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# MUC4 Based Immunotherapy for Pancreatic Cancer

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# MUC4 BASED IMMUNOTHERAPY FOR PANCREATIC CANCER

By

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# DEDICATION

*I dedicate my PhD dissertation to my father Mr. Chandan Banerjee, my mother Mrs. Sarbasree Banerjee, my younger sister Ms. Sanchari Banerjee*

*And my late grandmother Mrs. Kalyani Banerjee*

*For providing me constant love and support.*

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# ABSTRACT

## MUC4 based immunotherapy for pancreatic cancer

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Pancreatic Cancer (PC) is a lethal disease claiming approximately 45000 lives in the US in 2018, and it establishes an elaborate immunosuppressive tumor microenvironment that aids in disease pathogenesis. Immunotherapy has emerged as a strategy to target tumor cells by reprogramming patient's immune system. Challenges present in PC immunotherapy are: i) identifying a tumor-associated antigen that could be targeted, ii) identifying adjuvants that could efficiently deliver antigens, iii) eliciting robust anti-tumor responses and iv) overcoming peripheral tolerance and immunosuppression elicited by the tumor.

Firstly, we detected circulating autoantibodies to MUC4 present in PC patients and observed that IgM autoantibodies to MUC4 peptides significantly correlate with overall PC patient survival, thus suggesting that MUC4 could potentially be targeted for PC immunotherapy. Our group is the first to successfully purify recombinant MUC4β protein and characterize its immunogenic activity. We addressed the challenge of protein delivery by encapsulating MUC4β in novel polyanhydride nanoparticles (MUC4 nanovaccine). In the second part of the dissertation, we characterized MUC4 nanovaccine in both *in vitro* and *in vivo* system. Our studies showed that MUC4 nanovaccine could robustly activate dendritic cells (DCs) and induce secretion of Th1 cytokines *in vitro*. High levels of Th1 IgG2b anti-MUC4β antibodies were detected in MUC4 nanovaccine-immunized mice.

As described in the third part of the dissertation, we observed that *ex vivo* T-cells activated by MUC4 nanovaccine-pulsed DCs showed enhanced cytotoxic killing of miniMUC4 tumor cells, when compared to soluble MUC4β mixed with empty nanoparticles (MUC4+NP). We validated our data in an *in vivo* subcutaneous PC tumor mouse model, and observed enhanced immune cells infiltration and corresponding levels of necrosis in miniMUC4 tumors corroborated with low tumor volume of miniMUC4 tumor (in comparison to contralateral vector control tumor) in MUC4-immunized mice. Furthermore, the presence of PD-L1 surface expression on miniMUC4 tumor cells indicated active immunosuppression lodged by tumor cells in response to IFNγ-secreting infiltrating cytotoxic T-cells.

Taken together, studies in this dissertation demonstrate that MUC4 nanovaccine could serve as a potential strategy for PC immunotherapy.

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# LIST OF ABBREVIATIONS

ACT: Adoptive T-cell

ADCC: Antibody-Dependent Cellular Cytotoxicity

AMOP: Adhesion-associated Domain in MUC4 and Other Proteins

ANOVA: Analysis of Variance

APCs: Antigen presenting cells

BSA: Bovine Serum Albumin

CAR: Chimeric Antigen Receptors

CDNs: Cyclic Dinucleotides

CEA: Carcinoembryonic Antigen

ChARs: Chromatin-Accessible-Regions

CNP: Cationic Nanoparticles

CP: Chronic Pancreatitis

CPH: 1, 6-bis (*p*-carboxy phenoxy) hexane

CPTEG: 1, 8-bis (*p*-carboxy phenoxy)-3, 6-dioxaoctane

CR: Complete Response

CRS: Cytokine Release Syndrome

CTLA-4: Cytotoxic T-lymphocyte-associated protein 4

CTLs: Cytotoxic T-cells

DCs: Dendritic Cells

ECM: Extracellular Matrix

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

ELISA: Enzyme-Linked Immunosorbent Assay

EMT: Epithelial to Mesenchymal

FAK: Focal Adhesion Kinase

$FAP^{+}$: Fibroblast Activation Protein-positive

FBS: Fetal Bovine Serum

FGFR: Fibroblast Growth Factor Receptor

FNA: Fine Needle Aspirates

GAL-1: Galectin-1

Gal3: Galectin-3

GDPH: Glycine-Aspartate-Proline-Histidine

GM-CSF/neu: Granulocyte-Macrophage Colony-Stimulating Factor/neu

HER1: Human Epidermal Growth Factor Receptor 1

HER2: Human Epidermal Growth Factor Receptor 2

HLA: Human Leukocyte Antigen

HRP: Horseradish-peroxidase

IACUC: Institutional Animal Care and Use Committee

IDO: Indoleamine 2, 3-dioxygenase

IEDB: Immune Epitope Database and Analysis Resource

IF: Immunofluorescence

IFNs: Interferons

IFNβ: Interferon-beta

IFNγ: Interferon-gamma

IHC: Immunohistochemistry

IL: Interleukin

IRB: Institutional Review Board

JAK: Janus Kinases

JNK: c-Jun N-terminal kinases

KIF20A: Kinesin-like Protein 20A

KPC: K-ras$^{LSL.G12D/+}$; p53$^{R172H/+}$; Pdx-1Cre mice

LAG3: Lymphocyte Activation Gene 3

LAK: Lymphokine-activated Killer

LM: *Listeria monocytogenes*

LPS: Lipopolysaccharides

mAbs: Monoclonal Antibodies

MALP-2: Macrophage-Activating Lipopeptide-2

MAPK: Mitogen-Activated Protein Kinases

MDSCs: Myeloid-Derived Suppressor Cells

MHC-I: Major Histocompatibility Complex I

MHCII- Major Histocompatibility Complex II

MMAE: Monomethyl Auristatin E

MMPs: Matrix-Metallopeptidase

MSD: Mean Survival Days

MSLN: Mesothelin

MST: Mean Survival Time

MUC: Mucin

NF-κB: Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells

NIDO: Nidogen-like Domain

NK cells: Natural Killer Cells

NR: Not Reported

NRI: Nebraska Research Initiative

OVA - Ovalbumin

PanIn: Pancreatic Intraepithelial Neoplasm

PBMC: Peripheral Blood Mononuclear Cells

PBS: Phosphate Buffered Saline

PC: Pancreatic Cancer

PD-1: Programmed Cell Death Protein 1

PD-L1: Programmed Death Ligand 1

PEG: Polyethylene Glycol

PI3K: Phosphatidylinositide 3-Kinases

PKA: Protein Kinase A

PMA: Phorbol 12-myristate 13-acetate

PR: Partial Response

PSCA: Prostate Stem Cell Antigen

PSCs: Pancreatic Stellate Cells

RA: Retinoic Acid

RIT: RetroNectinVR (CH296)-induced T-cell

RT: Room Temperature

RTK: Receptor Tyrosine Kinase

STAT-1: Signal transducer and activator of transcription 1

STING: Stimulator of interferon genes

TAAs: Tumor Associated Antigens

TAMs: Tumor Associated Macrophages

TBS: Tris-buffered Saline

TCGA: The Cancer Genome Atlas

TCR: T-cell Receptor

TGFβ: Tumor Growth Factor-beta

TLRs: Toll-like Receptors

TMB: 3,3',5,5'-Tetramethylbenzidine

TME: Tumor Microenvironment

TNF: Tumor Necrosis Factor

TNFR-1: TNF Receptor 1

TNF-α: Tumor Necrosis Factor Alpha

UNMC: University of Nebraska Medical Center

UPMC: University of Pittsburgh Medical Center

VEGF: Vascular Endothelial Growth Factor

VEGFR1/2: VEGF Receptor1/2

VNTR: Variable Number Tandem Repeat

vWD: von Willebrand factor type D domain

WT1: Wilm’s Tumor protein 1

# CHAPTER 1A: INTRODUCTION

The material covered in this chapter is the subject of one review article

1. **Banerjee K.**, Kumar S., Ross K. A., Gautam S., Poelaert B., Nasser M. W., Aithal A., Bhatia R., Wannemuehler M.J., Narasimhan B., Solheim J. C., Batra S. K. and Jain M., *Emerging trends in the immunotherapy of pancreatic cancer*; **Cancer Letters, 417 (2018) 35-46**

## 1. Synopsis

Pancreatic cancer (PC) is the fourth leading cause of cancer-related deaths in the U.S., claiming approximately 45,000 lives every year. Much like other solid tumors, PC evades the host immune system and establishes itself by manipulating the immune system to establish an immunosuppressive tumor microenvironment (TME). Therefore, targeting and reinstating patient's immune system could serve as a powerful therapeutic tool. Indeed, immunotherapy has emerged in recent years as a potential adjunct treatment for solid tumors including PC. Immunotherapy modulates the host's immune response to tumor-associated antigens (TAAs), eradicates cancer cells by reducing host tolerance against TAAs and provides both short- and long-term protection against the disease. Passive immunotherapy like monoclonal antibodies or adoptive T-cell based therapy focuses on direct targeting of tumor cells, whereas, active immunotherapy like cancer vaccines specifically activates the patient's immune cells against cancer cells. Such strategies have been tested for antitumor responses alone and in combination with standard care in multiple preclinical and clinical studies. In this review, we discuss various immunotherapy strategies used currently and their efficacy in abrogating self-antigen tolerance and immunosuppression, as well as their ability to eradicate PC.

## 2. Introduction to Immunotherapy

It's forecasted that by 2030, pancreatic cancer (PC) will become the second leading cause of cancer-related deaths in the United States [1]. The current overall survival of PC patients by stage of disease progression is no more than 14% at Stage I, and the overall 5-year survival is approximately 8% [2]. The success rate of various treatment modalities for PC including surgery, chemotherapy, and radiation is limited and reoccurrence is typically inevitable [3]. In addition, late diagnosis of the disease further compounds the problem leading to high mortality rate. Recently immunotherapy has revolutionized cancer treatment especially in melanoma [4, 5]. It is increasingly being felt that immunotherapy if applied properly in combination with the standard of care can lead to better outcomes in solid tumors including PC. Studies support immunotherapy as a viable and metamorphic approach, which can boost and restore the immune system's ability to fight against cancer.

In this review, we describe the current understanding of different immunotherapeutic approaches including anti-cancer monoclonal antibodies (mAbs), T-cell-mediated immunotherapies and cancer vaccines, as powerful strategies for PC treatment. We will also discuss the clinical efficacy of immunotherapeutic strategies, challenges and assess their feasibility as next-generation treatment options, either alone or in combination with chemotherapy for PC treatment.

## 3. Immunoediting and Immunosuppressive Microenvironment

The interaction of the immune system with cancer cells is comprised of the three phases: Elimination, Equilibrium and Escape [6-8]. During the Elimination phase, the immune system can recognize and eliminate transformed cells. PC cells shed MICA that impairs cell surface expression of NKG2D receptor on T-cell and Natural Killer (NK) cells,

thus, helping tumor cells to escape immune surveillance by inhibiting the cytolytic activity of T-cell and NK cells [9, 10]. Transformed cells that escape the Elimination phase enter the Equilibrium phase, in which cancer cells undergo genomic editing and establish the tumor microenvironment (TME) that supports the growth of the early lesions. Finally, in the Escape phase, PC recruit immunosuppressive cells like myeloid-derived-suppressor cells (MDSCs), regulatory T-cells ($T_{reg}$ cells) and tumor-associated macrophages (TAMs) [11, 12]. These immature MDSCs induce apoptosis in activated T-cells. Depletion of MDSCs in an autochthonous PC mouse model results in the unmasking of adaptive immune responses, tumor cell death and remodeling of tumor stroma [13, 14]. PC conditioned media-treated $CD4^+$ T-cells favored expansion of $CD69^+$ activated $T_{reg}$ subset, which is known to suppress $CD4^+$ T-cell proliferation, thus promoting immunosuppression [15].

PC tumor and stromal cells secrete angiogenic chemokines like vascular endothelial growth factor (VEGF), signaling molecules like galectin-1 (GAL-1) [16], anti-inflammatory cytokines like Interleukin (IL)-10, IL-13 [17], and immune-inhibitory ligands like programmed death ligand 1 (PD-L1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which further facilitate escape from cytotoxic cell-mediated death by cluster of differentiation (CD)$8^+$ T-cells and NK cells [13, 14, 18-22] (**Figure 1**). Additionally, exhausted $CD8^+$ T-cells have chromatin-accessible-regions (ChARs) that serve as an enhancer to maintain high levels of programmed cell death protein 1 (PD-1), which further keeps $CD8^+$ T-cells in an immunosuppressive state [23]. After escaping the immunosurveillance, immunologically and genetically modified tumor cells proliferate, which further prevent immunological [6, 8, 24]. Overall, cancer cells modify itself genetically and exploit the immunosuppressive behavior of the immune system to facilitate its escape from cell death mediated by $CD8^+$ T lymphocytes and NK cells.

## 4. PC Microenvironment and Immune Suppression

PC cells harbor limited genetic alterations and simultaneously modulate the TME to escape antitumor immune response. Several studies suggest that immunotherapeutic approaches are more effective in cancers with high mutation profile, displaying varied neoantigens, and having permeable tumor matrix architecture allowing better effector $CD8^+$ T-cell infiltration [25, 26]. Furthermore, PC is an immunologically 'cold' tumor due to its low mutational load, dense desmoplasia and rigid extracellular matrix architecture, which restricts the access of effector immune cells to tumor islands, a phenomenon known as excluded infiltrate TME [27-31].

Activated PC stromal compartment excludes $CD8^+$ T-cells, $CD20^+$ B-cells, and $CD56^+$ NK cells from the juxtatumoral region of the tumor. Administration of all-trans retinoic acid in a spontaneous K-$ras^{LSL.G12D/+}$; $p53^{R172H/+}$; Pdx-1Cre mice (KPC) mouse model induced quiescence in stromal cells, reduced fibronectin expression and correspondingly increased high influx of $CD8^+$ T-cells into juxtatumoral compartment [32]. In PC tumor-bearing transgenic mice, ~55% of fibroblast activation protein-positive (FAP+) stromal cells were depleted by selective expression of the diphtheria toxin receptor, showed slow tumor growth only in the presence of $CD4^+$ and $CD8^+$ T-cells in the TME. Administration of anti PD1 and anti CTLA-4 antibodies along with $FAP^+$ stromal cell deletion further reduced tumor growth in these mice [33]. Jiang et al. targeted focal adhesion kinase (FAK) using a selective FAK inhibitor VS-4718 and observed reduced tumor fibrosis and significantly increased survival of KPC mice, compared to untreated control mice. The authors demonstrated that FAK was hyperactive in neoplastic PC tumor and contributed to immunosuppressive TME by restricting cytotoxic $CD8^+$ T-cells infiltration. Inhibition of FAK made TME penetrable, which increased adoptive cytotoxic anti-ovalbumin (OVA) $CD8^+$ T-cells infiltration in the tumor and the efficacy of PD-1

antagonists in KPC mice when combined with low dose gemcitabine (25mg/kg) compared to combined treatment with vehicle, gemcitabine and anti PD-1 antibody [34]. These studies suggest that the presence of stroma in PC adds an obstacle to current immunotherapy strategies.

Modulation of stromal cells and their effects are influenced by galectins, which are soluble immune-modulating glycoproteins involved in T-cell homeostasis. For example, GAL-1 is expressed primarily by pancreatic stellate cells (PSCs), and GAL-1 overexpressing PSCs have been shown to induce apoptosis in co-cultured $CD4^+$ and $CD8^+$ T-cells compared to normal or quiescent PSCs [35, 36]. Another member of the galectin family, Galectin3 (Gal3), has also been demonstrated to play an important role in $CD8^+$ T-cell biology by inducing the apoptosis in activated T-cells [37, 38]. Jaffee et al. found that neu-specific $CD8^+$ T-cells have a high surface expression of lymphocyte activation gene 3 (LAG3), and Gal3 interaction with LAG3 induces immunosuppressive signaling in T-cells through the Gal3-LAG3 axis. This interaction reduces Interferon γ (IFNγ) production by $CD8^+$ T-cells and abrogates their infiltration into the TME [39]. Immunization with GM-CSF/neu (granulocyte-macrophage colony-stimulating factor/neu) vaccine has higher efficacy in Gal3 knockout tumor-bearing mice when compared to wild-type tumor-bearing mice. After 60 days of treatment, approximately 80% of Gal-3-knockout mice were disease free, compared to 20% of wild-type mice. Neu-specific $CD8^+$ T-cells produced high amounts of interferon-gamma (IFNγ) and granzyme B. In addition, Gal3 knockout mice had increased infiltration of plasmacytoid dendritic cells (DCs), which have a higher potential to activate $CD8^+$ T-cells than conventional DCs. Thus, galectins play an important role not only in modulating T-cell function but also in the recruitment of immunosuppressive myeloid-derived suppressor cells (MDSCs) [39].

Apart from the establishment of stroma, tumor cells also modulate the cellular composition of their microenvironment. Tumor-associated macrophages (TAMs), which are M2 (pro-tumor) differentiated macrophages, express tolerance-inducing ligands such as PD-L1/L2, promote angiogenesis, and suppress adaptive immune responses through matrix remodeling carried out by matrix-metallopeptidases (MMPs) [40]. In addition, immunosuppressive $CD4^+FOXP3^+$ $T_{reg}$ cells heavily infiltrate human PC tumors. In the KPC mouse model $T_{reg}$ infiltration increases during the progression from pancreatic intraepithelial neoplasm (PanIN) to the advanced PC stage [13]. Similarly, increased numbers of $CD68^+$ TAMs and MDSCs in circulation and TME are associated with disease invasiveness in PC patients [41, 42]. MDSCs also produce reactive oxygen species that further inhibit the antigen-specific response of $CD8^+$ T-cells in TME [43].

PC tumor cells secrete anti-inflammatory cytokines and angiogenic chemokines that promote an immunosuppressive TME while facilitating metastasis [3, 32, 41, 44]. These cytokines activate $T_{reg}$ and Th2 cells that prohibit anti-tumor responses elicited by other immune cells [24, 45-47]. Likewise, Indoleamine 2, 3-dioxygenase (IDO) enzyme is secreted by PC tumor cells that upregulate the growth of tumor cells by catabolizing tryptophan into kynurenine, and in turn inhibits T-cell and NK cells activation as well as induces $T_{reg}$ differentiation leading to immunosuppressive TME [3].

Cytotoxic T-cells (CTLs), helper T-cells and DCs are functionally impaired in the immunosuppressive TME of PC, thus skewing to Th2 (tumor-tolerating) responses. Therefore, a proper understanding of these intricate cancer-immune system interactions is very essential to develop and monitor efficacious immunotherapies. The primary goal of PC immunotherapies is to target these interactions and the reprogramming of the immune system against PC tumor microenvironment

## 5. Immunotherapy Based Approaches

The goal of immunotherapy is to induce antitumor responses by reprogramming and augmenting immune surveillance and removing immune suppression. These anti-cancer immunotherapeutic approaches are divided into ‘passive’ and ‘active’ immunotherapies. Passive immunotherapeutic strategies involve mAbs, adoptive T-cell transfers and genetically engineered T-cells. Whereas the active immunotherapeutic approaches include vaccine-mediated immunity induced by the administration of tumor-associated antigens (TAAs) [48]. The TAAs could be delivered in the form of DNA or peptide vaccines, as well as modified tumor cells or antigen-pulsed DCs. Due to genetic alterations or post-translational modifications of proteins (such as glycosylation, phosphorylation, etc.), tumor cells can express proteins that differ from their counterpart in the normal cells or are aberrantly overexpressed in tumor tissues [49].

### 5.1 Passive Immunotherapeutic Strategies

Passive immunotherapy attacks cancer by directly targeting TAAs by the administration of diverse immune components that are engineered *ex vivo*. Following are the major passive immunotherapeutic strategies ongoing in preclinical studies or clinical trials and have been summarized in **Table II**.

#### 5.1.1 Antibody-Mediated Passive Immunotherapy

Antibody-mediated immunotherapy involves targeting tumors using monoclonal antibodies (mAbs), antibody fragments, antibody-drug conjugates, or radio-immunotherapy conjugates to inhibit tumor signaling, immune suppression, or immune checkpoint blockade.

*Anti CD40 mAbs*

CD40 is a member of the tumor necrosis factor (TNF) receptor superfamily and is expressed primarily on APCs such as DCs, macrophages, monocytes, B-cells and some non-immune cells like cancer cells [50]. Anti-CD40 antibodies mimic the co-stimulatory signal of the CD40 ligand (CD40L). Tumor-bearing KPC mice with constitutive K-ras activation and gain-of-function p53 mutation when treated with anti-CD40 (clone FGK45, endotoxin-free), either alone or in combination with gemcitabine, showed detectable tumor regression. Treatment with anti-CD40 mAb bypassed the requirement for Toll-Like Receptors (TLRs), inflammasome, Type I interferons (IFNs), and stimulator of interferon genes (STING) to effectively prime adaptive T-cell responses against PC in these animals [51]. The mechanistic role of agonistic anti-CD40 mAb is to activate host antigen presenting cells (APCs) especially DCs, and also to induce clinically relevant antitumor T-cell responses, reverse tumor-induced immune suppression and induce T-cell-independent but macrophage-dependent tumor regression in PC patients [52]. In a clinical trial, 22 naïve patients with advanced PC were administered weekly doses of anti-CD40 mAb in combination with gemcitabine, which led to the increased B-cell surface expression of co-stimulatory molecules CD86, HLA-DR, and CD54 at 24-48 h post-treatment [52-55]. In another study on 21 chemotherapy-naïve and surgically incurable PC patients, treatment with gemcitabine and a human agonist anti-CD40 mAb (CP-870,893) for three weekly cycles showed the enhanced overall survival of 7.4 months compared to those who received gemcitabine alone with the median overall survival of 5.7 months. Upon biopsy, the tumors of anti-CD40 mAb-treated patients showed higher infiltration of macrophages, however, with accompanying absence of lymphocytes [55, 56].

*Anti PD-L1mAbs*

PD-1 (CD279) is a T-cell co-inhibitory receptor expressed on the surface of activated T-cells, $T_{regs}$ and monocytes had extensively been exploited for immunotherapy.

PD-1 on T-cells interacts with two B7 family ligands, PD-L1 (CD274) and PD-L2 (CD273) expressed on tumor cells that leads to T-cell anergy or death and thus leading to tumor survival [57]. Presence of infiltrating PD-$1^+$ T-cells in densely or loosely desmoplastic pancreatic tumors suggests tumor antigen-specific T-cell activation that correlates with increased overall survival, progression-free survival and distant-metastasis-free survival of PC patients [58]. Activated T lymphocytes infiltrating the TME express inflammatory cytokines like IFNγ that further stimulates PD-L1/L2 expression in the tumor cells [59, 60]. Blockade of PD-1 by an mAb abrogates the PD-1/PD-L1 axis and restore T-cell cytotoxic function [61]. In a preclinical study, combined treatment of Panc02 tumor-bearing mice with anti-PD-L1 mAb and gemcitabine significantly reduced average tumor volumes compared to gemcitabine and anti-PD-L1 mAb alone [62]. Due to the relative success of anti-PD-1 antibodies in both preclinical and clinical studies on selective solid tumors, the FDA recently approved two anti-PD-1 antibodies, pembrolizumab and nivolumab, for head and neck cancer, renal, melanoma, and non-small cell lung cancer treatment [63, 64].

*Anti CTLA-4 mAbs*

CTLA-4 is another co-inhibitory molecule expressed on the surface of activated T-cells and $T_{reg}$ cells. CTLA-4 present on T-cells interacts with B7-1/B7-2 ligands on APCs, resulting in depletion and suppression of CD68-mediated T-cell-activation [65]. Ipilimumab, an antagonist mAb against CTLA-4, inhibits immunosuppression by $T_{reg}$ cells and enhances the antitumor activity of effector T lymphocytes and innate immune cells. In a preclinical study, *in-vitro* treatment with ipilimumab significantly enhanced T-cell proliferation (preferentially promoting $CD8^+$ T-cell expansion), Th1 cytokines release (IFNγ, IL-2, and IL-12), and increased cytotoxicity of $CD8^+$ T-cells against Colo356/FG PC cells [66, 67]. In a Phase Ib clinical trial, patients with previously treated or histologically proven PC were given ipilimumab alone or in combination with GVAX. Post-treatment,

both the single and combination treatments enhanced mesothelin (MSLN) specific$CD8^+$ T-cell populations that correlated with increased survival of >4.3 months, as well as a decline in CA-19.9 levels in 7 out of 15 patients compared to patients treated with ipilimumab alone (0 out of 15 patients) [68]. Combination therapy of anti-CD40, anti-CTLA-4 and anti-PD-1 antibodies with chemotherapy/nab-paclitaxel in KPC mice resulted in tumor regression in 39% of the animals (17 out of 44 mice), along with increased $CD8^+$ T-cell infiltration and reduction in $T_{reg}$ cells (7-fold CD8: $T_{reg}$ ratio) in the PC TME. Furthermore, PC tumor cells implanted on the opposite flank were rejected with no additional treatment in 67-86% of mice, suggesting the development of immunological memory [69].

*Other targeted therapy antibodies*

Additionally, antibodies like cetuximab, pertuzumab, and trastuzumab that bind to the extracellular domain of the human epidermal growth factor receptor 1 **(**HER1)/ epidermal growth factor receptor (EGFR) and inhibit their dimerization and internalization are under clinical investigation [70, 71]. In Phase II and III clinical studies, patients with metastatic PC were administered cetuximab in combination with gemcitabine leading to a stable disease in 63.4% of PC patients and a partial response in 12.2% of patients however there was no increase in the median overall survival of metastatic PC patients. [72]. Contrarily in IMPaCT clinical trial using next-generation sequencing technologies, personalized treatment of 5 PC patient's tumors with human epidermal growth factor receptor 2 (HER2) amplification with trastuzumab showed no successful outcome [73]. Similarly, another Phase II clinical trial showed no responses in 33 advanced PC patients treated with trastuzumab (3.0 or 4.0 mg/kg then 1.5 or 2mg/kg, weekly) and cetuximab (400mg/$m^2$ and then 250mg/$m^2$) [74].

Scales et al., developed an anti-mesothelin (anti-MSLN) antibody to the unfolded, non-glycosylated MSLN extracellular domain and conjugated it to the microtubule-disrupting drug monomethyl auristatin E (MMAE). Humanized versions of anti-mesothelin (MSLN)-MMAE induced regression of MSLN-expressing HPAC xenografts in nude mice with a doubling delay to ≥74 days. Due to its robust and durable efficacy in mouse models, humanized anti-MSLN-MMAE is in Phase I clinical trials for PC [75]. Similarly, a Phase II clinical trial with the VEGF antibody bevacizumab in 19 out of 30 patients showed stable disease and partial response in 1 patient, although the antibody treatment alone did not improve the overall survival of patients [76].

MDSCs are known to secrete tumor-promoting factors, such as prokineticin 2 (PK2/Bv8). Anti-Bv8 antibody targeting the extracellular domain of Bv8 given in combination with gemcitabine reduced growth of orthotopic metastatic PC tumors, significantly reduced MDSCs infiltration, hypoxia and angiogenesis compared to mice treated with gemcitabine alone, indicating the significant potential of the anti-Bv8 antibody as a combinatorial or post-chemotherapy treatment in PC patients [77]. PC cells express antigens that are either unique to cancer or are being shared with other cancers with similar epithelial origin. The widely studied TAAs of PC that are currently utilized in vaccines in clinical trials are listed in **Table I**.

### 5.1.2 Passive T-cell-Mediated Immunotherapy

Monoclonal antibody (mAbs) based targeted therapy can elicit direct killing of tumor cells but has not provided long-term benefit to PC patients. Multiple studies are evaluating the strategies to develop passive T-cell-mediated immunotherapies including increasing the number of antigen-specific $CD8^+$ T-cells, the responsiveness of the antigen-specific T-cells, or the affinity of the antigen-specific T-cell receptor (TCR) (e.g., with

transfected TCRs). Additional summary regarding the current clinical trials utilizing these strategies is provided in **Table III**.

a) *Adoptive T-cell transfer (ACT)*

The primary objective of ACT therapy is to isolate and expand T-cells *ex vivo* and transfer these autologous lymphocytes with antitumor activity in cancer patients. This method leads to the expansion of antitumor T-cell populations in the patient resulting in increased cytokine release and tumor cell targeting.

Kawaoka et al., developed CTLs by isolating T-cells from the blood of healthy volunteers expressing human leukocyte antigen (HLA)-A phenotype 24/26 and stimulating them with the MUC1-expressing human PC cell line YPK-1 (HLA-A phenotype 24/02) in combination with IL-2. The MUC1-specific CTLs killed five MUC1-expressing PC cell lines, irrespective of their HLA phenotype. 20 patients with either non-resectable or resectable PC were treated with MUC1-specific CTLs. Patients with non-resectable tumor did not show any improvement (median survival time (MST) of 5 months), however, 18 out of 20 patients with resectable PC responded with an MST of 17.8 months [78].

Murine PC cell lines have significant overexpression of telomerase activity. C57BL/6 mice were immunized with $H2^{b}$-restricted telomerase peptide emulsified with incomplete Freund's adjuvant, in complex with macrophage-activating lipopeptide-2 (MALP-2, a Toll-like receptor 2/6 agonist) to drive the generation of telomerase-specific CTLs. Orthotopically implanted syngeneic tumor-bearing mice were treated with IL-2-expanded anti-telomerase CTLs, which significantly reduced tumor volume compared to untreated mice. In addition, anti-telomerase CTL-treated mice developed higher numbers of both $CD8^{+}$ central memory and effector antigen-specific T-cells [79]. Furthermore, in a clinical study, 46 PC patients with non-resectable and recurrent tumors received anti-CD3-stimulated lymphokine-activated killer (CD3-LAK) therapy (25 patients) or RetroNectinVR

(CH296)-induced T-cell (RIT) therapy (21 patients) at 2-week intervals. The ACT treated patients showed an increased circulating levels of IFNγ, IL-12, and IL-2, suggesting that the combined circulatory levels of these cytokines may serve as a predictive marker of the clinical response to ACT in patients [80].

b) *Chimeric antigen receptors (CAR) T-cells*

Highly antigen-specific autologous T-cells that are genetically engineered to express tumor antigen-specific TCRs or immunoglobulin-based fusion proteins are known as chimeric antigen receptors (CAR) T-cells. These engineered CAR T-cells are then cultured and expanded *ex vivo* for therapeutic purposes (**Figure 2**).

The differential glycosylation pattern of mucins provides a unique repertoire of antigenic epitopes that can be exploited for developing tumor-specific CAR T-cells. Posey et al. designed a scFv of a high-affinity antibody (5E5) to detect truncated O-glycopeptide MUC1 epitopes (GSTAP with one or two Tn O-glycans on the Ser/Thr residues) that are not expressed in normal tissues. MUC1 CAR T-cells (composed of 5E5 mAb scFV on a CAR backbone of CD8α, transmembrane domain and costimulatory domains of 4-1BB and CD3ζ) were generated that target the Tn/STn glycopeptide epitope on MUC1, and upon recognition of MUC1-9Tn, secreted high quantities of IL-2 and IFNγ, but not in response to the non-glycosylated MUC1-60-mer. Hs766T pancreatic tumor-bearing mice when treated with 5E5 CAR T-cells showed potent responses and improved survival to 113 days with 100% animals surviving compared to 40% and 33% of mice treated with non-transduced and CD19 CAR T-cells, respectively. In addition, many 5E5 CAR T-cells specifically accumulated in Hs766T tumors, in contrast to a small percentage of CD19 CAR T-cells [81, 82].

Carcinoembryonic antigen (CEA) is highly overexpressed on the surface of PC cells. Murine CEA binding domain (SCA431scFv)-containing CAR T-cells with intracellular

CD28-CD3 signaling domain were adoptively transferred into Panc02 $CEA^+$ tumor-bearing CEA transgenic mice. Anti-CEA CAR T-cells significantly reduced the size of pancreatic tumors and produced long-term tumor elimination in 67% of the mice without inducing an autoimmune reaction. Upon re-challenge with $CEA^+$ C15A3 cells, the animals rejected the cells and demonstrated increased serum levels of IL-1β and IL-5 [83]. A similar study reported the eradication of $CEA^+$ tumors in CEA-transgenic mice as a primary response to anti-CEA CAR T-cells with CD3ζ endo-domain and rejection of $CEA^+$ PC cells upon re-challenge. Based on the CAR T-cell model, there is evidence that antigen-specific $CD8^+$ T-cells can be induced to overcome self-tolerance and eliminate cancer cells while sparing normal cells [84].

Prostate stem cell antigen (PSCA) is another highly expressed TAAs in PC patients as well as in tumor-derived cell lines. In a recent study, PSCA-specific CAR T-cells showed specific targeting and lysing of PSCA-expressing PC cells (ASPC1, Capan-1) while PSCA-negative 293T-cells showed no cytotoxicity [85]. In another study, anti-PSCA CAR T-cells were engineered using antigen-recognition domains derived from mouse or human antibodies with either one (CD28) or two (CD28 + 4-1BB) T-cell co-stimulatory molecules linked to the CD3ζ endo-domain. These anti-PSCA CAR T-cells elicited antitumor responses in established human PC-derived xenograft tumors and 2 out of 5 mice showed complete tumor eradication [86].

MSLN is highly overexpressed on PC cells compared to its negligible expression in normal pancreas. Hingorani et al. developed MSLN peptide-specific high-affinity $TCR_{1045}$ expressing $CD8^+$ CAR T-cells that lysed KPC tumor cells *in vitro* and secreted IFNγ upon antigen recognition. A study in KPC mice showed that $TCR_{1045}$ CAR T-cells infiltrated the pancreatic tumors four days post-injection and induced apoptosis of cancer cells after eight days of infusion. Upon the second infusion, $TCR_{1045}$ CAR T-cells showed

10-fold increased retention in pancreatic tumor compared to non-specific ($TCR_{gag}$) T-cells. However, mice in both treatment groups developed progressive disease. $TCR_{1045}$ cell recipient mice showed less metastasis (46%) and overall survival of 96 days compared to 64% metastatic lesions and survival of 54 days in $TCR_{gag}$ treated mice. Overall, these data suggest that tumor antigen-specific engineered T-cell therapies are viable options for the treatment of invasive PC [87].

## 5.2 Active Immunotherapeutic Strategies

Active immunotherapy relies on stimulation of the immune system through immunological recognition of TAAs by T and B lymphocytes. TAAs have been widely explored as cancer vaccines for the treatment of PC in both *in vivo* mouse models and clinical trials. Cancer vaccines can be whole cancer cell-based vaccines, antigenic-peptide pulsed vaccines or DC-based vaccines. These vaccines are developed to exploit and activate both innate and active immune arms to eradicate tumor cells and evade future recurrence of the disease. Cancer vaccines currently being investigated in clinical trials in PC are summarized in **Table IV**.

### 5.2.1 Cancer Vaccines

*Mucin (MUC) Vaccines*

Mucins (MUCs) are glycoproteins that are differentially overexpressed in pancreatic tumor but is absent in normal pancreas. Some mucins (e.g., MUC1 and MUC4) have also been demonstrated to contribute to chemoresistance, and to enhance proliferation and survival of PC cells [88]. Therefore, mucins are being studied as potential candidates for vaccine development for PC. Studies conducted in human MUC1-transgenic (MUC1.Tg) mice treated with MUC1 cancer vaccines failed to show any detectable responses against $MUC1^{+}$ tumor cells despite, MUC1-specific T-cells

generating IFNγ, IL-4 and IL-10 cytokines. The immune responses in these mice were not skewed to either type 1 or type 2 immune response thus rendering the vaccine ineffective against the B16.MUC1 tumor.[89]. The $CD8^+$ T-cell killing of MUC1-expressing tumor cells was found to be mediated by perforin and FasL cytolytic pathways. Also, lymphotoxin-α, but not TNF receptor-1 (TNFR-1), played a critical and non-redundant role in the cell-mediated rejection of MUC1 expressing tumor cells [90]. In a Phase I clinical trial, MUC1-peptide (GVTSAPDTRPAPGSTAPPAH)-pulsed DC vaccines were administered to 7 patients with advanced PC. 2 out of 7 patients showed significantly increased mature DCs and peripheral blood mononuclear cell (PBMC)-mediated immune responses that were characterized by high IL-12p40 and IFNγ secretion, respectively. However, there was no tumor rejection in these patients [91]. A similar Phase I clinical trial in 20 advanced PC patients was performed with MUC1 peptide-pulsed DCs in combination with MUC1-specific CTLs. One patient with lung metastases showed complete remission, while five other patients demonstrated stable disease for at least six months post-therapy [92]. A study was conducted with 6 PC metastatic patient-derived DCs that were co-transfected with MUC4 and survivin mRNAs. These mRNA-loaded DCs activated CTLs against the MUC4 protein. Anti-MUC4 CTLs effectively targeted a human PC cell line (Capan-2) via major-histocompatibility-complex-I (MHC-I)-restricted recognition and released IFNγ. MUC4-mRNA-pulsed DCs stimulated more CTLs than survivin-mRNA-pulsed DCs, but comparatively elicited fewer CTLs activated by MUC4-survivin-mRNA-loaded DCs [93]. In another study, mature DCs were pulsed with a MUC4 epitope peptide (LLGVGTFVV) and co-cultured with $CD8^+$ T-cells to generate MUC4-specific CTLs that could effectively kill HCT-116 colorectal cancer cells ($MUC4^+$, HLA-A2+). However, the intensity of MUC4 surface expression on PC cell-line HPAC proportionally increased the apoptosis of MUC4-specific T-cells, thus rendering the therapy ineffective [94]. Based on these studies, other

mucins like MUC5AC, and MUC16 that are significantly overexpressed in PC may serve as potential vaccine candidates to develop novel immunotherapies.

*Telomerase Vaccines*

Due to overexpression of telomerase in PC patients, cancer vaccine containing telomerase-derived peptide (GV1001) vaccine is under clinical studies. However, so far PC patients treated with a combination of the GV1001 vaccine, GM-CSF, and gemcitabine showed transitory and weak Th1-type immune response, reduced infiltration of $T_{reg}$ cells, and no significant increase in median overall survival. In a related clinical trial, the GV1001 vaccine failed to enhance the effects of chemotherapy (gemcitabine and capecitabine) [95, 96].

*CEA Vaccines*

CEA is overexpressed in >90% of PC patients making it a potential immunotherapeutic target. A Phase I clinical trial with CAP1-6D, an altered CEA peptide ligand/Montanide/GM-CSF vaccine, administered to 66 PC patients elicited robust $CD8^+$ T-cell-mediated. In addition, 8 of the patients were found to have high IFNγ production, of which four patients showed progressive disease, three patients had stable disease, and one patient showed a complete response [97].

*KIF20A Vaccines*

Another cancer vaccine candidate is kinesin-like protein 20A (KIF20A), a member of the kinesin super family, which is significantly upregulated in PC. KIF20A-66, an HLA-A24-restricted epitope peptide derived from KIF20A peptide vaccine was injected into 29 PC patients in Phase II clinical trial. The tumor size (as confirmed by CT scan) was reduced in 8 patients and 16 patients showed increased CTL responses, which correlated with the increased overall survival of vaccinated patients [98]. In a similar Phase I trial,

nine advanced PC patients who previously received chemo/radiotherapy were treated with KIF20A-10-66 (KVYLRVRPLL) peptide vaccine along with gemcitabine. These patients showed increased activation of KIF20A-specific IFNγ-producing T-cells, had stable diseases and longer overall survival, indicating that KIF20A-based vaccines are possible immunotherapy candidates for PC [99].

*K-ras Vaccines*

K-ras is mutated in the majority of PC patients and is currently under investigation as an immunotherapy target. Inactivation of the oncogenic mutant K-ras enhances MHC I presentation [100]. In an *in vivo* study, mice were treated with lysates of human PANC-1 PC cells expressing mutant K-ras, with lysate in combination with K-ras mutant peptide (K-ras+peptide), or with lysate and peptide plus cationic nanoparticles (CNP) encapsulating K-ras mutant peptide (K-ras+peptide-CNP). The K-ras+peptide-CNP activated CTLs induced specific killing of K-ras-positive tumors efficiently and improved the survival time of K-ras mutant tumor-bearing nude mice compared to the K-ras mutant lysate and K-ras peptide treatment group (132). In addition, a clinical trial testing a mutant Ras peptide vaccine (administered in combination with GM-CSF) in 11 patients with advanced PC showed higher antitumor responses (92% exhibiting an immune response) with two patients having a complete response and overall survival of 20.8 months [101, 102].

*WT1 Vaccines*

The Wilm's Tumor protein 1 (WT1) is another suitable vaccine target for PC due to its differential overexpression in tumor cells but not in the normal pancreas [103]. In a recent study, 32 HLA-A*24:02$^{+}$ advanced PC patients were treated with an HLA-A*24:02-restricted, modified 9-mer WT1 peptide (CYTWNQMNL) emulsified with Montanide ISA51 adjuvant (WT1 vaccine). The MST of patients who responded to the WT1 vaccine was

10.9 months. Further, these patients developed strong effector T-cell responses along with generation of WT1-specific $CD8^+$ memory T-cells, whereas unresponsive patients showed MST of only 3.9 months [104]. In a separate clinical trial, nine patients with advanced PC were vaccinated with WT1 peptide vaccine and 8 out of 9 patients demonstrated stable disease. However, no WT1-specific T-cells were observed in the circulation of these patients [105].

*VEGF Vaccines*

VEGF is another protein that is overexpressed in PC. In a trial, nine advanced PC patients were vaccinated with four peptides comprised of KIF20A, cell division cycle-associated 1 (CDCA1), VEGFR1, and VEGFR2. Patients demonstrated increased anti-CDCA1 and anti-VEGFR2 $CD8^+$ T-cells in circulation. Additionally, 4 out of 9 patients presented with stable disease post-vaccination [106]. In a subsequent study, vaccination with WT1 and VEGFR2 peptides generated HLA-A24-restricted CTLs, which demonstrated strong cytotoxicity towards PC cells that were HLA-A24-positive and expressed corresponding TAAs [107].

*Prophylactic Cancer Vaccines*

Prophylactic vaccines have recently been investigated as immunotherapy tools to target endogenous neoantigens by utilizing attenuated bacteria/virus to stimulate antitumor adaptive immune responses [108]. *Listeria monocytogenes* (LM) is a gram-positive bacterium that induces robust $CD4^+$ and $CD8^+$ T-cell responses by its selective infection of APCs (via *actA* virulence gene) over non-phagocytic cells (*inlB* gene). An LM ΔactA/ΔinlB strain engineered to express human MSLN (CRS-207 vaccine) was administered to 10 PC patients, which resulted in induction of expression of Th1 cytokines (IL-12, TNF-α). In addition, 6 out of the 10 patients developed MSLN-specific $CD8^+$ T-cells [109, 110]. Jaffee et al. conducted a clinical trial on 93 metastatic PC patients, in which 69

patients received two doses of cyclophosphamide with GVAX (Cy/GVAX) followed by four doses of CRS207 (Arm A) and 21 patients received six doses of Cy/GVAX (Arm B). Patients in Arm A showed increased overall survival of 9.7 months compared to 4.6 months inArm B, suggesting that the success of this immunotherapy might depend on the proper patient selection [111]. The $Kras^{G12D}$ oncogene prophylactic vaccine (LM-K-ras vaccine) has been studied in KPC mice, either alone or in combination with $T_{reg}$ depletion (anti-CD25 antibody, PC61, and low-dose Cy). KPC mice that received the vaccine at early PanIN 1 stages in combination with $T_{reg}$ depletion showed prolonged survival compared to mice that received the vaccine alone at either early or late PanINs stage, suggesting the potential of $T_{reg}$ depletion therapy as the prophylactic approach for PC [112].

