We chose HLA-A2 as it is the highest frequency class I HLA allele in Caucasian populations (268). Two highly immunogenic peptides, CEA691 and Mucin-12, were identified which generated Ag-specific CD8+ T cell responses in 13 PC patients. These two peptides may be candidates for vaccine studies.

Again, the short term cultures (stimulations) enabled us to quickly focus on peptides of interest, but were not helpful in assessing the T cells cytotoxic function or to obtain the sequence of a high affinity TCR. Thus, in Chapter 4, long-term stimulation cultures were subsequently established to characterize the function of TAA-specific CD8+ T cells from PC patients. Due to the limited number of samples stored from each patient, we selected three HLA-A2 binding peptides pCEA691, pMSLN547 and pWT1-126 to stimulate the T cells. These three TAAs are also considered clinically important. We included MSLN547 as it was examined in the initial experimental work described in Chapter 3, and our lab has extensive experience targeting WT126 in other malignancies. As a result, CEA691, WT1-126 and MSLN547 specific CTL lines were generated from PBMCs of PC patients and healthy controls, and all three peptides were able to trigger CD8+ T cell responses, but more PC patients had CEA691 specific T cells, and functional CEA691 specific T cell lines with Ag-specific cytokine production and cytotoxicity were generated from patients with pancreatic cancer and were able to kill pancreatic cancer cell lines in vitro. Hence, of the three peptide
specificities tested, CEA691 specific T cells may have been the ones to more readily expand to detectable levels in PC patients.

In our patient group, the frequency of IFN-producing CEA691-specific CTL after in vitro peptide stimulation was higher in patients with localized PC (Stage II-III) compared to healthy controls and patients with advanced PC (stage IV, metastatic disease). One potential explanation is that prolonged TAA exposure in cancer environment may exhaust antigen-specific immune responses of CEA691 specific T cells. However, we were unable to test PBMCs at different time points over the courses of PC progression for individual patients. Thus, the differentiation of anti-CEA691T cell responses in patients at different stages may be attributed to individual differentiation, the lack of immune cells in advanced patients, the treatment they received, or the degrees of immunosuppressive conditions in different people. Another possible interpretation could be that the PC patients without CEA691 specific CD8+ T cell responses may develop more aggressive and/or quickly progressing cancers, and that is why when the patients were diagnosed, they were at the end stage of the disease process. To address these issues, bigger sample sizes allowing enough samples in each subsection and more information related to treatment would be needed. Nevertheless, we can conclude from our study that patients with CEA691-specific CTL may have been enriched for patients at relatively earlier stages of PC.

We are not the first group to generate a CTL lines specific for CEA691. Rosenberg’s group has tested the efficacy of the CEA691-specific engineered high-affinity TCR, based on a HLA-A2 transgenic mouse model (380), in patients with metastatic colorectal carcinoma. Tumour regression was observed in one out of three patients, but patients also suffered from severe transient colitis, limiting the development of this particular T cell therapy (308). Noticeably, Rosenberg’s T cell clones had been isolated from transgenic mice, which typically generate higher affinity T cells than identified in humans - as the sequences of protein in mice is usually different from humans and the tolerance to CEA tends to be incomplete (278). The disadvantage of the high affinity TCR is that it can recognize low levels of CEA691 expressed on colonic epithelial cells, and there may be increased cross-reactivity recognizing other antigens in the gut. Moreover, T cells at the location of healthy tissues can be subjected to less immunosuppressive factors, compared to those found in tumour sites, further increasing their effector function against CEA691-presenting cells in normal tissues.
However, the T cell lines we generated were isolated from tumour patients, and the human T cells were theoretically previously subjected to central tolerance, in which the majority of high affinity T cells towards TAA should have been deleted. Our cell lines generated from CA07 and CA11 have demonstrated cytotoxicity towards pancreatic cancer lines in vitro, but no colitis was reported in these patients, suggesting that the cells did not attack cells expressing normal levels of CEA-HLA molecules. In the absence of in vivo experiments, it may be too early to answer if the T cells we isolated from the patients have the ability to specifically target tumour cells (with abnormally high CEA expression), but ignore the normal tissues (with relatively low CEA expression) and thus reduce potential off-target toxicity. However, one confirmed advantage of our T cells (directly isolated from patients) is that they can reflect the state (such as inhibitory receptor expression and cytokine production abilities) of T cells from PC patients, and therefore allow us to test if the checkpoint treatment could improve the anti-tumour function of T cells.