*STING activation as an adjuvant to vaccines and other immunotherapies*

STING is a transmembrane protein that resides on the endoplasmic reticulum, which upon activation through cyclic dinucleotides (CDNs), synthetic CDNs, or bacterial infection in the host induces interferon beta (IFNβ) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pro-inflammatory responses via IRF3 and Stat6 pathways [113]. It has been shown that endogenous STING activation via CDNs in the tumor microenvironment enhanced inflammatory responses, thereby inhibiting tumor progression and distant metastasis [114]. Recently, intraperitoneal injection of DMXAA, an activator of the mouse STING pathway, activated $CD8^+$ T-cells that led to tumor rejection [114, 115]. Similarly, synthetic STING activators known as RR-CDGs have shown efficacy in the regression of primary pancreatic tumors, and distant metastatic lesions through T-cells recruitment in a TNF-α-dependent manner [116]. Furthermore, these novel synthetic activators of STING have demonstrated enhanced adjuvant activity to accelerate adoptive immune responses in the presence of radiation therapy [116]. The

cGAMP-induced activation of endothelial cell-specific STING enhanced the antitumor responses of $CD8^+$ T-cells and improved the responses of anti-CTLA-4 and anti-PD-1 immunotherapies [117].

## 6. Challenges in Pancreatic Cancer (PC) Immunotherapy

The immune system has the potential to selectively target tumor cells upon strategic activation in cancer patients leading to better therapeutic outcomes. However, tumors employ extensive measures to escape immune surveillance, suggesting the necessity to develop novel counteracting strategies for the improved efficacy. Therefore, recent immunotherapeutic approaches alone or in combination with conventional treatment modalities need to be re-evaluated for successful therapeutic outcome in terms of improved patient survival.

### 6.1 Identification of Tumor Associated Antigens for PC Immunotherapy

Tumor-associated antigens are autologous cellular antigens that are specifically expressed by cancer cells and are negligibly expressed by or absent in normal cells. Mutation-derived tumor antigens are generated by somatic mutations inherited by tumor cells during malignant transformation that may be identified by immune surveillance [118]. The Cancer Genome Atlas (TCGA) database analysis showed that notably PC afflicted patients carry limited genetic mutations between 4 to 4000 neoantigens [119, 120]. When compared to melanoma patients who could likely express 14000 neoantigens that is a significantly low number of neoantigen repertoire in PC patients. Further expression neoantigens mutation load correlated negatively with T-cell tumor infiltration and corresponding overall survival of PC patients [119]. Differentially and spatiotemporally overexpressed or post-translationally antigens could potentially serve as tumor antigens for immunotherapy purposes [121]. A preferred way utilized to validate the likelihood of

these tumor antigens to be immunogenic in patients, is to detect autoantibodies against them in cancer patient serums and this strategy has met with moderate success [122, 123]. Multiple proteins like mucins (MUC1), WT1, VEGF, etc. overexpressed in PC patients, have been extensively exploited for PC immunotherapy but have achieved limited success as discussed previously in the review. It has been challenging to identify eligible TAAs and thus, necessitates coming up with a strategy to discover novel tumor antigen that could be targeted efficaciously by immunotherapy in PC patients.

### 6.2 Generation of Anti-Tumor Responses Against PC Microenvironment

Strong immunosuppressive TME, rigid tumor matrix architecture, $T_{regs}$ infiltration, constant antigen exposure mediated T-cell exhaustion, and upregulation of inhibitory receptors like PD-1 provide physical obstacles to effector T-cells function and generates tolerance towards tumors [124, 125]. Thus, checkpoint inhibitors and depletion of $T_{regs}$ could potentially reverse T-cell exhaustion of effector T-cells. Improper homing and inefficient infiltration of CAR T-cells to the tumor bed occur due to tumor blood vessels not responding to inflammatory stimuli. Anti-angiogenic therapy matures tumor blood vessels, facilitating pericyte recruitment and increasing tumor perfusion, which consequently increases the efficacy of CAR T-cell immunotherapy [126]. Apart from improper homing, CARs recognize TAAs that are also found at a lower level in normal tissues (which can cause toxicities in PC patients), thus if CAR T-cells survive for long periods of time in patients they increase the risk of developing autoimmunity in the future [127]. Furthermore, activated CAR T-cells containing co-stimulatory domains like CD27, CD28 or 4-1BB release a variety of inflammatory cytokines like IL-2, IL-6, and IFNγ after encountering tumor cells which induce macrophages to release more inflammatory cytokines thus establishing a positive cytokine-based feedback loop to enhance T-cell activity causing cytokine release syndrome (CRS), which could be fatal for patients [128, 129].

Development of short-lived CAR T-cells or combination treatment with an IL-6 receptor inhibitor like tocilizumab could be effective in reversing the effects of CRS without affecting the activity of CAR T-cells [128, 130].

Human PC malignancy is complex because of its TME architecture and cytokine milieu that is inefficiently recapitulated in KPC or xenograft mouse models. Secondly, both tumor cells as well as normal cells express common antigens (though usually at a lower level than normal cells), and therefore cancer vaccines can potentially cause toxicities in the patients. Thirdly, peptide-based cancer vaccines do not capture all unique immunogenic epitopes present on original tumor antigens. Pancreas-specific transgene expressing spontaneous PC mouse models, and either protein fragments or intact proteins as immunogens could address the limitations faced in this field, thereby increasing the cancer vaccine efficacy. Additionally, selection of PC patient based on both tumor stage and tumor infiltrating lymphocytes (TILs) like $CD8^+$ and $PD\text{-}1^+$ T-cells could further increase the response to cancer vaccines [131].

### 6.3 Overcoming Immunosuppression in PC Microenvironment

Nanoparticles are capable of encapsulating multiple proteins, ligands, nucleic acids and other materials, thus increasing the epitope repertoire. Nanoparticles can also incorporate immune-stimulatory adjuvants (such as TLR agonists) or chemotherapeutic drugs to enhance the overall immunogenicity, stability, delivery and/or direct cytotoxicity of the vaccine, therefore overcoming the limitations of current cancer immunotherapies [132]. For example, mice immunized with Doxorubicin-CpG-PLGA microparticles showed a reduced tumor burden at lower drug concentrations compared to mice that received doses of the soluble drug. When combined with an anti-CTLA-4 antibody, the treatment successfully reduced aggressive tumor burden at both the injected and distant tumor sites in tumor-bearing mice [133]. This co-encapsulation of multiple therapeutics and immune

stimulatory molecules may provide dose-sparing capabilities, reducing the cost and toxicity of cancer therapeutics [134].

Encapsulation into biodegradable nanoparticles protects the payload until release [135, 136]. In addition, tuning the polymer chemistry enables sustained and controlled release of encapsulated payloads [137] and immunomodulatory capabilities [138]. Particularly, it has been demonstrated that varying chemistries of polyanhydride nanoparticles were efficiently internalized by APCs, leading to the upregulation of MHC I, major histocompatibility complex II (MHC II) and costimulatory molecules, as well as inducing the secretion of cytokines [139-141]. In addition, amphiphilic nanoparticles promoted the production of long-lived, high avidity antibody [142] with suboptimal doses of antigen [143], suggesting the development of long-lived plasma cells. Polyanhydride nanoparticles loaded with OVA-induced memory $CD8^+$ T-cells that were recruited and responded to subsequent challenges with OVA-secreting tumor cells [144]. Finally, many nanoparticles can be functionalized with ligands or antibodies that may increase selectivity and reduce the side effects of chemotherapeutics on healthy tissues [145]. Targeting moieties are often attached to the nanoparticle surface via a polyethylene glycol (PEG) linker [145]. This method of PEGylation allows for flexibility of the targeting moiety and may enhance interactions with cancer cell receptors [145, 146]. For example, PLGA nanoparticles covalently modified with folate via PEG demonstrated an increased association and uptake with cancer cells *in vivo* [147].

The limited success of immunotherapeutic studies performed in PC provides a generous room for improvement. Tailoring immunotherapy to PC patients by identifying unique tumor-specific antigens through genetic screening and expression studies [49, 148]and combining it with continuous collection and screening of tumor samples in clinical

trials to understand immunotherapy resistance, will further improve the response rates and survival benefits of PC immunotherapy.

**Table I: Tumor associated antigens targeted for PC immunotherapy.**

| Tumor Associated Antigen (Cancer Antigen) | Expression | | Description |
|---|---|---|---|
| | Normal Pancreas | Pancreatic Cancer | |
| **Mucins**<br>**MUC1**<br>**MUC4**<br>**MUC5AC** | MUC4 and MUC5AC are undetectable, while MUC1 is expressed at low levels. | Aberrantly overexpressed and glycosylated in PC patients | Mucins are glycoproteins that are differentially overexpressed in PC but are not expressed in the normal pancreatic epithelium (except for MUC1 which is expressed at low level). These mucins (e.g., MUC1, MUC4, and MUC5AC) are involved in PC pathogenesis, provide chemoresistance and enhance proliferation and survival of PC cells. Their overexpression has been correlated with poor prognosis in patients.<br>[Ref. No. 54,64,69-70] |
| **Telomerase** | Absent | Expressed in 80-90% of PC patients | Telomerase is a ribonucleoprotein enzyme that catalyzes the synthesis of telomeric DNA. It is involved in the formation and protection of the telomere, which prevents cells from undergoing senescence. Telomerase activity has been detected in pancreatic juice samples of PC patients. hTERT expression and telomerase activity are predictors of poor outcome in pancreatic cancer.<br>[Ref. no. 55, 71-72] |
| **Carcinoembryonic antigen (CEA)** | Absent | Expressed in 77% of PC patients and detected in patient serum. | Carcinoembryonic antigen (CEA), a glycosylated protein of MW 180 kDa, is related to tumor burden of PC due to its close association with cancer |

| | | | |
|---|---|---|---|
| | | | cell adhesion, metabolism, and proliferation. In clinical practice, CEA is often used to predict the outcomes of patients with resectable PC.<br>[Ref. no. 60,73] |
| **Mutated K-ras (G12D)** | Absent | Expressed in 89.8-94.9% of PC patients | K-ras is mutated in PC cells and inactivation of the oncogenic mutant K-ras enhances MHC I presentation. K-RAS belongs to the superfamily of small G proteins and plays crucial roles in signal transduction in cells. K-RAS mutations in PC transform and alter the biological behavior in PC cells including metabolism reprogramming, thus playing a crucial role in PC pathogenesis.<br>[Ref. no. 77-80] |
| **Vascular endothelial growth factor (VEGF)** | Absent | Expressed in 77-93% of PC patients | VEGF, primarily VEGF-A and its receptors (VEGFR1 & VEGFR2), are primarily involved in the angiogenesis process in PC cancer. Increased vascularization of pancreatic tumors promotes their growth and metastasis by providing nutritional flow. Neovascularization also facilitates infiltration of pro-tumor immune cells (e.g., MDSCs).<br>[Ref. no. 82-83] |
| **Mesothelin** | Absent | Expressed in ~86% of PC patients | Mesothelin (MSLN) is a glycoprotein overexpressed in various epithelial cancers like mesothelioma and pancreatic, ovarian, and lung cancers. MSLN is synthesized as a 71 kDa precursor protein, which is processed to a 30 kDa megakaryocyte-potentiating factor and a 40 kDa MSLN |

| | | | |
|---|---|---|---|
| | | | protein. It is attached to the plasma membrane by a glycosylphosphatidylinositol anchor and is involved in cell adhesion. MSLN serves as a marker of neoplastic transformation of pancreatic epithelial cells.<br>[Ref. no. 86-87] |

**Table II: Clinical trials testing antibody-based immunotherapies for pancreatic cancer.**

| Antigen & Drug | Clinical Trials .gov Identifier | Phase | No. of Patients | Status | Median overall survival (months) | Median progression-free survival (months) | Objective response rate (%) |
|---|---|---|---|---|---|---|---|
| CD40 (CP-870,893) 0.2 mg/kg | NCT00711191 [Ref. No. 35] | I | 22 | Completed | 7.4 (5.5 to 12.8) | 5.6 (4.0 to 7.4) | 7.7 (0.2 to 36.0) |
| CD40 (CP-870,893) + Gemcitabine | NCT01456585 * | I | 10 | Completed | NR[b] | NR | NR |
| PD-1 (CT-011) alone or in combination with Gemcitabine | NCT01313416* | II | 29 | Suspended | NR | NR | NR |
| PD-L1 (pembrolizumab) | NCT02362048 * | II | 73 | Active | NR | NR | NR |
| PD-L1 (pembrolizumab) | NCT02009449 * | I | 350 | Active | NR | NR | NR |
| CTLA-4 (ipilimumab) | NCT00112580 | II | 27 | Completed | NR | NR | 1 patient had PR[c] |
| CTLA-4 (ipilimumab) + Pancreatic Cancer Vaccine | NCT00836407 * | I | 30 | Completed | 5.7 (4.3 to 14.7) | NR | NR |

*** Data obtained from https://clinicaltrials.gov/**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| HER1 (Cetuximab) + Irinotecan + Docetaxel | NCT00042939* | II | 87 | Completed | 5.3 (4.5 to 9.4) | 4.5 (2.7 to 5.6) | 0.07 (0.024 to 0.198) |
| HER1 (Cetuximab) + Gemcitabine + Radiotherapy | NCT00225784*8 | II | 37 | Completed | 17.3 (2 to N/A) | 9.1 (2 to N/A) | 10 out of 37 had PR |
| HER1 (Cetuximab) + Ixabepilone | NCT0038314* | II | 54 | Completed | 7.6 (5.5 to 12.2) | 3.9 (2.6 to 4.4) | 4 patients had PR |
| HER1 (Cetuximab) + Irinotecan + Oxaliplatin | NCT00871169* | II | 58 | Completed | NR | NR | 6.9 (1.91 to 16.7) |
| HER1 (Cetuximab) + Gemcitabine + VEGF (Bevacizumab) | NCT00326911* | II | 30 | Terminated | 5.41 (3.84 to 6.74) | 3.55 (2.00 to 5.59) | 4 patients had either PR or CR[d] |
| HER1 (Cetuximab) + Gemcitabine + VEGF (Bevacizumab) | NCT00091026* | II | 71 | Completed | 7.9 (5.5 to 9.5) | 5.0 (3.7 to 5.5) | 21 (12 to 32) |
| HER1 (Cetuximab) + Gemcitabine + Oxaliplatin | NCT00338039* | II | 69 | Completed | 19.2 (14.2 to 24.2) | NR | NR |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| HER1 (Cetuximab) + Gemcitabine + Capecitabine + Radiation | NCT00305877 * | II | 65 | Completed | 0.38 (0.26 to 0.50) | 0.17 (0.08 to 0.26) | 0.30 (0.19 to 0.42) |
| HER2 (Trastuzumab) + Interleukin 12 | NCT00004074 * | I | 15 | Completed | NR | NR | NR |
| HER2 (Trastuzumab) + HER1 (Cetuximab) | NCT00923299 [Ref. No. 51] | I & II | 44 | Completed | 4.6 (2.7-6.6) | 1.8 (1.7-2.0) | NR |
| Mesothelin (SS1(dsFv)-PE38 immunotoxin) | NCT00006981 * | I | NR | Completed | NR | NR | NR |
| VEGF (Bevacizumab) + Gemcitabine + accelerated Radiation Therapy | NCT00557492 * | II | 43 | Ongoing | 19.7 (16.5 to 28.2) | 12.9 (7.0 to 18.7) | 2.3 (0.1 to 12) |
| VEGF (Bevacizumab) + Octreotide Acetate + Everolimus | NCT01229943 * | II | 75 | Ongoing | 36.7 (31.8 to N/A) | 16.7 (12.6 to 19.7) | 31 |

*** Data obtained from https://clinicaltrials.gov/**

[b] NR (not reported).
[c] PR (partial response).
[d] CR (complete response).

**Table III: Clinical trials testing T cell-mediated immunotherapies for pancreatic cancer**.

| Antigen & Drug | ClinicalTrials.gov Identifier | Phase | No. of Patients | Status |
|---|---|---|---|---|
| Anti-CEA CAR-T cells | NCT02416466* | I | 8 | Ongoing |
| Autologous T cells transfected with chimeric anti-mesothelin immunoreceptor SS1 | NCT01897415* | I | 16 | Ongoing |
| CART-meso-19 T cells + Cyclophosphamide | NCT02465983* | I | 12 | Ongoing |
| GI-4000 Vaccine + Activated T Cells | NCT00837135* | I | NR[b] | Withdrawn |
| MFE23 scFv-expressing autologous anti-CEA MFEz T lymphocytes | NCT01212887* | I | 14 | Terminated due to safety concerns and lack of efficacy |
| Autologous Natural Killer / Natural Killer T Cell Immunotherapy | NCT00909558* | I | 24 | Suspended |
| Prostate Stem Cell Antigen (PSCA) Specific CAR T Cells (BPX-601) + Rimiducid | NCT02744287* | I | 30 | Recruiting |

*** Data obtained from https://clinicaltrials.gov/** [b] NR (not reported).

**Table IV: Clinical trials testing cancer vaccines for pancreatic cancer.**

| Antigen & Drug | ClinicalTrials.gov Identifier | Phase | No. of Patients | Status |
|---|---|---|---|---|
| MUC1 Vaccine (Cvac vaccine) | NCT02310971* | II | 0 | Withdrawn |
| Falimarev (MUC1 PANVAC-F vaccine) + Inalimarev (MUC1 PANVAC-V vaccine) + Sargramostim (GM-CSF vaccine) | NCT00669734* | I | 18 | Ongoing |
| Telomerase vaccine (GV1001) + gemcitabine + Sargramostim + tadalafil + Radiation Therapy | NCT01342224* | I | 11 | Active |
| Telomerase vaccine (GV1001) + Sargramostim + capecitabine + gemcitabine | NCT00425360* | III | 1110 | Completed |
| CEA vaccine (ALVAC + vaccinia) + aldesleukin (IL-2) + Sargramostim | NCT00003125* | II | 24 | Completed |
| CEA vaccine (AVX701) | NCT00529984* | I & II | 28 | Completed |
| Recombinant fowlpox-CEA(6D)/TRICOM vaccine + GM-CSF vaccine + Sargramostim | NCT00028496* | I | 48 | Completed |
| Recombinant fowlpox-CEA(6D)/TRICOM vacci | NCT00128622* | I | 24 | Completed |

| | | | | |
|---|---|---|---|---|
| ne + denileukin diftitox + therapeutic autologous dendritic cells | | | | |
| CEA vaccine (TRICOM-CEA(6D)) | NCT00027534* | I | 14 | Completed |
| CEA RNA-pulsed DC cancer vaccine | NCT00004604* | I | 24 | Completed |
| CEA vaccine (carcinoembryonic antigen peptide 1-6D) + incomplete Freund's adjuvant + sargramostim | NCT00012246* | II | 7 | Terminated |
| K-ras vaccine (TG01) + GM-CSF + Gemcitabine | NCT02261714* | I & II | 32 | Active |
| Aldesleukin + ras peptide cancer vaccine + sargramostim + DetoxPC | NCT00019331* | II | 11 | Completed |
| HLA-A*02:01-restricted VEGFR1-derived peptide vaccination | NCT00683085* | I & II | 2 | Terminated |
| VEGFR-2 DNA vaccine VXM01 | NCT01486329* | I | 72 | Completed |
| Mesothelin vaccine (CRS-207) + GVAX vaccine + gemcitabine + capecitabine + 5-FU + irinotecan or erlotinib + cyclophosphamide | NCT02004262 [Ref no. 87, 88] | II | 303 | Completed |
| GVAX Pancreas + Mesothelin vaccine | NCT01417000 | II | 93 | Ongoing |

| | | | | |
|---|---|---|---|---|
| (CRS-207) + Cyclophosphamide | [Ref. no. 88] | | | |
| Mesothelin vaccine (CRS-207) | NCT00585845* | I | 17 | Terminated |
| Cancer stem cell vaccine | NCT02074046* | I & II | 40 | Completed |

*** Data obtained from https://clinicaltrials.gov/**

**Figure 1: Pancreatic cancer cells establish an immunosuppressive TME**.

Cancer cells secrete various anti-inflammatory cytokines like IL-10, TGF-β, IL-23, along with angiogenic chemokines (e.g., CXCL1-3, CXCL5, CXCL12, CCL2, and VEGF-A), which generate an immunosuppressive tumor microenvironment (TME) and facilitate cancer initiation, progression and metastasis. Upregulation of the expression of these cytokines shifts the balance in TME, which facilitates the evasion from immune surveillance during PC progression [6, 8, 20, 28]. The PC immunosuppressive microenvironment also includes crosstalk between cancer cells and various myeloid and lymphoid subsets. Tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) express immuno-inhibitory ligands and reactive oxygen species that inhibit infiltration and activation of T and NK cells [3, 11-13]. MDSCs and cancer cells also secrete VEGFs that promote angiogenesis, which aids in the metastasis of the cancer cells [17]. PC tumor cells and pancreatic stellate cells (desmoplasia) secrete inhibitory cytokines and chemokines, and express inhibitory surface ligands such as programmed death ligand-1 (PD-L1) and galectin-1 (Gal-1) that lead to inactivation and apoptosis of cytotoxic ($CD8^+$) and helper ($CD4^+$) T-cells by programmed death receptor-1(PD-1) or Gal-1 binding receptor respectively [16, 18, 21, 22]. $T_{reg}$ cells suppress the functions of activated T-cells and NK cells in the TME [13, 125]. In addition, the rigid architecture of pancreatic tumor bed provides a physical barrier to T-cells infiltration thereby excluding them to the edge/boundary of the tumor and thus rendering the pancreatic tumor as an immunologically ‘cold’ tumor [28-31]. All these cells are involved in the maintenance of the immunosuppressive TME, and cancer progression.

# Figure 1

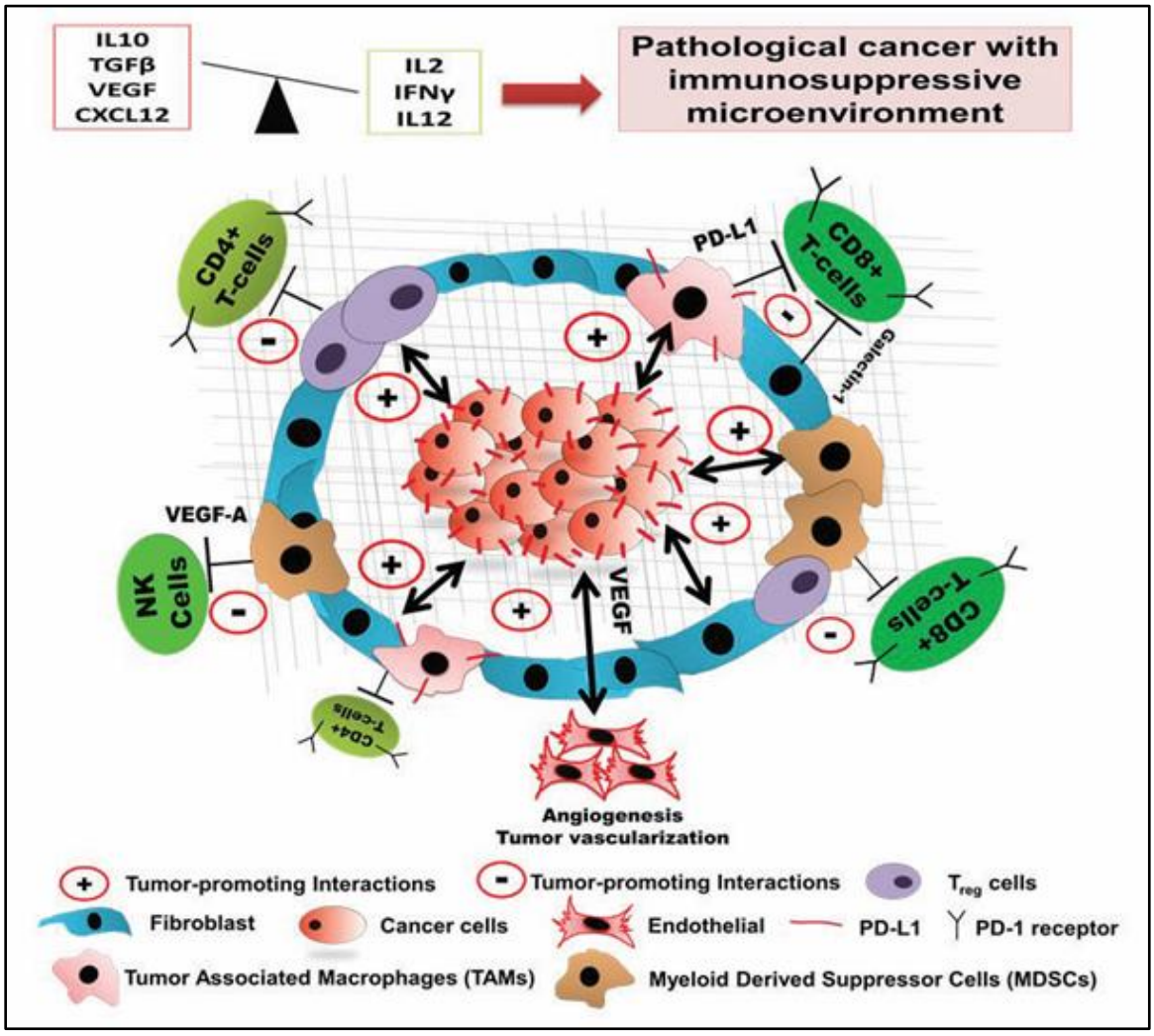
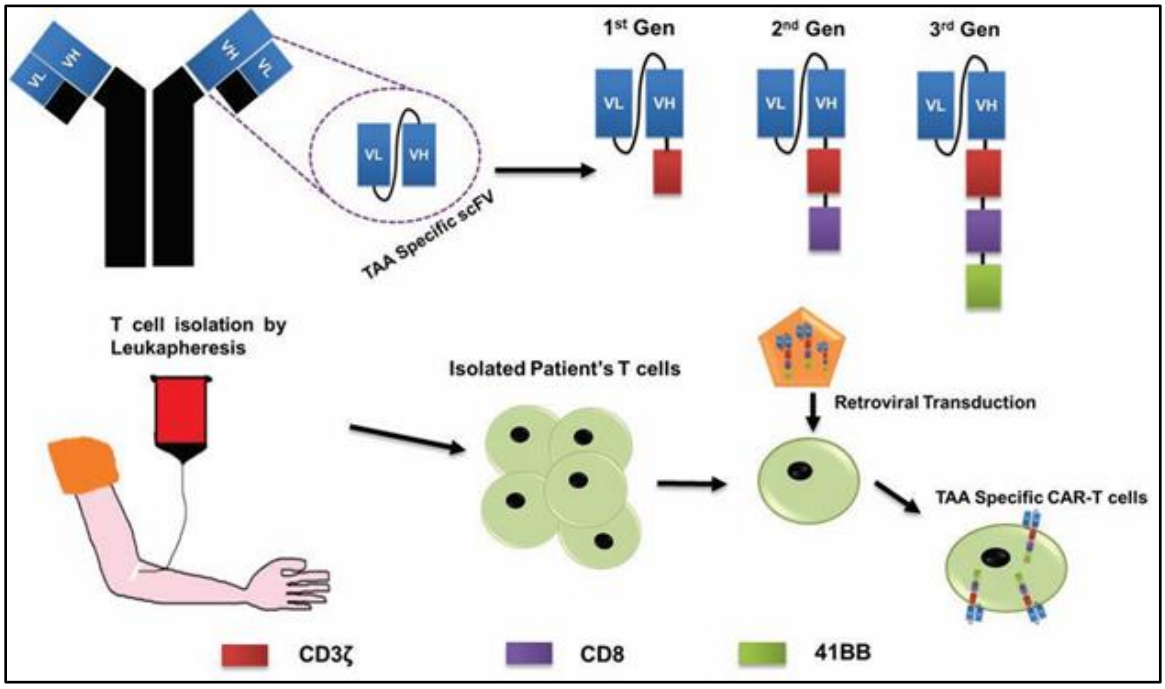

IL10
TGFβ
VEGF
CXCL12
IL2
IFNγ
IL12
Pathological cancer with immunosuppressive microenvironment
CD4+ T-cells
PD-L1
CD8+ T-cells
Galectin-1
VEGF-A
NK Cells
VEGF
CD8+ T-cells
CD4+ T-cells
Angiogenesis
Tumor vascularization
Tumor-promoting Interactions
Tumor-promoting Interactions
Treg cells
Fibroblast
Cancer cells
Endothelial
PD-L1
PD-1 receptor
Tumor Associated Macrophages (TAMs)
Myeloid Derived Suppressor Cells (MDSCs)

**Figure 2: Schematic representation of engineering of CAR-T cells.**

CAR T-cells are genetically engineered T-cells expressing tumor antigen-specific chimeric TCR [149, 150]. The modified receptor is a chimera of a signaling domain of the TCR complex and an antigen-recognizing domain, such as a single chain fragment (scFv) of an antibody [151, 152]. CAR T-cells are not dependent on antigen presentation by MHC molecules expressed on APCs for antigen-specific activation. Adoptive cell transfer of CAR T-cells involves the isolation, stimulation, expansion, transduction, and ultimately re-infusion of human T lymphocytes [153, 154]. First-generation TCRs included only the intracellular domain of the CD3ζ chain but did not show any significant *in vivo* efficacy in transgenic mouse model studies [155]. Second-generation CARs introduced additional co-stimulatory domains such as CD28, which significantly augmented CAR signaling, and improved cytokine production and T-cell proliferation, as well as differentiation, and survival [156, 157]. Third-generation CARs contain multiple co-stimulatory domains such as 4-1BB (CD137), and whether they have a clinical benefit over second-generation CAR T-cells is still under investigation [152, 158, 159].

# Figure 2


1st Gen
2nd Gen
3rd Gen
VL
VH
TAA Specific scFV
T cell isolation by Leukapheresis
Isolated Patient's T cells
Retroviral Transduction
TAA Specific CAR-T cells
CD3ζ
CD8
41BB

# Reference List


[1] L. Rahib, B.D. Smith, R. Aizenberg, A.B. Rosenzweig, J.M. Fleshman, and L.M. Matrisian, Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res. 74 (2014) 2913-2921.

[2] Pancreatic Cancer Survival Rates, by Stage. 5-31-2016.

[3] K. Sideras, H. Braat, J. Kwekkeboom, C.H. van Eijck, M.P. Peppelenbosch, S. Sleijfer, and M. Bruno, Role of the immune system in pancreatic cancer progression and immune modulating treatment strategies. Cancer Treat. Rev. 40 (2014) 513-522.

[4] D. McDermott, C. Lebbe, F.S. Hodi, M. Maio, J.S. Weber, J.D. Wolchok, J.A. Thompson, and C.M. Balch, Durable benefit and the potential for long-term survival with immunotherapy in advanced melanoma. Cancer Treat. Rev. 40 (2014) 1056-1064.

[5] H. Gogas, A. Polyzos, and J. Kirkwood, Immunotherapy for advanced melanoma: fulfilling the promise. Cancer Treat. Rev. 39 (2013) 879-885.

[6] D. Mittal, M.M. Gubin, R.D. Schreiber, and M.J. Smyth, New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. Curr. Opin. Immunol. 27 (2014) 16-25.

[7] A. Bhatia and Y. Kumar, Cellular and molecular mechanisms in cancer immune escape: a comprehensive review. Expert. Rev. Clin. Immunol. 10 (2014) 41-62.

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

[9] Z. Dambrauskas, H. Svensson, M. Joshi, A. Hyltander, P. Naredi, and B.M. Iresjo, Expression of major histocompatibility complex class I-related chain A/B (MICA/B) in pancreatic carcinoma. Int. J. Oncol. 44 (2014) 99-104.

[10] X. Duan, L. Deng, X. Chen, Y. Lu, Q. Zhang, K. Zhang, Y. Hu, J. Zeng, and W. Sun, Clinical significance of the immunostimulatory MHC class I chain-related molecule A and NKG2D receptor on NK cells in pancreatic cancer. Med. Oncol. 28 (2011) 466-474.

[11] H. Kurahara, H. Shinchi, Y. Mataki, K. Maemura, H. Noma, F. Kubo, M. Sakoda, S. Ueno, S. Natsugoe, and S. Takao, Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. J. Surg. Res. 167 (2011) e211-e219.

[12] S. Kusmartsev, Y. Nefedova, D. Yoder, and D.I. Gabrilovich, Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. J. Immunol. 172 (2004) 989-999.

[13] C.E. Clark, G.L. Beatty, and R.H. Vonderheide, Immunosurveillance of pancreatic adenocarcinoma: insights from genetically engineered mouse models of cancer. Cancer Lett. 279 (2009) 1-7.

[14] I.M. Stromnes, J.S. Brockenbrough, K. Izeradjene, M.A. Carlson, C. Cuevas, R.M. Simmons, P.D. Greenberg, and S.R. Hingorani, Targeted depletion of an MDSC subset unmasks pancreatic ductal adenocarcinoma to adaptive immunity. Gut 63 (2014) 1769-1781.

[15] P. Fogar, D. Basso, E. Fadi, E. Greco, G. Pantano, A. Padoan, D. Bozzato, M. Facco, M.C. Sanzari, S. Teolato, C.F. Zambon, F. Navaglia, G. Semenzato, S. Pedrazzoli, and M. Plebani, Pancreatic cancer alters human CD4+ T lymphocyte function: a piece in the immune evasion puzzle. Pancreas 40 (2011) 1131-1137.

[16] D. Tang, J. Gao, S. Wang, Z. Yuan, N. Ye, Y. Chong, C. Xu, X. Jiang, B. Li, W. Yin, Y. Miao, D. Wang, and K. Jiang, Apoptosis and anergy of T cell induced by pancreatic stellate cells-derived galectin-1 in pancreatic cancer. Tumour. Biol 36 (2015) 5617-5626.

[17] R.F. Gabitass, N.E. Annels, D.D. Stocken, H.A. Pandha, and G.W. Middleton, Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13. Cancer Immunol. Immunother. 60 (2011) 1419-1430.

[18] K. Okudaira, R. Hokari, Y. Tsuzuki, Y. Okada, S. Komoto, C. Watanabe, C. Kurihara, A. Kawaguchi, S. Nagao, M. Azuma, H. Yagita, and S. Miura, Blockade of B7-H1 or B7-DC induces an anti-tumor effect in a mouse pancreatic cancer model. Int. J. Oncol. 35 (2009) 741-749.

[19] L. Geng, D. Huang, J. Liu, Y. Qian, J. Deng, D. Li, Z. Hu, J. Zhang, G. Jiang, and S. Zheng, B7-H1 up-regulated expression in human pancreatic carcinoma tissue associates with tumor progression. J. Cancer Res. Clin. Oncol. 134 (2008) 1021-1027.

[20] B. Burkholder, R.Y. Huang, R. Burgess, S. Luo, V.S. Jones, W. Zhang, Z.Q. Lv, C.Y. Gao, B.L. Wang, Y.M. Zhang, and R.P. Huang, Tumor-induced perturbations of cytokines and immune cell networks. Biochim. Biophys. Acta 1845 (2014) 182-201.

[21] K.C. Soares, A.A. Rucki, A.A. Wu, K. Olino, Q. Xiao, Y. Chai, A. Wamwea, E. Bigelow, E. Lutz, L. Liu, S. Yao, R.A. Anders, D. Laheru, C.L. Wolfgang, B.H. Edil, R.D. Schulick, E.M. Jaffee, and L. Zheng, PD-1/PD-L1 blockade together with vaccine therapy facilitates effector T-cell infiltration into pancreatic tumors. J. Immunother. 38 (2015) 1-11.

[22] W. Peng, H.Y. Wang, Y. Miyahara, G. Peng, and R.F. Wang, Tumor-associated galectin-3 modulates the function of tumor-reactive T cells. Cancer Res. 68 (2008) 7228-7236.

[23] D.R. Sen, J. Kaminski, R.A. Barnitz, M. Kurachi, U. Gerdemann, K.B. Yates, H.W. Tsao, J. Godec, M.W. LaFleur, F.D. Brown, P. Tonnerre, R.T. Chung, D.C. Tully, T.M. Allen, N. Frahm, G.M. Lauer, E.J. Wherry, N. Yosef, and W.N. Haining, The epigenetic landscape of T cell exhaustion. Science 354 (2016) 1165-1169.

[24] B. Burkholder, R.Y. Huang, R. Burgess, S. Luo, V.S. Jones, W. Zhang, Z.Q. Lv, C.Y. Gao, B.L. Wang, Y.M. Zhang, and R.P. Huang, Tumor-induced perturbations of cytokines and immune cell networks. Biochim. Biophys. Acta 1845 (2014) 182-201.

[25] N. McGranahan, A.J. Furness, R. Rosenthal, S. Ramskov, R. Lyngaa, S.K. Saini, M. Jamal-Hanjani, G.A. Wilson, N.J. Birkbak, C.T. Hiley, T.B. Watkins, S. Shafi, N. Murugaesu, R. Mitter, A.U. Akarca, J. Linares, T. Marafioti, J.Y. Henry, E.M. Van Allen, D. Miao, B. Schilling, D. Schadendorf, L.A. Garraway, V. Makarov, N.A. Rizvi, A. Snyder, M.D. Hellmann, T. Merghoub, J.D. Wolchok, S.A. Shukla, C.J. Wu, K.S. Peggs, T.A. Chan, S.R. Hadrup, S.A. Quezada, and C. Swanton, Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. Science 351 (2016) 1463-1469.

[26] M.S. Lawrence, P. Stojanov, P. Polak, G.V. Kryukov, K. Cibulskis, A. Sivachenko, S.L. Carter, C. Stewart, C.H. Mermel, S.A. Roberts, A. Kiezun, P.S. Hammerman, A. McKenna, Y. Drier, L. Zou, A.H. Ramos, T.J. Pugh, N. Stransky, E. Helman, J. Kim, C. Sougnez, L. Ambrogio, E. Nickerson, E. Shefler, M.L. Cortes, D. Auclair, G. Saksena, D. Voet, M. Noble, D. DiCara, P. Lin, L. Lichtenstein, D.I. Heiman, T. Fennell, M. Imielinski, B. Hernandez, E. Hodis, S. Baca, A.M. Dulak, J. Lohr, D.A. Landau, C.J. Wu, J. Melendez-Zajgla, A. Hidalgo-Miranda, A. Koren, S.A. McCarroll, J. Mora, R.S. Lee, B. Crompton, R. Onofrio, M. Parkin, W. Winckler, K. Ardlie, S.B. Gabriel, C.W. Roberts, J.A. Biegel, K. Stegmaier, A.J. Bass, L.A. Garraway, M. Meyerson, T.R. Golub, D.A. Gordenin, S. Sunyaev, E.S. Lander, and G. Getz, Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature 499 (2013) 214-218.

[27] M.S. Lawrence, P. Stojanov, P. Polak, G.V. Kryukov, K. Cibulskis, A. Sivachenko, S.L. Carter, C. Stewart, C.H. Mermel, S.A. Roberts, A. Kiezun, P.S. Hammerman, A. McKenna, Y. Drier, L. Zou, A.H. Ramos, T.J. Pugh, N. Stransky, E. Helman, J. Kim, C. Sougnez, L. Ambrogio, E. Nickerson, E. Shefler, M.L. Cortes, D. Auclair, G. Saksena, D. Voet, M. Noble, D. DiCara, P. Lin, L. Lichtenstein, D.I. Heiman, T. Fennell, M. Imielinski, B. Hernandez, E. Hodis, S. Baca, A.M. Dulak, J. Lohr, D.A. Landau, C.J. Wu, J. Melendez-Zajgla, A. Hidalgo-Miranda, A. Koren, S.A. McCarroll, J. Mora, R.S. Lee, B. Crompton, R. Onofrio, M. Parkin, W. Winckler, K. Ardlie, S.B. Gabriel, C.W. Roberts, J.A. Biegel, K. Stegmaier, A.J. Bass, L.A. Garraway, M. Meyerson, T.R. Golub, D.A. Gordenin, S. Sunyaev, E.S. Lander, and G. Getz, Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature 499 (2013) 214-218.

[28] J.A. Wargo, S.M. Reddy, A. Reuben, and P. Sharma, Monitoring immune responses in the tumor microenvironment. Curr. Opin. Immunol. 41 (2016) 23-31.

[29] R.S. Herbst, J.C. Soria, M. Kowanetz, G.D. Fine, O. Hamid, M.S. Gordon, J.A. Sosman, D.F. McDermott, J.D. Powderly, S.N. Gettinger, H.E. Kohrt, L. Horn, D.P. Lawrence, S. Rost, M. Leabman, Y. Xiao, A. Mokatrin, H. Koeppen, P.S. Hegde, I. Mellman, D.S. Chen, and F.S. Hodi, Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 515 (2014) 563-567.

[30] H. Salmon, K. Franciszkiewicz, D. Damotte, M.C. Dieu-Nosjean, P. Validire, A. Trautmann, F. Mami-Chouaib, and E. Donnadieu, Matrix architecture defines the preferential localization and migration of T cells into the stroma of human lung tumors. J. Clin. Invest 122 (2012) 899-910.

[31] J. Watt and H.M. Kocher, The desmoplastic stroma of pancreatic cancer is a barrier to immune cell infiltration. Oncoimmunology. 2 (2013) e26788.

[32] A. Ene-Obong, A.J. Clear, J. Watt, J. Wang, R. Fatah, J.C. Riches, J.F. Marshall, J. Chin-Aleong, C. Chelala, J.G. Gribben, A.G. Ramsay, and H.M. Kocher, Activated pancreatic stellate cells sequester CD8+ T cells to reduce their infiltration of the juxtatumoral compartment of pancreatic ductal adenocarcinoma. Gastroenterology 145 (2013) 1121-1132.

[33] C. Feig, J.O. Jones, M. Kraman, R.J. Wells, A. Deonarine, D.S. Chan, C.M. Connell, E.W. Roberts, Q. Zhao, O.L. Caballero, S.A. Teichmann, T. Janowitz, D.I. Jodrell, D.A. Tuveson, and D.T. Fearon, Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. Proc. Natl. Acad. Sci. U. S. A 110 (2013) 20212-20217.

[34] H. Jiang, S. Hegde, B.L. Knolhoff, Y. Zhu, J.M. Herndon, M.A. Meyer, T.M. Nywening, W.G. Hawkins, I.M. Shapiro, D.T. Weaver, J.A. Pachter, A. Wang-Gillam, and D.G. DeNardo, Targeting focal adhesion kinase renders pancreatic cancers responsive to checkpoint immunotherapy. Nat. Med. 22 (2016) 851-860.

[35] D. Tang, J. Gao, S. Wang, Z. Yuan, N. Ye, Y. Chong, C. Xu, X. Jiang, B. Li, W. Yin, Y. Miao, D. Wang, and K. Jiang, Apoptosis and anergy of T cell induced by pancreatic stellate cells-derived galectin-1 in pancreatic cancer. Tumour. Biol 36 (2015) 5617-5626.

[36] D. Tang, Z. Yuan, X. Xue, Z. Lu, Y. Zhang, H. Wang, M. Chen, Y. An, J. Wei, Y. Zhu, Y. Miao, and K. Jiang, High expression of Galectin-1 in pancreatic stellate cells plays a role in the development and maintenance of an immunosuppressive microenvironment in pancreatic cancer. Int J Cancer 130 (2012) 2337-2348.

[37] A. Grigorian, S. Torossian, and M. Demetriou, T-cell growth, cell surface organization, and the galectin-glycoprotein lattice. Immunol. Rev. 230 (2009) 232-246.

[38] W. Peng, H.Y. Wang, Y. Miyahara, G. Peng, and R.F. Wang, Tumor-associated galectin-3 modulates the function of tumor-reactive T cells. Cancer Res. 68 (2008) 7228-7236.

[39] T. Kouo, L. Huang, A.B. Pucsek, M. Cao, S. Solt, T. Armstrong, and E. Jaffee, Galectin-3 Shapes Antitumor Immune Responses by Suppressing CD8+ T Cells

via LAG-3 and Inhibiting Expansion of Plasmacytoid Dendritic Cells. Cancer Immunol. Res. 3 (2015) 412-423.

[40] J.G. Quatromoni and E. Eruslanov, Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. Am. J. Transl. Res. 4 (2012) 376-389.

[41] R.F. Gabitass, N.E. Annels, D.D. Stocken, H.A. Pandha, and G.W. Middleton, Elevated myeloid-derived suppressor cells in pancreatic, esophageal and gastric cancer are an independent prognostic factor and are associated with significant elevation of the Th2 cytokine interleukin-13. Cancer Immunol. Immunother. 60 (2011) 1419-1430.

[42] H. Kurahara, H. Shinchi, Y. Mataki, K. Maemura, H. Noma, F. Kubo, M. Sakoda, S. Ueno, S. Natsugoe, and S. Takao, Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. J. Surg. Res. 167 (2011) e211-e219.

[43] S. Kusmartsev, Y. Nefedova, D. Yoder, and D.I. Gabrilovich, Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. J. Immunol. 172 (2004) 989-999.

[44] L. Geng, D. Huang, J. Liu, Y. Qian, J. Deng, D. Li, Z. Hu, J. Zhang, G. Jiang, and S. Zheng, B7-H1 up-regulated expression in human pancreatic carcinoma tissue associates with tumor progression. J. Cancer Res. Clin. Oncol. 134 (2008) 1021-1027.

[45] J. Watt and H.M. Kocher, The desmoplastic stroma of pancreatic cancer is a barrier to immune cell infiltration. Oncoimmunology. 2 (2013) e26788.

[46] K.S. Inman, A.A. Francis, and N.R. Murray, Complex role for the immune system in initiation and progression of pancreatic cancer. World J Gastroenterol. 20 (2014) 11160-11181.

[47] M. Jinushi, S. Chiba, H. Yoshiyama, K. Masutomi, I. Kinoshita, H. Dosaka-Akita, H. Yagita, A. Takaoka, and H. Tahara, Tumor-associated macrophages regulate tumorigenicity and anticancer drug responses of cancer stem/initiating cells. Proc. Natl. Acad. Sci. U. S. A 108 (2011) 12425-12430.

[48] D. Baxter, Active and passive immunization for cancer. Hum. Vaccin. Immunother. 10 (2014) 2123-2129.

[49] S. Battaglia and J.B. Muhitch, Unmasking targets of antitumor immunity via high-throughput antigen profiling. Curr. Opin. Biotechnol. 42 (2016) 92-97.

[50] R. Elgueta, M.J. Benson, V.C. de Vries, A. Wasiuk, Y. Guo, and R.J. Noelle, Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunol. Rev. 229 (2009) 152-172.

[51] K.T. Byrne and R.H. Vonderheide, CD40 Stimulation Obviates Innate Sensors and Drives T Cell Immunity in Cancer. Cell Rep. 15 (2016) 2719-2732.

[52] R.H. Vonderheide and M.J. Glennie, Agonistic CD40 antibodies and cancer therapy. Clin. Cancer Res. 19 (2013) 1035-1043.

[53] K.T. Byrne and R.H. Vonderheide, CD40 therapy and surgery: a potential immunologic partnership. J. Immunother. 36 (2013) 359-361.

[54] R.H. Vonderheide, D.L. Bajor, R. Winograd, R.A. Evans, L.J. Bayne, and G.L. Beatty, CD40 immunotherapy for pancreatic cancer. Cancer Immunol. Immunother. 62 (2013) 949-954.

[55] G.L. Beatty, E.G. Chiorean, M.P. Fishman, B. Saboury, U.R. Teitelbaum, W. Sun, R.D. Huhn, W. Song, D. Li, L.L. Sharp, D.A. Torigian, P.J. O'Dwyer, and R.H. Vonderheide, CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. Science 331 (2011) 1612-1616.

[56] A. Mohammed, N.B. Janakiram, S. Pant, and C.V. Rao, Molecular Targeted Intervention for Pancreatic Cancer. Cancers. (Basel) 7 (2015) 1499-1542.

[57] L.V. Riella, A.M. Paterson, A.H. Sharpe, and A. Chandraker, Role of the PD-1 pathway in the immune response. Am. J Transplant. 12 (2012) 2575-2587.

[58] A. Diana, L.M. Wang, Z. D'Costa, P. Allen, A. Azad, M.A. Silva, Z. Soonawalla, S. Liu, W.G. McKenna, R.J. Muschel, and E. Fokas, Prognostic value, localization and correlation of PD-1/PD-L1, CD8 and FOXP3 with the desmoplastic stroma in pancreatic ductal adenocarcinoma. Oncotarget. 7 (2016) 40992-41004.

[59] S.T. Haile, J.J. Bosch, N.I. Agu, A.M. Zeender, P. Somasundaram, M.K. Srivastava, S. Britting, J.B. Wolf, B.R. Ksander, and S. Ostrand-Rosenberg, Tumor cell programmed death ligand 1-mediated T cell suppression is overcome by coexpression of CD80. J Immunol. 186 (2011) 6822-6829.

[60] S.J. Lee, B.C. Jang, S.W. Lee, Y.I. Yang, S.I. Suh, Y.M. Park, S. Oh, J.G. Shin, S. Yao, L. Chen, and I.H. Choi, Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-gamma-induced upregulation of B7-H1 (CD274). FEBS Lett. 580 (2006) 755-762.

[61] J. Duraiswamy, K.M. Kaluza, G.J. Freeman, and G. Coukos, Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. Cancer Res. 73 (2013) 3591-3603.

[62] T. Nomi, M. Sho, T. Akahori, K. Hamada, A. Kubo, H. Kanehiro, S. Nakamura, K. Enomoto, H. Yagita, M. Azuma, and Y. Nakajima, Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. Clin. Cancer Res. 13 (2007) 2151-2157.

[63] K.T. Byrne, R.H. Vonderheide, E.M. Jaffee, and T.D. Armstrong, Special Conference on Tumor Immunology and Immunotherapy: A New Chapter. Cancer Immunol. Res.2015).

[64] J.R. Brahmer, S.S. Tykodi, L.Q. Chow, W.J. Hwu, S.L. Topalian, P. Hwu, C.G. Drake, L.H. Camacho, J. Kauh, K. Odunsi, H.C. Pitot, O. Hamid, S. Bhatia, R.

Martins, K. Eaton, S. Chen, T.M. Salay, S. Alaparthy, J.F. Grosso, A.J. Korman, S.M. Parker, S. Agrawal, S.M. Goldberg, D.M. Pardoll, A. Gupta, and J.M. Wigginton, Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N. Engl. J. Med. 366 (2012) 2455-2465.

[65] G. Goel and W. Sun, Cancer immunotherapy in clinical practice -- the past, present, and future. Chin J. Cancer 33 (2014) 445-457.

[66] H. Yano, A. Thakur, E.N. Tomaszewski, M. Choi, A. Deol, and L.G. Lum, Ipilimumab augments antitumor activity of bispecific antibody-armed T cells. J. Transl. Med. 12 (2014) 191.

[67] R.E. Royal, C. Levy, K. Turner, A. Mathur, M. Hughes, U.S. Kammula, R.M. Sherry, S.L. Topalian, J.C. Yang, I. Lowy, and S.A. Rosenberg, Phase 2 trial of single agent Ipilimumab (anti-CTLA-4) for locally advanced or metastatic pancreatic adenocarcinoma. J. Immunother. 33 (2010) 828-833.

[68] D.T. Le, E. Lutz, J.N. Uram, E.A. Sugar, B. Onners, S. Solt, L. Zheng, L.A. Diaz, Jr., R.C. Donehower, E.M. Jaffee, and D.A. Laheru, Evaluation of ipilimumab in combination with allogeneic pancreatic tumor cells transfected with a GM-CSF gene in previously treated pancreatic cancer. J. Immunother. 36 (2013) 382-389.

[69] R. Winograd, K.T. Byrne, R.A. Evans, P.M. Odorizzi, A.R. Meyer, D.L. Bajor, C. Clendenin, B.Z. Stanger, E.E. Furth, E.J. Wherry, and R.H. Vonderheide, Induction of T-cell Immunity Overcomes Complete Resistance to PD-1 and CTLA-4 Blockade and Improves Survival in Pancreatic Carcinoma. Cancer Immunol. Res. 3 (2015) 399-411.

[70] R. Nahta, Molecular Mechanisms of Trastuzumab-Based Treatment in HER2-Overexpressing Breast Cancer. ISRN. Oncol 2012 (2012) 428062.

[71] C. Larbouret, N. Gaborit, T. Chardes, M. Coelho, E. Campigna, C. Bascoul-Mollevi, J.P. Mach, D. Azria, B. Robert, and A. Pelegrin, In pancreatic carcinoma, dual EGFR/HER2 targeting with cetuximab/trastuzumab is more effective than treatment with trastuzumab/erlotinib or lapatinib alone: implication of receptors' down-regulation and dimers' disruption. Neoplasia. 14 (2012) 121-130.

[72] G. Thomas, T. Chardes, N. Gaborit, C. Mollevi, W. Leconet, B. Robert, N. Radosevic-Robin, F. Penault-Llorca, C. Gongora, P.E. Colombo, Y. Lazrek, R. Bras-Goncalves, A. Savina, D. Azria, H. Bazin, A. Pelegrin, and C. Larbouret, HER3 as biomarker and therapeutic target in pancreatic cancer: new insights in pertuzumab therapy in preclinical models. Oncotarget. 5 (2014) 7138-7148.

[73] L.A. Chantrill, A.M. Nagrial, C. Watson, A.L. Johns, M. Martyn-Smith, S. Simpson, S. Mead, M.D. Jones, J.S. Samra, A.J. Gill, N. Watson, V.T. Chin, J.L. Humphris, A. Chou, B. Brown, A. Morey, M. Pajic, S.M. Grimmond, D.K. Chang, D. Thomas, L. Sebastian, K. Sjoquist, S. Yip, N. Pavlakis, R. Asghari, S. Harvey, P. Grimison, J. Simes, and A.V. Biankin, Precision Medicine for Advanced Pancreas Cancer: The Individualized Molecular Pancreatic Cancer Therapy (IMPaCT) Trial. Clin. Cancer Res. 21 (2015) 2029-2037.

[74] E. Assenat, D. Azria, C. Mollevi, R. Guimbaud, N. Tubiana-Mathieu, D. Smith, J.P. Delord, E. Samalin, F. Portales, C. Larbouret, B. Robert, F. Bibeau, J.P. Bleuse, E. Crapez, M. Ychou, and A. Pelegrin, Dual targeting of HER1/EGFR and HER2 with cetuximab and trastuzumab in patients with metastatic pancreatic cancer after gemcitabine failure: results of the "THERAPY"phase 1-2 trial. Oncotarget. 6 (2015) 12796-12808.

[75] S.J. Scales, N. Gupta, G. Pacheco, R. Firestein, D.M. French, H. Koeppen, L. Rangell, V. Barry-Hamilton, E. Luis, J. Chuh, Y. Zhang, G.S. Ingle, A. Fourie-O'Donohue, K.R. Kozak, S. Ross, M.S. Dennis, and S.D. Spencer, An anti-mesothelin-monomethyl auristatin E conjugate with potent anti-tumor activity in ovarian, pancreatic and mesothelioma models. Mol. Cancer Ther.2014).

[76] K. Sahora, M. Schindl, I. Kuehrer, A. Eisenhut, G. Werba, C. Brostjan, B. Telek, A. Ba'ssalamah, J. Stift, S.F. Schoppmann, and M. Gnant, A phase II trial of two durations of Bevacizumab added to neoadjuvant gemcitabine for borderline and locally advanced pancreatic cancer. Anticancer Res. 34 (2014) 2377-2384.

[77] E. Hasnis, D. Alishekevitz, S. Gingis-Veltski, R. Bril, E. Fremder, T. Voloshin, Z. Raviv, A. Karban, and Y. Shaked, Anti-Bv8 antibody and metronomic gemcitabine improve pancreatic adenocarcinoma treatment outcome following weekly gemcitabine therapy. Neoplasia. 16 (2014) 501-510.

[78] T. Kawaoka, M. Oka, M. Takashima, T. Ueno, K. Yamamoto, N. Yahara, S. Yoshino, and S. Hazama, Adoptive immunotherapy for pancreatic cancer: cytotoxic T lymphocytes stimulated by the MUC1-expressing human pancreatic cancer cell line YPK-1. Oncol. Rep. 20 (2008) 155-163.

[79] H. Hassanin, S. Serba, J. Schmidt, and A. Marten, Ex vivo expanded telomerase-specific T cells are effective in an orthotopic mouse model for pancreatic adenocarcinoma. Clin. Exp. Immunol. 158 (2009) 125-132.

[80] T. Ishikawa, S. Kokura, N. Sakamoto, T. Okayama, M. Endo, R. Tsuchiya, M. Okajima, T. Matsuyama, S. Adachi, K. Kamada, K. Katada, K. Uchiyama, O. Handa, T. Takagi, N. Yagi, T. Ando, K. Uno, Y. Naito, and T. Yoshikawa, Whole blood interferon-gamma levels predict the therapeutic effects of adoptive T-cell therapy in patients with advanced pancreatic cancer. Int. J. Cancer 133 (2013) 1119-1125.

[81] A.D. Posey, Jr., R.D. Schwab, A.C. Boesteanu, C. Steentoft, U. Mandel, B. Engels, J.D. Stone, T.D. Madsen, K. Schreiber, K.M. Haines, A.P. Cogdill, T.J. Chen, D. Song, J. Scholler, D.M. Kranz, M.D. Feldman, R. Young, B. Keith, H. Schreiber, H. Clausen, L.A. Johnson, and C.H. June, Engineered CAR T Cells Targeting the Cancer-Associated Tn-Glycoform of the Membrane Mucin MUC1 Control Adenocarcinoma. Immunity. 44 (2016) 1444-1454.

[82] A.D. Posey, Jr., H. Clausen, and C.H. June, Distinguishing Truncated and Normal MUC1 Glycoform Targeting from Tn-MUC1-Specific CAR T Cells: Specificity Is the Key to Safety. Immunity. 45 (2016) 947-948.

[83] M. Chmielewski, O. Hahn, G. Rappl, M. Nowak, I.H. Schmidt-Wolf, A.A. Hombach, and H. Abken, T cells that target carcinoembryonic antigen eradicate orthotopic pancreatic carcinomas without inducing autoimmune colitis in mice. Gastroenterology 143 (2012) 1095-1107.

[84] M. Chmielewski, G. Rappl, A.A. Hombach, and H. Abken, T cells redirected by a CD3zeta chimeric antigen receptor can establish self-antigen-specific tumour protection in the long term. Gene Ther. 20 (2013) 177-186.

[85] U.L. Katari, J.M. Keirnan, A.C. Worth, S.E. Hodges, A.M. Leen, W.E. Fisher, and J.F. Vera, Engineered T cells for pancreatic cancer treatment. HPB (Oxford) 13 (2011) 643-650.

[86] D. Abate-Daga, K.H. Lagisetty, E. Tran, Z. Zheng, L. Gattinoni, Z. Yu, W.R. Burns, A.M. Miermont, Y. Teper, U. Rudloff, N.P. Restifo, S.A. Feldman, S.A. Rosenberg, and R.A. Morgan, A Novel Chimeric Antigen Receptor Against Prostate Stem Cell Antigen Mediates Tumor Destruction in a Humanized Mouse Model of Pancreatic Cancer. Hum. Gene Ther.2014).

[87] I.M. Stromnes, T.M. Schmitt, A. Hulbert, J.S. Brockenbrough, H.N. Nguyen, C. Cuevas, A.M. Dotson, X. Tan, J.L. Hotes, P.D. Greenberg, and S.R. Hingorani, T Cells Engineered against a Native Antigen Can Surmount Immunologic and Physical Barriers to Treat Pancreatic Ductal Adenocarcinoma. Cancer Cell 28 (2015) 638-652.

[88] M.P. Torres, S. Chakraborty, J. Souchek, and S.K. Batra, Mucin-based targeted pancreatic cancer therapy. Curr. Pharm. Des 18 (2012) 2472-2481.

[89] M.L. VanLith, K.G. Kohlgraf, C.L. Sivinski, R.M. Tempero, and M.A. Hollingsworth, MUC1-specific anti-tumor responses: molecular requirements for CD4-mediated responses. Int. Immunol. 14 (2002) 873-882.

[90] C.L. Sivinski, K.G. Kohlgraf, M.L. VanLith, K. Morikane, R.M. Tempero, and M.A. Hollingsworth, Molecular requirements for CD8-mediated rejection of a MUC1-expressing pancreatic carcinoma: implications for tumor vaccines. Cancer Immunol. Immunother. 51 (2002) 327-340.

[91] Y. Rong, X. Qin, D. Jin, W. Lou, L. Wu, D. Wang, W. Wu, X. Ni, Z. Mao, T. Kuang, Y.Q. Zang, and X. Qin, A phase I pilot trial of MUC1-peptide-pulsed dendritic cells in the treatment of advanced pancreatic cancer. Clin. Exp. Med. 12 (2012) 173-180.

[92] H. Kondo, S. Hazama, T. Kawaoka, S. Yoshino, S. Yoshida, K. Tokuno, M. Takashima, T. Ueno, Y. Hinoda, and M. Oka, Adoptive immunotherapy for pancreatic cancer using MUC1 peptide-pulsed dendritic cells and activated T lymphocytes. Anticancer Res. 28 (2008) 379-387.

[93] J. Chen, X.Z. Guo, H.Y. Li, X. Liu, L.N. Ren, D. Wang, and J.J. Zhao, Generation of CTL responses against pancreatic cancer in vitro using dendritic cells co-transfected with MUC4 and survivin RNA. Vaccine 31 (2013) 4585-4590.

[94] Y. Zhu, J.J. Zhang, W.B. Liang, R. Zhu, B. Wang, Y. Miao, and Z.K. Xu, Pancreatic cancer counterattack: MUC4 mediates Fas-independent apoptosis of antigen-specific cytotoxic T lymphocyte. Oncol Rep. 31 (2014) 1768-1776.

[95] C. Staff, F. Mozaffari, J.E. Frodin, H. Mellstedt, and M. Liljefors, Telomerase (GV1001) vaccination together with gemcitabine in advanced pancreatic cancer patients. Int. J. Oncol. 45 (2014) 1293-1303.

[96] G. Middleton, P. Silcocks, T. Cox, J. Valle, J. Wadsley, D. Propper, F. Coxon, P. Ross, S. Madhusudan, T. Roques, D. Cunningham, S. Falk, N. Wadd, M. Harrison, P. Corrie, T. Iveson, A. Robinson, K. McAdam, M. Eatock, J. Evans, C. Archer, T. Hickish, A. Garcia-Alonso, M. Nicolson, W. Steward, A. Anthoney, W. Greenhalf, V. Shaw, E. Costello, D. Naisbitt, C. Rawcliffe, G. Nanson, and J. Neoptolemos, Gemcitabine and capecitabine with or without telomerase peptide vaccine GV1001 in patients with locally advanced or metastatic pancreatic cancer (TeloVac): an open-label, randomised, phase 3 trial. Lancet Oncol. 15 (2014) 829-840.

[97] D.M. Geynisman, Y. Zha, R. Kunnavakkam, M. Aklilu, D.V. Catenacci, B.N. Polite, C. Rosenbaum, A. Namakydoust, T. Karrison, T.F. Gajewski, and H.L. Kindler, A randomized pilot phase I study of modified carcinoembryonic antigen (CEA) peptide (CAP1-6D)/montanide/GM-CSF-vaccine in patients with pancreatic adenocarcinoma. J. Immunother. Cancer 1 (2013) 8.

[98] S. Asahara, K. Takeda, K. Yamao, H. Maguchi, and H. Yamaue, Phase I/II clinical trial using HLA-A24-restricted peptide vaccine derived from KIF20A for patients with advanced pancreatic cancer. J. Transl. Med. 11 (2013) 291.

[99] N. Suzuki, S. Hazama, T. Ueno, H. Matsui, Y. Shindo, M. Iida, K. Yoshimura, S. Yoshino, K. Takeda, and M. Oka, A phase I clinical trial of vaccination with KIF20A-derived peptide in combination with gemcitabine for patients with advanced pancreatic cancer. J. Immunother. 37 (2014) 36-42.

[100] J.J. El-Jawhari, Y.M. El-Sherbiny, G.B. Scott, R.S. Morgan, R. Prestwich, P.A. Bowles, G.E. Blair, T. Tanaka, T.H. Rabbitts, J.L. Meade, and G.P. Cook, Blocking oncogenic RAS enhances tumour cell surface MHC class I expression but does not alter susceptibility to cytotoxic lymphocytes. Mol. Immunol. 58 (2014) 160-168.

[101] O.E. Rahma, J.M. Hamilton, M. Wojtowicz, O. Dakheel, S. Bernstein, D.J. Liewehr, S.M. Steinberg, and S.N. Khleif, The immunological and clinical effects of mutated ras peptide vaccine in combination with IL-2, GM-CSF, or both in patients with solid tumors. J. Transl. Med. 12 (2014) 55.

[102] G. Tan, Z. Wang, X. Zhang, Z. Cai, and J. Zhang, Induction of CTLs by DCs pulsed with K-ras mutant peptide on the surface of nanoparticles in the treatment of pancreatic cancer. Oncol. Rep. 26 (2011) 215-221.

[103] Y. Oji, S. Nakamori, M. Fujikawa, S. Nakatsuka, A. Yokota, N. Tatsumi, S. Abeno, A. Ikeba, S. Takashima, M. Tsujie, H. Yamamoto, M. Sakon, R. Nezu, K. Kawano, S. Nishida, K. Ikegame, M. Kawakami, A. Tsuboi, Y. Oka, K. Yoshikawa, K. Aozasa, M. Monden, and H. Sugiyama, Overexpression of the Wilms' tumor gene WT1 in pancreatic ductal adenocarcinoma. Cancer Sci. 95 (2004) 583-587.

[104] S. Nishida, S. Koido, Y. Takeda, S. Homma, H. Komita, A. Takahara, S. Morita, T. Ito, S. Morimoto, K. Hara, A. Tsuboi, Y. Oka, S. Yanagisawa, Y. Toyama, M. Ikegami, T. Kitagawa, H. Eguchi, H. Wada, H. Nagano, J. Nakata, Y. Nakae, N. Hosen, Y. Oji, T. Tanaka, I. Kawase, A. Kumanogoh, J. Sakamoto, Y. Doki, M. Mori, T. Ohkusa, H. Tajiri, and H. Sugiyama, Wilms tumor gene (WT1) peptide-based cancer vaccine combined with gemcitabine for patients with advanced pancreatic cancer. J. Immunother. 37 (2014) 105-114.

[105] M. Kaida, Y. Morita-Hoshi, A. Soeda, T. Wakeda, Y. Yamaki, Y. Kojima, H. Ueno, S. Kondo, C. Morizane, M. Ikeda, T. Okusaka, Y. Takaue, and Y. Heike, Phase 1 trial of Wilms tumor 1 (WT1) peptide vaccine and gemcitabine combination therapy in patients with advanced pancreatic or biliary tract cancer. J Immunother. 34 (2011) 92-99.

[106] R. Okuyama, A. Aruga, T. Hatori, K. Takeda, and M. Yamamoto, Immunological responses to a multi-peptide vaccine targeting cancer-testis antigens and VEGFRs in advanced pancreatic cancer patients. Oncoimmunology. 2 (2013) e27010.

[107] T. Terashima, E. Mizukoshi, K. Arai, T. Yamashita, M. Yoshida, H. Ota, I. Onishi, M. Kayahara, K. Ohtsubo, T. Kagaya, M. Honda, and S. Kaneko, P53, hTERT, WT-1, and VEGFR2 are the most suitable targets for cancer vaccine therapy in HLA-A24 positive pancreatic adenocarcinoma. Cancer Immunol. Immunother. 63 (2014) 479-489.

[108] N.J. Chu, T.D. Armstrong, and E.M. Jaffee, Nonviral oncogenic antigens and the inflammatory signals driving early cancer development as targets for cancer immunoprevention. Clin. Cancer Res. 21 (2015) 1549-1557.

[109] G.M. Springett, Novel pancreatic cancer vaccines could unleash the army within. Cancer Control 21 (2014) 242-246.

[110] D.T. Le, D.G. Brockstedt, R. Nir-Paz, J. Hampl, S. Mathur, J. Nemunaitis, D.H. Sterman, R. Hassan, E. Lutz, B. Moyer, M. Giedlin, J.L. Louis, E.A. Sugar, A. Pons, A.L. Cox, J. Levine, A.L. Murphy, P. Illei, T.W. Dubensky, Jr., J.E. Eiden, E.M. Jaffee, and D.A. Laheru, A live-attenuated Listeria vaccine (ANZ-100) and a live-attenuated Listeria vaccine expressing mesothelin (CRS-207) for advanced cancers: phase I studies of safety and immune induction. Clin Cancer Res. 18 (2012) 858-868.

[111] D.T. Le, A. Wang-Gillam, V. Picozzi, T.F. Greten, T. Crocenzi, G. Springett, M. Morse, H. Zeh, D. Cohen, R.L. Fine, B. Onners, J.N. Uram, D.A. Laheru, E.R. Lutz, S. Solt, A.L. Murphy, J. Skoble, E. Lemmens, J. Grous, T. Dubensky, Jr., D.G. Brockstedt, and E.M. Jaffee, Safety and survival with GVAX pancreas prime and Listeria Monocytogenes-expressing mesothelin (CRS-207) boost vaccines for metastatic pancreatic cancer. J. Clin. Oncol. 33 (2015) 1325-1333.

[112] B.P. Keenan, Y. Saenger, M.I. Kafrouni, A. Leubner, P. Lauer, A. Maitra, A.A. Rucki, A.J. Gunderson, L.M. Coussens, D.G. Brockstedt, T.W. Dubensky, Jr., R. Hassan, T.D. Armstrong, and E.M. Jaffee, A Listeria vaccine and depletion of T-regulatory cells activate immunity against early stage pancreatic intraepithelial neoplasms and prolong survival of mice. Gastroenterology 146 (2014) 1784-1794.

[113] T. Li, H. Cheng, H. Yuan, Q. Xu, C. Shu, Y. Zhang, P. Xu, J. Tan, Y. Rui, P. Li, and X. Tan, Antitumor Activity of cGAMP via Stimulation of cGAS-cGAMP-STING-IRF3 Mediated Innate Immune Response. Sci. Rep. 6 (2016) 19049.

[114] L. Corrales and T.F. Gajewski, Molecular Pathways: Targeting the Stimulator of Interferon Genes (STING) in the Immunotherapy of Cancer. Clin. Cancer Res. 21 (2015) 4774-4779.

[115] L. Corrales, L.H. Glickman, S.M. McWhirter, D.B. Kanne, K.E. Sivick, G.E. Katibah, S.R. Woo, E. Lemmens, T. Banda, J.J. Leong, K. Metchette, T.W. Dubensky, Jr., and T.F. Gajewski, Direct Activation of STING in the Tumor Microenvironment Leads to Potent and Systemic Tumor Regression and Immunity. Cell Rep. 11 (2015) 1018-1030.

[116] J.R. Baird, D. Friedman, B. Cottam, T.W. Dubensky, Jr., D.B. Kanne, S. Bambina, K. Bahjat, M.R. Crittenden, and M.J. Gough, Radiotherapy Combined with Novel STING-Targeting Oligonucleotides Results in Regression of Established Tumors. Cancer Res. 76 (2016) 50-61.

[117] O. Demaria, G.A. De, S. Coso, N. Gestermann, D.J. Di, L. Flatz, O. Gaide, O. Michielin, P. Hwu, T.V. Petrova, F. Martinon, R.L. Modlin, D.E. Speiser, and M. Gilliet, STING activation of tumor endothelial cells initiates spontaneous and therapeutic antitumor immunity. Proc. Natl. Acad. Sci. U. S. A 112 (2015) 15408-15413.

[118] J.P. Finnigan, Jr., A. Rubinsteyn, J. Hammerbacher, and N. Bhardwaj, Mutation-Derived Tumor Antigens: Novel Targets in Cancer Immunotherapy. Oncology (Williston. Park) 29 (2015) 970-975.

[119] P. Bailey, D.K. Chang, M.A. Forget, F.A. Lucas, H.A. Alvarez, C. Haymaker, C. Chattopadhyay, S.H. Kim, S. Ekmekcioglu, E.A. Grimm, A.V. Biankin, P. Hwu, A. Maitra, and J. Roszik, Exploiting the neoantigen landscape for immunotherapy of pancreatic ductal adenocarcinoma. Sci. Rep. 6 (2016) 35848.

[120] T.N. Schumacher and R.D. Schreiber, Neoantigens in cancer immunotherapy. Science 348 (2015) 69-74.

[121] N. Vigneron, Human Tumor Antigens and Cancer Immunotherapy. Biomed. Res. Int. 2015 (2015) 948501.

[122] J.Y. Zhang, K.S. Looi, and E.M. Tan, Identification of tumor-associated antigens as diagnostic and predictive biomarkers in cancer. Methods Mol. Biol. 520 (2009) 1-10.

[123] E.M. Tan, Autoantibodies as reporters identifying aberrant cellular mechanisms in tumorigenesis. J. Clin. Invest 108 (2001) 1411-1415.

[124] C. Bauer, B. Kuhnemuth, P. Duewell, S. Ormanns, T. Gress, and M. Schnurr, Prevailing over T cell exhaustion: New developments in the immunotherapy of pancreatic cancer. Cancer Lett.2016).

[125] N. Hiraoka, K. Onozato, T. Kosuge, and S. Hirohashi, Prevalence of FOXP3+ regulatory T cells increases during the progression of pancreatic ductal adenocarcinoma and its premalignant lesions. Clin. Cancer Res. 12 (2006) 5423-5434.

[126] A. Ager, H.A. Watson, S.C. Wehenkel, and R.N. Mohammed, Homing to solid cancers: a vascular checkpoint in adoptive cell therapy using CAR T-cells. Biochem. Soc. Trans. 44 (2016) 377-385.

[127] V. Alonso-Camino, S.L. Harwood, A. Alvarez-Mendez, and L. Alvarez-Vallina, Efficacy and toxicity management of CAR-T-cell immunotherapy: a matter of responsiveness control or tumour-specificity? Biochem. Soc. Trans. 44 (2016) 406-411.

[128] S.L. Maude, D. Barrett, D.T. Teachey, and S.A. Grupp, Managing cytokine release syndrome associated with novel T cell-engaging therapies. Cancer J. 20 (2014) 119-122.

[129] X.J. Xu and Y.M. Tang, Cytokine release syndrome in cancer immunotherapy with chimeric antigen receptor engineered T cells. Cancer Lett. 343 (2014) 172-178.

[130] D.T. Teachey, S.R. Rheingold, S.L. Maude, G. Zugmaier, D.M. Barrett, A.E. Seif, K.E. Nichols, E.K. Suppa, M. Kalos, R.A. Berg, J.C. Fitzgerald, R. Aplenc, L. Gore, and S.A. Grupp, Cytokine release syndrome after blinatumomab treatment related to abnormal macrophage activation and ameliorated with cytokine-directed therapy. Blood 121 (2013) 5154-5157.

[131] C. Ogi and A. Aruga, Approaches to improve development methods for therapeutic cancer vaccines. Immunol. Lett. 164 (2015) 100-108.

[132] O.C. Farokhzad and R. Langer, Impact of nanotechnology on drug delivery. ACS Nano. 3 (2009) 16-20.

[133] A. Makkouk, V.B. Joshi, A. Wongrakpanich, C.D. Lemke, B.P. Gross, A.K. Salem, and G.J. Weiner, Biodegradable microparticles loaded with doxorubicin and CpG ODN for in situ immunization against cancer. AAPS. J. 17 (2015) 184-193.

[134] K. Cho, X. Wang, S. Nie, Z.G. Chen, and D.M. Shin, Therapeutic nanoparticles for drug delivery in cancer. Clin Cancer Res. 14 (2008) 1310-1316.

[135] S. Gao, F. Dagnaes-Hansen, E.J. Nielsen, J. Wengel, F. Besenbacher, K.A. Howard, and J. Kjems, The effect of chemical modification and nanoparticle formulation on stability and biodistribution of siRNA in mice. Mol. Ther. 17 (2009) 1225-1233.

[136] M.P. Torres, A.S. Determan, G.L. Anderson, S.K. Mallapragada, and B. Narasimhan, Amphiphilic polyanhydrides for protein stabilization and release. Biomaterials 28 (2007) 108-116.

[137] T. Musumeci, C.A. Ventura, I. Giannone, B. Ruozi, L. Montenegro, R. Pignatello, and G. Puglisi, PLA/PLGA nanoparticles for sustained release of docetaxel. Int J Pharm. 325 (2006) 172-179.

[138] M.J. Kipper, J.H. Wilson, M.J. Wannemuehler, and B. Narasimhan, Single dose vaccine based on biodegradable polyanhydride microspheres can modulate immune response mechanism. J. Biomed. Mater. Res. A 76 (2006) 798-810.

[139] A.V. Chavez-Santoscoy, R. Roychoudhury, N.L. Pohl, M.J. Wannemuehler, B. Narasimhan, and A.E. Ramer-Tait, Tailoring the immune response by targeting C-type lectin receptors on alveolar macrophages using "pathogen-like" amphiphilic polyanhydride nanoparticles. Biomaterials 33 (2012) 4762-4772.

[140] L.K. Petersen, A.E. Ramer-Tait, S.R. Broderick, C.S. Kong, B.D. Ulery, K. Rajan, M.J. Wannemuehler, and B. Narasimhan, Activation of innate immune responses in a pathogen-mimicking manner by amphiphilic polyanhydride nanoparticle adjuvants. Biomaterials 32 (2011) 6815-6822.

[141] M.P. Torres, J.H. Wilson-Welder, S.K. Lopac, Y. Phanse, B. Carrillo-Conde, A.E. Ramer-Tait, B.H. Bellaire, M.J. Wannemuehler, and B. Narasimhan, Polyanhydride microparticles enhance dendritic cell antigen presentation and activation. Acta Biomater. 7 (2011) 2857-2864.

[142] S.L. Haughney, K.A. Ross, P.M. Boggiatto, M.J. Wannemuehler, and B. Narasimhan, Effect of nanovaccine chemistry on humoral immune response kinetics and maturation. Nanoscale. 6 (2014) 13770-13778.

[143] L. Huntimer, J.H. Wilson Welder, K. Ross, B. Carrillo-Conde, L. Pruisner, C. Wang, B. Narasimhan, M.J. Wannemuehler, and A.E. Ramer-Tait, Single immunization with a suboptimal antigen dose encapsulated into polyanhydride microparticles promotes high titer and avid antibody responses. J. Biomed. Mater. Res. B Appl. Biomater. 101 (2013) 91-98.

[144] Lucas M.Huntimer. Polyanhydride nanovaccine platform enhances antigen-specific cytotoxic T cell responses. Kathleen A.Ross, Ross J.Darling, Nicole E.Winterwood, Paola Boggiatto, Balaji Narasimhan, Amanda E.Ramer-Tait, and Michael J.Wannemuehler. TECHNOLOGY 02(02), 171-175. 5-30-2014.

[145] M. Wang and M. Thanou, Targeting nanoparticles to cancer. Pharmacol. Res. 62 (2010) 90-99.

[146] D. Pozzi, V. Colapicchioni, G. Caracciolo, S. Piovesana, A.L. Capriotti, S. Palchetti, G.S. De, A. Riccioli, H. Amenitsch, and A. Lagana, Effect of polyethyleneglycol (PEG) chain length on the bio-nano-interactions between PEGylated lipid nanoparticles and biological fluids: from nanostructure to uptake in cancer cells. Nanoscale. 6 (2014) 2782-2792.

[147] R.I. El-Gogary, N. Rubio, J.T. Wang, W.T. Al-Jamal, M. Bourgognon, H. Kafa, M. Naeem, R. Klippstein, V. Abbate, F. Leroux, S. Bals, T.G. Van, A.O. Kamel, G.A. Awad, N.D. Mortada, and K.T. Al-Jamal, Polyethylene glycol conjugated polymeric

nanocapsules for targeted delivery of quercetin to folate-expressing cancer cells in vitro and in vivo. ACS Nano. 8 (2014) 1384-1401.

[148] Olivier Gires and Barbara Seliger. Tumor-associated Antigens (TAAs): Subclasses of TAAs. Tumor-Associated Antigens , 3-22. 2009. WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

[149] H. Shi, M. Sun, L. Liu, and Z. Wang, Chimeric antigen receptor for adoptive immunotherapy of cancer: latest research and future prospects. Mol. Cancer 13 (2014) 219.

[150] D.M. Barrett, N. Singh, D.L. Porter, S.A. Grupp, and C.H. June, Chimeric antigen receptor therapy for cancer. Annu. Rev. Med. 65 (2014) 333-347.

[151] H. Singh, J.S. Moyes, M.H. Huls, and L.J. Cooper, Manufacture of T cells using the Sleeping Beauty system to enforce expression of a CD19-specific chimeric antigen receptor. Cancer Gene Ther. 22 (2015) 95-100.

[152] J. Wang, M. Jensen, Y. Lin, X. Sui, E. Chen, C.G. Lindgren, B. Till, A. Raubitschek, S.J. Forman, X. Qian, S. James, P. Greenberg, S. Riddell, and O.W. Press, Optimizing adoptive polyclonal T cell immunotherapy of lymphomas, using a chimeric T cell receptor possessing CD28 and CD137 costimulatory domains. Hum. Gene Ther. 18 (2007) 712-725.

[153] G. Dotti, S. Gottschalk, B. Savoldo, and M.K. Brenner, Design and development of therapies using chimeric antigen receptor-expressing T cells. Immunol. Rev. 257 (2014) 107-126.

[154] S.S. Kenderian, M. Ruella, S. Gill, and M. Kalos, Chimeric antigen receptor T-cell therapy to target hematologic malignancies. Cancer Res. 74 (2014) 6383-6389.

[155] T. Brocker, Chimeric Fv-zeta or Fv-epsilon receptors are not sufficient to induce activation or cytokine production in peripheral T cells. Blood 96 (2000) 1999-2001.

[156] A. Loskog, V. Giandomenico, C. Rossig, M. Pule, G. Dotti, and M.K. Brenner, Addition of the CD28 signaling domain to chimeric T-cell receptors enhances chimeric T-cell resistance to T regulatory cells. Leukemia 20 (2006) 1819-1828.

[157] J. Maher, R.J. Brentjens, G. Gunset, I. Riviere, and M. Sadelain, Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta /CD28 receptor. Nat. Biotechnol. 20 (2002) 70-75.

[158] M.C. Milone, J.D. Fish, C. Carpenito, R.G. Carroll, G.K. Binder, D. Teachey, M. Samanta, M. Lakhal, B. Gloss, G. Danet-Desnoyers, D. Campana, J.L. Riley, S.A. Grupp, and C.H. June, Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy in vivo. Mol. Ther. 17 (2009) 1453-1464.

[159] C. Carpenito, M.C. Milone, R. Hassan, J.C. Simonet, M. Lakhal, M.M. Suhoski, A. Varela-Rohena, K.M. Haines, D.F. Heitjan, S.M. Albelda, R.G. Carroll, J.L. Riley,

I. Pastan, and C.H. June, Control of large, established tumor xenografts with genetically retargeted human T cells containing CD28 and CD137 domains. Proc. Natl. Acad. Sci. U. S. A 106 (2009) 3360-3365.

# CHAPTER 1B: MUCINS-BASED VACCINES AND MUC4 IN PANCREATIC CANCER

# 1. Synopsis

Mucins are high-molecular-weight, heavily O-glycosylated glycoproteins that are differentially overexpressed in pancreatic cancer. Literature surveys has shown the instrumental role mucins play in the pathobiology of PC disease. Overexpression, aberrant glycosylation and pathological role proposes mucins as lucrative targets for developing targeted therapies. Immunotherapy is one of the strategies that specifically target tumor-associated antigens and simultaneously reprograms the patient's immune system towards the targeted killing of tumor cells. MUC1 has been one of the well-explored mucins for developing PC immunotherapies but achieved limited success in PC patients. MUC4 is one of the differentially overexpressed mucins in PC and unlike MUC1, MUC4 has unique tumor specificity since it is undetectable in normal pancreas. MUC4 plays a crucial role in PC disease progression and metastasis. Further MUC4's expression has been demonstrated to be regulated by cytokines such as IFNγ and tumor growth factor beta (TGFβ), suggesting a possible interrelationship between immune tumor microenvironment and PC disease aggressiveness. Previous studies have provided evidence that makes a strong case to evaluate MUC4 as an immunotherapeutic candidate, but to date only limited studies have been made. Therefore, in this section we have reviewed existing literature and summarized their findings to make a case for investigating MUC4 as a potential candidate for PC immunotherapy.

## 2. Mucins: A Target for Pancreatic Cancer Immunotherapy

PC cells undergo a cellular and genetic transformation that contributes to the expression of proteins that are mostly absent in *de novo* pancreas cells. One of the families of proteins that is aberrantly overexpressed in PC tumor cells and has been reported to play a crucial role in the pathogenesis of the disease is mucins. MUCs are high molecular weight glycoproteins with O-glycosylated variable number tandem repeat (VNTR) region and consists of 21 family members. Fourteen MUC proteins out of 21 mucin family members fall under the category of classical mucins, which is characterized by high molecular weight O-glycoprotein, secreted in the mucus layer, presence of VNTR sequence, predicted peptide domain containing high percentage of serine and threonine residues, and lastly a complex mRNA expression of these mucins [1]. MUC glycoproteins are classified according to their structure and function into two categories: transmembrane/membrane-bound mucins comprising of MUC1, MUC3A, MUC3B, MUC4, MUC12 and MUC17; and secretory/gel-forming mucins consisting of MUC6, MUC2, MUC5AC and MUC5B [2].