Numerous obstacles exist which may prevent the development of a CEA691-specific T cell therapy. Firstly, as we used a high concentration of CEA peptide to stimulate the T cell culture, we might have selected out low avidity T cells rather than high avidity ones. The high avidity of therapeutic T cell is crucial in adoptively transferred T cell therapy. The tumour cells are known to down-regulate HLA molecule expression, and the therapeutic T cells therefore are required to recognize tumour cells presenting relatively lower levels of peptide/HLA-A2 complexes than in the experimental environment. Although we knew that the CEA691-tetramer could help us to select out clones with high affinity (which may benefit to further functional test), our tetramer expired (no longer effective at binding CEA691-specific T cells) before we performed the planned experiments. Secondly, we have not had sufficient time to develop a robust in vivo model of PC. The microenvironment of pancreatic cancer is known to be far more complicated than in many other tumours, partly due to the desmoplasia. The effector T cells may be blocked by the dense stroma, and the cytokines may mediate a highly suppressive tumour microenvironment compared to other kinds of tumours. Thus, to make adoptive T cell therapy more effective, we have to solve the problems of desmoplasia. In theory, depletion of the stroma can be a solution. Some investigators also believe the depletion of PC stroma may reduce the high incidence of drug resistance observed in pancreatic cancer (29), and enhance CTLA-4 blockade and antiangiogenic therapy (436). However, more studies are in demand to identify the dual roles—both protective and
detrimental-of stoma in PC (437). Thirdly, we still need more evidence to correlate the efficacy of CEA specific T cells to improved survival of cancer patients.

**Improve the function of T cells from PC**

The expression of PD-L1 was reported in 80% of PC cases, and the upregulation of PD-L1 expression is related to malignancy of PC (178, 179). Our data has demonstrated that patients not responding to CEA691 had larger numbers of PD-1 expressing T cells in their unstimulated PBMCs, compared to the T cells isolated from responding patients or healthy controls. T cells isolated from tumour draining LNs expressed more PD-1 and TIM-3 than T cells from the peripheral blood (Chapter 4). In other experiments, an increase in serum IL-10 concentration was observed in PC patients, compared to patients with benign pancreatic disease or healthy controls (Chapter 3). Not surprisingly, these findings support the existence of an immunosuppressive environment in PC patients. It is therefore likely that future therapies involving adoptively transferred T cells will require them to be engineered to function in such conditions. It may also support the development of combination therapies with checkpoint inhibitors and antigen specific T cells.

For that reason, we examined the effect of IL-10, PD-L1 and TIM3 blockade on the function of Ag-specific T cells isolated from PC patients. Anti-IL10, anti PD-L1 and anti-TIM3 were able to enhance the function of antigen specific T cells in vitro. IL-10 blockade improved the performance of MSLN-specific CD4+ T cells (statistically significant). PD-L1 blockade had a greater impact on restoring the function of CEA691 specific CD8+ T cells than TIM3-blockade. This may be because TIM-3 levels were generally low in freshly isolated T cells.