Transmembrane mucins like MUC1, MUC4 and MUC16 are differentially overexpressed by PC cancer cells and their expression gradually increases with the progression of the disease [3-9]. MUC1 and MUC4 interact with various receptors such as receptor tyrosine kinases (RTKs) including epidermal growth factor receptor (EGFR), ERBB2 & ERBB3, and fibroblast growth factor receptor (FGFR), that promotes proliferation, invasion, metastasis and resistance to therapeutic anti-RTK (Receptor Tyrosine Kinase) antibodies [10-14]. MUCs expressed on the PC cancer cell surface due to their large structure interact with the TME that induce immune evasion and oncogenic signaling-mediated angiogenesis and metastasis [2]. Studies have demonstrated that MUCs like MUC1 on PC cells interact with TAMs through sialoadhesin [15]. Interaction

between M2 macrophages with PC cells via CA125 (carbohydrate epitope located on MUC16) differentiates these macrophages to immunosuppressive phenotype and induces secretion of IL10 cytokine [16]. In addition to MUC's crucial role in pathogenesis and immunosuppression of PC, they undergo aberrant glycosylation due to the altered expression and localization of glycosyltransferases [17]. Thus, these aspects make MUCs an important therapeutic target for PC immunotherapy strategies.

## 2.1 Mucin-based Cancer Vaccines for PC

Due to their contributions in PC pathogenesis, MUCs have been targeted by immunotherapy strategies like cancer vaccines to efficaciously treat PC patients. We have previously discussed MUC vaccines studied and tested in both pre-clinical and clinical trials for PC immunotherapy in our previous chapter [18]. In addition, a recent pre-clinical study with MUC1-based DNA vaccine (pVAX1-MUC1-$VNTR_n$ DNA vaccine) showed that MUC1-$VNTR_6$- and MUC1-$VNTR_9$- transfected DCs were able to activate IFN-γ producing CTLs, increase the cytotoxicity of CTLs and suppress Panc02-MUC1 PC tumor cell's growth in tumor-bearing mice [19]. Mice immunized with MUC1-tandem repeat B-cell peptide conjugated with Ttox (MUC1-Ttox vaccine) produced high titers of anti MUC1 IgG antibodies. These IgG MUC1 antibodies could specifically differentiate between human normal and PC tumor cells [20]. A clinical trial (NCT03114631) with DCs pulsed with MUC1/WT-1 peptides is currently under investigation for treating both resectable and unresectable PC patients.

## 2.2 Limitations of Mucin Vaccines for PC Immunotherapy

Efficacious MUC cancer vaccines have been a challenge to develop because tumor antigens like MUC1 are also expressed by normal cells (self-antigens) causing the immune system to develop self-tolerance against them, which leads to

hyporesponsiveness of APCs upon exposure to MUC1 cancer vaccines. Efficient activation of DCs is instrumental to activate cellular and humoral responses in an antigen-specific phenotype. In a very detailed study done in MUC1.Tg mice, it was observed that MUC1p cancer vaccine couldn't successfully activate APCs post vaccination in MUC1 expressing MUC1.Tg mice and was unable to restrict the growth of both transplanted and spontaneous PC tumors [21]. Whereas in WT mice, MUC1p cancer vaccination activated DCs and induced significant expression of surface markers like MHC-II, CD80, CD86 and CD40. Upon further investigation they discovered that MUC1.Tg immunized mice had a higher percentage of $Fox3^{+}$ $T_{regs}$ cells and these $T_{regs}$ inhibited the expression of pancreatic enzymes such as trypsin and CBP1 transcript in DCs recovered from spleens of MUC1.Tg mice. Specific depletion of $T_{regs}$ by anti-CD25 treatment or blocking of IL-10 by anti-IL10R antibody prior to MUC1p cancer vaccination rescued the expression of these enzymes in DCs to the similar levels of DCs isolated from WT mice [21]. This study suggests the necessity of discovering TAAs that are distinct from their counterparts being expressed on normal cells to prevent the formation of self-tolerance against them. A strategy to recognize MUCs against which self-tolerance is likely compromised in naïve PC patients who haven't received any treatments or surgery, could enable identifying the immunogenic MUC candidate that will probably overcome the hyporesponsiveness of APCs and generate corresponding anti-tumor responses.

Another limitation of MUC vaccines is the number of available epitopes to activate APCs. Peptides from VNTR region of MUC proteins have been utilized to develop cancer vaccines like peptide-toxin vaccines [20] or DC vaccines [19]. But these vaccines have achieved only limited success in clinical trials. Peptide-based vaccines have certain limitations such as peptides are mostly designed to have an MHC-I restriction that leads to the presentation of individual peptides on only certain HLA types. In addition, if selected

peptides have low affinity for MHC, they may be poorly immunogenic and induce weak or transient immune responses by APCs upon exposure [22]. As previously discussed in our review [18], protein provides an array of epitopes having multiple immunogenicities and thus could be presented by different MHC-I HLA types, potentially leading to the generation of strong immune responses. Expression, isolation and purification of transmembrane MUC glycoproteins come with its challenges. Due to the high molecular weight of MUCs, their purification and maintenance of their native antigenicity have not been feasible to date.

## 3. MUC4 as Tumor-Associated Antigen for PC

### 3.1 MUC4 in Pancreatic Cancer

In contrast to MUC1, MUC4 expression in normal organs is mostly restricted and is undetectable in normal pancreas and inflammatory diseases of the pancreas [23, 24] thus providing a better tumor specificity for targeting purposes. MUC4 is one of the most differentially overexpressed multi-domain transmembrane glycoprotein in PC [2, 7, 23, 25]. MUC4 protein carries a high percentage of allelic polymorphism in the VNTR domain that is rich in serine, threonine, and proline residues and it extensively undergoes mucin-type O-linked glycosylation [2, 23, 26] thus contributing to the high molecular weight of the apoprotein. MUC4 has a putative cleavage site comprising of Glycine-Aspartate-Proline-Histidine (GDPH) that can undergo autocatalysis and generate two subunits: MUC4α upstream of GDPH cleavage site and MUC4β downstream of the site [26-28]. MUC4α subunit is the large N-terminal domain that is composed of VNTR domain, nidogen-like domain (NIDO), and adhesion-associated domain in MUC4 and other proteins (AMOP). MUC4β on the other hand is a smaller subunit composed of von Willebrand factor type D

domain (vWD), three epidermal growth factor (EGF)-like domains, a transmembrane domain and a short cytoplasmic tail [23].

MUC4 has been demonstrated to play a fundamental role in the pathobiology of PC. Over the years studies have shown that MUC4's interaction with EGFR family members, HER2 and HER3 is mediated by the 3 EGF-like domains present in the MUC4β subunit [29-31]. These interactions subsequently activate an intracellular cascade of signaling events including mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinases (JNK), and signal transducer and activator of transcription 1 (STAT-1) that promote cell proliferation, migration and metastasis [12, 29-32]. The NIDO domain on MUC4 interacts with fibulin-2 that competitively disrupts its interaction with extracellular matrix (ECM) proteins present in the basement membrane, thus abrogating normal ECM protein-protein interactions [33]. An overall survey of the literature shows that MUC4 can modulate diverse pathways such as drug resistance [34], epithelial to mesenchymal transition (EMT) and metastasis [35], tumor cell proliferation and invasion [12], and thus PC patients with a MUC4 expression on tumors have a worse prognosis [36]. Given the tumor-promoting nature and PC-specific expression of MUC4, various studies have focused on utilizing MUC4 as a diagnostic/prognostic marker. MUC4 has emerged as a useful diagnostic tool for PC in fine needle aspirates (FNAs) [37, 38]. Also studies with patient serum samples for MUC4 serving as biomarkers have been investigated [39, 40]. In summary, MUC4 has been undisputedly established as a PC tumor-specific molecule that plays an instrumental role in progression and metastasis of PC disease, as well as serves as a useful diagnostic marker.

### 3.2 Immune Regulation of MUC4 in Pancreatic Cancer

Multiple signaling pathways regulate MUC4 expression in PC through the binding of different transcription factors at the MUC4 promoter site [41, 42]. Previous studies have

shown cytokine-mediated expression of MUC4 in PC cells. IFNγ and retinoic acid (RA) synergistically upregulates the expression of MUC4 in PC cells through the dual activation of STAT-1 and transforming growth factor beta-2 (TGFβ) pathways [43, 44]. TGFβ can upregulate MUC4 expression either by cooperative activation of Smad3 and Smad4 signaling pathways, or by activating MAPK, phosphatidylinositide 3-kinases (PI3K) and protein kinase A (PKA) signaling pathways [45]. Further, in other cancers such as gastric and colon cancer cell lines, it has been observed that interleukin (IL)-4, IL9, IL6, & IL24 could upregulate MUC4 expression by activating JAK/STAT pathway [46, 47] or in a STAT-3 dependent manner [48, 49]. PC has an elaborate TME composed of multiple immune cells such as TAMs, MDSCs, M2 macrophages etc. that secrete Th2 cytokines such as IL4, IL9 and IL24 (an IL-10 cytokine family member) to establish and maintain immunosuppression [18]. In addition, aberrant glycosylation of MUC4 appears to induce MUC4-specific immune responses such as generation of both Th1 and Th2 cytokines in MUC4-expressing pancreatic tumors [44]. Cumulatively these studies demonstrate that immune cells in TME can regulate the expression of MUC4 on PC cells by secretion of cytokines, which reveals an unexplored relationship between MUC4 and PC immunosuppression.

## 4. Summary and Conclusion

PC immunotherapy faces some major challenges in the selection of a suitable antigen. It has to meet three criteria: tumor-specific expression, antigen availability due to sufficient expression, and high immunogenicity to elicit strong immune responses. Due to its deregulated overexpression in PC and functional role in pathogenesis, MUC4 has emerged as a promising diagnostic and therapeutic candidate. MUC4's central role in eliciting tumor proliferation, invasiveness, drug-resistance and metastasis, has led to considerable interest in targeting MUC4 to avail therapeutic benefits. Further, due to

aberrant glycosylation [50] and cleavage [26-28], MUC4 expressed on PC tumor cells will be distinct from the expression on normal tissues. Circulating autoantibodies to MUC4 has been detected in colorectal patients [51] and preliminary study with MUC4 peptide-induced DNA vaccine showed that MUC4 DC vaccine could efficiently present the antigen and elicit potent MUC4-specific CTL response [52].

Further, the structural complexity and various glycoforms of MUC4 adds a challenge in designing strategies for immunotherapy. Previous studies have been focused on peptides located in the VNTR region. Since MUC4 could putatively get cleaved at the GDPH site into alpha and beta subunits, thus there is a possibility of MUC4α (containing VNTR domain) to be not attached to the cell surface of tumor cells because MUC4 fragments have been discovered in secretions [53, 54].

In conclusion, MUC4 is a cancer-specific tumor-promoting glycoprotein that could serve as an effective antigen to develop vaccines against PC. However, investigations exploring MUC4 as a candidate for immunotherapy are in infancy. It requires multiple optimizations like identifying optimal biodegradable adjuvant delivery systems, identification of helper epitopes and developing platforms with good adjuvant properties that conserve the antigenicity of the epitopes. Thus, further studies are necessary to establish MUC4 as a suitable target for PC immunotherapy.

## References

[1] N. Moniaux, M. Andrianifahanana, R.E. Brand, S.K. Batra, Multiple roles of mucins in pancreatic cancer, a lethal and challenging malignancy, Br J Cancer, 91 (2004) 1633-1638.

[2] S. Kaur, S. Kumar, N. Momi, A.R. Sasson, S.K. Batra, Mucins in pancreatic cancer and its microenvironment, Nat Rev Gastroenterol Hepatol, 10 (2013) 607-620.

[3] M.J. Swartz, S.K. Batra, G.C. Varshney, M.A. Hollingsworth, C.J. Yeo, J.L. Cameron, R.E. Wilentz, R.H. Hruban, P. Argani, MUC4 expression increases progressively in pancreatic intraepithelial neoplasia, Am J Clin Pathol, 117 (2002) 791-796.

[4] M. Saitou, M. Goto, M. Horinouchi, S. Tamada, K. Nagata, T. Hamada, M. Osako, S. Takao, S.K. Batra, T. Aikou, K. Imai, S. Yonezawa, MUC4 expression is a novel prognostic factor in patients with invasive ductal carcinoma of the pancreas, J Clin Pathol, 58 (2005) 845-852.

[5] H. Yamasaki, S. Ikeda, M. Okajima, Y. Miura, T. Asahara, N. Kohno, F. Shimamoto, Expression and localization of MUC1, MUC2, MUC5AC and small intestinal mucin antigen in pancreatic tumors, Int J Oncol, 24 (2004) 107-113.

[6] D. Haridas, S. Chakraborty, M.P. Ponnusamy, I. Lakshmanan, S. Rachagani, E. Cruz, S. Kumar, S. Das, S.M. Lele, J.M. Anderson, U.A. Wittel, M.A. Hollingsworth, S.K. Batra, Pathobiological implications of MUC16 expression in pancreatic cancer, PLoS One, 6 (2011) e26839.

[7] M.A. Hollingsworth, J.M. Strawhecker, T.C. Caffrey, D.R. Mack, Expression of MUC1, MUC2, MUC3 and MUC4 mucin mRNAs in human pancreatic and intestinal tumor cell lines, Int J Cancer, 57 (1994) 198-203.

[8] Y. Hinoda, Y. Ikematsu, M. Horinochi, S. Sato, K. Yamamoto, T. Nakano, M. Fukui, Y. Suehiro, Y. Hamanaka, Y. Nishikawa, H. Kida, S. Waki, M. Oka, K. Imai, S. Yonezawa, Increased expression of MUC1 in advanced pancreatic cancer, J Gastroenterol, 38 (2003) 1162-1166.

[9] D.C. Chhieng, E. Benson, I. Eltoum, M.A. Eloubeidi, N. Jhala, D. Jhala, G.P. Siegal, W.E. Grizzle, U. Manne, MUC1 and MUC2 expression in pancreatic ductal carcinoma obtained by fine-needle aspiration, Cancer, 99 (2003) 365-371.

[10] K.G. Kohlgraf, A.J. Gawron, M. Higashi, J.L. Meza, M.D. Burdick, S. Kitajima, D.L. Kelly, T.C. Caffrey, M.A. Hollingsworth, Contribution of the MUC1 tandem repeat and cytoplasmic tail to invasive and metastatic properties of a pancreatic cancer cell line, Cancer Res, 63 (2003) 5011-5020.

[11] H. Tsutsumida, B.J. Swanson, P.K. Singh, T.C. Caffrey, S. Kitajima, M. Goto, S. Yonezawa, M.A. Hollingsworth, RNA interference suppression of MUC1 reduces the growth rate and metastatic phenotype of human pancreatic cancer cells, Clin Cancer Res, 12 (2006) 2976-2987.

[12] P. Chaturvedi, A.P. Singh, N. Moniaux, S. Senapati, S. Chakraborty, J.L. Meza, S.K. Batra, MUC4 mucin potentiates pancreatic tumor cell proliferation, survival, and invasive properties and interferes with its interaction to extracellular matrix proteins, Mol Cancer Res, 5 (2007) 309-320.

[13] A.P. Singh, N. Moniaux, S.C. Chauhan, J.L. Meza, S.K. Batra, Inhibition of MUC4 expression suppresses pancreatic tumor cell growth and metastasis, Cancer Res, 64 (2004) 622-630.

[14] P.K. Singh, M.A. Hollingsworth, Cell surface-associated mucins in signal transduction, Trends Cell Biol, 16 (2006) 467-476.

[15] D. Nath, A. Hartnell, L. Happerfield, D.W. Miles, J. Burchell, J. Taylor-Papadimitriou, P.R. Crocker, Macrophage-tumour cell interactions: identification of MUC1 on breast cancer cells as a potential counter-receptor for the macrophage-restricted receptor, sialoadhesin, Immunology, 98 (1999) 213-219.

[16] P. Allavena, M. Chieppa, G. Bianchi, G. Solinas, M. Fabbri, G. Laskarin, A. Mantovani, Engagement of the mannose receptor by tumoral mucins activates an immune suppressive phenotype in human tumor-associated macrophages, Clin Dev Immunol, 2010 (2010) 547179.

[17] L. Tu, D.K. Banfield, Localization of Golgi-resident glycosyltransferases, Cell Mol Life Sci, 67 (2010) 29-41.

[18] K. Banerjee, S. Kumar, K.A. Ross, S. Gautam, B. Poelaert, M.W. Nasser, A. Aithal, R. Bhatia, M.J. Wannemuehler, B. Narasimhan, J.C. Solheim, S.K. Batra, M. Jain, Emerging trends in the immunotherapy of pancreatic cancer, Cancer Lett, 417 (2018) 35-46.

[19] Y.F. Gong, Q.B. Zhou, Y.D. Liao, C. Mai, T.J. Chen, Y.Q. Tang, R.F. Chen, Optimized construction of MUC1-VNTRn DNA vaccine and its anti-pancreatic cancer efficacy, Oncol Lett, 13 (2017) 2198-2206.

[20] B. Palitzsch, N. Gaidzik, N. Stergiou, S. Stahn, S. Hartmann, B. Gerlitzki, N. Teusch, P. Flemming, E. Schmitt, H. Kunz, A Synthetic Glycopeptide Vaccine for the Induction of a Monoclonal Antibody that Differentiates between Normal and Tumor Mammary Cells and Enables the Diagnosis of Human Pancreatic Cancer, Angew Chem Int Ed Engl, 55 (2016) 2894-2898.

[21] A.M. Farkas, D.M. Marvel, O.J. Finn, Antigen choice determines vaccine-induced generation of immunogenic versus tolerogenic dendritic cells that are marked by differential expression of pancreatic enzymes, J Immunol, 190 (2013) 3319-3327.

[22] C.L. Slingluff, Jr., The present and future of peptide vaccines for cancer: single or multiple, long or short, alone or in combination?, Cancer J, 17 (2011) 343-350.

[23] P. Chaturvedi, A.P. Singh, S.K. Batra, Structure, evolution, and biology of the MUC4 mucin, FASEB J, 22 (2008) 966-981.

[24] M. Andrianifahanana, N. Moniaux, B.M. Schmied, J. Ringel, H. Friess, M.A. Hollingsworth, M.W. Buchler, J.P. Aubert, S.K. Batra, Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumor marker of diagnostic significance, Clin Cancer Res, 7 (2001) 4033-4040.

[25] S.K. Gautam, S. Kumar, A. Cannon, B. Hall, R. Bhatia, M.W. Nasser, S. Mahapatra, S.K. Batra, M. Jain, MUC4 mucin- a therapeutic target for pancreatic ductal adenocarcinoma, Expert Opin Ther Targets, 21 (2017) 657-669.

[26] N. Moniaux, S. Nollet, N. Porchet, P. Degand, A. Laine, J.P. Aubert, Complete sequence of the human mucin MUC4: a putative cell membrane-associated mucin, Biochem J, 338 ( Pt 2) (1999) 325-333.

[27] N. Moniaux, F. Escande, S.K. Batra, N. Porchet, A. Laine, J.P. Aubert, Alternative splicing generates a family of putative secreted and membrane-associated MUC4 mucins, Eur J Biochem, 267 (2000) 4536-4544.

[28] N. Moniaux, F. Escande, N. Porchet, J.P. Aubert, S.K. Batra, Structural organization and classification of the human mucin genes, Front Biosci, 6 (2001) D1192-1206.

[29] K.L. Carraway, 3rd, E.A. Rossi, M. Komatsu, S.A. Price-Schiavi, D. Huang, P.M. Guy, M.E. Carvajal, N. Fregien, C.A. Carraway, K.L. Carraway, An intramembrane modulator of the ErbB2 receptor tyrosine kinase that potentiates neuregulin signaling, J Biol Chem, 274 (1999) 5263-5266.

[30] I. Lakshmanan, P. Seshacharyulu, D. Haridas, S. Rachagani, S. Gupta, S. Joshi, C. Guda, Y. Yan, M. Jain, A.K. Ganti, M.P. Ponnusamy, S.K. Batra, Novel HER3/MUC4 oncogenic signaling aggravates the tumorigenic phenotypes of pancreatic cancer cells, Oncotarget, 6 (2015) 21085-21099.

[31] N. Jonckheere, N. Skrypek, J. Merlin, A.F. Dessein, P. Dumont, E. Leteurtre, A. Harris, J.L. Desseyn, C. Susini, F. Frenois, I. Van Seuningen, The mucin MUC4 and its membrane partner ErbB2 regulate biological properties of human CAPAN-2 pancreatic cancer cells via different signalling pathways, PLoS One, 7 (2012) e32232.

[32] P. Seshacharyulu, M.P. Ponnusamy, S. Rachagani, I. Lakshmanan, D. Haridas, Y. Yan, A.K. Ganti, S.K. Batra, Targeting EGF-receptor(s) - STAT1 axis attenuates tumor growth and metastasis through downregulation of MUC4 mucin in human pancreatic cancer, Oncotarget, 6 (2015) 5164-5181.

[33] S. Senapati, V.S. Gnanapragassam, N. Moniaux, N. Momi, S.K. Batra, Role of MUC4-NIDO domain in the MUC4-mediated metastasis of pancreatic cancer cells, Oncogene, 31 (2012) 3346-3356.

[34] S. Bafna, S. Kaur, N. Momi, S.K. Batra, Pancreatic cancer cells resistance to gemcitabine: the role of MUC4 mucin, Br J Cancer, 101 (2009) 1155-1161.

[35] S. Rachagani, M.A. Macha, M.P. Ponnusamy, D. Haridas, S. Kaur, M. Jain, S.K. Batra, MUC4 potentiates invasion and metastasis of pancreatic cancer cells through stabilization of fibroblast growth factor receptor 1, Carcinogenesis, 33 (2012) 1953-1964.

[36] X. Huang, X. Wang, S.M. Lu, C. Chen, J. Wang, Y.Y. Zheng, B.H. Ren, L. Xu, Clinicopathological and prognostic significance of MUC4 expression in cancers: evidence from meta-analysis, Int J Clin Exp Med, 8 (2015) 10274-10283.

[37] N. Jhala, D. Jhala, S.M. Vickers, I. Eltoum, S.K. Batra, U. Manne, M. Eloubeidi, J.J. Jones, W.E. Grizzle, Biomarkers in Diagnosis of pancreatic carcinoma in fine-needle aspirates, Am J Clin Pathol, 126 (2006) 572-579.

[38] M. Higashi, S. Yokoyama, T. Yamamoto, Y. Goto, I. Kitazono, T. Hiraki, H. Taguchi, S. Hashimoto, Y. Fukukura, C. Koriyama, Y. Mataki, K. Maemura, H. Shinchi, M. Jain, S.K. Batra, S. Yonezawa, Mucin expression in endoscopic ultrasound-guided fine-needle aspiration specimens is a useful prognostic factor in pancreatic ductal adenocarcinoma, Pancreas, 44 (2015) 728-734.

[39] G. Wang, R.J. Lipert, M. Jain, S. Kaur, S. Chakraboty, M.P. Torres, S.K. Batra, R.E. Brand, M.D. Porter, Detection of the potential pancreatic cancer marker MUC4 in serum using surface-enhanced Raman scattering, Anal Chem, 83 (2011) 2554-2561.

[40] A.V. Krasnoslobodtsev, M.P. Torres, S. Kaur, I.V. Vlassiouk, R.J. Lipert, M. Jain, S.K. Batra, Y.L. Lyubchenko, Nano-immunoassay with improved performance for detection of cancer biomarkers, Nanomedicine, 11 (2015) 167-173.

[41] M. Perrais, P. Pigny, M.P. Ducourouble, D. Petitprez, N. Porchet, J.P. Aubert, I. Van Seuningen, Characterization of human mucin gene MUC4 promoter: importance of growth factors and proinflammatory cytokines for its regulation in pancreatic cancer cells, J Biol Chem, 276 (2001) 30923-30933.

[42] A.P. Singh, S.C. Chauhan, M. Andrianifahanana, N. Moniaux, J.L. Meza, M.C. Copin, I. van Seuningen, M.A. Hollingsworth, J.P. Aubert, S.K. Batra, MUC4 expression is regulated by cystic fibrosis transmembrane conductance regulator in pancreatic adenocarcinoma cells via transcriptional and post-translational mechanisms, Oncogene, 26 (2007) 30-41.

[43] M. Andrianifahanana, A. Agrawal, A.P. Singh, N. Moniaux, I. van Seuningen, J.P. Aubert, J. Meza, S.K. Batra, Synergistic induction of the MUC4 mucin gene by interferon-gamma and retinoic acid in human pancreatic tumour cells involves a reprogramming of signalling pathways, Oncogene, 24 (2005) 6143-6154.

[44] M. Andrianifahanana, S.C. Chauhan, A. Choudhury, N. Moniaux, R.E. Brand, A.A. Sasson, P.M. Pour, S.K. Batra, MUC4-expressing pancreatic adenocarcinomas show elevated levels of both T1 and T2 cytokines: potential pathobiologic implications, Am J Gastroenterol, 101 (2006) 2319-2329.

[45] N. Jonckheere, M. Perrais, C. Mariette, S.K. Batra, J.P. Aubert, P. Pigny, I. Van Seuningen, A role for human MUC4 mucin gene, the ErbB2 ligand, as a target of TGF-beta in pancreatic carcinogenesis, Oncogene, 23 (2004) 5729-5738.

[46] G. Damera, B. Xia, H.R. Ancha, G.P. Sachdev, IL-9 modulated MUC4 gene and glycoprotein expression in airway epithelial cells, Biosci Rep, 26 (2006) 55-67.

[47] G. Damera, B. Xia, G.P. Sachdev, IL-4 induced MUC4 enhancement in respiratory epithelial cells in vitro is mediated through JAK-3 selective signaling, Respir Res, 7 (2006) 39.

[48] R. Mejias-Luque, S. Peiro, A. Vincent, I. Van Seuningen, C. de Bolos, IL-6 induces MUC4 expression through gp130/STAT3 pathway in gastric cancer cell lines, Biochim Biophys Acta, 1783 (2008) 1728-1736.

[49] A. Andoh, M. Shioya, A. Nishida, S. Bamba, T. Tsujikawa, S. Kim-Mitsuyama, Y. Fujiyama, Expression of IL-24, an activator of the JAK1/STAT3/SOCS3 cascade, is enhanced in inflammatory bowel disease, J Immunol, 183 (2009) 687-695.

[50] S. Chugh, V.S. Gnanapragassam, M. Jain, S. Rachagani, M.P. Ponnusamy, S.K. Batra, Pathobiological implications of mucin glycans in cancer: Sweet poison and novel targets, Biochim Biophys Acta, 1856 (2015) 211-225.

[51] J.W. Pedersen, A. Gentry-Maharaj, A. Nostdal, E.O. Fourkala, A. Dawnay, M. Burnell, A. Zaikin, J. Burchell, J.T. Papadimitriou, H. Clausen, I. Jacobs, U. Menon, H.H. Wandall, Cancer-associated autoantibodies to MUC1 and MUC4--a blinded case-control study of colorectal cancer in UK collaborative trial of ovarian cancer screening, Int J Cancer, 134 (2014) 2180-2188.

[52] J. Wei, W. Gao, J. Wu, K. Meng, J. Zhang, J. Chen, Y. Miao, Dendritic cells expressing a combined PADRE/MUC4-derived polyepitope DNA vaccine induce multiple cytotoxic T-cell responses, Cancer Biother Radiopharm, 23 (2008) 121-128.

[53] B. Liu, G.D. Offner, D.P. Nunes, F.G. Oppenheim, R.F. Troxler, MUC4 is a major component of salivary mucin MG1 secreted by the human submandibular gland, Biochem Biophys Res Commun, 250 (1998) 757-761.

[54] J. Zhang, A. Perez, M. Yasin, P. Soto, M. Rong, G. Theodoropoulos, C.A. Carothers Carraway, K.L. Carraway, Presence of MUC4 in human milk and at the luminal surfaces of blood vessels, J Cell Physiol, 204 (2005) 166-177.

# CHAPTER 1C: DISSERTATION GENERAL HYPOTHESIS AND OBJECTIVES

# 1. Background and Rationale

PC is the third leading cause of cancer-related death in the U.S. claiming 45,000 lives every year because of its poor prognosis and resistance to conventional therapies [1]. Much like other solid tumors, PC evades immune surveillance by manipulating immune cells to establish an immunosuppressive TME. Immunotherapy has emerged as an alternative approach for PC immunotherapy. It reprograms the patient's immune system to selectively target and kill cancer cells thus reducing the non-specific side-effects. PC immunotherapy faces multiple challenges such as: i) identification of tumor-associated antigen that could be targeted; ii) preservation of TAA antigenicity and its sustained delivery; iii) generation of robust anti-tumor responses; and iv) overcoming immunosuppression on effector immune cells in PC tumor microenvironment.

PC harbor limited genetic alterations which render it as an immunologically 'cold' tumor. Overexpression and altered glycosylation of mucins in tumor cells can trigger humoral and cellular immune responses. Among various mucins, MUC4, heterodimeric transmembrane mucin is aberrantly overexpressed in PC but is undetectable in normal pancreas and is associated with poor prognosis in PC patients [2]. In PC, MUC4 is aberrantly glycosylated, and thus could serve as a potential source of neoantigenic epitopes, which could be exploited for cancer immunotherapy**.** Additionally, due to the transmembrane nature of MUC4 only a very limited amount is released into the blood, and that makes MUC4 a very strong candidate for vaccine [3-6]. A survey of literature has shown that mucins like MUC1 have been targeted by immunotherapy approaches for PC treatment but have not fared well in clinical trials. Further detection of circulating serum autoantibodies to MUC4 has shown its potential to be immunogenic in colorectal cancer patients [7]. However, MUC4 immunogenicity in PC patients has yet not been studied.

These summarized findings suggest that MUC4 has potential to be a candidate for PC immunotherapy.

Polyanhydride nanoparticles represent an ideal vaccine adjuvant/delivery platform. Polyanhydride nanoparticles have been demonstrated to provide sustained release and enhanced stability of encapsulated antigens (peptides, proteins etc.) [8]. The ratio of polyanhydride nanoparticle's formulation provides an immune modulation property that could be utilized to tune the immune responses to either Th1 or Th2 phenotype [9, 10]. In addition, their adjuvant properties enhance antigen internalization by antigen-presenting cells and stimulate both antibody- and cell-mediated immunity [11-13]. Due to their pathogen-mimicking, immune-modulation and biodegradable nature, the polyanhydride nanoparticles platform could be utilized to develop vaccines for PC immunotherapy.

## 2. Hypothesis

Based on previous studies demonstrating the tumor-specific overexpression of MUC4 in PC and suitable qualities of polyanhydride nanoparticles to serve as a vaccine delivery platform, we *hypothesized* that i) MUC4 is immunogenic in PC patients, and ii) delivery of encapsulated MUC4 in unique amphiphilic polyanhydride nanovaccine will elicit robust immune responses in an antigen-specific manner.

## 3. Objectives

**Aim 1:** To determine whether tolerance will be broken against MUC4 in pancreatic cancer patients and elucidating it as a suitable target it for immunotherapy studies.

**Aim 2:** To evaluate whether MUC4 nanovaccine can elicit robust immune responses.

**Aim 3:** To evaluate whether MUC4 nanovaccine can elicit antigen-specific anti-tumor responses in PC-subcutaneous tumor-bearing mouse model.

# REFERENCES

[1] K. Banerjee, S. Kumar, K.A. Ross, S. Gautam, B. Poelaert, M.W. Nasser, A. Aithal, R. Bhatia, M.J. Wannemuehler, B. Narasimhan, J.C. Solheim, S.K. Batra, M. Jain, Emerging trends in the immunotherapy of pancreatic cancer, Cancer Lett, 417 (2018) 35-46.

[2] X. Huang, X. Wang, S.M. Lu, C. Chen, J. Wang, Y.Y. Zheng, B.H. Ren, L. Xu, Clinicopathological and prognostic significance of MUC4 expression in cancers: evidence from meta-analysis, Int J Clin Exp Med, 8 (2015) 10274-10283.

[3] P. Chaturvedi, A.P. Singh, S.K. Batra, Structure, evolution, and biology of the MUC4 mucin, FASEB J, 22 (2008) 966-981.

[4] P. Chaturvedi, A.P. Singh, N. Moniaux, S. Senapati, S. Chakraborty, J.L. Meza, S.K. Batra, MUC4 mucin potentiates pancreatic tumor cell proliferation, survival, and invasive properties and interferes with its interaction to extracellular matrix proteins, Mol Cancer Res, 5 (2007) 309-320.

[5] S.K. Gautam, S. Kumar, A. Cannon, B. Hall, R. Bhatia, M.W. Nasser, S. Mahapatra, S.K. Batra, M. Jain, MUC4 mucin- a therapeutic target for pancreatic ductal adenocarcinoma, Expert Opin Ther Targets, 21 (2017) 657-669.

[6] S. Kaur, S. Kumar, N. Momi, A.R. Sasson, S.K. Batra, Mucins in pancreatic cancer and its microenvironment, Nat Rev Gastroenterol Hepatol, 10 (2013) 607-620.

[7] J.W. Pedersen, A. Gentry-Maharaj, A. Nostdal, E.O. Fourkala, A. Dawnay, M. Burnell, A. Zaikin, J. Burchell, J.T. Papadimitriou, H. Clausen, I. Jacobs, U. Menon, H.H. Wandall, Cancer-associated autoantibodies to MUC1 and MUC4--a blinded case-control study of colorectal cancer in UK collaborative trial of ovarian cancer screening, Int J Cancer, 134 (2014) 2180-2188.

[8] K.A. Ross, H. Loyd, W. Wu, L. Huntimer, M.J. Wannemuehler, S. Carpenter, B. Narasimhan, Structural and antigenic stability of H5N1 hemagglutinin trimer upon release from polyanhydride nanoparticles, J Biomed Mater Res A, 102 (2014) 4161-4168.

[9] B.D. Ulery, H.M. Kan, B.A. Williams, B. Narasimhan, K.W. Lo, L.S. Nair, C.T. Laurencin, Facile fabrication of polyanhydride/anesthetic nanoparticles with tunable release kinetics, Adv Healthc Mater, 3 (2014) 843-847.

[10] E.I. Wafa, S.M. Geary, J.T. Goodman, B. Narasimhan, A.K. Salem, The effect of polyanhydride chemistry in particle-based cancer vaccines on the magnitude of the anti-tumor immune response, Acta Biomater, 50 (2017) 417-427.

[11] L.K. Petersen, A.E. Ramer-Tait, S.R. Broderick, C.S. Kong, B.D. Ulery, K. Rajan, M.J. Wannemuehler, B. Narasimhan, Activation of innate immune responses in a pathogen-mimicking manner by amphiphilic polyanhydride nanoparticle adjuvants, Biomaterials, 32 (2011) 6815-6822.

[12] B.D. Ulery, D. Kumar, A.E. Ramer-Tait, D.W. Metzger, M.J. Wannemuehler, B. Narasimhan, Design of a protective single-dose intranasal nanoparticle-based vaccine platform for respiratory infectious diseases, PLoS One, 6 (2011) e17642.

[13] L. Huntimer, J.H. Wilson Welder, K. Ross, B. Carrillo-Conde, L. Pruisner, C. Wang, B. Narasimhan, M.J. Wannemuehler, A.E. Ramer-Tait, Single immunization with a suboptimal antigen dose encapsulated into polyanhydride microparticles promotes high titer and avid antibody responses, J Biomed Mater Res B Appl Biomater, 101 (2013) 91-98.

# CHAPTER 2:

# MATERIALS AND METHODS

## 1. Human Serum Sample Description

Human sera were obtained after written informed consent was acquired from individuals {the protocol was approved by the UPMC Review Board (IRB number PRO07030072), and UNMC Review Board (IRB number 209–00)} from the University of Pittsburgh Medical Center (UPMC) and shipped to UNMC. Patients with benign pathologies or abnormal imaging of pancreas were categorized as Healthy; PC staging was determined surgically by pathologists; chronic pancreatitis (CP) was defined based on standard clinical practices at UPMC. Sera from PC patients were collected close to the time of the first diagnosis of cancer, prior to surgery and before chemotherapy treatment. All samples collected at UPMC were shipped by overnight mail to the UNMC. After receiving the samples, they were stored following clotting of sera for 30 min, aliquoted and immediately frozen at -70°C within 60 min of collection. The sample set was comprised of 21 CP patients, 27 Healthy, and 31 PC patients, which could be divided among 16 early-stage patients (Stage I–II) and 15 late-stage patients (Stage III–IV) who were recruited at UPMC. To evaluate the presence of autoantibodies against MUC4 peptides, randomized patient sera was selected to make a cohort of 10 Healthy, 10 CP patients and 22 PC patients. Details on the groups of patients are provided in the supplementary section, and patient demographic data are described in **Supplementary Table 1 &2**.

## 2. Prediction of MUC4 Immunogenic Peptides by Bioinformatics

MUC4 immunogenic peptides were predicted by multiple software: T-cell epitopes for human MHC-I (most common: HLA-A*-02:01) were predicted and scored by Immune Epitope Database and Analysis Resource (IEDB) [24-31] and TepiTool [32], and combined human HLA isoforms scores were predicted by NetCTL-1.0 [33] online prediction tools. Human MUC4 peptides that could be loaded on C57BL/6 mouse MHC-I & MHC-II complexes were predicted and scored by IEDB online prediction tool; and linear B cell

epitopes were predicted and scored by IEDB and BCPred [34] online prediction tools. Lowest percentile rank scoring peptides were selected to be synthesized for our study. For our study, we selected the amino acid sequence derived from the MUC4 sequence isolated and characterized at www.uniprot.org (UniProtKB/Swiss-Prot: Q99102.4) [20, 22, 35, 36].

### 3. MUC4 Peptide and MUC4β Purification

Predicted MUC4 9-mer-amino-acid peptides, MUC4 VNTR peptide (TR) and pancreatic differentiation 2 (PD2) peptide used in this study were locally synthesized and purified at UNMC. Seven peptides from the randomized regions of MUC4α (excluding the TR region) and four peptides from the MUC4β region (upstream of the transmembrane domain) were used to detect autoantibodies in PC patients, CP patients and Healthy sera.

Recombinant human MUC4β protein was expressed in *E. coli* R-2 (DE3) strain transformed with MUC4β-6-His-Tag expression plasmid and purified by AKTA Ni-NTA affinity chromatography. Eluted protein fractions were assessed by Coomassie Blue stained 10% SDS-PAGE gels and by immunoblotting using anti-His tag antibody (Thermo Fisher, Pierce # 31430), confirming the presence of MUC4β fractions. MUC4β fractions were concentrated by using Amicon Ultra centrifuge filters and further purified by dialysis in ultra-purified endotoxin-free water. Purified MUC4β fractions were passed through an endotoxin removal spin column (Thermo Fisher, Pierce # 88277) and the final endotoxin level measured by Pierce LAL Chromogenic Endotoxin assay kit (Pierce, # 88282) was less than <1. The purified recombinant MUC4β protein fraction was quantified by BCA protein assay kit (Thermo Fisher, Pierce # 23225) for the study.

## 4. Encapsulation of MUC4β in Polyanhydride Nanoparticles

The 20:80 CPTEG:CPH polymer was synthesized via melt polycondensation [23]. Next, nanoparticles encapsulating MUC4β were synthesized using a solid-oil-oil double emulsion technique previously described in [28]. Briefly, purified MUC4β was dialyzed to nanopure water and lyophilized. Next, 20:80 CPTEG: CPH polymer containing three wt.% MUC4β was dissolved 20 mg/mL in methylene chloride. The solution was sonicated for 30 s to ensure even distribution of the protein. The nanoparticles were precipitated into chilled pentane (-10°C; 1:250 methylene chloride: pentane) and collected via vacuum filtration. Nanoparticle morphology was verified by scanning electron microscopy (FEI Quanta 250, FEI, Hillsboro, OR) and their size subsequently analyzed with ImageJ (ImageJ 1.48v, NIH). The release kinetics of MUC4β from 20:80 CPTEG: CPH nanoparticles were monitored as previously described [22]. Briefly, nanoparticles were incubated in PBS at 37°C. Periodically, the samples were centrifuged, supernatant collected, and particles were resuspended in fresh buffer. The amount of protein in the collected supernatant was quantified using a microBCA assay. At the end of approximately one month, the buffer was exchanged with 40 mM sodium hydroxide to quickly degrade the nanoparticles and release any remaining protein. The encapsulation efficiency was determined by comparing the total amount of protein released to the amount theoretically encapsulated.

## 5. Primary Dendritic cells (DCs) Isolation

C57BL/6 mice were kept under SPF conditions at UNMC animal facilities in accordance with UNMC Institutional Animal Care and Use Committee (IACUC) standards. Femurs and tibiae of female, 6-8 weeks old female C57BL/6 mice were removed and purified from the surrounding muscle tissue by rubbing with kleenex tissues. Thereafter intact bones were disinfected in phosphate buffered saline pH 7.2 (PBS) containing

penicillin (100 U/mL, Sigma) and streptomycin (100 m/mL, Sigma) solution for 2-5 min. Then both ends were cut with scissors and the marrow was flushed with PBS using a 1 mL syringe attached to 25 G needle. Homogenous pipetting disintegrated clusters within the marrow suspension. Cells were centrifuged at 2000 rpm for 2.5 min and the cell pellet was resuspended in 10 mL of 1X RBC lysis buffer in the dark for 5 min. Ten mL of RPMI was added to stop the lysis. Cells were centrifuged at 2000 rpm and washed 3 times in 10 mL of RPMI. After the last wash, cells were resuspended homogeneously in a 1 bone:1 mL of media ratio. Resuspended cells were passed through a single cell strainer to remove clumps. Single cell suspensions were poured in 10 mL RPMI containing 10% FBS, penicillin (100 U/mL, Sigma) and streptomycin (100 mg/mL, Sigma) media in a 10 cm tissue culture plate that was kept in an incubator for 3 h. After incubation, we collected all floating cells and washed the plate with media twice to collect rest of the suspended cells in a 50 mL falcon tube. The cells were pelleted by centrifugation and the media aspirated. The cells were resuspended in 10 mL RPMI media and transfered into T75 flasks for our *in vitro* studies.

## 6. DC Maturation and Pulsing

To enhance the DC population, we added 100 ng/µl of rmGM-CSF and 50 ng/µl rmIL-4 reconstituted and diluted in serum-free RPMI to freshly isolated DC cultures on Day 0. On Day 3, 5, and 7, we collected the nonadherent cells in 50 ml centrifuge tubes, pelleted them and resuspended them in a total volume of 10 ml having 75% of fresh media containing 10% FBS + antibiotics and 100 ng/µl of GM-CSF and 50 ng/µl IL-4 in a fresh T75 flask. At day 9, immature DCs were counted and seeded in 24 well plate for activation studies. Polyanhydride nanoparticles were suspended in complete culture medium, sonicated briefly (30s on ice), and added to the DC cultures at day 9 at a concentration of 100 µg/mL. DCs were pulsed in the following groups: 3 µg/ml free MUC4β protein

(MUC4), free MUC4β protein (3 µg/ml) mixed with blank nanoparticles (100 µg/mL) (MUC4+NP) and MUC4 nanovaccine (100 µg/mL). Unstimulated DCs (US) and DCs stimulated with LPS (200 ng/mL) were used as negative and positive controls, respectively. Cultures were incubated for 48 h (37 °C, 5% $CO_2$). Activated dendritic cells were harvested from 24-well plate and centrifuged at 1000 x g for 5 minutes to collect the culture medium and the pellet was processed further for flow cytometry studies. Supernatant was collected for ELISA studies from each treatment groups.

## 7. Flow Cytometry of Activated DCs

Dendritic cells were resuspended and washed 3 times in FACS buffer {1X PBS (pH 7.2) + 1% Fetal bovine serum (FBS)} to remove any residual culture medium. DCs were fixed in 4% paraformaldehyde for 15 min at RT and washed by centrifugation with excess FACs Buffer. After that, the $1X10^5$ DCs were resuspended in100 µl volume of conjugated antibody cocktail for detection of DC surface markers consisting of CD11c, MHC-I, MHC-II, CD40, CD80, CD86, and CD205 was prepared at 1:300 dilution in FACs Buffer. Corresponding isotype controls were also prepared at 1:300 dilution in FACs buffer. The washed DCs were suspended in either DC surface markers antibody cocktail or in isotype-antibody cocktail and incubated for 60 min on ice ($4^0$C) in the dark. The labelled cells were again washed and analyzed using a BD LSR-II Green Flow Cytometer (BD Biosciences). FlowJo® and BD FACSDIVA software were used to analyze the data.

## 8. Cytokine Analysis by ELISA

Supernatants were preserved at -$80^0$C and thawed on ice for cytokine analysis. IL-6, IFN-γ and IL-12/IL-23p40 cytokines in supernatants were measured by enzyme-linked immunosorbent assay (ELISA) kits from BioLegend and the manufacturer's protocol was followed for the assay. 96-well ELISA strips were coated with capture antibodies (1:200 dilution) diluted in carbonate-bicarbonate buffer (0.5M, pH 9.6) and incubated overnight

at $4^0$C. The next day coated strips were washed 4 times with 1X PBST (0.05% Tween 20) and blocked with 3% bovine serum albumin (BSA) in PBS for 2 hours at $37^0$C. Strips were then washed 4 times and 100 µl of supernatants were added to coated strips and incubated for 2 hours at $37^0$C. Plates were washed with PBST for 4 times, followed by incubation with detection antibody (1:200) for 1 hour at $37^0$C. Secondary antibody was washed away with 4 PBST washes. Avidin (1:1000) was added to ELISA strips and incubated for 30 min at RT in dark. Excess Avidin was washed away with 5 PBST washes, followed by addition of TMB (3,3',5,5'-Tetramethylbenzidine) substrate in the dark and incubated at RT for color to develop. Absorbance was measured after the reaction was stopped (~15 mins) with 1N $H_2SO_4$ at 450 nm using a SpectraMax® Plus384 microplate reader (Molecular Devices LLC, Sunnyvale, California). For serum cytokines analysis, serum samples were collected post-animal sacrifice and stored at $-80^0$C. Serum samples were added at 1:10 dilution to capture-antibody coated plates for cytokine analysis. IL-6, IFN-γ and IL-12/IL-23p40 were measured by ELISA kits from BioLegend using a similar manufacturer's protocol.

## 9. Mice Immunization for Anti-MUC4 Antibodies Generation

Eight weeks C57BL/6 mice were immunized with various formulations of recombinant MUC4β protein, including protein alone (20 µg/mouse/dose), encapsulated MUC4β protein (300 µg/mouse/dose), protein plus nanoparticle, and saline control, constituted in Freund's adjuvant. First booster dose was given after 2 weeks of primary immunization. The blood was withdrawn form submandibular vein after 1 week of a single booster dose. The serum was isolated from the blood after overnight storage at 4°C and stored at -20 °C for further use.

## 10. MUC4 Autoantibody ELISA

To detect circulating MUC4 autoantibodies in sera of the sample set, a novel modified sandwich ELISA-assay was developed. Briefly each 96-well plate was coated with five µg/mL of recombinant MUC4β protein or with one µg/mL of MUC4 peptides or control peptides (PD2 & TR) in carbonate-bicarbonate coating buffer (0.5 M, pH 9.6) and incubated overnight at $4^0$C. The plate was washed two times with 1X PBST and blocked with 3% BSA in PBS for 3 hours at $37^0$C. Further, the plate was washed four times and then a serial dilution (1:16000) of the serum samples were added to MUC4β-coated plate and a serial dilution of 1:2000 was added to MUC4 peptide-coated plates. ELISA plates with primary serum dilutions were incubated overnight at $4^0$C. Plates were washed with PBST for four times, followed by incubation with horseradish-peroxidase (HRP) conjugated goat anti-human IgM+ IgG+ IgA (H+L) (Jackson ImmunoResearch), or for isotyping HRP conjugated IgM, IgG (H+L) and IgA (Jackson ImmunoResearch) respectively for 1 hour at $37^0$C. The excess secondary antibody was washed away with 5 PBST washes and followed by addition of TMB substrate in the dark. Absorbance was measured after the reaction was stopped (~15mins) by adding 1N $H_2SO_4$ at 450 nm using a SpectraMax® Plus384 microplate reader (Molecular Devices LLC, Sunnyvale, California).

## 11. Anti-MUC4β Antibodies Detection in Immunized Mice Serum Using ELISA

Serum samples were collected post-animal sacrifice and stored at $-80^0$C for detection of anti-MUC4β antibodies using ELISA techniques. To detect anti-MUC4β antibodies, we used a modified ELISA protocol. A 96-well plate was coated with 5 µg/mL of recombinant MUC4β protein in carbonate-bicarbonate coating buffer (0.5 M, pH 9.6) and incubated

overnight at $4^0$C. Plate was washed 2 times with 1X PBST and blocked with 3% BSA in PBS for 3 hours at $37^0$C. Further, the plate was washed 4 times and then serial dilutions of the serum samples were incubated in MUC4β coated plate for 2 hours at $37^0$C. Plates were washed with PBST for 4 times, followed by incubation with horseradish-peroxidase (HRP) conjugated goat anti-mouse IgG (total H+L) (Thermo Fisher), IgG1 (Abcam) and IgG2b (Abcam) for 1 hour at $37^0$C. Excess secondary antibody was washed away with 5 PBST washes and followed by addition of TMB substrate in the dark. Absorbance was measured after the reaction was stopped (~15 min) with 1N $H_2SO_4$ at 450 nm using a SpectraMax® Plus384 microplate reader (Molecular Devices LLC, Sunnyvale, California).

## 12. Generation of MUC4-Expressing Murine PC Cell Lines

A MUC4-expressing mouse pancreatic cancer cell line derived from KPC mice (KPC960) was developed by transfection of KPC960 cell lines with miniMUC4-pSecTagC plasmid using the Lipofectamine method (Invitrogen) and single colonies were obtained by zeocin selection as published previously in Moniaux *et.al* 2007 and confirmed by immunoblotting with a MUC4 peptide mouse monoclonal antibody (8G7). The miniMUC4 expressing KPC960 cells were kindly developed, validated and gifted by Dr. Shailendra Gautam for our *in vivo* and *ex vivo* studies.

## 13. Tumor Implantation and Mice Immunization

For *in vivo* characterization of MUC4 nanovaccine in mice, 8 weeks C57BL/6-FBP mixed background mice were immunized with different formulations of recombinant MUC4β protein, including protein alone (MUC4- 35 µg/mouse/dose), free MUC4 protein mixed with nanoparticles (MUC4+ NP) (35 µg/mouse/dose of MUC4 + 500 µg/mouse/dose empty nanoparticles), MUC4 nanovaccine (500 µg/mouse/dose), and unimmunized mice (negative control). First booster dose was given after two weeks of primary immunization.

Two weeks after first booster, mice were injected with $1x10^6$ cells of mini-Muc4 or Vector expressing KPC derived mouse pancreatic cell lines(KPC960) into right and left flanks of mixed background mice (n=5), respectively. Tumor growth was monitored till the tumor volume reached to $100mm^3$ volume. Mice were then immunized with second booster dose but the MUC4 protein dose was reduced to 20 µg/mouse/dose in all treatment groups except for saline control. Tumors were harvested at $23^{rd}$-day post second booster immunization and preserved in formalin for further use.

## 14. T-cell Isolation and *In Vitro* Activation

Naïve T-cells were isolated from 8-10weeks old C57BL/6-FBP mixed background mice. Mice were sacrificed, and spleens were isolated in RPMI+10%FBS+Pen-Strp (RPMI) medium in 50 ml Falcon tube on ice. Media was removed by suction inside the laminar hood and one spleen was added to one of the wells of 6 well plates. Spleens were teased with a 25G syringe needle and 1 ml pipette tip. Teased-out splenocytes were transferred to 15 ml falcon tube in RPMI media and was centrifuged for 2-3mins at 2000 rpm. The supernatant was removed and the pellet was resuspended in 10-12ml of 1X RBC lysis buffer, then the tube was kept for 5 min in the dark. The tube was centrifuged again for 2-3 min at 2000 rpm and the supernatant was aspirated. The pellet was resuspended and splenocytes were washed for 3 times with RPMI media. After the last wash, the pellet was resuspended in 2 ml of RPMI media and counted total cells/ml. We centrifuged the cell suspension at 2000 rpm for 10 min and removed the supernatant completely. We resuspended the cell pellet in 40 µL of MACs Buffer (1X PBS pH 7.2, 0.5% BSA, 2mM EDTA) per $10^7$ cells. We added 10 µL of Pan T cell Biotin-Antibody Cocktail (MACS Miltenyi Biotech, Inc.) per $10^7$ cells and mixed it well. We incubated the mixture for 5 min in the refrigerator (2-8$^o$ C) and then added 30 µL of MACs buffer per $10^7$ cells, and followed by addition of 40 µL of Anti-Biotin Microbeads per $10^7$ cells. We mixed all the

components well & incubated for additional 10 min in the refrigerator (2-8$^{o}$ C). We took the mixture and processed the sample in an autoMACS® Pro Separator (MACS Miltenyi Biotech, Inc.). Tubes were placed in the following Chill Rack positions: position A = sample, position B = negative fraction position C = positive fraction. We prepared and primed the instrument and followed the instructions that were given in the user manual. T cells were isolated by magnetic separation and we used the 'Depletes' program to do the separation into positive (other splenocytes) and negative (T cells) population.

Naïve T-cells were then cultured and expanded in RPMI on day 0. On day 1, purified T cells were incubated with phorbol 12-myristate 13-acetate (PMA) (25 ng/mL) Ionomycin (500 ng/mL, Abcam) for 48 hours at 37°C. On day 3, these T-cells were co-cultured in 12-well plate with 48hour-pulsed DCs in the following groups: 3 µg/ml free MUC4β protein (MUC4), free MUC4β protein (3 µg/ml) mixed with blank nanoparticles (100 µg/mL) (MUC4+NP) and MUC4 nanovaccine (100 µg/mL). Unstimulated DCs (US) and DCs stimulated with lipopolysaccharides {LPS} (200 ng/mL) were used as negative and positive controls, respectively. T-cells were co-cultured for 48 hours with pulsed DCs and then collected for further analysis. The experiment was done in triplicates.

Since the Pan T cell Isolation kit can purify both naïve and activated T cells, a similar T-cell isolation protocol was utilized to isolate T-cells from immunized mice and expanded *in vitro* by treatment with PMA/Ionomycin (25 ng/mL/500ng/mL, Abcam) for 48 hours at 37°C. On day 3, the expanded T-cells were analyzed by flow cytometry for phenotyping T-cells and cytokine secretion studies.

## 15. Cytotoxicity Assay of T-cells

The miniMUC4 KPC960 cells and KPC960 vector control cells (500 cells/well) were seeded in 96-U bottom plates and cultured overnight in a 37$^{0}$C incubator. The plate

was centrifuged and the media was removed. Activated T-cells were added from each of the treatment groups to target cancer cells at 10:1 dilution into their respectively labeled wells. T-cell mediated cytotoxicity was measured by the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega) and the manufacturer's protocol was followed for the assay. The plate was centrifuged at 250 × g for 4 minutes and the plate was incubated at 37°C for 4 hours. At 45 minutes prior to supernatant harvest, Lysis Solution was added to wells of both cancer cell lines that didn't have T-cells added to them, which served as our Target Cell Maximum LDH Release Control. The plate was centrifuged at 250 × g for 4 minutes and 50 µl of the supernatant from each well of the assay plate was transferred to the corresponding well of a flat-bottom 96-well enzymatic assay plate. Substrate Mix was reconstituted using Assay Buffer and 50 µl of the reconstituted Substrate Mix was added to each well of the plate. The plate was incubated at room temperature in the dark for 30 min. Absorbance was measured after the reaction was stopped with 50 µl of the Stop Solution at 450 nm using a SpectraMax® Plus384 microplate reader (Molecular Devices LLC, Sunnyvale, California).

## 16. Flow cytometry of Activated T-cells

T-cells isolated from immunized mice were expanded by PMA + Ionomycin treatment. Brefeldin A (2 ug/mL) was added to the treated flask and untreated flask and cells were incubated for 4 hours in a 37ºC incubator. T-cells were resuspended and washed three times in FACS buffer {1X PBS (pH 7.2) + 1% Fetal bovine serum (FBS)} to remove any residual culture medium. After that, the $1X10^5$ T-cells were resuspended in 100 µl volume of conjugated antibody cocktail for detection of T-cell surface markers (consisting of Th1 phenotype CD4, CD8, and Tbet) which was prepared at 1:300 dilution in FACs buffer. Corresponding isotype controls were also prepared at 1:300 dilution in FACs buffer. The washed T-cells were suspended in either DC surface marker's antibody

cocktail or in isotype-antibody cocktail and incubated for 60 min on ice ($4^0$C) in the dark. The labelled cells were again washed with FACs buffer. T-cells labelled with surface antibodies were fixed in 4% paraformaldehyde for 15 min at RT and washed by centrifugation with excess FACs buffer. For intracellular labeling of cytokines, antibodies and their corresponding isotype controls were prepared in pre-chilled Phosflow Buffer™ (BD Biosciences) at 1:300 dilution and keep it on ice. Fixed T-cells were centrifuged, and 4% paraformaldehyde was discarded by gentle flicking and washed one time with FACs buffer. Fixed T-cells were permeabilized by adding 100 ul of PhosFlow Buffer™ in the dark and on ice for 30 min. T-cells were centrifuged and the permeabilization buffer was decanted. For intracellular cytokine staining, 100 ul of IL2, IL12, TNFα and IFNγ (eBioscience) antibody cocktail or isotype control antibody cocktail added to T-cells and incubated for 60 min on ice ($4^0$C) in the dark. The labelled cells were again washed with FACs buffer and analyzed using a BD LSR-II Green Flow Cytometer (BD Biosciences). FlowJo® and BD FACSDIVA software were used to analyze the data.

## 17. Immunohistochemistry and Immunofluorescence staining of tumor tissue sections

The miniMUC4-expressing and vector control murine subcutaneous tumor sections were evaluated for PD-L1 expression by immunohistochemistry (IHC) and for CD4 and CD8 T-cell's infiltration by immunofluorescence (IF). Harvested tumors were fixed in 10% neutral buffered formalin, embedded in paraffin, and 4 µm thick sections were placed on slides. After deparaffinizing with xylene and rehydrating with decreasing alcohol gradients with final rehydration in MilliQ water, for IHC staining, tumor sections were treated with 5% $H_2O_2$ in 100% methanol for 1 hour at room temperature in the dark for quenching of endogenous peroxidases. For IF, tumor sections were treated with 100% methanol for 1 hour at room temperature in the dark for fixing the tissue. Antigen retrieval

was done using Tris-EDTA buffer, pH 9.0 in the microwave (1100W) at high power for 15 min. For simple hematoxylin and eosin staining of tumor tissue sections, tissue slides were counterstained with hematoxylin and eosin, followed by dehydration with increasing alcohol gradients, xylene washes, and mounting with a cover slip. All hematoxylin and eosin-stained slides were shown to the pathologist for analysis and quantification.

Tissues were blocked with 2.5% normal horse serum for 3 hours, and then incubated with a primary anti-mouse PD-L1 antibody (cc-50298, Santa Cruz Biotechnology Inc.) at 1:200 dilution in Tris-buffered saline (TBS) at 4°C overnight. For T-cell's IF staining, two separate tissue sections were incubated with anti-mouse CD4 or CD8 antibodies (Thermo Fisher Scientific) respectively at 1:400 in PBS at 4°C overnight. PD-L1 stained slides were rinsed with TBST and T-cells stained slides were washed with PBST. After wash, PD-L1 labeled slides were incubated with an HRP-conjugated goat anti-rabbit secondary antibody for 1 hour. T-cell's labeled sections were incubated with Alexa Fluor 488 conjugated goat-anti-mouse secondary antibody for 1 hour in the dark. IHC slides were rinsed with 2 washes of TBST and 3 washes of TBS, and subsequently, using DAB substrate kit (Vector Laboratories) the color was developed. Sections were counterstained with hematoxylin, followed by dehydration with increasing alcohol gradients, then xylene washes and mounting with a cover slip. IF slides were again washed with 4 washes with PBST and two washes with PBS, followed by covering the section with a glass slide and the anti-fade VECTASHIELD mounting media (Vector Laboratories Inc., Burlingame, CA, USA). IF staining was observed under the Zeiss 510 LASER SCAN confocal microscope and quantified by ImageJ software (https://imagej.nih.gov/).

## 18. Statistical Analysis

For our autoantibodies analysis, the differences among group means were tested by one-way analysis of variance (ANOVA) F-test using JMP® Data Analysis Software (SAS Institute Inc., NC). If the F-test was significant, paired Wilcoxon sign rank t-tests were performed for pairwise comparisons of group means. Significance was defined as $p < 0.001$.

For our nanovaccine *in vitro*, *ex vivo* and *in* vivo studies differences among group means were tested by one-way analysis of variance (ANOVA) F-test using GraphPad Prism v. 7.0 (GraphPad, La Jolla, CA). If the F-test was significant, Tukey's t-tests and Student's t-tests were performed for pairwise comparisons of group means. Significance was defined as $p < 0.05$.

## 19. Table I: List of antibodies

| S.No. | Antibodies | Company | Catalogue No. |
|---|---|---|---|
| 1 | Alexa Fluor® 700 anti-mouse CD11c (clone N418) | eBioscience (San Diego, CA) | 56-0114-80 |
| 2 | FITC conjugated anti-mouse/rat MHC Class II (I-Ek, clone 14-4-4S) | eBioscience (San Diego, CA) | 11-5980-81 |
| 3 | (APC) anti-mouse CD40 (clone 1C10) | eBioscience (San Diego, CA) | 17-0401-81 |
| 4 | (PE)-Cy7 conjugated anti-mouse F4/80 (clone BM8) | eBioscience (San Diego, CA) | 25-4801-82 |
| 5 | Anti-Mouse MHC Class I (H-2Kd) eFluor® 450 (Clone: SF1-1.1.1) | eBioscience (San Diego, CA) | 48-5957-80 |
| 6 | Alexa Fluor® 700 conjugated Armenian hamster IgG (clone eBio299Arm) | eBioscience (San Diego, CA) | 56-4888-80 |
| 7 | FITC IgG2a κ (clone eBR2a) | eBioscience (San Diego, CA) | 11-4321-80 |
| 8 | Rat IgG2a K Isotype Control APC (clone eBR2a) | eBioscience (San Diego, CA) | 17-4321-41<br>17-4321-81 |
| 9 | PE-conjugated rat IgG2a (clone eBR2a) | eBioscience (San Diego, CA) | 12-4321-80 |
| 10 | Rat IgG2a K Isotype Control PE-Cyanine7 (clone eBR2a) | eBioscience (San Diego, CA) | 25-4321-81 |
| 11 | PE/Cy7 anti-mouse CD86 (Clone GL1) | BD Biosciences | 560582 |
| 12 | PE conjugated anti-mouse CD80 (clone 16-10A1) | eBioscience (San Diego, CA) | 12-0801-81 |

| | | | |
|---|---|---|---|
| **13** | **PE/Cy 5.5 antimouse CD205 (MMR, clone NLDC-145)** | **BioLegend** | **138207** |
| **14** | **PerCP/Cy5.5 Rat IgG2a, κ Isotype Ctrl Antibody** | **BioLegend** | **400531** |
| **15** | **Rat IgG2a K Isotype Control eFluor® 450 (clone eBR2a)** | **eBioscience (San Diego, CA)** | **48-4321-80** |
| **16** | **T-bet (Anti-Human/Mouse T-bet PerCP-Cyanine5.5** | **eBioscience (San Diego, CA)** | **45-5825-80** |
| **17** | **GATA-3 (Anti-Human/Mouse Gata-3 eFluor® 660)** | **eBioscience (San Diego, CA)** | **50-9966-41** |
| **18** | **CD4 (Anti-Mouse CD4 eFluor® 450)** | **eBioscience (San Diego, CA)** | **48-0042-80** |
| **19** | **CD8 (Anti-Mouse CD8a PE)** | **eBioscience (San Diego, CA)** | **12-0081-81** |
| **20** | **IL-2 (Anti-Mouse IL-2 PE-Cyanine7)** | **eBioscience (San Diego, CA)** | **25-7021-80** |
| **21** | **IL-10 (Anti-Mouse IL-10 FITC)** | **eBioscience (San Diego, CA)** | **11-7101-41** |
| **22** | **IL-12 (Anti-Mouse IL-12/IL-23 p40 Alexa Fluor® 488)** | **eBioscience (San Diego, CA)** | **53-7123-80** |
| **23** | **IL-13 (Anti-Mouse IL-13 PE-Cyanine7)** | **eBioscience (San Diego, CA)** | **25-7133-80** |
| **24** | **IFNγ (Anti-Mouse IFN gamma APC)** | **eBioscience (San Diego, CA)** | **17-7311-81** |
| **25** | **Rat IgG2b Isotype Control eFluor® 660** | **eBioscience (San Diego, CA)** | **50-4031-80** |
| **26** | **Rat IgG2a K Isotype Control Alexa Fluor® 488** | **eBioscience (San Diego, CA)** | **53-4321-80** |

| | | | |
|---|---|---|---|
| **27** | **Rat IgG2b K Isotype Control FITC** | **eBioscience (San Diego, CA)** | **11-4031-81** |
| **28** | **Rat IgG1 K Isotype Control eFluor® 450** | **eBioscience (San Diego, CA)** | **48-4301-80** |
| **29** | **CD4 Monoclonal Antibody (RIV6)** | **ThermoFisher Scientific** | **MA1-7631** |
| **30** | **CD8 Monoclonal Antibody (RIV11)** | **ThermoFisher Scientific** | **MA1-7632** |
| **31** | **Pdcd-1L1 (H-130) {Discontinued Antibody}** | **SantaCruz Biotechnology INC.** | **Sc-50298** |
| **32** | **Goat Anti-mouse IgG2b-HRP** | **ThermoFisher Scientific** | **M32407** |
| **33** | **Goat Anti-mouse IgG1-HRP** | **ThermoFisher Scientific** | **A10551** |
| **34** | **Peroxidase AffiniPure Goat Anti-Human IgM, Fc5μfragment specific** | **Jackson ImmunoResearch Laboratories, INC.** | **109-035-129** |
| **35** | **Peroxidase AffiniPure Goat Anti-Human IgG (H+L)** | **Jackson ImmunoResearch Laboratories, INC.** | **109-035-003** |
| **36** | **Peroxidase AffiniPure Goat Anti-Human IgA + IgG + IgM (H+L)** | **Jackson ImmunoResearch Laboratories, INC.** | **109-035-064** |
| **37** | **Peroxidase AffiniPure Goat Anti-Human Serum IgA, α Chain Specific** | **Jackson ImmunoResearch Laboratories, INC.** | **109-035-011** |
| **38** | **Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488** | **ThermoFisher Scientific** | **A-11001** |
| **39** | **Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP** | **ThermoFisher Scientific** | **31430** |
| **40** | **Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP** | **ThermoFisher Scientific** | **31460** |

**Supplemental Table 1. Patient characteristics, all subjects**

| | | PC (n=22) | Healthy (n=27) | CP (n=21) | p-value |
|---|---|---|---|---|---|
| Age | Median (range) | 71 (56-85) | 64 (28-90) | 54 (30-81) | 0.0003 |
| Gender | Female<br>Male | 9 (41%)<br>13 (59%) | 18 (67%)<br>9 (33%) | 8 (38%)<br>13 (62%) | 0.12 |
| Race | African American<br>Caucasian | 1 (5%)<br>21 (95%) | 1 (4%)<br>26 (96%) | 5 (24%)<br>16 (76%) | 0.090 |
| Stage | I/II<br>III/IV | 12 (55%)<br>10 (45%) | - | - | |

**Supplemental Table 2. Patient characteristics, limited subjects**

| | | PC (n=22) | Healthy (n=10) | CP (n=10) | p-value |
|---|---|---|---|---|---|
| Age | Median (range) | 71 (56-85) | 62 (33-86) | 48.5 (30-81) | 0.016 |
| Gender | Female<br>Male | 9 (41%)<br>13 (59%) | 6 (60%)<br>4 (40%) | 5 (50%)<br>5 (50%) | 0.61 |
| Race | African American<br>Caucasian | 1 (5%)<br>21 (95%) | 0<br>10 (100%) | 2 (20%)<br>8 (80%) | 0.27 |
| Stage | I/II<br>III/IV | 12 (55%)<br>10 (45%) | - | - | |

# CHAPTER 3: MUC4 AS A VACCINE CANDIDATE FOR PC IMMUNOTHERAPY

The material covered in this chapter is the subject of a research article

**2. Banerjee K.**, Kshirsagar P., Kaur S., Brand RE., Smith L., Batra SK., and Jain M. *Presence of MUC4 autoantibodies in the serum of pancreatic cancer patients.* (Manuscript under Preparation)

# 1. Synopsis

A major challenge in pancreatic cancer (PC) immunotherapy is identifying tumor-associated-antigens (TAAs) that can be targeted. In PC, Mucin-4 (MUC4) is differentially overexpressed and aberrantly glycosylated, thus potentially serving as a source of neo-antigenic epitopes, which could be exploited for cancer immunotherapy. Literature review has shown that an immune response developed in the form of autoantibodies to various tumor antigens could serve as a surrogate for immunogenicity (and compromised self-tolerance) in cancer patients. The presence of MUC4 autoantibodies was analyzed in serum samples taken from patients at the time of pancreatic cancer diagnosis prior to any treatments, from patients with chronic pancreatitis, and healthy controls. By using indirect-ELISA techniques we were able to detect autoantibodies against recombinant human MUC4-beta (MUC4β) protein in 77.41% of PC patient's sera, whereas only 33.33% of age-matched healthy controls and 23.81% of chronic pancreatitis (CP) patients had positive sera. In addition, we used a panel of 7 MUC4-alpha region peptides and 4 MUC4β peptides to identify highly immunogenic MUC4 epitopes. Furthermore, isotyping these autoantibodies in PC patient sera indicates that IgM antibodies to peptides A2, D4 and B1 correlate with better prognosis. Our novel study suggests that MUC4 expressed by pancreatic tumors is immunogenic in PC patients and could be used as a target for cancer immunotherapy. Further these studies suggest that segregation of PC patients based on the presence of MUC4 autoantibodies against multiple MUC4 peptide, could be helpful in identifying patients for personalized MUC4-based immunotherapy treatment of pancreatic cancer.

## 2. Background and Rationale

Pancreatic cancer (PC) is a fatal disease with a poor 5-year overall survival of merely 8% [1]. Conventional therapies for PC such as chemotherapy, radiation therapy and surgery have fared with limited success, thus necessitating the need to develop alternative treatment strategies [2, 3]. Immunotherapy has emerged as a viable option but had stunted success in the efficacious treatment of pancreatic cancer patients [4, 5]. PC has limited genetic mutations, notably at the *KRAS* codon position with 12 mutations, and bear at the maximum 4000 potential immunogenic neoantigens, whereas melanoma has a predicted neoantigen count of 14000 that corroborates with success seen in melanoma immunotherapy [6-8]. Thus, one of the major challenges in PC immunotherapy is the identification of tumor-associated antigens (TAAs) that could be specifically targeted to cancer with negligible side-effects on healthy cells/organs.

TAAs expressed by cancer cells are mostly recognized as self-antigen due to the overriding of the immune system by immunoediting, therefore establishing a potent cancer-mediated tolerance against its TAAs [3, 9, 10]. Abnormal expression of antigens by cancer cells being recognized by immune surveillance that leads to the generation of autoantibodies is reflective of the immune response generated in cancer patients [10]. Expression of the antigen at the aberrant location where it is neither expressed in normal organ nor during other malignancy affecting that organ makes it a lucrative target for immune cells. In addition, post-translational modifications of such antigens affect their processing and loading on major histocompatibility complex (MHC molecules). The modified antigen-loaded MHC interacts with T cell receptors (TCRs), hence activating $CD4^+$ T helper cells to mount a humoral autoantibody response, overriding immunological tolerance against the TAA [10, 11].

Mucin 4 (MUC4), a high molecular weight glycoprotein, is aberrantly overexpressed by and glycosylated in PC cancer cells, whereas it is negligibly present in normal pancreas and chronic pancreatitis (CP) patients [12-14]. Presence of autoantibodies against the VNTR region of another aberrantly overexpressed mucin in PC, Mucin 1 (MUC1), has been detected in platinum based drug resistant lung and ovarian cancers [15, 16], but there were no significant increase in MUC1 autoantibodies detected in PC patients when compared to age-matched controls [17]. Unlike MUC1, the MUC4 expression is restricted in most of the normal organs except for low levels being expressed in urogenital tracts and trachea in lungs [18]. In addition, autoantibodies against MUC4-TR region glycopeptides have been detected in colorectal cancer patients [19]. Thus, we hypothesized that MUC4 could be potentially immunogenic because of its spatial expression and post-translational modifications leading to tolerance against MUC4 being compromised in PC patients.

MUC4 has a potential autocatalytic Gly-AspPro-His (GDPH) cleavage site that generates the N-terminal MUC4α subunit (containing the TR region) and a membrane-tethered MUC4β subunit [20-22]. In contrast to the published studies on MUC4 autoantibodies [19], our study encompassed the entire length of MUC4 to demonstrate that MUC4 is immunogenic in PC patients. This design provided us a unique opportunity to assess the development of the humoral response to the entire protein backbone of MUC4 in PC patient serum. Due to the large molecular weight of MUC4α we were unable to express and purify the recombinant protein in a bacterial system. We successfully purified the MUC4β recombinant protein, which was used in our autoantibody study. Our lab is interested in utilizing MUC4 to develop an immunotherapy strategy to treat PC. Therefore, we also predicted potential major histocompatibility complex-I (MHC-I) HLA-binding immunogenic peptides using a reverse immunology approach [23] across the

entire protein sequence of MUC4. Most of the available studies on cancer vaccine immunotherapies primarily focus on HLA-binding peptides to induce T-cell mediated responses. Humoral response analysis against such peptides have not been investigated and thus excludes an equivalently crucial arm from factoring in the overall clinical outcome status of vaccine-immunized patients. We predicted the B-cell probability score for the same peptides and were keen to observe if an autoantibody signature was present against these T-cell peptides. Our study reported the presence of circulating autoantibodies against both MUC4β protein and MUC4 peptides present in PC patient serum when compared to CP patient's and healthy individual's serum. This interesting observation suggests that MUC4 protein could potentially stimulate both T-cells and B-cells that may affect the overall clinical outcome. Further, we elucidated the various isotypes of autoantibodies present produced against MUC4 in PC patients. In addition, since the humoral response against MUC4 serves as a surrogate of compromised tolerance, we stratified and predicted patients who could have a higher probability of response to personalized MUC4-based immunotherapy.

# 3. Results

## 3.1 Analysis of Circulating MUC4 Autoantibody in PC patients

The primary aim of this study was to develop a novel platform for selective detection of circulating autoantibodies against the human MUC4β recombinant protein. To our knowledge we are the first group to purify a recombinant human MUC4β protein, which we have been utilized to develop the assay. Circulating autoantibodies against recombinant human MUC4β protein was observed to be upregulated in PC patients as compared to healthy individuals and chronic pancreatitis patients. The low level of antibody positive sera and high level of antibody positive sera was defined at its cut-off values of <1.283 and ≥1.283 respectively **(Figure 1A)**. Based on the cut-off values we

were able to determine that 76.7% of PC patients had high levels of MUC4 autoantibodies, whereas only 37% of healthy and 28.6% of CP patients showed high levels of MUC4β autoantibodies **(Figure 1A)**. We didn't observe a significant advantage in survival between PC patients with high levels of MUC4 autoantibodies and low levels of MUC4 autoantibodies **(Figure 1B)**. Further we performed stage-wise analysis and observed that 86.7% of early-stage PC patients and 66.7% of late-stage patients had circulating MUC4 autoantibodies in their sera **(Figure 1C)**. PC patient survival days analysis demonstrated that patients with higher serum reactivity had mean survival days (MSD) of 179 days for Stage 1-II and 309 days for Stage II-IV, compared to the patients with lower serum reactivity had MSD of 131 days for Stage 1-II and 244 days for Stage II-IV **(Figure 1C)**.

### 3.2 Bioinformatics Analysis Predicts Potential MUC4 Peptides

Due to the difficulty in purification of the MUC4α domain, we predicted potential adequate HLA-binding immunogenic MHC-I peptides inside both MUC4α and MUC4β domains to capture the entire MUC4 protein. A prerequisite for an immune response to arm, immunogenic peptides of MUC4 must be loaded on major histocompatibility complexes that would be cross-represented to effector immune cells (i.e. T-cells and B-cells). HLA-A2 is one of the widely used HLA-subtype for peptide predictions that have been used in designing vaccinations for melanoma and lung cancer [29, 37-39]. We used 3 prediction tools NetCTL1.0 and IEDB (Tepi Tool and Peptide processing) for determining HLA A2-binding T cell epitopes. NetCTL1.0 (based on a combined prediction of peptide MHC binding, proteasomal C terminal cleavage and TAP transport efficiency) software analyzed MUC4 sequence and predicted potential HLA-binding 9-mer amino acid sequences scored across 10 major human HLA-A2 isoforms. Peptides with combined high scores have higher specificity, sensitivity and higher affinity binding to HLA-A2 MHC-I complex. We further analyzed these peptides using IEDB (http://tools.iedb.org/tepitool/)

prediction TepiTool which is based on consensus method employing artificial neural network, combinatorial library networks and SMM-align. TepiTool scored the NetCTL1.0 predicted peptides based on their likelihood to bind to human HLA-A2*02.1 (mostly used in vaccine studies [39]), as well as, C57BL/6 mouse HLA-$D^b$ and HLA-$K^b$ MHC-I isotypes. In addition IEDB Processing (http://tools.iedb.org/processing/) analysis, which scores based on proteasomal C terminal cleavage, TAP transport efficiency and MHC-I complex loading, ranked these predicted peptides for both mouse and human HLA MHC-I loading. Lower predicted rank score suggests a higher probability of these peptides to be loaded on MHC-I complex and cross-presented to T-cells for activating them in an antigen-specific manner **(Figure 2)**.

Our autoantibody analysis demonstrated MUC4β specific antibodies being generated specifically in PC patients. Bioinformatics analysis predicted MHC-I peptides in MUC4β region that made us curious to analyze the potential of these peptides to serve as B-cell immunogenic epitopes. For the same, we ran and scored these peptides on the IEDB B-cell prediction tool (http://tools.iedb.org/bcell/) and performed BCPred analysis (http://ailab.ist.psu.edu/bcpred/predict.html). Our B-cell epitope analysis showed that there was a difference in predicted scores between each peptide. Only 13 out of 24 predicted peptides were picked up by both prediction software and scored for the potential of them getting recognized by B-cells. We ranked these peptides based on their BCPred scores **(Figure 3)**, and randomly selected peptides that represent all differentially scored B-cell epitope regions of both MUC4α and MUC4β to elucidate the plausibility of autoantibodies generated against these predicted peptides.

### 3.3 Screening of Autoantibody Signature against MUC4 Peptides

Our initial experiment elucidated the presence of autoantibodies against a MUC4β recombinant protein which provides a large repertoire of epitopes that could be recognized

by B cells. In addition, our bioinformatics analysis predicted peptides that have the potential to serve as both T-cell and B-cell epitopes. To validate our observations, we developed a modified indirect ELISA to detect autoantibodies if any, generated against these epitopes. Our ELISA studies revealed differential serum reactivity to each of the 7 MUC4α peptides whereas significantly low/ reactivity was seen against TR and PD2 peptides and 5 µg/ml of BSA protein **(Figure 4A)**. Further, we were curious to understand whether any peptide specifically fared better when compared to the survival status of PC patients. Our study showed that patients with high serum reactivity to peptides D3 and F3 survived longer than their low serum reactivity counterparts, but the rest of the 5 MUC4α peptides didn't show similar relationship **(Figure 4B)**.

Similarly, PC patient serum significantly reacted with all 4 peptides from MUC4β region and low reactivity was observed with control peptides and protein **(Figure 5A)**. In addition, high serum reactivity to MUC4β peptides didn't provide a survival advantage **(Figure 5B)**. Thus, our study shows for the first time that MUC4 MHC-I HLA-binding T-cell peptides could potentially be recognized by B-cells and might have a dual (activate both cellular and humoral immune pathway) epitope property. Further, we stratified PC patients with high autoantibody levels detected in their sera and observed that we could potentially design a selection strategy to elucidate patients who likely might have compromised peripheral tolerance **(Table I)**. To ensure the specificity of the detected antibodies, we compared the serum reactivity of randomly selected 10 healthy and 10 chronic pancreatitis individuals and we observed that PC patient specific reactivity was seen to all 11 MUC4 peptides and negative in healthy or CP individuals **(Supplemental Figure 1 & 2)**.

### 3.4 Isotype Analysis of MUC4 Autoantibodies in PC Patients

Autoantibody isotypes such as IgM autoantibodies in cancer patient serum have been shown to correlate with poor prognosis and overall survival of those patients [15].

Activation of B-cells to its mature form after receiving secondary stimulation from $CD4^+$ T-cells induces isotype switching to generate various classes of antibodies with the same variable antigen binding region as the original antibody generated to the antigen by the VD (J) recombination pathway [40, 41]. Our observations demonstrated that autoantibodies are present against both recombinant MUC4 protein and its peptides, but higher levels of these autoantibodies didn't significantly correlate with patient survival data. Isotyping of mucin autoantibodies could provide some insight into understanding whether activation of B-cells confers any survival advantage to PC patients. We measured different isotypes (IgA, IgM and IgG) of MUC4 autoantibodies present in PC patient sera and correlated with overall patient survival and prognosis. All three MUC4β protein autoantibody isotypes were detected, however presence of IgG isotype autoantibodies in serum segregated PC patients in two groups. Despite the segregation, we didn't observe any significant correlation between autoantibody isotypes and patient survival **(Supplemental Figure 3)**.

Isotype analysis of autoantibodies against MUC4α peptides demonstrated differential levels of isotypes present in PC patient serum **(Figure 6A)**. Our data further elucidated that high levels of IgM autoantibody against A2 and D4 MUC4α peptides correlate strongly with overall patient survival ($P<0.001$) **(Figure 6B)**. Analysis of MUC4β peptides also demonstrated that all isotypes were detected **(Figure 7A)**. Similarly, Kaplan-Meier graph shows that high levels of IgM autoantibody to B1 peptide correlated strongly with PC survival **(Figure 7B)**. Our data showed that MUC4 is not only immunogenic in PC patients but also induces maturation of B-cells. Interestingly, for all MUC4 peptides, patients with IgM isotype autoantibodies had better median survival than patients with either IgG or IgA autoantibodies **(Supplemental Figure 4)**.

# 4. Discussion

The present study investigated the possibility of MUC4 being immunogenic in PC patients. We developed a modified ELISA strategy and tested both MUC4 recombinant protein and peptides to determine the presence of autoantibodies in patient sera. MUC4 is aberrantly overexpressed in PC patients and its expression increases with gradual progression of the malignancy [42]. MUC4 overexpression and aberrant glycosylation in PC and its absence in the normal pancreas [14] makes it a potential antigen that might get recognized by the immune system (specifically by B-cells). The modified indirect ELISA discovery platform for detecting human serum autoantibodies was developed for efficient antigen-antibody binding and provide a remarkably low background. Cancer-associated autoantibodies to recombinant MUC4β protein were identified in PC patient sera, whereas healthy individuals and CP patients were negligible for MUC4 autoantibodies, although survival analysis didn't reveal any robust correlation with levels of autoantibodies present in PC patient sera. Presence of circulating MUC4 autoantibodies in PC patients therefore supports the hypothesis that immunological tolerance is compromised against MUC4 and gets recognized by B-cells.

In our study, we utilized both recombinant protein and peptides to investigate whether different domains/regions of MUC4 is immunogenic other than just the TR region sequence that is well studied. Bioinformatics analysis predicted HLA-binding peptides that have potential to serve as a dual epitopes for both cellular and humoral immune pathways in human as well as in mouse. To capture MUC4α region we randomly selected peptides corresponding to that region due to unavailability of purified recombinant MUC4α protein. The rationale behind using peptides of MUC4β region was to recognize immunodominant region present in MUC4β protein and it was investigated on a peptide-based ELISA platform. Our study revealed that only PC patient serum contained autoantibodies against

MUC4 peptides, whereas there was negligible reactivity detected in control individuals. Our patient stratification strategy based on their serum reactivity with respective MUC4 peptides elucidate that the tolerance against each peptide is compromised in patient-to-patient basis. Such case basis-compromised tolerance might correlate with the differential response of PC patients to MUC4-based immunotherapy that needs to be investigated in the future.

Detection of specific autoantibody isotypes suggests activation and maturation of B-cells leading to the systemic availability of neutralizing antibodies protecting against cancer, which could be translated to the observed negative correlation of immature IgM antibodies with the survival of patients [15, 19]. Our data in contrast showed that high levels of IgM autoantibodies against A2, D4 and B1 have a statistically strong correlation with longer survival of PC patients. Further, PC patients with high levels of IgM autoantibodies have better survival than those who have IgG/IgA circulating autoantibodies, thus making a case for a better understanding of the role of humoral responses in the survival of patients.

Overall our studies provide enough evidence to suggest that entire MUC4 is immunogenic and peripheral tolerance to MUC4 is compromised in PC patients. In a recent study, it has been shown that $CD8^+$ T cell-specific MUC16 neoantigen epitopes provide a survival advantage to PC patients [43]. Interestingly our data for the first time reveals that T-cell epitopes located on various regions of the MUC4 protein sequence (excluding VNTR region), could simultaneously be recognized by B-cells and activate the humoral arm of the immune system. Further, circulating IgM MUC4 autoantibodies against particular peptides, showed to provide protection to the PC patients and correlates with increased overall survival status. Cancer vaccines have emerged as an alternative treatment modality for cancer patients who respond poorly to traditional therapies. An

efficient vaccine-mediated immunity requires strong activation of both humoral (antibody and memory B-cell generation) and cellular responses (activation of both $CD4^+$ helper T-cells, $CD8^+$ cytotoxic T cells and memory T-cells), which provides optimal protection from the disease [44]. We propose that the dual antigenic (activate both B & T cells) epitope quality and aberrant expression of MUC4 in all the stages of PC tumor development qualifies it to be a strong candidate for immunotherapy strategies like cancer vaccines.