Currently, the efficacy of anti-CTLA-4 and anti-PD1/PDL1 antibodies in treating several types of cancers has been demonstrated in the clinical setting (214). Checkpoint blockade is considered to have improved efficacy in treating cancers with high mutational loads, e.g., melanoma and lung cancer, because such cancers may generate more neoantigens and checkpoint therapy can revive the function of the T cells (typically TILs) which recognize such neoantigens (24). On average, the neoantigen repertoire of pancreatic cancer is 1 mutation per megabase, which is less than that observed in melanoma (with more than 10 mutations per magabase on average). In theory, therefore, pancreatic cancer should not be very sensitive to checkpoint therapy. To date, limited positive results in PC patients treated
with checkpoint blockers have been published (235). However, our in vitro results suggest to us the PD-1/PD-L1 pathway may play a part in inhibiting the anti-tumour functions of CD8+ T cells from PC patients. Following the addition of PD-L1 blocking antibodies, the T cells’ ability to produce cytokines (including IFN-γ and TNF-α) after peptide stimulation was improved. The restoring effect of anti-PD-L1 antibody is more impressive when using CEA691 to stimulate T cells from LNs than PBMCs, and the percentages of T cells expressing PD-1 in LNs is higher than in PBMCs, suggesting PD-L1 blockade could contribute to improve the function of TAA-specific T cells with higher PD-1 expression, such as in the draining LNs. Thus, the unsatisfactory results of checkpoint blocking research in PC may due to the relatively small neoantigen repertoire, but the PD-1/PD-L1 blockade may still be a potential methodology to augment the function of TILs or other therapeutic T cells.

General summary of my PhD

My PhD project relied on the collection of patient samples, thus, I met some difficulties in securing a large enough sample size. Prior to starting this project, there were no published data characterizing functional MSLN547-specific CTL lines isolated from PC patients. Others have previously generated CEA691 specific T cells using different methods. Adverse events occurred due to off-target, off-tumour toxicity, namely severe colitis. It remains important to identify the reasons for the adverse effects observed, and there may be value in trying to identify T cells functioning within an optimal affinity range. Despite this, CEA remains a valuable target, especially in pancreatic cancer.

Due to time constraints, I was unable to carry out further experiments to identify which TCR could be considered the optimal CEA691 TCRs (from the sequenced dominant TCRs), to maximize anti-tumour effects whilst minimizing toxicity associated with low level CEA expression in normal tissues. Unfortunately, repeat or serial PBMC samples were unavailable on the majority of patients studied in this project. I was therefore not able to follow up the functional and phenotypic changes in the Ag-specific T cell repertoire at different stages of the disease (except for patient CA11). Extended analysis would enable to answer whether the CEA/MSLN-specific T cell responses were able to influence on the disease-free survival of the patients.

Further work
To carry on this project, an expanded sample size, including serial samples from the same patients, would help test further our hypothesis that plasma mesothelin levels have a diagnostic value and that during the progression of PC there is a change in expression of inhibitory receptors.

Also, we are especially interested in potential therapeutic methodology. Based on the MSLN-specific HLA-class II epitopes that we have identified, we plan to do the HLA-class II typing and match the epitope to particular HLA types, which may allow us to design peptide vaccine to expand CD4+ T cell responses in PC patients.

Moreover, one of the most important goals of our study was to isolate high avidity TCR clones and utilize them for treatment purposes. To achieve that, future work includes selecting out the high avidity clones, sequencing the TCR for such clones and redirecting T cells. We have obtained the sequence information of the dominant TCRs from the T cell cultures in CA07 and CA11 (Chapter 5), but we cannot guarantee they are high avidity TCR, as we used high-concentration peptides to stimulate these cells. Tetramers can facilitate the isolation of T cell clones bearing high affinity TCR, and be used in further functional tests. An alternative approach is to use software to predict sequences with the highest binding strength for the sequences obtained.

Once the genetic information regarding putative high avidity TCR is available, we could move to redirect T cells using TCR gene transfer protocols. A series of T cells recognizing the same peptide/HLA molecules could then be assessed for their functional avidity and anti-tumour effects in vitro and in vivo, through cytotoxicity assays based on peptide loaded T2 cells and pancreatic cancer lines, and through in vivo animal experiments to acquire survival curves. Then, the exact TCR clones with highest avidity and anti-tumour effect could be identified. It would also be worthwhile to check if the combination of checkpoint inhibition and redirected CEA691 specific T cells could work synergistically to improve survival of patients with pancreatic cancer.
Chapter 7

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