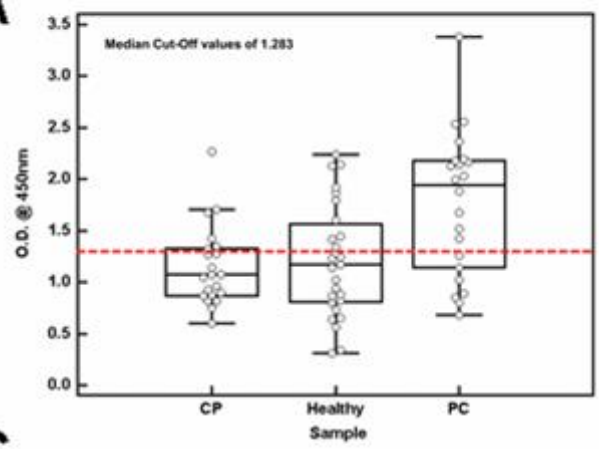
**Table I: Autoantibodies reactivity against MUC4 peptides can predict patients with compromised tolerance**

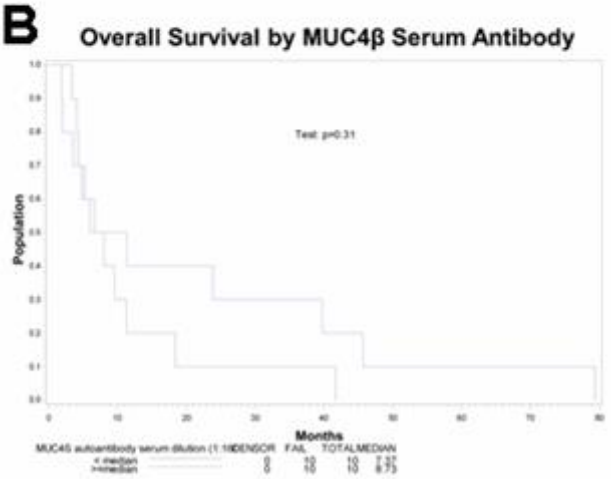
| Patient No. | Stage | Alpha peptide recognized | Beta peptide recognized |
|---|---|---|---|
| 12 | IIB | A2, E4, D3, C4, F1, F3 | B1, B3, B4, E3 |
| 6 | III | A2, E4, D3, C4, F1 | B1, B3, B4, E3 |
| 18 | IIB | A2, C4, D4, F3 | B1, E3 |
| 5 | IIB | A2, E4, F3 | B1, B4 |
| 7 | IV | E4, D3, C4, F1 | B4 |
| 9 | IIA | A2, D4, F1, F3 | B1 |
| 30 | III | A2, E4 | B4, E3 |
| 16 | IV | E4 | B3, B4 |
| 25 | IIB | D4, C4 | B3 |
| 22 | IIB | F3 | E3 |
| 15 | IIA | D4, F1, F3 | |
| 3 | III | D4, F1 | |
| 24 | IV | C4 | |
| 10 | IV | | B1 |
| 20 | IIB | | E3 |

**Figure 1: Autoantibodies against MUC4β were detected in PC patient serum.**

Indirect ELISA study revealed that PC patients had high expression of circulating autoantibodies to recombinant human MUC4β protein (A). The high levels of MUC4 autoantibodies didn't correlate with PC survival as seen in Kaplan-Meier graph (B). Further analysis showed that patients with high levels of MUC4 autoantibodies, both at early (Stage I-II) and late (Stage IV) stages of PC, had longer survival than patients with low levels of autoantibodies (C).

# Figure 1




C

| | | Diagnosis | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | PC (stage I-II) | | PC (stage III- IV) | | CP | | Healthy | |
| | | MUC4.strat | | MUC4.strat | | MUC4.strat | | MUC4.strat | |
| | | High Expression | Low Expression | High Expression | Low Expression | High Expression | Low Expression | High Expression | Low Expression |
| Age | N | 13 | 2 | 10 | 5 | 6 | 15 | 10 | 17 |
| | Median | 83 | 62 | 71 | 71 | 55 | 54 | 58.5 | 65 |
| Survival time (days) | N | 13 | 2 | 10 | 5 | | | | |
| | Median | 179 | 131 | 309 | 244 | | | | |

| | | Stage | | | | Stage | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | stage IIA | | stage IIB | | stage III | | stage IV | |
| | | Stage | | | | MUC4.strat | | MUC4.strat | | MUC4.strat | | MUC4.strat | |
| | | stage IIA | stage IIB | stage III | stage IV | High Expression | Low Expression | High Expression | Low Expression | High Expression | Low Expression | High Expression | Low Expression |
| Age | N | 4 | 10 | 4 | 11 | 4 | 1 | 9 | 1 | 2 | 2 | 8 | 3 |
| | Median | 82 | 71 | 70 | 66 | 83 | 62 | 71 | 71 | 69 | 74.5 | 67 | 59 |
| | Interquartile Range | 22 | 14 | 12.25 | 14 | 2 | 0 | 17 | 0 | 6 | 13 | 18.75 | 17 |
| Survival time (days) | N | 1 | 10 | 4 | 10 | 1 | 0 | 9 | 1 | 2 | 2 | 7 | 3 |
| | Median | 204 | 169 | 130.5 | 326.5 | 204 | . | 179 | 131 | 115 | 685.5 | 347 | 244 |
| | Interquartile Range | 0 | 304.75 | 912.75 | 582.75 | 0 | . | 425.5 | 0 | 88 | 1167 | 1063 | 222 |

**Figure 2: Bioinformatics analysis predicted T-cell immunodominant epitopes of MUC4.**

NetCTL1.0 and IEDB software analysis based on combined prediction of peptide MHC binding, proteasomal C terminal cleavage and TAP transport efficiency, predicted and ranked T-cell immunodominant epitopes. Lower predicted rank score suggests a higher probability of these peptides to be loaded on MHC-I complex and cross-represented to T-cells for activating them in an antigen-specific manner.

# Figure 2

| Name | Peptide Seq | Human T cell Score (NetCTL 2.0) | Human MHC-I (HLA-A*02:01) TepiTool Percentile rank | Human MHC-I (HLA-A*02:01) Peptide Processing rank | Murine MHC-I (H-2-Kb & H-2- Db) TepiTool Percentile rank | Murine MHC-I (H-2-Kb & H-2-Db) Peptide Processing rank |
|---|---|---|---|---|---|---|
| A2 | MMTSEKITV | 0.5474 | 1.8 | -0.52 | H-2-Kb : 8.1<br>H-2-Db : 16.0 | H-2-Kb : -2.62<br>H-2-Db : -2.74 |
| E1 | QATDTFSTV | 0.4943 | 14 | -2.64 | H-2-Kb : 3.45<br>H-2-Db : 17.0 | H-2-Kb : -2.56<br>H-2-Db : -3.05 |
| F4 | TLANSVVST | 0.2844 | 6.4 | -2.15 | H-2-Kb : 52<br>H-2-Db : 63 | H-2-Kb : -3.60<br>H-2-Db : -3.83 |
| C3 | AAMTHTHQA | 04335 | 8.4 | -2.80 | H-2-Kb : 10.1<br>H-2- Db : 2.1 | H-2-Kb : -2.78<br>H-2-Db : -2.43 |
| E2 | SVHNVTGTV | 0.5395 | 5.5 | -2.35 | H-2-Kb : 11.1<br>H-2- Db : 16.0 | H-2-Kb : -2.52<br>H-2-Db : -3.19 |
| D4 | VMETVTQET | 0.4484 | 15 | -3.02 | H-2-Kb : 33.5<br>H-2- Db : 37.0 | H-2-Kb : -3.58<br>H-2-Db : -3.77 |
| C4 | NVMETVTQE | 0.2021 | 30 | -4.12 | H-2-Kb : 43<br>H-2- Db : 48 | H-2-Kb : -4.23<br>H-2-Db : -4.31 |
| A1 | MMTSKTITM | 0.5697 | 3.6 | -0.86 | H-2-Kb : 1.35<br>H-2- Db : 3.2 | H-2-Kb : -1.98<br>H-2-Db : -2.25 |
| F1 | SVHNVMETV | 0.5137 | 6.7 | -1.36 | H-2-Kb : 12.6<br>H-2- Db : 5.7 | H-2-Kb : -2.36<br>H-2-Db : -2.65 |
| D2 | SVIQMIKTA | 0.3433 | 14.0 | -2.58 | H-2-Kb : 18.0<br>H-2- Db : 5.3 | H-2-Kb : -2.93<br>H-2-Db : -3.03 |
| F2 | TLTQMMTST | 0.2905 | 8.3 | -3.34 | H-2-Kb : 44.5<br>H-2- Db : 32.0 | H-2-Kb : -3.97<br>H-2-Db : -4.22 |
| C2 | HTSQQITTA | 0.6948 | 23 | -3.49 | H-2-Kb : 77.5<br>H-2- Db : 29.0 | H-2-Kb : -3.66<br>H-2-Db : -3.67 |
| D3 | HTTQSTTEL | 0.8685 | 22 | -2.71 | H-2-Kb : 26.5<br>H-2- Db : 8.7 | H-2-Kb : -2.56<br>H-2-Db : -2.49 |
| C1 | MTSKTITMT | 0.7004 | 21 | -3.66 | H-2-Kb : 41<br>H-2- Db : 61 | H-2-Kb : -3.78<br>H-2-Db : -4.05 |
| A3 | VTSAALVTV | 0.6861 | 4.4 | -1.03 | H-2-Kb : 9.1<br>H-2- Db : 16.0 | H-2-Kb : -2.42<br>H-2-Db : -2.98 |
| B3 | VVEAFLYHV | 0.5953 | 2.7 | -0.74 | H-2-Kb : 1.15<br>H-2- Db : 26.0 | H-2-Kb : -1.85<br>H-2-Db : -3.32 |
| B4 | QLLAAVVEA | 0.3776 | 1.8 | -0.71 | H-2-Kb : 59.5<br>H-2- Db : 26.0 | H-2-Kb : -3.32<br>H-2-Db : -3.34 |
| E3 | CLYNQTSRV | 0.4851 | 0.2 | -0.80 | H-2-Kb : 3.3<br>H-2- Db : 35 | H-2-Kb : -2.05<br>H-2-Db : -3.34 |
| A4 | GVSQEIFTL | 0.4879 | 5.4 | -1.07 | H-2-Kb : 30.0<br>H-2- Db : 27.0 | H-2-Kb : -2.30<br>H-2-Db : -2.52 |
| D1 | LVTSKVFRM | 0.4660 | 12.0 | -2.61 | H-2-Kb : 2.8<br>H-2- Db : 25.0 | H-2-Kb : -2.31<br>H-2-Db : -3.32 |
| B2 | TLIQYTSNA | 0.3345 | 3.1 | -1.31 | H-2-Kb : 14.6<br>H-2- Db : 40.0 | H-2-Kb : -2.41<br>H-2-Db : -3.50 |
| B1 | SMAEVNASV | 0.6073 | 0.3 | 0.57 | H-2-Kb : 6.6<br>H-2- Db : 15.0 | H-2-Kb : -2.27<br>H-2-Db : -2.67 |
| F3 | TISAITSKV | 0.5102 | 3.2 | -1.54 | H-2-Kb : 26.3<br>H-2- Db : 62.0 | H-2-Kb : -2.81<br>H-2-Db : -3.29 |
| E4 | AITSKVSTI | 0.4367 | 12.0 | -2.31 | H-2-Kb : 7.1<br>H-2- Db : 11.0 | H-2-Kb : -2.42<br>H-2-Db : -2.86 |

**Figure 3: Bioinformatics analysis predicted B-cell immunodominant epitopes of MUC4.**

IEDB B-cell prediction software analysis based on sequence characteristics of the antigen using amino acid scales and HMMs, predicted and ranked B-cell immunodominant epitopes. High predicted rank score suggests higher probability of these peptides to be recognized by B-cells.

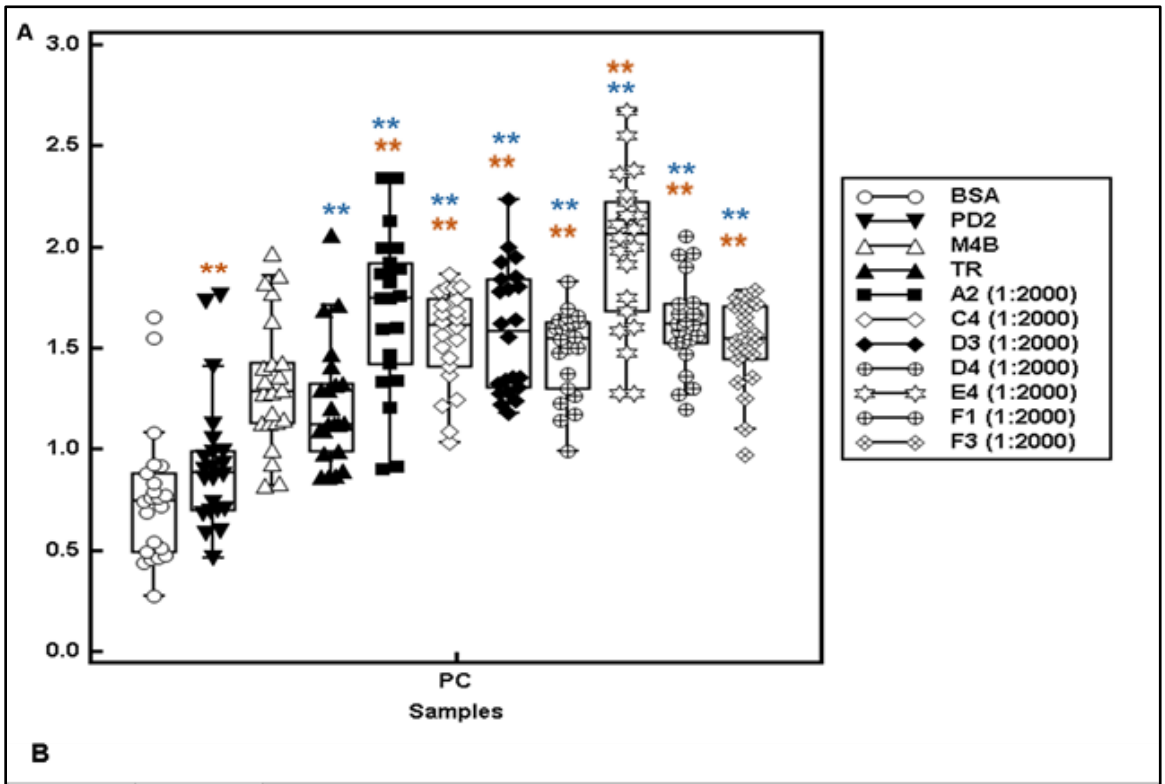
# Figure 3

| Name | Peptide Seq | BCPRED | IEDB (Epitope) |
|---|---|---|---|
| A2 | MMTSEKITV | MMTSEKITVTTSTGSTLGNP (0.999) | - |
| E1 | QATDTFSTV | SQATDTFSTVPPTPPSITSS (0.998) | MAQTQRTGTSRGSDTISLASQATDTFSTVPPTPP SITSSGLTSPQTQTHTLSPSGSGKTF |
| F4 | TLANSVVST | TLANSVVSTPGGPEGQWTSA (0.998) | SVVSTPGGPEGQWTSASASTSPDTAAAMTHTHQ |
| C3 | AAMTHTHQA | SASASTSPDTAAAMTHTHQA (0.997) | SVVSTPGGPEGQWTSASASTSPDTAAAMTHTHQ AESTEASGQT |
| E2 | SVHNVTGTV | SPSVHNVTGTVSQKTSPSGE (0.985) | QTSTLTHRTTSTPSFSPSVHNVTGTVSQKTSPSG ETATSS |
| D4 | VMETVTQET | VHNVMETVTQETAPPDEMTT (0.975) | ETVTQETAPPDEMTTSFPSSVT |
| C4 | NVMETVTQE | VHNVMETVTQETAPPDEMTT (0.975) | ETVTQETAPPDEMTTSFPSSVT |
| A1 | MMTSKTITM | MMTSKTITMTTSTDSTLGNT (0.969) | TSMMTS |
| F1 | SVHNVMETV | SPSVHNVMETVTQETAPPDE (0.966) | - |
| D2 | SVIQMIKTA | YSVIQMIKTATSPSSSPMLD (0.963) | - |
| F2 | TLTQMMTST | TETTSKAQTDTLTQMMTSTL (0.959) | - |
| C2 | HTSQQITTA | MLDRHTSQQITTAPSTNHST (0.958) | TATSPSSSPMLDRHTSQQITTAPSTNHSTIHSTSTS P |
| D3 | HTTQSTTEL | FSSNPSRDSHTTQSTTELLS (0.946) | EGISTSGETTRFSSNPSRDSHTTQSTTELLSASAS HGAIPVSTGMASSIVPG |
| C1 | MTSKTITMT | TNTLMMTSKTITMTTSTDST (0.905) | - |
| A3 | VTSAALVTV | PGETSSVPVTGSLMPVTSAA (0.822) | KITVTTSTGSTLGNPGETSSVPVTGSLMPV |
| B3 | VVEAFLYHV | VVEAFLYHVPRRSEEPRNDV (0.901) | - |
| B4 | QLLAAVVEA | EFQYRPRGPVIDFLNNQLLAAVVEAFLYHV PRRSEEPRNDV (0.901) | - |
| E3 | CLYNQTSRV | HCNAESQCLYNQTSRVGNSS (0.848) | CLYN |
| A4 | GVSQEIFTL | GRPHERSSFSPGVSQEIFTL (0.763) | TLSTALSPSSLPPKISTAFHTQQSEGAETTGRPHE RSSFSPGVSQ |
| D1 | LVTSKVFRM | TISSPSSVSNTFLVTSKVFR (0.758) | - |
| B2 | TLIQYTSNA | GRVIEAYKGQTTLIQYTSNA (0.745) | REVSKNYEQANATLNQYPPSINGGRVIEAYKGQT TLIQYTSNAEDANFT |
| B1 | SMAEVNASV | - | EENASMAEVN |
| F3 | TISAITSKV | - | TISAI |
| E4 | AITSKVSTI | - | - |

**Figure 4: Reactivity against predicted MUC4α epitopes was found in PC patients.**

Indirect ELISA study demonstrated that PC patient serum contains circulating autoantibodies against MUC4α immunodominant predicted epitopes, whereas low reactivity to PD2 internal protein control was observed. The serum reactivity to MUC4 α was observed to be significantly higher than PD2 and TR control peptides (A). Presence of autoantibodies against these peptides didn't confer any survival advantage (B). ANOVA analysis of group means was significant ($P<0.001$), ****** and ****** denotes paired T-test between PD2 vs. MUC4 peptides and TR vs peptides respectively ($P<0.001$)

# Figure 4



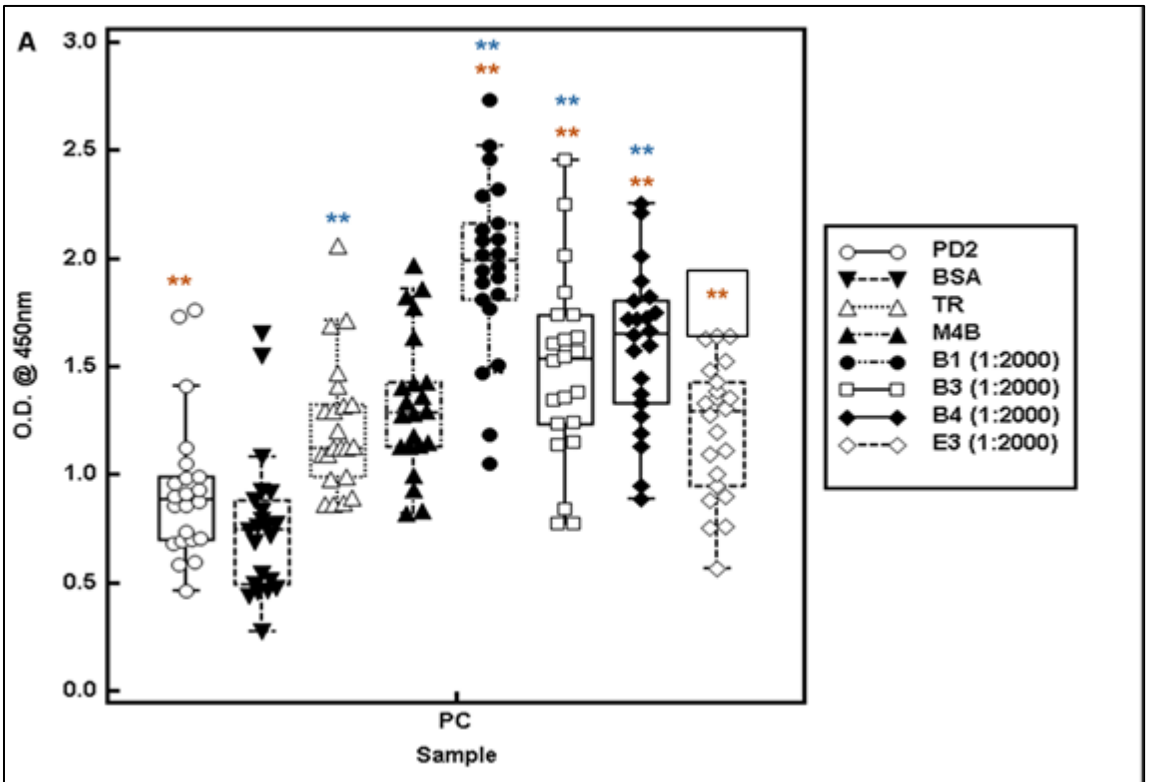

B

| | | MUC4α Peptides | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Autoantibodies to MUC4α Peptides (High Expression) | | | | | | |
| | | A2 | C4 | D3 | D4 | E4 | F1 | F3 |
| Survival time (days) | N | 10 | 10 | 11 | 10 | 10 | 11 | 10 |
| | Median | 236.5 | 194 | 289 | 224 | 164.5 | 204 | 266.5 |
| | | | | | | | | |
| | | Autoantibodies to MUC4α Peptides (Low Expression) | | | | | | |
| | | A2 | C4 | D3 | D4 | E4 | F1 | F3 |
| Survival time (days) | N | 10 | 10 | 9 | 10 | 10 | 9 | 10 |
| | Median | 294 | 316.5 | 184 | 264 | 294 | 344 | 164.5 |

**Figure 5: Reactivity against predicted MUC4β epitopes was found in PC patients**.

Indirect ELISA study demonstrated that PC patient serum contains circulating autoantibodies against MUC4β immunodominant predicted epitopes, whereas low reactivity to PD2 internal protein control was observed. The serum reactivity to MUC4 α was observed to be significantly higher than PD2 and TR control peptides (A). Presence of autoantibodies against these peptides didn't confer any survival advantage (B). ANOVA analysis of group means was significant ($P<0.001$), ****** and ****** denotes paired T-test between PD2 vs. MUC4 peptides and TR vs peptides respectively ($P<0.001$)

# Figure 5




B

| | | MUC4β Peptides | | | |
|---|---|---|---|---|---|
| | | Autoantibodies to MUC4β Peptides (High Expression) | | | |
| | | B1 | B3 | B4 | E3 |
| Survival time (days) | N | 10 | 10 | 10 | 10 |
| | Median | 214 | 157.5 | 138 | 194 |
| | | Autoantibodies to MUC4β Peptides (Low Expression) | | | |
| | | B1 | B3 | B4 | E3 |
| Survival time (days) | N | 10 | 10 | 10 | 10 |
| | Median | 274 | 345.5 | 345.5 | 452 |

**Figure 6: IgM autoantibodies to MUC4α peptides show a better prognosis in PC patients.**

Isotyping analysis revealed that differential levels of all three IgM, IgG and IgA isotypes have been detected against all MUC4α peptides (A). Kaplan-Meier graph shows that high levels of IgM autoantibodies to A2 and D4 (blue arrows) significantly correlated with PC patient survival (MSD: 344 days & 535 days respectively).

# Figure 6

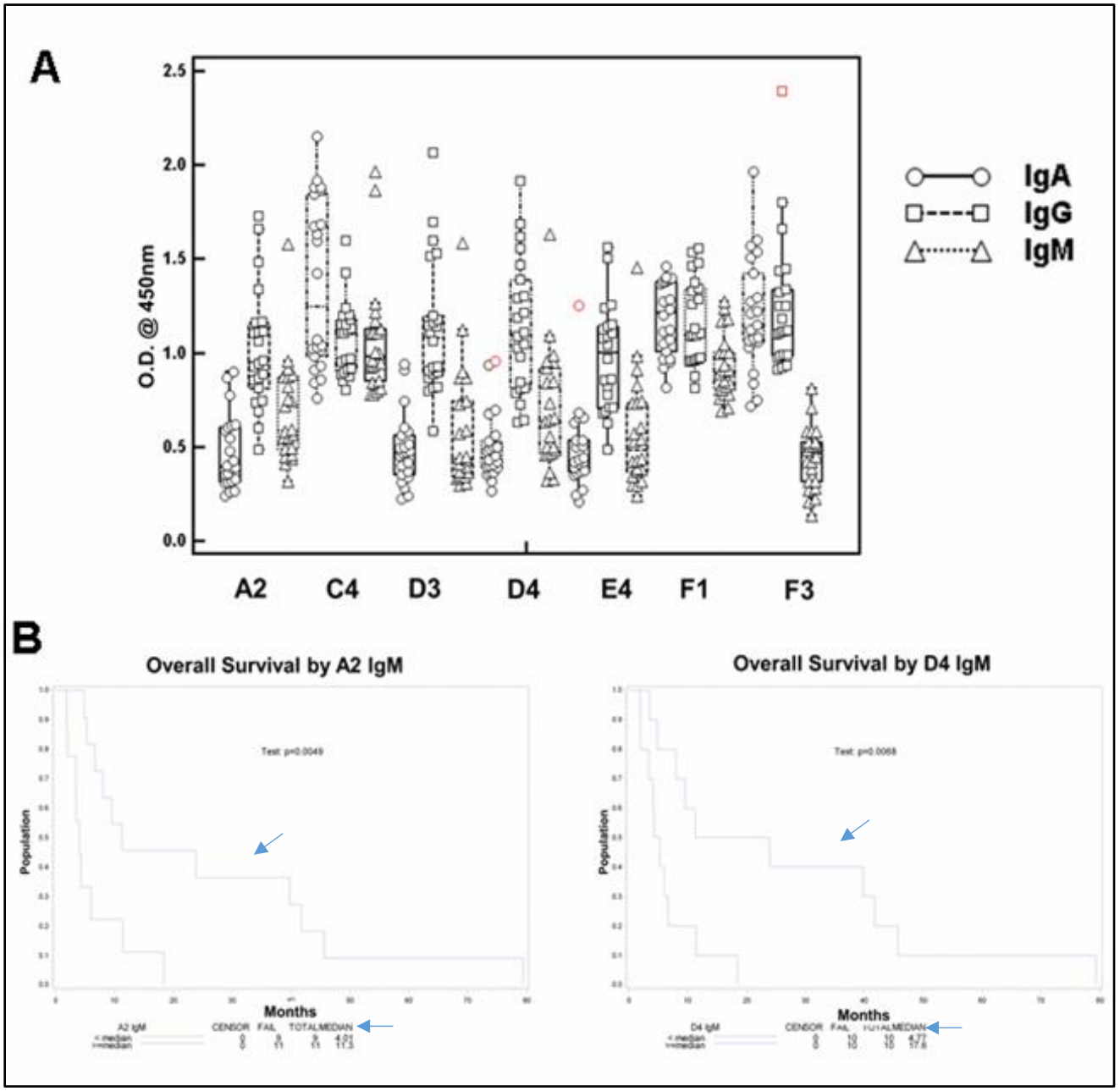
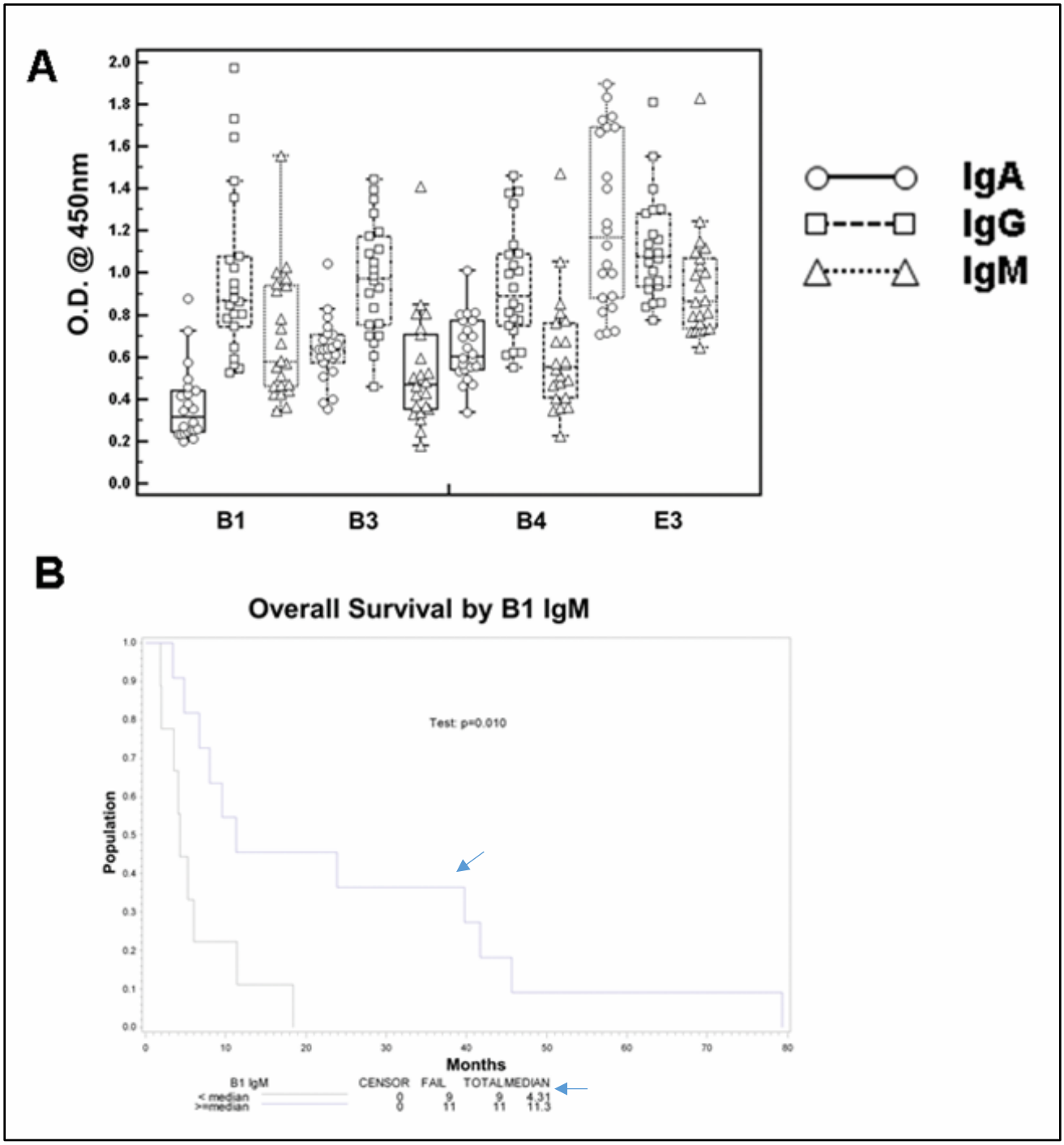

A
O.D. @ 450nm
2.5
2.0
1.5
1.0
0.5
0.0
A2
C4
D3
D4
E4
F1
F3
IgA
IgG
IgM
B
Overall Survival by A2 IgM
Overall Survival by D4 IgM
Population
Months
Months

**Figure 7: IgM autoantibodies to MUC4β peptides show a better prognosis in PC patients.**

Isotype analysis revealed that differential levels of all three IgM, IgG and IgA isotypes have been detected against all MUC4β peptides (A). Kaplan-Meier graph shows that high levels of IgM autoantibodies to B1 (blue arrow) significantly correlated with PC patient survival (MSD: 344 days).

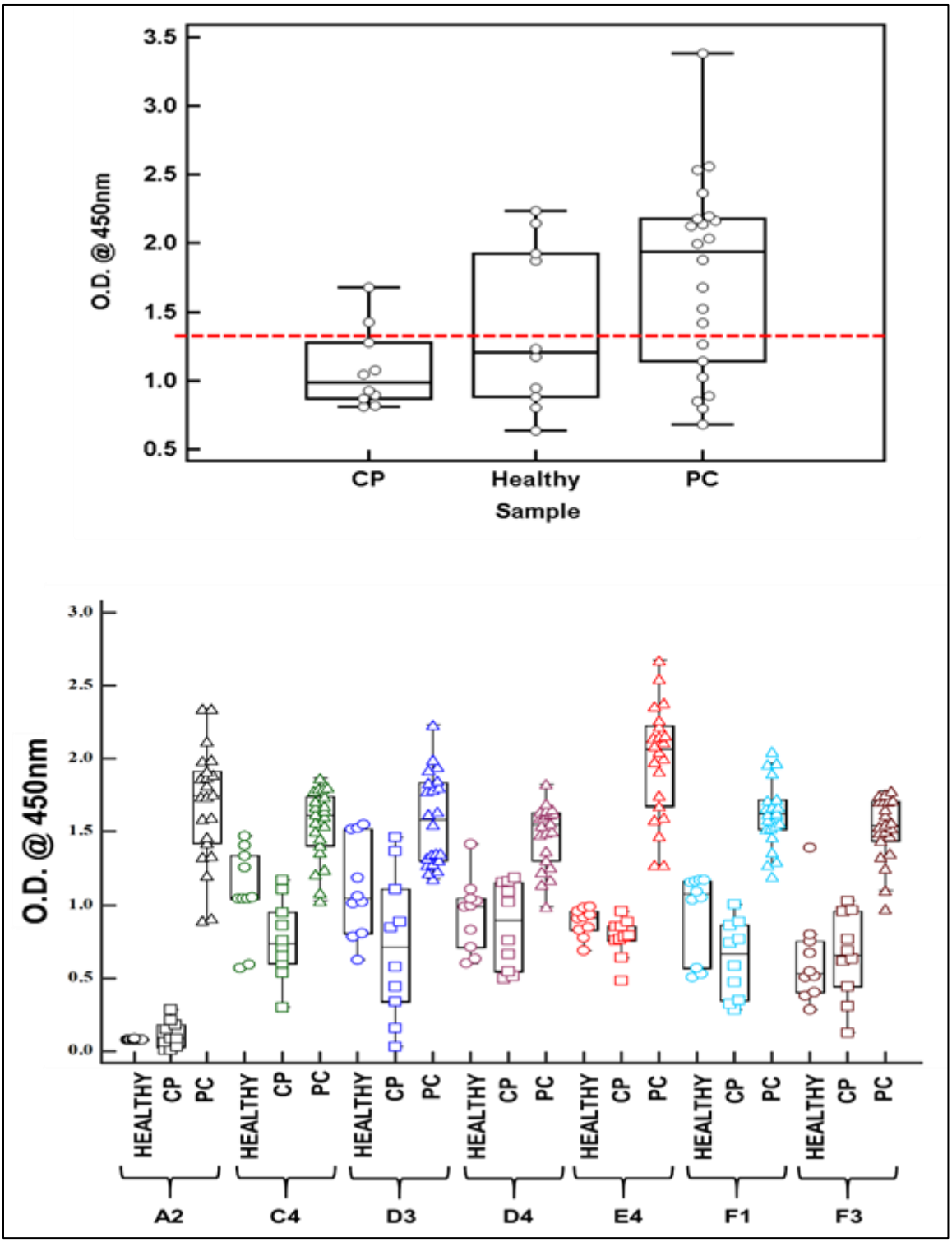
## Figure 7


A
O.D. @ 450nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
B1
B3
B4
E3
IgA
IgG
IgM
B
Overall Survival by B1 IgM
Test: p=0.010
Population
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
80
Months
B1 IgM
CENSOR
FAIL
TOTAL
MEDIAN
< median
0
9
9
4.31
>=median
0
11
11
11.3

**Supplemental Figure 1: Reactivity against predicted MUC4α epitopes was found only in PC patients.**

PC specific reactivity was found against MUC4α peptides with significantly low reactivity in both healthy and CP controls, suggesting that these peptides could serve as unique immunodominant epitopes being immunologically recognized specifically by PC patient's immune system.

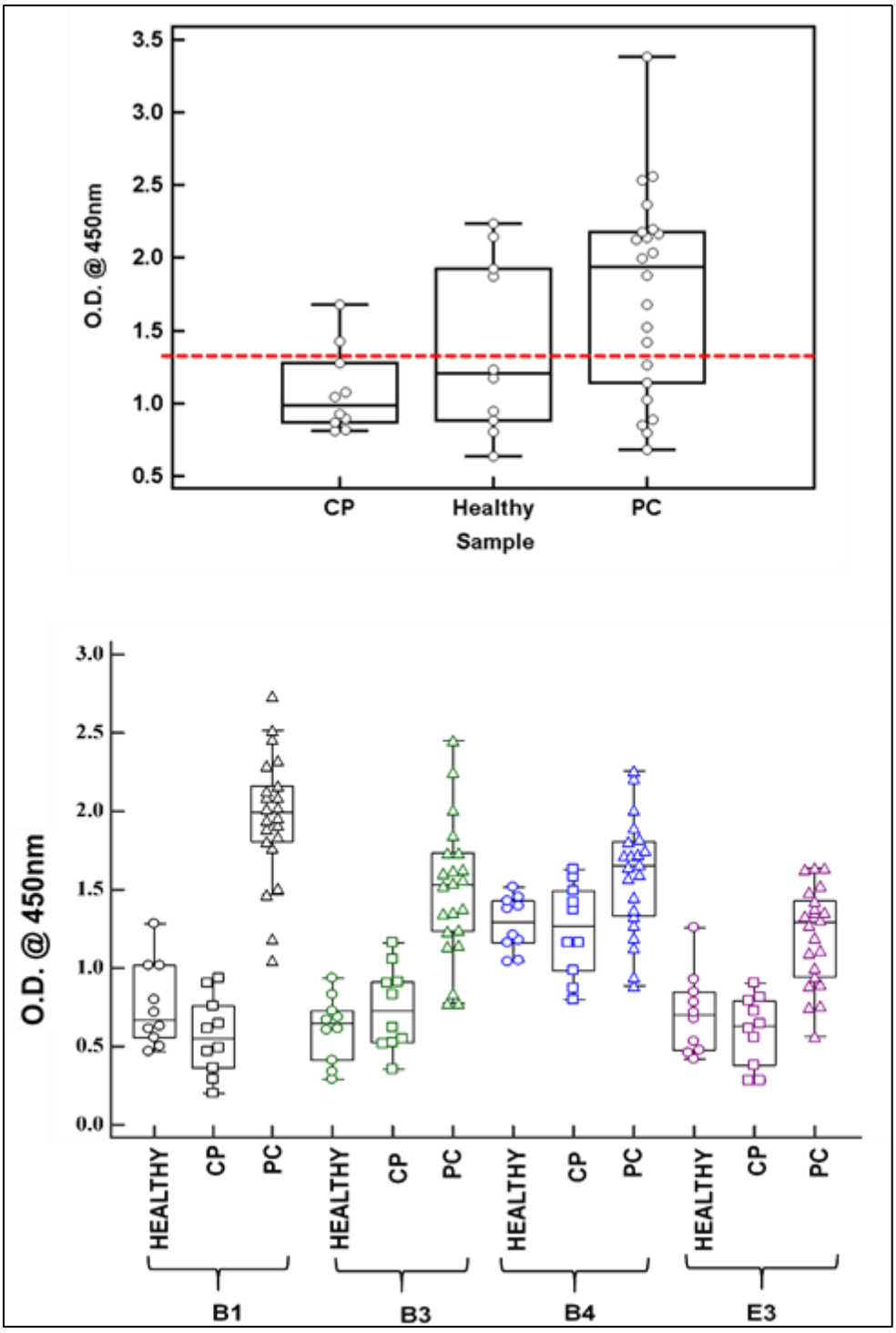
# Supplemental Figure 1



**Supplemental Figure 2: Reactivity against predicted MUC4β epitopes was found only in PC patients.**

PC specific reactivity was found against MUC4β peptides with significantly low reactivity in both healthy and CP controls, suggesting that these peptides could serve as unique immunodominant epitopes being immunologically recognized specifically by PC patient's immune system.

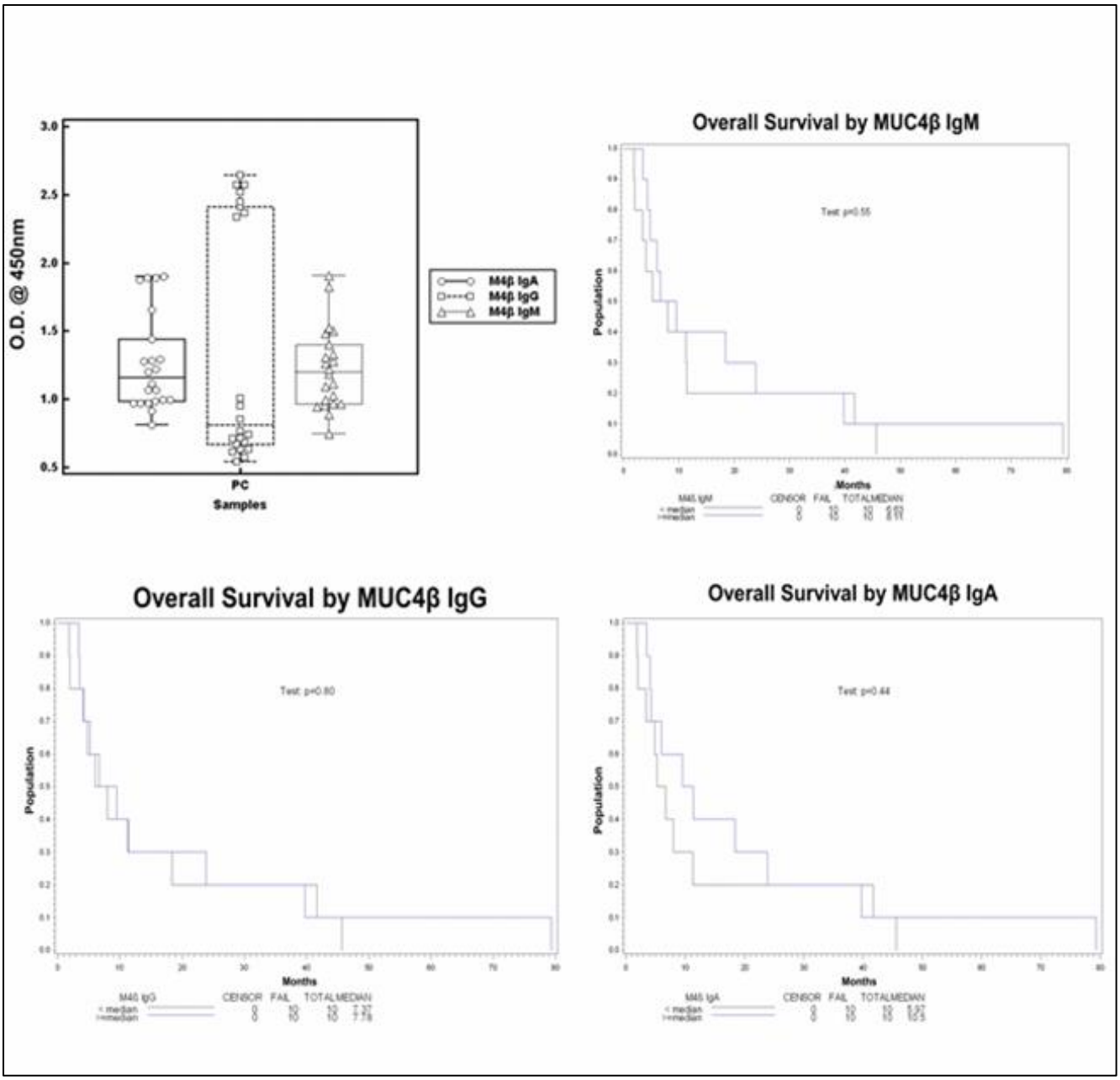
## Supplemental Figure 2



**Supplemental Figure 3: Isotyping of MUC4 circulating antibodies didn't show any correlation with survival status of PC patients.**

Isotype analysis of autoantibodies to recombinant human MUC4β protein showed that PC patients could be segregated on the levels of IgG autoantibodies present in PC patient serum. However, none of these isotypes correlated with PC patient survival.

# Supplemental Figure 3



**Supplemental Figure 4: Autoantibodies isotype to MUC4α and MUC4β peptides reveals a differential correlation with patient survival.**

Correlation of PC patient survival with isotypes of autoantibodies to MUC4 peptides present in patient sera demonstrates that high levels of IgM autoantibodies seem to positively correlate with patient survival compared to IgG or IgA autoantibodies for all peptides.

# Supplemental Figure 4

| | | A2 | | | C4 | | | D3 | | | D4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IgM | IgG | IgA | IgM | IgG | IgA | IgM | IgG | IgA | IgM | IgG | IgA |
| Survival time (days) | N | 11 | 10 | 10 | 10 | 10 | 11 | 10 | 10 | 11 | 10 | 10 | 11 |
| | Median | 344 | 246.5 | 174.5 | 316.5 | 266.5 | 204 | 316.5 | 266.5 | 184 | 535.5 | 266.5 | 204 |

| | | E4 | | | F1 | | | F3 | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | IgM | IgG | IgA | IgM | IgG | IgA | IgM | IgG | IgA |
| Survival time (days) | N | 11 | 10 | 10 | 10 | 11 | 10 | 9 | 11 | 9 |
| | Median | 289 | 266.5 | 194 | 266.5 | 204 | 236.5 | 244 | 244 | 204 |

| | | B1 | | | B3 | | | B4 | | | E3 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IgM | IgG | IgA | IgM | IgG | IgA | IgM | IgG | IgA | IgM | IgG | IgA |
| Survival time (days) | N | 11 | 11 | 11 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 11 | 10 |
| | Median | 344 | 289 | 184 | 316.5 | 224 | 224 | 316.5 | 266.5 | 246.5 | 266.5 | 244 | 266.5 |

# References

[1] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2018, CA Cancer J Clin, 68 (2018) 7-30.

[2] K. Banerjee, S. Kumar, K.A. Ross, S. Gautam, B. Poelaert, M.W. Nasser, A. Aithal, R. Bhatia, M.J. Wannemuehler, B. Narasimhan, J.C. Solheim, S.K. Batra, M. Jain, Emerging trends in the immunotherapy of pancreatic cancer, Cancer Lett, 417 (2018) 35-46.

[3] K. Sideras, H. Braat, J. Kwekkeboom, C.H. van Eijck, M.P. Peppelenbosch, S. Sleijfer, M. Bruno, Role of the immune system in pancreatic cancer progression and immune modulating treatment strategies, Cancer Treat Rev, 40 (2014) 513-522.

[4] L.R. Brunet, T. Hagemann, G. Andrew, S. Mudan, A. Marabelle, Have lessons from past failures brought us closer to the success of immunotherapy in metastatic pancreatic cancer?, Oncoimmunology, 5 (2016) e1112942.

[5] L.M. Matrisian, J.D. Berlin, The Past, Present, and Future of Pancreatic Cancer Clinical Trials, Am Soc Clin Oncol Educ Book, 35 (2016) e205-215.

[6] P. Bailey, D.K. Chang, M.A. Forget, F.A. Lucas, H.A. Alvarez, C. Haymaker, C. Chattopadhyay, S.H. Kim, S. Ekmekcioglu, E.A. Grimm, A.V. Biankin, P. Hwu, A. Maitra, J. Roszik, Exploiting the neoantigen landscape for immunotherapy of pancreatic ductal adenocarcinoma, Sci Rep, 6 (2016) 35848.

[7] L.B. Alexandrov, S. Nik-Zainal, D.C. Wedge, P.J. Campbell, M.R. Stratton, Deciphering signatures of mutational processes operative in human cancer, Cell Rep, 3 (2013) 246-259.

[8] M.S. Lawrence, P. Stojanov, P. Polak, G.V. Kryukov, K. Cibulskis, A. Sivachenko, S.L. Carter, C. Stewart, C.H. Mermel, S.A. Roberts, A. Kiezun, P.S. Hammerman, A. McKenna, Y. Drier, L. Zou, A.H. Ramos, T.J. Pugh, N. Stransky, E. Helman, J. Kim, C. Sougnez, L. Ambrogio, E. Nickerson, E. Shefler, M.L. Cortes, D. Auclair, G. Saksena, D. Voet, M.

Noble, D. DiCara, P. Lin, L. Lichtenstein, D.I. Heiman, T. Fennell, M. Imielinski, B. Hernandez, E. Hodis, S. Baca, A.M. Dulak, J. Lohr, D.A. Landau, C.J. Wu, J. Melendez-Zajgla, A. Hidalgo-Miranda, A. Koren, S.A. McCarroll, J. Mora, B. Crompton, R. Onofrio, M. Parkin, W. Winckler, K. Ardlie, S.B. Gabriel, C.W.M. Roberts, J.A. Biegel, K. Stegmaier, A.J. Bass, L.A. Garraway, M. Meyerson, T.R. Golub, D.A. Gordenin, S. Sunyaev, E.S. Lander, G. Getz, Mutational heterogeneity in cancer and the search for new cancer-associated genes, Nature, 499 (2013) 214-218.

[9] D. Delitto, S.M. Wallet, S.J. Hughes, Targeting tumor tolerance: A new hope for pancreatic cancer therapy?, Pharmacol Ther, 166 (2016) 9-29.

[10] P. Zaenker, E.S. Gray, M.R. Ziman, Autoantibody Production in Cancer--The Humoral Immune Response toward Autologous Antigens in Cancer Patients, Autoimmun Rev, 15 (2016) 477-483.

[11] H.H. Wandall, O. Blixt, M.A. Tarp, J.W. Pedersen, E.P. Bennett, U. Mandel, G. Ragupathi, P.O. Livingston, M.A. Hollingsworth, J. Taylor-Papadimitriou, J. Burchell, H. Clausen, Cancer biomarkers defined by autoantibody signatures to aberrant O-glycopeptide epitopes, Cancer Res, 70 (2010) 1306-1313.

[12] M. Andrianifahanana, N. Moniaux, B.M. Schmied, J. Ringel, H. Friess, M.A. Hollingsworth, M.W. Buchler, J.P. Aubert, S.K. Batra, Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumor marker of diagnostic significance, Clin Cancer Res, 7 (2001) 4033-4040.

[13] S. Carrara, M.G. Cangi, P.G. Arcidiacono, F. Perri, M.C. Petrone, G. Mezzi, C. Boemo, A. Talarico, E.D. Cin, G. Grassini, C. Doglioni, P.A. Testoni, Mucin expression pattern in pancreatic diseases: findings from EUS-guided fine-needle aspiration biopsies, Am J Gastroenterol, 106 (2011) 1359-1363.

[14] S.K. Gautam, S. Kumar, A. Cannon, B. Hall, R. Bhatia, M.W. Nasser, S. Mahapatra, S.K. Batra, M. Jain, MUC4 mucin- a therapeutic target for pancreatic ductal adenocarcinoma, Expert Opin Ther Targets, 21 (2017) 657-669.

[15] R.A. Budiu, G. Mantia-Smaldone, E. Elishaev, T. Chu, J. Thaller, K. McCabe, D. Lenzner, R.P. Edwards, A.M. Vlad, Soluble MUC1 and serum MUC1-specific antibodies are potential prognostic biomarkers for platinum-resistant ovarian cancer, Cancer Immunol Immunother, 60 (2011) 975-984.

[16] Y. Hirasawa, N. Kohno, A. Yokoyama, K. Kondo, K. Hiwada, M. Miyake, Natural autoantibody to MUC1 is a prognostic indicator for non-small cell lung cancer, Am J Respir Crit Care Med, 161 (2000) 589-594.

[17] B. Burford, A. Gentry-Maharaj, R. Graham, D. Allen, J.W. Pedersen, A.S. Nudelman, O. Blixt, E.O. Fourkala, D. Bueti, A. Dawnay, J. Ford, R. Desai, L. David, P. Trinder, B. Acres, T. Schwientek, A. Gammerman, C.A. Reis, L. Silva, H. Osorio, R. Hallett, H.H. Wandall, U. Mandel, M.A. Hollingsworth, I. Jacobs, I. Fentiman, H. Clausen, J. Taylor-Papadimitriou, U. Menon, J.M. Burchell, Autoantibodies to MUC1 glycopeptides cannot be used as a screening assay for early detection of breast, ovarian, lung or pancreatic cancer, Br J Cancer, 108 (2013) 2045-2055.

[18] P. Chaturvedi, A.P. Singh, S.K. Batra, Structure, evolution, and biology of the MUC4 mucin, FASEB J, 22 (2008) 966-981.

[19] J.W. Pedersen, A. Gentry-Maharaj, A. Nostdal, E.O. Fourkala, A. Dawnay, M. Burnell, A. Zaikin, J. Burchell, J.T. Papadimitriou, H. Clausen, I. Jacobs, U. Menon, H.H. Wandall, Cancer-associated autoantibodies to MUC1 and MUC4--a blinded case-control study of colorectal cancer in UK collaborative trial of ovarian cancer screening, Int J Cancer, 134 (2014) 2180-2188.

[20] N. Moniaux, F. Escande, S.K. Batra, N. Porchet, A. Laine, J.P. Aubert, Alternative splicing generates a family of putative secreted and membrane-associated MUC4 mucins, Eur J Biochem, 267 (2000) 4536-4544.

[21] N. Moniaux, F. Escande, N. Porchet, J.P. Aubert, S.K. Batra, Structural organization and classification of the human mucin genes, Front Biosci, 6 (2001) D1192-1206.

[22] N. Moniaux, S. Nollet, N. Porchet, P. Degand, A. Laine, J.P. Aubert, Complete sequence of the human mucin MUC4: a putative cell membrane-associated mucin, Biochem J, 338 ( Pt 2) (1999) 325-333.

[23] N. Vigneron, Human Tumor Antigens and Cancer Immunotherapy, Biomed Res Int, 2015 (2015) 948501.

[24] M. Andreatta, M. Nielsen, Gapped sequence alignment using artificial neural networks: application to the MHC class I system, Bioinformatics, 32 (2016) 511-517.

[25] Y. Kim, J. Ponomarenko, Z. Zhu, D. Tamang, P. Wang, J. Greenbaum, C. Lundegaard, A. Sette, O. Lund, P.E. Bourne, M. Nielsen, B. Peters, Immune epitope database analysis resource, Nucleic Acids Res, 40 (2012) W525-530.

[26] C. Lundegaard, K. Lamberth, M. Harndahl, S. Buus, O. Lund, M. Nielsen, NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8-11, Nucleic Acids Res, 36 (2008) W509-512.

[27] J. Sidney, E. Assarsson, C. Moore, S. Ngo, C. Pinilla, A. Sette, B. Peters, Quantitative peptide binding motifs for 19 human and mouse MHC class I molecules derived using positional scanning combinatorial peptide libraries, Immunome Res, 4 (2008) 2.

[28] P. Wang, J. Sidney, C. Dow, B. Mothe, A. Sette, B. Peters, A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach, PLoS Comput Biol, 4 (2008) e1000048.

[29] P. Wang, J. Sidney, Y. Kim, A. Sette, O. Lund, M. Nielsen, B. Peters, Peptide binding predictions for HLA DR, DP and DQ molecules, BMC Bioinformatics, 11 (2010) 568.

[30] M. Nielsen, C. Lundegaard, P. Worning, S.L. Lauemoller, K. Lamberth, S. Buus, S. Brunak, O. Lund, Reliable prediction of T-cell epitopes using neural networks with novel sequence representations, Protein Sci, 12 (2003) 1007-1017.

[31] B. Peters, A. Sette, Generating quantitative models describing the sequence specificity of biological processes with the stabilized matrix method, BMC Bioinformatics, 6 (2005) 132.

[32] S. Paul, J. Sidney, A. Sette, B. Peters, TepiTool: A Pipeline for Computational Prediction of T Cell Epitope Candidates, Curr Protoc Immunol, 114 (2016) 18 19 11-18 19 24.

[33] M.V. Larsen, C. Lundegaard, K. Lamberth, S. Buus, S. Brunak, O. Lund, M. Nielsen, An integrative approach to CTL epitope prediction: a combined algorithm integrating MHC class I binding, TAP transport efficiency, and proteasomal cleavage predictions, Eur J Immunol, 35 (2005) 2295-2303.

[34] J.E. Larsen, O. Lund, M. Nielsen, Improved method for predicting linear B-cell epitopes, Immunome Res, 2 (2006) 2.

[35] A. Choudhury, N. Moniaux, J.P. Winpenny, M.A. Hollingsworth, J.P. Aubert, S.K. Batra, Human MUC4 mucin cDNA and its variants in pancreatic carcinoma, J Biochem, 128 (2000) 233-243.

[36] F. Escande, L. Lemaitre, N. Moniaux, S.K. Batra, J.P. Aubert, M.P. Buisine, Genomic organization of MUC4 mucin gene. Towards the characterization of splice variants, Eur J Biochem, 269 (2002) 3637-3644.

[37] M.A. Player, K.C. Barracchini, T.B. Simonis, L. Rivoltini, F. Arienti, C. Castelli, A. Mazzocchi, F. Belli, G. Parmiani, F.M. Marincola, Differences in frequency distribution of HLA-A2 subtypes between North American and Italian white melanoma patients: relevance for epitope specific vaccination protocols, J Immunother Emphasis Tumor Immunol, 19 (1996) 357-363.

[38] L. Rivoltini, D.J. Loftus, K. Barracchini, F. Arienti, A. Mazzocchi, W.E. Biddison, M.L. Salgaller, E. Appella, G. Parmiani, F.M. Marincola, Binding and presentation of peptides derived from melanoma antigens MART-1 and glycoprotein-100 by HLA-A2 subtypes. Implications for peptide-based immunotherapy, J Immunol, 156 (1996) 3882-3891.

[39] P.O. Flores-Villanueva, M. Ganachari, H. Guio, J.A. Mejia, J. Granados, An Isolated TCR alphabeta Restricted by HLA-A*02:01/CT37 Peptide Redirecting CD8(+) T Cells To Kill and Secrete IFN-gamma in Response to Lung Adenocarcinoma Cell Lines, J Immunol, (2018).

[40] E. Market, F.N. Papavasiliou, V(D)J recombination and the evolution of the adaptive immune system, PLoS Biol, 1 (2003) E16.

[41] J. Stavnezer, C.T. Amemiya, Evolution of isotype switching, Semin Immunol, 16 (2004) 257-275.

[42] M.J. Swartz, S.K. Batra, G.C. Varshney, M.A. Hollingsworth, C.J. Yeo, J.L. Cameron, R.E. Wilentz, R.H. Hruban, P. Argani, MUC4 expression increases progressively in pancreatic intraepithelial neoplasia, Am J Clin Pathol, 117 (2002) 791-796.

[43] V.P. Balachandran, M. Luksza, J.N. Zhao, V. Makarov, J.A. Moral, R. Remark, B. Herbst, G. Askan, U. Bhanot, Y. Senbabaoglu, D.K. Wells, C.I.O. Cary, O. Grbovic-Huezo, M. Attiyeh, B. Medina, J. Zhang, J. Loo, J. Saglimbeni, M. Abu-Akeel, R. Zappasodi, N. Riaz, M. Smoragiewicz, Z.L. Kelley, O. Basturk, I. Australian Pancreatic Cancer Genome, R. Garvan Institute of Medical, H. Prince of Wales, H. Royal North Shore, G. University of, H. St Vincent's, Q.B.M.R. Institute, C.f.C.R. University of Melbourne, I.f.M.B. University of Queensland, H. Bankstown, H. Liverpool, C.O.B.L. Royal Prince Alfred Hospital, H. Westmead, H. Fremantle, H. St John of God, H. Royal Adelaide, C. Flinders Medical, P. Envoi, H. Princess Alexandria, H. Austin, I. Johns Hopkins Medical, A.R.-N.C.f.A.R.o. Cancer, M. Gonen, A.J. Levine, P.J. Allen, D.T. Fearon, M. Merad, S. Gnjatic, C.A. Iacobuzio-Donahue, J.D. Wolchok, R.P. DeMatteo, T.A. Chan, B.D. Greenbaum, T.

Merghoub, S.D. Leach, Identification of unique neoantigen qualities in long-term survivors of pancreatic cancer, Nature, 551 (2017) 512-516.

[44] I.J. Amanna, M.K. Slifka, Contributions of humoral and cellular immunity to vaccine-induced protection in humans, Virology, 411 (2011) 206-215.

# CHAPTER 4: DEVELOPMENT OF MUC4 NANOVACCINE

The material covered in this chapter is the subject of a research article

**2. Banerjee K.**[1#], Gautam S.[1#], Kshirsagar P., Ross KA., Spagnol G., Sorgen P., Wannemuehler MJ., Narasimhan B., Solheim J., Kumar S., Batra SK., and Jain M. *Recombinant MUC4 nanovaccine activates dendritic cells and induces potent anti-tumor immunity*. (Submitted)

# 1. Synopsis

Mucin 4 (MUC4) is high molecular weight glycoprotein that is differentially overexpressed in pancreatic cancer (PC) and functionally contributes to disease progression while its expression correlates with poor survival. Further, due to its aberrant glycosylation and extensive splicing in cancer, MUC4 is a potential target for cancer immunotherapy. Our previous studies have demonstrated the utility of amphiphilic polyanhydride nanoparticles as a useful platform for the development of protein-based prophylactic and therapeutic vaccines. In present study, we encapsulated purified recombinant human MUC4 (MUC4β) protein in 20:80 ratio of amphiphilic polyanhydride (CPTEG & CPH) adjuvants (MUC4-nanovaccine) and evaluated its ability to activate dendritic cells and induce anti-tumor immunity. Immature dendritic cells when pulsed with MUC4-nanovaccine exhibited more than 2-fold increase in surface expression of activation markers (MHC-II and MHC-I) and 1.5-fold increase in pro-inflammatory cytokines (IFN-γ, IL-6, and IL-2) levels as compared to cells exposed to MUC4β alone or MUC4β mixed with blank nanoparticles (MUC4 +NP). Further, the antibody analysis in the sera collected from immunized mice showed two-fold higher levels of IgG2b antibodies than IgG1 antibodies, suggesting a predominantly Th1-type of immune response in MUC4-nanovaccine group. Thus, our findings demonstrate MUC4-nanoformulation as a novel platform for PC vaccine development.

## 2. Background and Rationale

Pancreatic cancer (PC) has a dismal prognosis with an overall survival rate of 8%, due to the limited efficacy of existing treatment modalities like surgery, chemotherapy, and radiation [1, 2]. Furthermore, PC has an elaborate immunosuppressive tumor microenvironment comprising of high desmoplasia, immune-suppressive cells and an anti-inflammatory cytokine *milieu* [3]. Due to high level of chemotherapy-induced toxicity, PC patients seldom benefit from chemotherapy. Recent studies have shown that immunotherapy-based strategies like cancer vaccines can provide therapeutic benefit by breaking the tolerance, overcoming immunosuppression and thereby, improving the overall survival and quality of life [4, 5]. However, the development of anti-cancer vaccines are rather more arduous due to the challenges in finding optimal tumor-associated antigens (TAAs), because the majority of these antigens behave as “self”, and therefore, are immunologically ignored by the host immune system leading to development of tolerance against TAAs [4].

Mucins are high molecular-weight glycoproteins that are overexpressed on pancreatic tumor cells and have oncogenic functions in PC pathogenesis. MUC family members have emerged as TAAs for PC and are currently being exploited for cancer immunotherapy. Mucin1 (MUC1) is one of the well-studied targets for PC vaccine studies [6]. MUC1 peptide and glycopeptide vaccine studies have shown the potential of reprogramming the immune system against mucins and generating anti-tumor responses in various malignancies [7-12]. However, limited immunogenic epitopes provided by peptide-based MUC1 vaccines have achieved suboptimal clinical success in PC patients [9, 13, 14]. Unlike MUC1, Mucin4 (MUC4) is undetectable in normal pancreatic tissue and its expression progressively increases with disease progression and [15]. MUC4 is putatively cleaved at a Gly-AspPro-His (GDPH) site in an autocatalytic manner into two

subunits: a large N-terminal MUC4α containing the tandem repeat region and a smaller membrane-tethered MUC4β [16-18]. The membrane-tethered MUC4β region is considered functionally important as it has 3 EGF-like domains that interact with HER-2 and promote cell proliferation. Further, targeting MUC4β is simplified since the subunit will still be present on the cell surface of PC tumor cells post-cleavage due to its transmembrane region. In a study, mice immunized with MUC4 glycopeptides mixed with tetanus toxin induced strong immune responses and predominantly produced IgG1 antibodies [19]. Such “cherry-picked” immunodominant peptides limit the epitopes that can be employed to elicit immune response in an unbiased manner, are of limited translation values. While large size of MUC4 can potentially provide a large epitope repertoire for eliciting potent immune response, it also makes the production and purification of intact protein equally challenging. We thus investigated the utility of recombinant MUC4β-domain for cancer vaccine development. To circumvent these, we investigated the utility of recombinant MUC4β subunit for tumor vaccine development.

One of the major challenges of vaccine delivery vehicles is to ensure protein stability and release over a sustained period in circulation [20, 21]. Amphiphilic polyanhydride nanoparticles, composed of 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis(*p*-carboxyphenoxy)hexane (CPH), have been shown to stabilize the structure and activity of encapsulated proteins while providing sustained release via a surface erosion mechanism [22, 23]. Furthermore, these nanoparticles have been shown to be readily internalized by antigen presenting cells (APCs), such as dendritic cells and macrophages, leading to the upregulation of surface activation markers including major histocompatibility complexes class I and II (MHC-I & MHC-II), co-activating ligands (CD86, CD40), secretion of inflammatory cytokines and generation of humoral responses [24-27].

In the present study, we encapsulated endotoxin free recombinant human MUC4β in 20:80 CPTEG:CPH nanoparticles (MUC4-nanovaccine). We investigated the relationships between antigen release kinetics, immunological activity in terms of APC activation, and induction of humoral responses by the MUC4-nanovaccine. Our study demonstrates that the MUC4-nanovaccione activated mature DCs, eliciting a Th1 type of immune response. We further observe that MUC4 nanovaccine-immunized mice generate more IgG2b anti-MUC4β serum antibodies than IgG1 antibodies, suggesting that MUC4-nanovaccine elicits a predominantly Th1-type response. Therefore, recombinant human MUC4β-based polyanhydride nanovaccine has the potential to be an effective immunotherapy against pancreatic cancer and other MUC4 overexpressing malignancies.

## 3. Results

### 3.1 Encapsulation of MUC4β into Polyanhydride Nanoparticles Provides Sustained Antigen Release Kinetics

The 20:80 CPTEG:CPH nanoparticles loaded with 3 wt. % MUC4β were synthesized via solid-oil-oil double emulsion. Scanning electron microscopy showed the nanoparticles to be relatively spherical with a geometric mean diameter of 147 nm (with a geometric standard deviation of 1.3) **(Figure 1A)**. The release kinetics of MUC4β from 20:80 showed a burst of ca. 20% at early time points followed by slow, sustained release with smaller amount of protein released over 30 days. The data showed that after one month, the hydrophobic 20:80 CPTEG:CPH particles released ca. 25% of the encapsulated protein in a near-zero order release profile, which was consistent with previous work on protein release kinetics from CPTEG:CPH polyanhydrides formulations [22, 23, 27-29]. Finally, the encapsulation efficiency of the MUC4β was determined to be 32 ± 1% **(Figure 1B)**.

## 3.2 MUC4 Nanovaccine Enhances Surface Expression of MHC and Co-Stimulatory Molecules on DCs

While the functional role of MUC4 in pancreatic cancer pathobiology has been studied extensively, the immunogenicity of MUC4 protein has not been assessed until now. To examine the antigenicity of MUC4β protein (MUC4) and characterize the potential of MUC4 nanovaccine in activating $CD86^+$ $CD11c^+$ DCs **(Supplementary Figure 1)**, flow cytometry was used to measure expression of cell surface markers such as major histocompatibility complex molecule class II (MHC II) and class I (MHC I), co-stimulatory molecule CD80, and C-type lectin CD205 (DEC-205: DC maturation marker). Recombinant MUC4β protein alone or delivered with empty nanoparticles did not upregulate surface expression of MHC I and II on DCs over controls. However, a significant 4-fold increase in mean fluorescence intensity (MFI) of MHC I and II was observed on DCs cultured with MUC4 nanovaccine in contrast to unstimulated DCs. Moreover, DCs cultured with MUC4 nanovaccine expressed high levels of MHC II (2-fold higher) than LPS-stimulated DCs **(Figure 2 A & B)**. Furthermore, MUC4 nanovaccine enhanced the DC surface expression of the co-stimulatory molecule CD80 by 75% when compared to MUC4 only treated DCs, but no significant difference was observed in CD40 expression **(Figure 2 C & D)**. In addition, DC surface expression of CD205 was 60%, 20% and 25% higher on MUC4-nanovaccine stimulated DCs when compared to unstimulated, LPS- and MUC4β protein-treated DCs **(Figure 2E)** suggesting a higher proliferation and maturation of DCs which is shown in **Figure 2F**. Altogether, these results demonstrate that the MUC4 nanovaccine significantly enhanced the expression of surface markers and co-stimulatory molecules involved in DC maturation and antigen presentation.

### 3.3 MUC4 Nanovaccine Induces Pro-Inflammatory DC Cytokine Secretion

Dendritic cells direct immune responses by not only cross-presenting antigens to effector T and B cells, but also by secreting an array of cytokines to modulate these responses. After culturing DCs with various treatment groups for 48 h, we observed that the MUC4 nanovaccine significantly enhanced DC secretion of the cytokines IL-12p40, IL-6, and IFNγ in comparison to untreated DCs and free MUC4 simulated DCs **(Figure 3)**. The amounts of IL-12p40 and IL-6 in culture supernatants of DCs treated with MUC4 nanovaccine were 40% and 30% higher than that of DCs stimulated with LPS respectively **(Fig 3A & B),** and the levels of IFNγ were comparable between these two stimulants **(Fig 3C)**. DCs treated with MUC4β alone (MUC4) or MUC4β mixed with empty nanoparticles (MUC4+NP) expressed low or undetectable levels of cytokines, which were no different from unstimulated DCs. Similar to the data obtained for surface expression of MHC II and DC co-stimulatory molecules, encapsulation of MUC4β protein in 20:80 CPTEG:CPH nanoparticles significantly enhanced DC cytokine production.

### 3.4 Single Immunization with MUC4 Nanovaccine Elicit Robust Anti-MUC4 Humoral Responses

Polyanhydride nanovaccines have been shown to generate germinal centers and B cells thus leading to sustained serum antibody responses in a single dose [30]. Apart from measuring antigen-specific antibody levels (which indicates the degree of humoral stimulation), isotyping of antibodies (IgG1 and IgG2b levels) demonstrate the type of immune responses (Th1 or Th2) generated through the determination of IgG2b:IgG1 ratios. The presence of high levels of antigen-specific IgG2b antibodies over IgG1 antibodies indicates preferred isotype-switching to Th1 type responses, whereas low IgG2b:IgG1 ratio is indicative of a Th2 type response [31]. To investigate if a single immunization with MUC4 nanovaccine induced robust humoral immune responses, animals were immunized

subcutaneously with MUC4β-loaded 20:80 CPTEG:CPH nanoparticles. Serum antibodies were detected at 1:1000 serum dilution in mice that were immunized with MUC4β alone, MUC4β mixed with empty nanoparticles and MUC4 nanovaccine. Antibodies against MUC4β were detected at higher dilutions in mice administered MUC4β only and MUC4 nanovaccine, but not when free protein was mixed with empty particles **(Figure 4A)**.

Since it has been shown that MUC4 peptides induce primarily IgG1 Th2 antibodies and not IgG2b [19], we evaluated the isotypes of the antibodies in MUC4β-immunized mice. Mice immunized with the MUC4 nanovaccine demonstrated a high IgG2b:IgG1 ratio, whereas mice immunized with MUC4β alone had a low IgG2b:IgG1 antibody ratio **(Figure 4B)**. These results indicate that encapsulation of MUC4β protein into 20:80 CPTEG:CPH nanoparticles modulates the immune response towards a Th1 phenotype , which could likely provide anti-tumor protection.

### 3.5 Immunization with MUC4 Nanovaccine Enhances the Presence of Inflammatory Cytokines in Sera

Since the analysis of antibody isotypes demonstrated an induction of a Th1 immune response, we also investigated the presence of Th1 cytokines (IL-12p40, IL-6, IL1β, and IFNγ) in sera of immunized mice. Sera from MUC4 nanovaccine immunized mice had a slightly higher increased mean amount of IL-6 compared to MUC4 and MUC4+NP immunized mice **(Figure 5A)**. In addition, sera from MUC4 nanovaccine immunized mice had significant amounts (~50 fold increase) of IL1β Th1 cytokine when compared to other treatment groups and PBS-treated mice (negative control) **(Figure 5B)** that correlates with Th1 isotype switching of antibodies in these mice. The amounts of IL-12p40 and IFNγ were below detection levels in all treatment groups (data not shown).

## 4. Discussion

To date vaccine studies involving mucins have been based on selected peptides which have limited repertoire of immunogenic epitopes. In pre-clinical studies, these vaccines have not shown promising results which could be attributed to studies done only with the tandem repeat regions of mucins including MUC4. Recombinant proteins could address these limitations by presenting the entire spectrum of possible epitopes present on the original antigens in an unbiased manner [3]. In this study, the β-subunit of MUC4 was expressed in a bacterial system and its immunogenicity was investigated. The data presented showed that MUC4β (MUC4) induces a Th2 type immune responses such as low expression of MHC-I and II complexes and co-stimulatory molecule CD80 **(Fig 2 A-C)**, and low levels of inflammatory cytokine generation by pulsed DCs **(Fig 3 A-C)**. In addition, mice immunized with only MUC4 produced significantly high levels of Th2 IgG1 antibodies to MUC4 **(Fig 4B)**. This indicated that utilizing the free protein by itself will likely not provide an effective immunotherapeutic response. Th2 immune responses have been well established to promote tumor pathogenesis and aggressiveness, whereas shifting the immune response to Th1 phenotype provides anti-tumor protection [32-34]. Thus, we encapsulated MUC4β into 20:80 CPTEG:CPH nanoparticles (MUC4 nanovaccine) and investigated whether the MUC4 nanovaccine could enhance activation of dendritic cells and modulate Th1 type humoral responses *in vivo*.

The MUC4 nanovaccine enhanced surface expression of MHC I and MHC II in $CD86^+$ $CD11c^+$ DCs **(Figure 3 A-B)**, which are implicated in presentation of antigen to T cells and B cells, along with CD80 (**Figure 3C**), a co-stimulatory molecule required for activation of naïve CD4 helper T cells. Proper antigen presentation followed by secondary activation signal provided by CD80 is crucial in programming effector immune cells to specifically target the cancer cells. Upregulation of these markers by MUC4-nanovaccine,

when compared to the free MUC4 pulsed DCs, suggests that the encapsulated formulation was able to reprogram the DCs from Th2 to a Th1 response phenotype. Further, we observed that only stimulation with MUC4-nanovaccine enhanced DC secretion of the cytokines IL-12p40, IL-6 and IFNγ *in vitro* when compared to free MUC4 or free protein mixed with empty nanoparticle (MUC4+NP) that corroborated with the DC activation marker expression data. These results indicate that the encapsulation of MUC4β protein in CPTEG:CPH nanoparticles is crucial to enhancing the immunogenicity of recombinant MUC4β. Thus, the result suggests that the encapsulation of MUC4β protein in CPTEG:CPH polymers is important for modulating the immunogenic response of Th2-inducing recombinant MUC4β.

Previously, it has been shown that a single immunization of polyanhydride nanovaccines can induce high antibody titers and provide protective immunity against multiple pathogens in mice [28, 35]. Additionally, it is important to consider the quality of antibody response generated by the nanovaccine, which may be characterized by the specificity, avidity and isotype profile of the antibody response [36]. It is therefore noteworthy that MUC4-nanovaccine immunized mice had the highest IgG2b:IgG1 ratio which indicates Th1 type antibodies, whereas MUC4β alone or in combination with blank nanoparticles preferentially induced Th2 type IgG1 anti-MUC4β antibodies in alignment with its immunogenic nature **(Figure 4B)**. This observation supports our *in vitro* observation that encapsulation of MUC4β into polyanhydride nanoparticles likely plays a crucial role in activating dendritic cells in favor of Th1 type immune responses, which was further validated with the detection of higher levels of IL-6 and IL-1β cytokines **(Figure 5)**. The data herein shows encapsulating MUC4β in 20:80 CPTEG:CPH nanoparticles is an effective strategy to activate dendritic cells in MUC4-specific manner and modulate the response towards an anti-tumor Th1 phenotype. The presence of IgG2b antibodies could

possibly further provide immunity against MUC4-expressing tumors by inducing antibody-dependent cellular cytotoxicity (ADCC) or antigen-mediated tumor killing by NK cells that can recognize the FcR region of antibodies bound to tumor cells [37, 38]. The current studies provide a basis for investigating the use of the MUC4 nanovaccine as an immunotherapeutic strategy in cancer models that overexpress MUC4 as a tumor-associated antigen.

## 5. Conclusion

Our data have clearly demonstrated that the MUC4-nanovaccine enhances DC surface expression of both MHC molecules and co-stimulatory ligands, and Th1 cytokine secretion. Further, in *in vivo* studies, the MUC4 nanovaccine effectively induced production of anti-MUC4β antibodies and isotype-switching of these antibodies to primarily IgG2b (Th1 type) isotypes, which typically correlates to anti-tumor immune responses [39]. Thus, this work demonstrates that polyanhydride MUC4 nanovaccines are a promising platform for immunotherapies against pancreatic cancer.

**Acknowledgments**

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The UNMC Flow Cytometry Research Facility is administrated through the Office of the Vice Chancellor for Research and supported by state funds from the Nebraska Research Initiative (NRI) and The Fred and Pamela Buffett Cancer Center's National Cancer Institute Cancer Support Grant. Major instrumentation has been provided by the Office of the Vice Chancellor for Research, The University of Nebraska Foundation, the Nebraska Banker's Fund, and by the NIH-NCRR Shared Instrument Program.

**Figure 1: Synthesis, encapsulation and release kinetics of MUC4 nanovaccine.**

Endotoxin-free recombinant MUC4β protein was isolated from Rosetta bacteria and purified by affinity chromatography. 20:80 CPTEG:CPH nanoparticles encapsulating 3% MUC4 were synthesized via a solid/oil/oil double emulsion flash nanoprecipitation process. SEM images of 20:80 CPTEG :CPH nanoparticle encapsulated recombinant mucin fragment: MUC4-β **(A).** Antigen release kinetics were characterized by incubating the nanoparticles in PBS and measuring MUC4 released at regular intervals with a microBCA assay. 3% MUC4-loaded 20:80 CPTEG:CPH nanoparticles exhibited an initial burst (20%) release of protein followed by sustained release. The encapsulation efficiency of protein was determined to be 32%, suggesting low affinity between the polymer and MUC4 protein.

# Figure 1

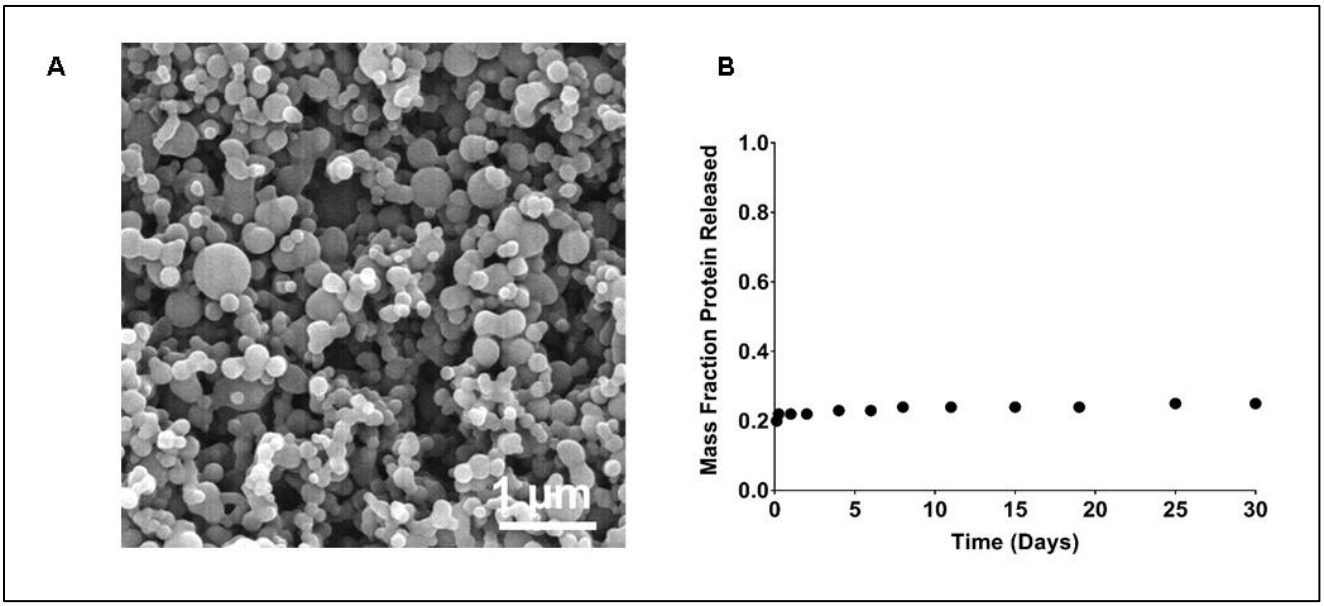

A
1 µm
B
Mass Fraction Protein Released
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Time (Days)

**Figure 2: MUC4 nanovaccine activates DCs and induces expression of MHC II and co-stimulatory molecules.**

Flow cytometry analysis demonstrated that MUC4 nanovaccine have activated DCs robustly like the LPS positive control. MUC4β (MUC4) protein has Th2 immunogenic activity that abrogates DC activation and expression of MHC and co-stimulatory molecules. Free MUC4 mixed with empty nanoparticles (MUC4+NP) slightly increases the immunogenicity of MUC4β, but only MUC4 nanovaccine could significantly activate DCs, suggesting encapsulation of MUC4β is crucial for inducing a Th1 immune responses **(A-E).** Increased DC proliferation was observed in MUC4 nanovaccine group compared to unstimulated DCs and other MUC4 treatment groups **(F)**. Statistical significance was set at $p<0.05$. ANOVA analysis of the data was $p<0.05$. Statistical comparison between MUC4, MUC4+NP & US with MUC4 nanovaccine is denoted by #, * & + respectively.

# Figure 2

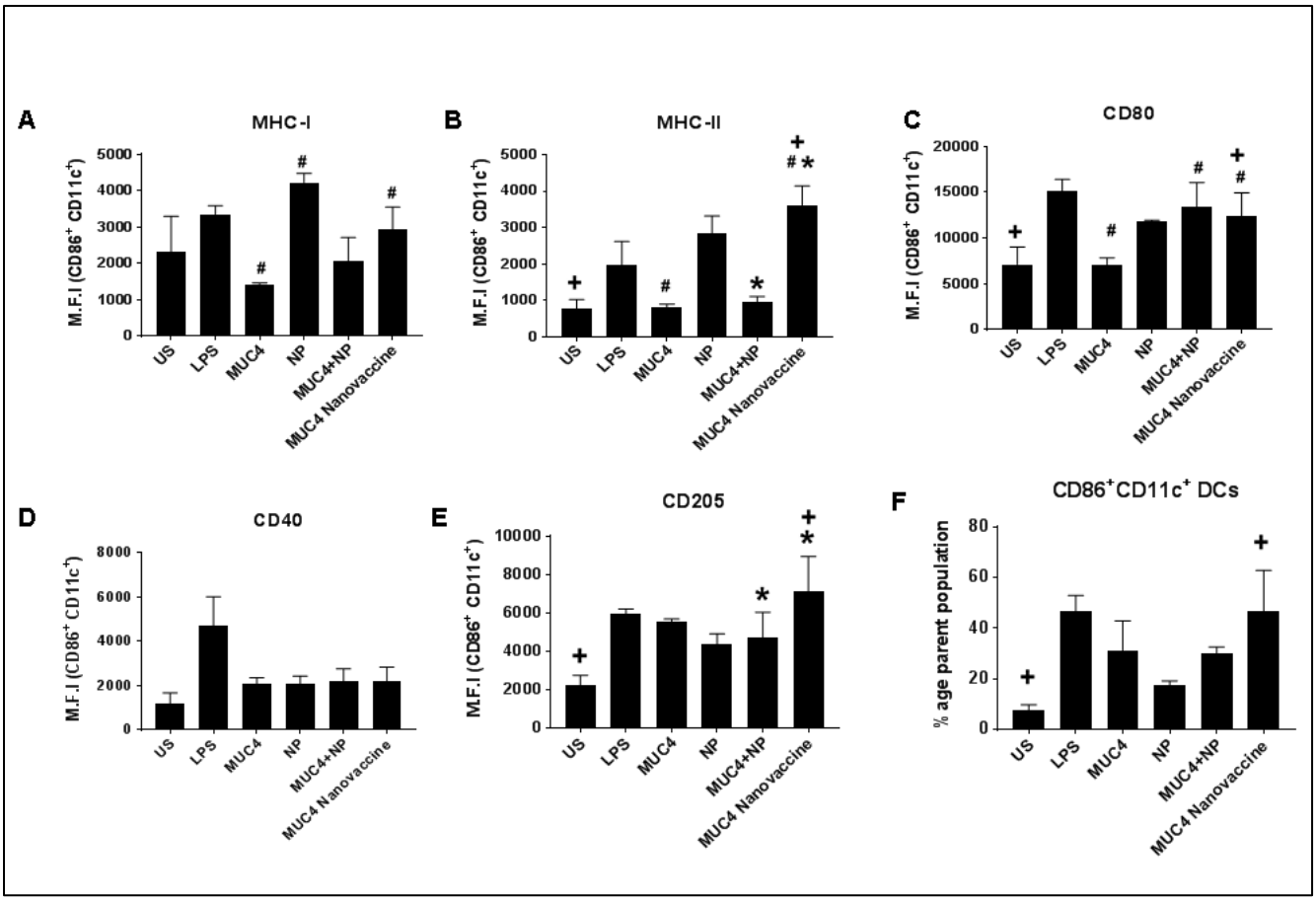

A
MHC-I
M.F.I (CD86+ CD11c+)
US
LPS
MUC4
NP
MUC4+NP
MUC4 Nanovaccine
B
MHC-II
C
CD80
D
CD40
E
CD205
F
CD86+CD11c+ DCs
% age parent population

**Figure 3: MUC4 nanovaccine induced robust Th1 DC cytokine secretion.**

Cytokine analysis demonstrated that MUC4 nanovaccine activated DCs secrete Th1 cytokines. Free MUC4 mixed with empty nanoparticles (MUC4+NP) and MUC4 pulsed DCs have low expression of Th1 cytokines, suggesting that mixing of a strong adjuvant was not able to modulate the immune response induced by MUC4. Only encapsulation of MUC4 could modulate and reprogram DCs to secrete IL12, IL6 and IFNγ Th1 cytokines **(A-C)**. Statistical significance was set at $p<0.05$. ANOVA analysis of the data was $p<0.05$. Statistical comparison between MUC4, MUC4+NP & US with MUC4 nanovaccine is denoted by #, * & + respectively.

# Figure 3

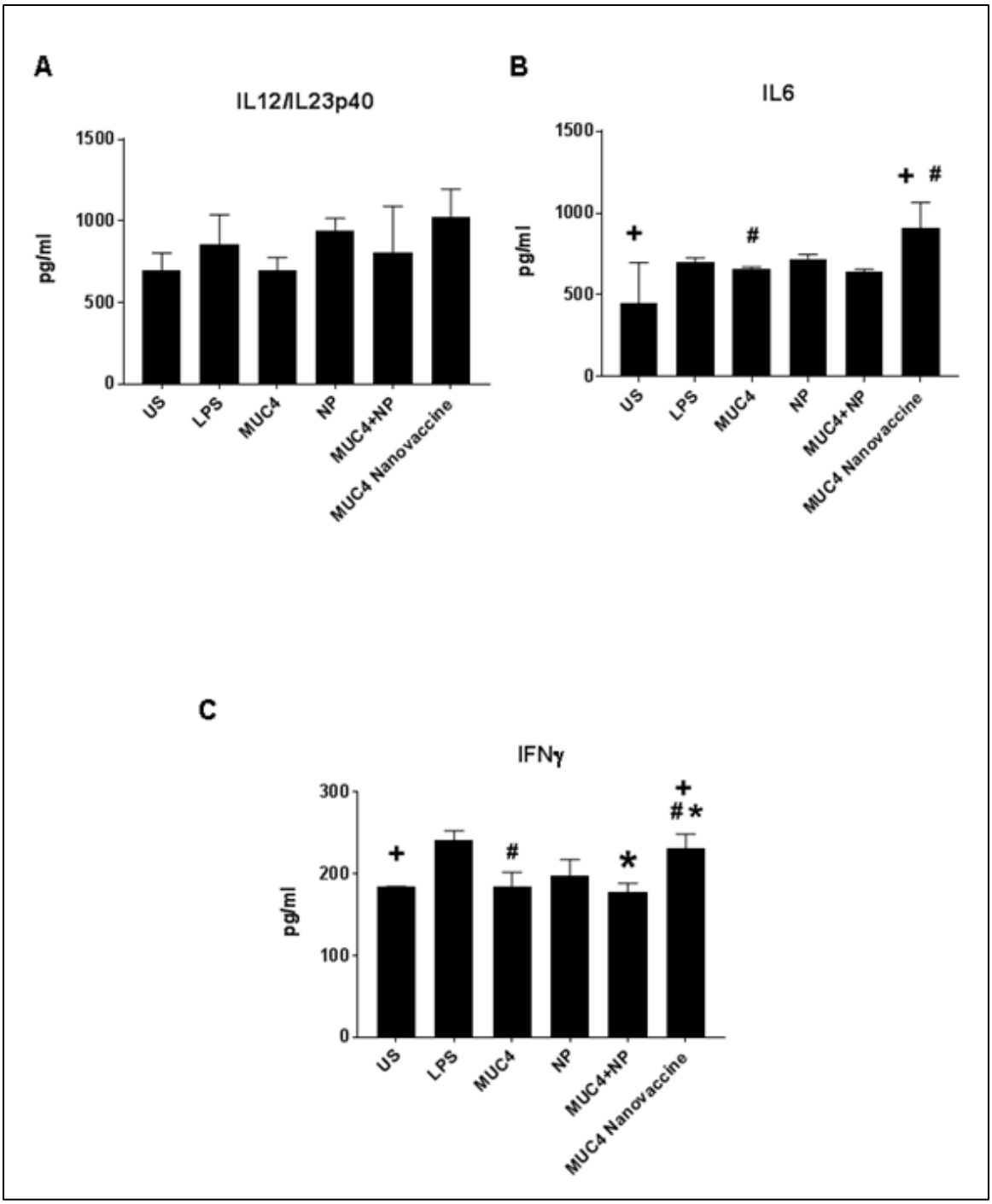

A
IL12/IL23p40
pg/ml
1500
1000
500
0
US
LPS
MUC4
NP
MUC4+NP
MUC4 Nanovaccine
B
IL6
pg/ml
1500
1000
500
0
+
#
+ #
US
LPS
MUC4
NP
MUC4+NP
MUC4 Nanovaccine
C
IFNγ
pg/ml
300
200
100
0
+
#
*
+
#*
US
LPS
MUC4
NP
MUC4+NP
MUC4 Nanovaccine

**Figure 4: Mice immunized with MUC4β nanovaccine generated anti-MUC4β Th1 humoral response.**

Eight week-old C57BL/6 mice were immunized with a single dose and serum was collected for detecting antibodies. ELISA studies showed that MUC4, MUC4+NP and MUC4 nanovaccine immunized mice serum carried MUC4β antibodies **(A)**. Further, isotyping of MUC4β antibodies demonstrated that isotype switching was predominantly to Th1 IgG2b in MUC4 nanovaccine immunized mice, whereas in MUC4 immunized mice it was predominantly in IgG1 Th2 isotype **(B)**. Statistical significance was set at $p<0.05$. ANOVA analysis of the data was $p<0.05$. Statistical comparison between MUC4, MUC4+NP and US with MUC4 nanovaccine is denoted by #, * & + respectively.

# Figure 4

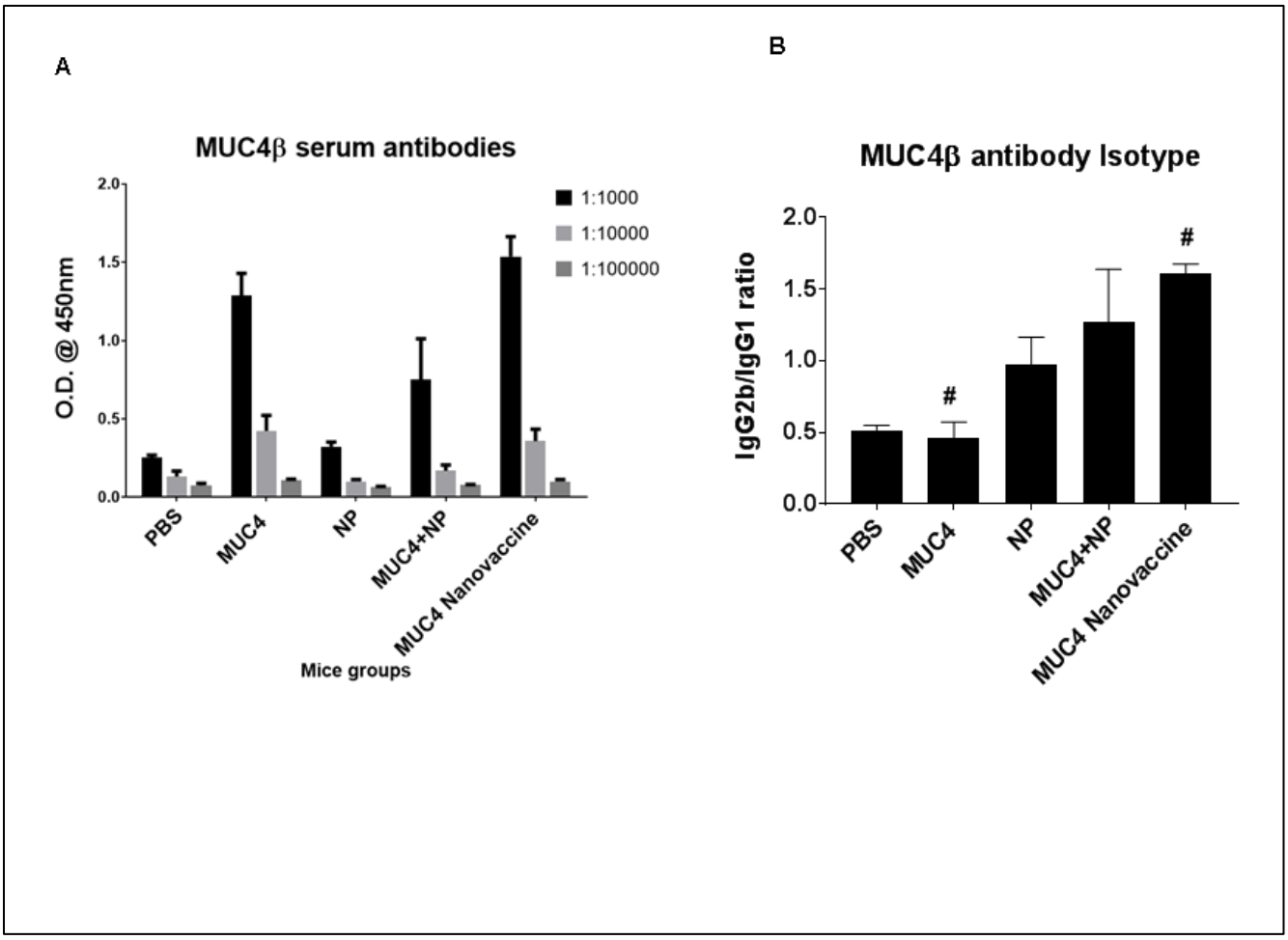
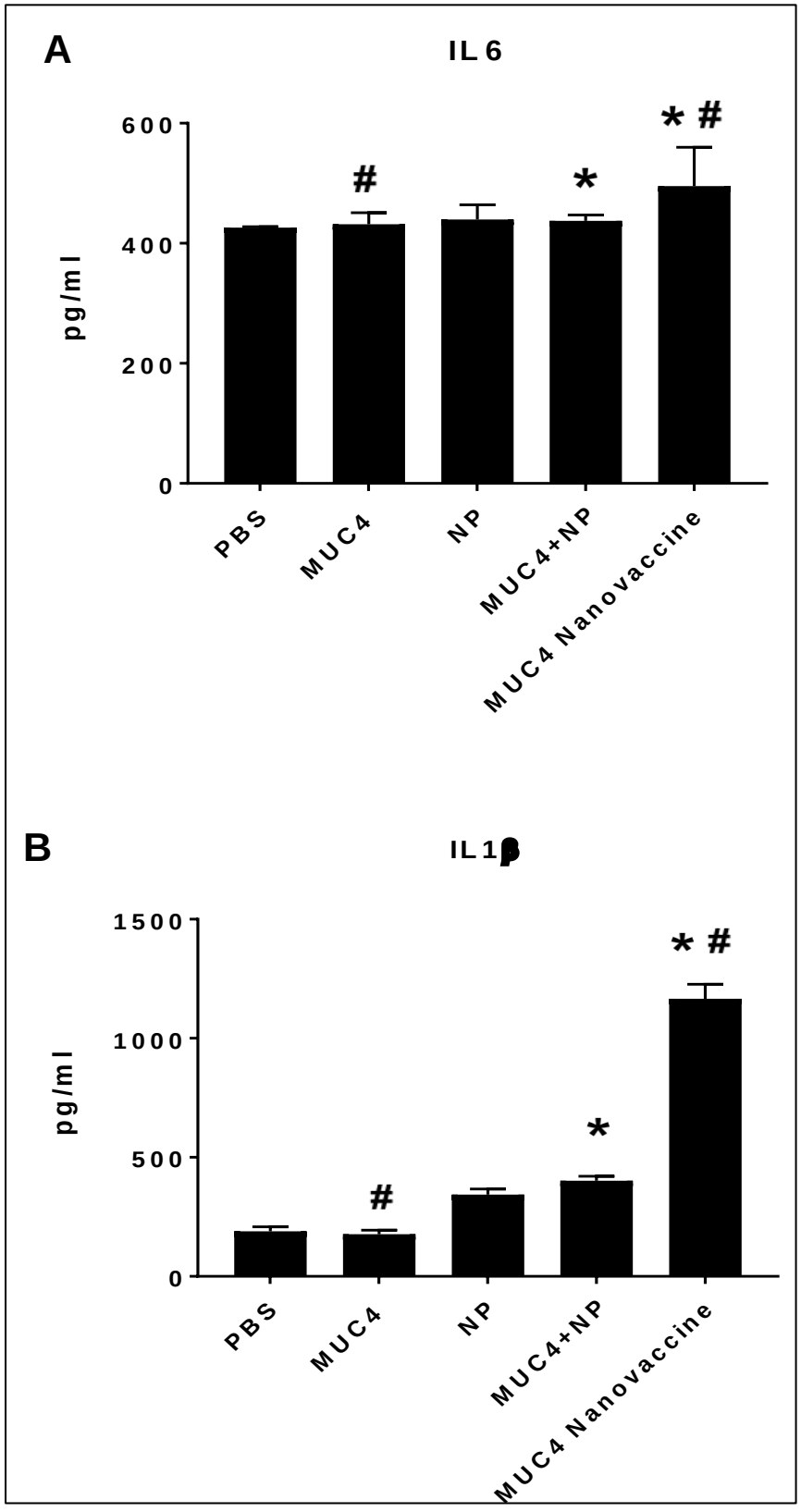

A
MUC4β serum antibodies
O.D. @ 450nm
2.0
1.5
1.0
0.5
0.0
1:1000
1:10000
1:100000
PBS
MUC4
NP
MUC4+NP
MUC4 Nanovaccine
Mice groups
B
MUC4β antibody Isotype
IgG2b/IgG1 ratio
2.0
1.5
1.0
0.5
0.0
#
#
PBS
MUC4
NP
MUC4+NP
MUC4 Nanovaccine

**Figure 5: Presence of Th1 cytokines in mice immunized with MUC4β nanovaccine.**

MUC4 nanovaccine immunized mice serum had significantly higher levels of IL6 **(A)** and IL1β **(B)** Th1 cytokines that correlated with the predominant Th1 IgG2b MUC4β antibodies present in these mice. Statistical significance was set at $p<0.05$. ANOVA analysis of the data was $p<0.05$. Statistical comparison between MUC4, and MUC4+NP with MUC4 nanovaccine is denoted by # & * respectively.

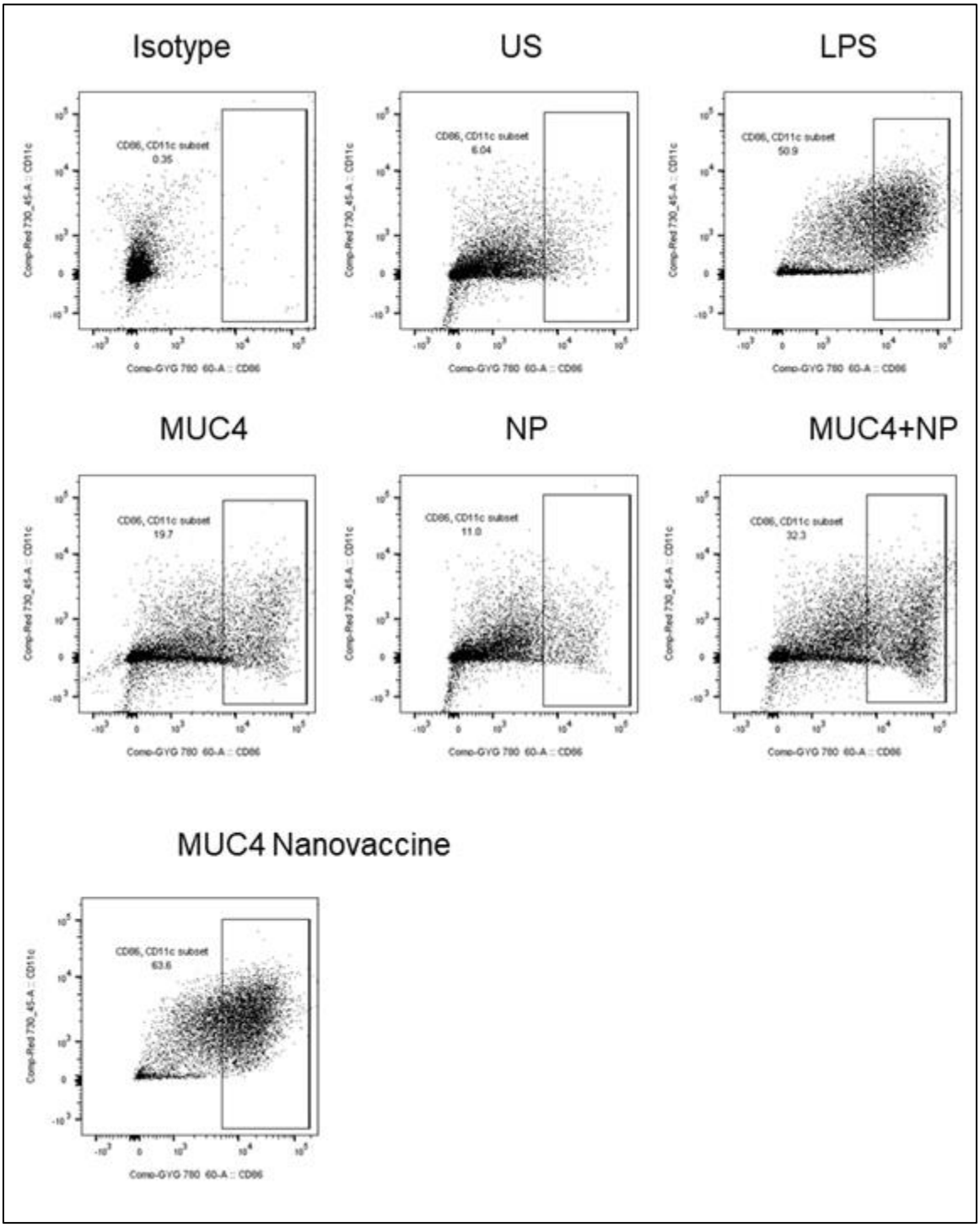
## Figure 5


A
IL 6
600
400
200
0
pg/ml
#
*
* #
PBS
MUC4
NP
MUC4+NP
MUC4 Nanovaccine
B
IL1β
1500
1000
500
0
pg/ml
#
*
* #
PBS
MUC4
NP
MUC4+NP
MUC4 Nanovaccine

**Supplementary Figure 1: MUC4 nanovaccine elicits robust proliferation of dendritic cells (DCs)**.

Total of $1X10^5$ DCs were pulsed with free MUC4β protein (MUC4), free MUC4β protein mixed with empty nanoparticles (MUC4+NP) and MUC4-nanovaccine. Unstimulated DCs (US) and LPS-treated DCs serve as negative and positive controls. Antigen-matured DCs were characterized as $CD86^{hi}$ CD11c positive ($CD86^{+}CD11c^{+}$) cells. MUC4 nanovaccine strongly stimulates DCs and robust 3-fold proliferation is observed when compared to MUC4-pulsed DCs.

## Supplementary Figure 1



## REFERENCES

1. Sideras K, Braat H, Kwekkeboom J, van Eijck CH, Peppelenbosch MP, Sleijfer S, Bruno M. Role of the immune system in pancreatic cancer progression and immune modulating treatment strategies. Cancer Treat Rev. 2014; 40: 513-22. doi: 10.1016/j.ctrv.2013.11.005.

2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin. 2018; 68: 7-30. doi: 10.3322/caac.21442.

3. Banerjee K, Kumar S, Ross KA, Gautam S, Poelaert B, Nasser MW, Aithal A, Bhatia R, Wannemuehler MJ, Narasimhan B, Solheim JC, Batra SK, Jain M. Emerging trends in the immunotherapy of pancreatic cancer. Cancer Lett. 2018; 417: 35-46. doi: 10.1016/j.canlet.2017.12.012.

4. Delitto D, Wallet SM, Hughes SJ. Targeting tumor tolerance: A new hope for pancreatic cancer therapy? Pharmacol Ther. 2016; 166: 9-29. doi: 10.1016/j.pharmthera.2016.06.008.

5. Kajihara M, Takakura K, Kanai T, Ito Z, Matsumoto Y, Shimodaira S, Okamoto M, Ohkusa T, Koido S. Advances in inducing adaptive immunity using cell-based cancer vaccines: Clinical applications in pancreatic cancer. World J Gastroenterol. 2016; 22: 4446-58. doi: 10.3748/wjg.v22.i18.4446.

6. Torres MP, Chakraborty S, Souchek J, Batra SK. Mucin-based targeted pancreatic cancer therapy. Curr Pharm Des. 2012; 18: 2472-81. doi:

7. Glaffig M, Stergiou N, Schmitt E, Kunz H. Immunogenicity of a Fully Synthetic MUC1 Glycopeptide Antitumor Vaccine Enhanced by Poly(I:C) as a TLR3-Activating Adjuvant. ChemMedChem. 2017; 12: 722-7. doi: 10.1002/cmdc.201700254.

8. Liu Y, Wang Y, Yu F, Zhang Z, Yang Z, Zhang W, Wang PG, Zhao W. Potentiating the immune response of MUC1-based antitumor vaccines using a peptide-based

nanovector as a promising vaccine adjuvant. Chem Commun (Camb). 2017; 53: 9486-9. doi: 10.1039/c7cc04386d.

9. Ramanathan RK, Lee KM, McKolanis J, Hitbold E, Schraut W, Moser AJ, Warnick E, Whiteside T, Osborne J, Kim H, Day R, Troetschel M, Finn OJ. Phase I study of a MUC1 vaccine composed of different doses of MUC1 peptide with SB-AS2 adjuvant in resected and locally advanced pancreatic cancer. Cancer Immunol Immunother. 2005; 54: 254-64. doi: 10.1007/s00262-004-0581-1.

10. Scheikl-Gatard T, Tosch C, Lemonnier F, Rooke R. Identification of new MUC1 epitopes using HLA-transgenic animals: implication for immunomonitoring. J Transl Med. 2017; 15: 154. doi: 10.1186/s12967-017-1254-0.

11. Stergiou N, Glaffig M, Jonuleit H, Schmitt E, Kunz H. Immunization with a Synthetic Human MUC1 Glycopeptide Vaccine against Tumor-Associated MUC1 Breaks Tolerance in Human MUC1 Transgenic Mice. ChemMedChem. 2017; 12: 1424-8. doi: 10.1002/cmdc.201700387.

12. Tosch C, Bastien B, Barraud L, Grellier B, Nourtier V, Gantzer M, Limacher JM, Quemeneur E, Bendjama K, Preville X. Viral based vaccine TG4010 induces broadening of specific immune response and improves outcome in advanced NSCLC. J Immunother Cancer. 2017; 5: 70. doi: 10.1186/s40425-017-0274-x.

13. Kondo H, Hazama S, Kawaoka T, Yoshino S, Yoshida S, Tokuno K, Takashima M, Ueno T, Hinoda Y, Oka M. Adoptive immunotherapy for pancreatic cancer using MUC1 peptide-pulsed dendritic cells and activated T lymphocytes. Anticancer Res. 2008; 28: 379-87. doi:

14. Rong Y, Qin X, Jin D, Lou W, Wu L, Wang D, Wu W, Ni X, Mao Z, Kuang T, Zang YQ, Qin X. A phase I pilot trial of MUC1-peptide-pulsed dendritic cells in the treatment of advanced pancreatic cancer. Clin Exp Med. 2012; 12: 173-80. doi: 10.1007/s10238-011-0159-0.

15. Singh AP, Moniaux N, Chauhan SC, Meza JL, Batra SK. Inhibition of MUC4 expression suppresses pancreatic tumor cell growth and metastasis. Cancer Res. 2004; 64: 622-30. doi:

16. Moniaux N, Escande F, Batra SK, Porchet N, Laine A, Aubert JP. Alternative splicing generates a family of putative secreted and membrane-associated MUC4 mucins. Eur J Biochem. 2000; 267: 4536-44. doi:

17. Moniaux N, Escande F, Porchet N, Aubert JP, Batra SK. Structural organization and classification of the human mucin genes. Front Biosci. 2001; 6: D1192-206. doi:

18. Moniaux N, Nollet S, Porchet N, Degand P, Laine A, Aubert JP. Complete sequence of the human mucin MUC4: a putative cell membrane-associated mucin. Biochem J. 1999; 338 ( Pt 2): 325-33. doi:

19. Cai H, Palitzsch B, Hartmann S, Stergiou N, Kunz H, Schmitt E, Westerlind U. Antibody induction directed against the tumor-associated MUC4 glycoprotein. Chembiochem. 2015; 16: 959-67. doi: 10.1002/cbic.201402689.

20. Bilati U, Allemann E, Doelker E. Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles. Eur J Pharm Biopharm. 2005; 59: 375-88. doi: 10.1016/j.ejpb.2004.10.006.

21. Bilati U, Allemann E, Doelker E. Nanoprecipitation versus emulsion-based techniques for the encapsulation of proteins into biodegradable nanoparticles and process-related stability issues. AAPS PharmSciTech. 2005; 6: E594-604. doi: 10.1208/pt060474.

22. Torres MP, Determan AS, Anderson GL, Mallapragada SK, Narasimhan B. Amphiphilic polyanhydrides for protein stabilization and release. Biomaterials. 2007; 28: 108-16. doi: 10.1016/j.biomaterials.2006.08.047.

23. Torres MP, Vogel BM, Narasimhan B, Mallapragada SK. Synthesis and characterization of novel polyanhydrides with tailored erosion mechanisms. J Biomed Mater Res A. 2006; 76: 102-10. doi: 10.1002/jbm.a.30510.

24. Chavez-Santoscoy AV, Roychoudhury R, Pohl NL, Wannemuehler MJ, Narasimhan B, Ramer-Tait AE. Tailoring the immune response by targeting C-type lectin receptors on alveolar macrophages using "pathogen-like" amphiphilic polyanhydride nanoparticles. Biomaterials. 2012; 33: 4762-72. doi: 10.1016/j.biomaterials.2012.03.027.

25. Haughney SL, Ross KA, Boggiatto PM, Wannemuehler MJ, Narasimhan B. Effect of nanovaccine chemistry on humoral immune response kinetics and maturation. Nanoscale. 2014; 6: 13770-8. doi: 10.1039/c4nr03724c.

26. Petersen LK, Ramer-Tait AE, Broderick SR, Kong CS, Ulery BD, Rajan K, Wannemuehler MJ, Narasimhan B. Activation of innate immune responses in a pathogen-mimicking manner by amphiphilic polyanhydride nanoparticle adjuvants. Biomaterials. 2011; 32: 6815-22. doi: 10.1016/j.biomaterials.2011.05.063.

27. Torres MP, Wilson-Welder JH, Lopac SK, Phanse Y, Carrillo-Conde B, Ramer-Tait AE, Bellaire BH, Wannemuehler MJ, Narasimhan B. Polyanhydride microparticles enhance dendritic cell antigen presentation and activation. Acta Biomater. 2011; 7: 2857-64. doi: 10.1016/j.actbio.2011.03.023.

28. Joshi VB, Geary SM, Carrillo-Conde BR, Narasimhan B, Salem AK. Characterizing the antitumor response in mice treated with antigen-loaded polyanhydride microparticles. Acta Biomater. 2013; 9: 5583-9. doi: 10.1016/j.actbio.2012.11.001.

29. Haughney SL, Petersen LK, Schoofs AD, Ramer-Tait AE, King JD, Briles DE, Wannemuehler MJ, Narasimhan B. Retention of structure, antigenicity, and biological function of pneumococcal surface protein A (PspA) released from polyanhydride nanoparticles. Acta Biomater. 2013; 9: 8262-71. doi: 10.1016/j.actbio.2013.06.006.

30. Vela Ramirez JE, Tygrett LT, Hao J, Habte HH, Cho MW, Greenspan NS, Waldschmidt TJ, Narasimhan B. Polyanhydride Nanovaccines Induce Germinal Center B Cell Formation and Sustained Serum Antibody Responses. J Biomed Nanotechnol. 2016; 12: 1303-11. doi:

31. Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. Nat Immunol. 2001; 2: 947-50. doi: 10.1038/ni712.

32. Deligne C, Metidji A, Fridman WH, Teillaud JL. Anti-CD20 therapy induces a memory Th1 response through the IFN-gamma/IL-12 axis and prevents protumor regulatory T-cell expansion in mice. Leukemia. 2015; 29: 947-57. doi: 10.1038/leu.2014.275.

33. Pradhan P, Qin H, Leleux JA, Gwak D, Sakamaki I, Kwak LW, Roy K. The effect of combined IL10 siRNA and CpG ODN as pathogen-mimicking microparticles on Th1/Th2 cytokine balance in dendritic cells and protective immunity against B cell lymphoma. Biomaterials. 2014; 35: 5491-504. doi: 10.1016/j.biomaterials.2014.03.039.

34. Huang CY, Chen JJ, Shen KY, Chang LS, Yeh YC, Chen IH, Chong P, Liu SJ, Leng CH. Recombinant lipidated HPV E7 induces a Th-1-biased immune response and protective immunity against cervical cancer in a mouse model. PLoS One. 2012; 7: e40970. doi: 10.1371/journal.pone.0040970.

35. Ulery BD, Kumar D, Ramer-Tait AE, Metzger DW, Wannemuehler MJ, Narasimhan B. Design of a protective single-dose intranasal nanoparticle-based vaccine platform for respiratory infectious diseases. PLoS One. 2011; 6: e17642. doi: 10.1371/journal.pone.0017642.

36. Wilson-Welder JH, Torres MP, Kipper MJ, Mallapragada SK, Wannemuehler MJ, Narasimhan B. Vaccine adjuvants: current challenges and future approaches. J Pharm Sci. 2009; 98: 1278-316. doi: 10.1002/jps.21523.

37. Richards JO, Albers AJ, Smith TS, Tjoe JA. NK cell-mediated antibody-dependent cellular cytotoxicity is enhanced by tamoxifen in HER2/neu non-amplified, but not HER2/neu-amplified, breast cancer cells. Cancer Immunol Immunother. 2016; 65: 1325-35. doi: 10.1007/s00262-016-1885-7.

38. Wang W, Erbe AK, Hank JA, Morris ZS, Sondel PM. NK Cell-Mediated Antibody-Dependent Cellular Cytotoxicity in Cancer Immunotherapy. Front Immunol. 2015; 6: 368. doi: 10.3389/fimmu.2015.00368.

39. Wittrup KD. Antitumor Antibodies Can Drive Therapeutic T Cell Responses. Trends Cancer. 2017; 3: 615-20. doi: 10.1016/j.trecan.2017.07.001.

# CHAPTER 5: COMBINING IMMUNE CHECKPOINT BLOCKADE WITH MUC4 NANOVACCINE FOR PC TREATMENT

# 1. Synopsis

Pancreatic cancer (PC) is a highly metastatic and therapy-resistant malignancy characterized by immunosuppressive tumor microenvironment (TME) mucin overexpression. Immunotherapy strategies for PC treatment are constantly being developed and tested in clinical trials but have achieved underwhelming outcomes. MUC4 is the most differentially overexpressed mucin and functionally contributes to PC disease aggressiveness. Our previous studies have demonstrated that MUC4 is immunogenic in PC patients and is thus an ideal candidate for targeted therapies. We developed a novel vaccine using recombinant MUC4 fragments and exploiting adjuvant-like properties of an amphiphilic polyanhydride-based nanoparticle delivery system. In addition, we showed that recombinant human MUC4β protein encapsulated in amphiphilic polyanhydride nanoparticles (MUC4 nanovaccine) successfully activates dendritic cells and induces Th1 anti-MUC4β antibodies in immunized C57BL/6 mice. Tumor cells are also known to express programmed death ligand 1 (PD-L1) to suppress effector immune cells activity via the PD-L1-PD-1 axis. Our preliminary study elucidated that MUC4 nanovaccine-immunized mice exhibited slower tumor growth kinetics than unimmunized control mice. However, we did not observe complete tumor regression and detected PD-L1 expression on MUC4-expressing tumors only. Based on this, we rationalized that PD-L1 expression by MUC4-expressing tumor cells suppressed and inhibited the therapeutic benefits of the nanovaccine in-vivo. The strong involvement of MUC4 in disease aggressiveness and PD-L1 in immunosuppression thus makes a compelling case for their combined targeting.

## 2. Background and Rationale

The PC tumor microenvironment is a complex relationship between cellular components, desmoplasia and cytokine milieu. PC's dense stroma/desmoplasia harbor immune cells such as TAMs, MDSCs, and $T_{reg}$ cells that directly inhibit the activity of effector $CD4^+$ and $CD8^+$ T-cells. These pro-tumor immune cells further secrete anti-inflammatory Th2 cytokines like IL-4, IL-10 and IL-13, which negatively regulate the infiltration and functionality of effector immune cells by precluding immunological recognition [1-3]. To overcome the immune suppression elicited by the TME of pancreatic cancer, reprogramming of patient's immune system and generation of strong anti-tumor Th1 responses are necessary. Therapeutic cancer vaccines have emerged as a tool to reprogram and activate the patient's immune system in a tumor antigen-specific manner to effectively target tumor cells [4, 5]. Cancer vaccines have seen limited clinical success in some solid tumors such as lung cancer, breast cancer and renal cancer. A major factor that could improve the efficacy of these vaccines is firstly identifying TAAs that could be targeted [5]. Our previous study has identified MUC4 to be immunogenic in PC patients, suggesting that these patients have compromised peripheral tolerance.

A second major factor is a generation of robust anti-tumor responses to cancer vaccines to overcome peripheral immune tolerance and escape immune suppression of TME. To break tolerance and constrain immune suppression, selection of a strong immunological adjuvant to be combined with the antigen is required [5-7]. Adjuvants are an instrumental component of a potent vaccine that enhances the immunogenicity of the antigen and increases the antigen-specific immune response. However, developing a cancer vaccine with a successful adjuvant is not easy because the adjuvant must preserve the antigen and needs to be safe, potent and economically viable [6, 7]. We developed a novel MUC4 nanovaccine by encapsulating human MUC4β recombinant protein in

pathogen-mimicking amphiphilic polyanhydride nanoparticles. We observed that the MUC4 nanovaccine elicited robust activation of DCs by upregulating surface expression of DC activation markers, as well as secretion of Th1 cytokines like IL12, IL6 and IFNγ. Further, we elucidated that mice immunized with MUC4 nanovaccine carried high levels of Th1 IgG2b anti-MUC4β antibodies circulating in their serum. These data provide initial evidence that MUC4 nanovaccine could potentially serve as a potent immunotherapy strategy to treat PC.

The PD-L1 co-inhibitory molecule expressed on tumor cells binds to its receptor programmed cell death protein 1 (PD-1) present on T cells and negatively regulates T-cell signaling and effector functions [8]. PD-1 is expressed only on antigen-experienced $CD4^+$ and $CD8^+$ T cells, as well as B cells, and is absent on resting T and B cells [9]. Its ligand PD-L1, also known as cluster of differentiation 274 (CD274) or B7 homolog 1 (B7-H1), is expressed on activated DCs and macrophages[10], as well as non-lymphoid tissues like cancer cells upon IFNγ stimulation [11]. PD-L1 is also expressed in peripheral tissues, thus suggesting its role in peripheral tolerance against self-reactive T and B cells, and may serve in regulating inflammatory responses at these sites [12]. PD-L1, upon binding to its receptor PD-1, inhibits T cell proliferation and its effector functions by inducing apoptosis. In addition, this axis promotes differentiation of $CD4^+$ T cells into $Foxp3^+$ regulatory T cells [13-15].

One of the major challenges that have emerged from recent preclinical studies is the counter PD-L1 mediated-suppression elicited on infiltrating effector lymphocytes by tumor cells, therefore inhibiting the efficacy of these vaccines [16-18]. Expression of PD-L1 by tumor cells is an indicator of an active immune interaction occurring between cytotoxic $CD8^+$ T-cells and tumor cells. Blockade of PD-1 receptor on effector T-cells to its ligand PD-L1 by PD-1 inhibitors have shown some promise in rescuing anti-tumor effects

as been discussed in the following review [1]. However, PC patients show differential expression of PD-L1 in tumors due to the various degree of effector T-cells infiltration found in the TME [19]. Combined high percentage of infiltrating $CD8^+$ T-cells and high PD-L1 expression is correlated with lymph node metastasis and poor survival of PC patients [20-22].

Based on the data, our working goal was to investigate whether MUC4 nanovaccine could provide immunity against MUC4 tumor-bearing mice. Our preliminary data suggest that MUC4 nanovaccine has the potential to induce anti-tumor responses. We observed a positive correlation between TILs and tumor regression. Accumulation of infiltrating CD8+ and CD4+ T cells was greater in mice receiving the MUC4 nanovaccine compared to soluble MUC4 delivered with blank nanoparticles (MUC4+NP), indicating the benefit of sustained availability of antigen via encapsulation. However, we didn't achieve complete clearing of the MUC4-expressing tumor and thus investigated the PD-L1 expression in them. We did observe that MUC4 tumors had high expression of PD-L1 on tumor cells whereas only low expression of PD-L1 was seen in vector control tumors. These results suggest that MUC4 nanovaccine combined with checkpoint blockade could have enhanced therapeutic potential.

# 3. Results

## 3.1 The Cytotoxic Killing of MUC4-Expressing Cell Lines by CTLs

miniMUC4 construct captures the entire structure of original human MUC4 protein but has approximately 90% of the VNTR region missing due to which it runs at a lower molecular weight of 250-300 kDa in 2% agarose SDS gel [23]. Our cytotoxicity assay demonstrated that T-cells activated *ex vivo* by MUC4 nanovaccine-pulsed DCs mediated 25% higher antigenic-specific killing of miniMUC4 expressing-KPC960 cells when

compared to T-cells activated by MUC4+NP-pulsed DCs **(Figure 1)**. Based on this, we proposed to elucidate the therapeutic potential of MUC4 nanovaccine in *in vivo* model.

### 3.2 MUC4 Nanovaccine Immunized Mice have Slower Tumor Growth Kinetics

To fully understand the preventive and therapeutic role of MUC4 nanovaccine, we pre-immunized the mice with primary dose and first booster. After tumor cells implantation, once the tumor volume reached 100 $mm^3$, we immunized the mice with a lower second booster dose **(Figure 2A)**. We observed that miniMUC4 tumors had slower growth kinetics with respect to its contralateral vector control KPC960 tumors. In addition, miniMUC4 tumors in MUC4 nanovaccine-immunized mice had overall lower tumor volume when compared to the unimmunized control mice **(Figure 2B & C)**.

### 3.3 MUC4 Nanovaccine Enhanced Immune Cell Infiltration in miniMUC4 Tumors

Our observation of slower tumor growth kinetics and overall smaller tumor volume of miniMUC4 tumors, when compared to vector control tumors, in MUC4 nanovaccine-immunized mice, made us curious to investigate the immune cell infiltration in miniMUC4 tumors. Hematoxylin and eosin staining of tumor tissue sections showed a MUC4-specific immune cell infiltration in miniMUC4 tumor cells in the MUC4 nanovaccine immunized mice **(Figure 3A)**. We did observe some antigen-specific immune cell infiltration in miniMUC4 tumors of free soluble MUC4-immunized mice, but the percentage of infiltration was significantly lower than MUC4 nanovaccine-immunized mice. The pathologist noticed an area of necrosis proximal to the immune infiltrate region, which were quantified. We found a positive correlation between tumor necrosis and the degree of immune cell infiltration in miniMUC4 tumors. MUC4 nanovaccine-immunized mice had 5% higher tumor necrosis than other groups where the MUC4 antigen was provided **(Figure 3B)**.

Further, we investigated the percentage of $CD8^+$ and $CD4^+$ T-cells infiltrated in miniMUC4 tumor cells by immunofluorescence and confocal microscopy **(Figure 4 A-B)**. We observed that in MUC4 nanovaccine-immunized mice, 65% $CD8^+$ and 25% $CD4^+$ of infiltrating T-cells were present in miniMUC4 tumors, whereas only 33% $CD8^+$ and 9% $CD4^+$ of infiltrating T-cells were detected in miniMUC4 tumors of MUC4+NP-immunized mice **(Figure 4 C-D)**. These data provide a rationale for the slower miniMUC4 tumor growth kinetics observed in MUC4 nanovaccine-immunized mice.

### 3.4 MUC4 Nanovaccine Induces IFNγ Mediated PD-L1 Expression on miniMUC4 Tumor Cells

Our data suggest that MUC4 nanovaccine is inducing immunological targeting of miniMUC4 tumors; however we did not achieve complete tumor regression. To overcome immunosuppression of PC tumor cells, it is well understood that cancer vaccines need to generate robust cellular responses as well as high levels of Th1 cytokines. To evaluate whether MUC4 nanovaccine was able to induce strong cytokine secretion, we analyzed through flow cytometry the levels of Th1 cytokines produced by effector T cells. Our analysis demonstrated that significantly high levels ($P<0.01$) of Th1 $CD4^+$ (Tbet-positive) T-cells were present in MUC4 nanovaccine-immunized mice. In addition, both $CD8^+$ and $CD4^+$ T-cells produced robust levels of IFNγ cytokine in contrast to unimmunized mice **(Figure 5 A-B)**.

Survey of literature has elucidated that IFNγ secreted by effector T-cells could potentially induce PD-L1 expression by tumor cell as a counterattack mechanism and inactivate the functionality of cytotoxic T-cells [11]. Since our data showed that significantly higher levels of IFNγ are produced by effector T-cells, we were curious to understand whether IFNγ was inducing PD-L1 expression on miniMUC4 tumor cells that lead to the limited success of MUC4 nanovaccine in our *in vivo* mouse model. IHC staining of PD-L1

revealed that PD-L1 was expressed on the surface of miniMUC4 tumor cells and stroma from MUC4 nanovaccine-immunized mice **(Figure 5C)**. There was some low-intensity stromal staining and no tumor cell staining was observed in vector control tumors, whereas cellular surface staining of PD-L1 was observed on miniMUC4 tumor cells, suggesting an active cross-talk between effector T-cells and tumor cells, which corroborates with the high degree of immune infiltration detected in miniMUC4 tumors removed from MUC4 nanovaccine-immunized mice.

## 4. Discussion

The present study investigated whether MUC4 nanovaccine could elicit anti-tumor responses and abrogate from tumor growth. Studies from our lab have shown that MUC4 nanovaccine could potentially activate dendritic cells and humoral responses. But whether the cancer vaccine could provide cellular immunity against MUC4 expressing tumor cells needed to be investigated. Thus, we did a preliminary study to investigate the preventive and therapeutic potential of MUC4 nanovaccine in a subcutaneous PC tumor mice model. Our *ex vivo* experiments provided evidence that MUC4 nanovaccine could activate effector T-cells in MUC4-specific manner and induce robust cytotoxic killing of MUC4 expressing KPC960 cells. Based on this data, we investigated whether MUC4 nanovaccine could inhibit the growth of MUC4 expressing tumors and lead to complete tumor regression. Our results suggest that even though MUC4 nanovaccine could significantly reduce the tumor growth kinetics and tumor volume along with increased immune cells infiltration and Th1 cytokine production, complete tumor regression was not attained in these immunized mice.

One of the reasons for not being able to attain a complete regression was that this study was done in C57BL/6-FBP mixed background mice, due to which different HLA

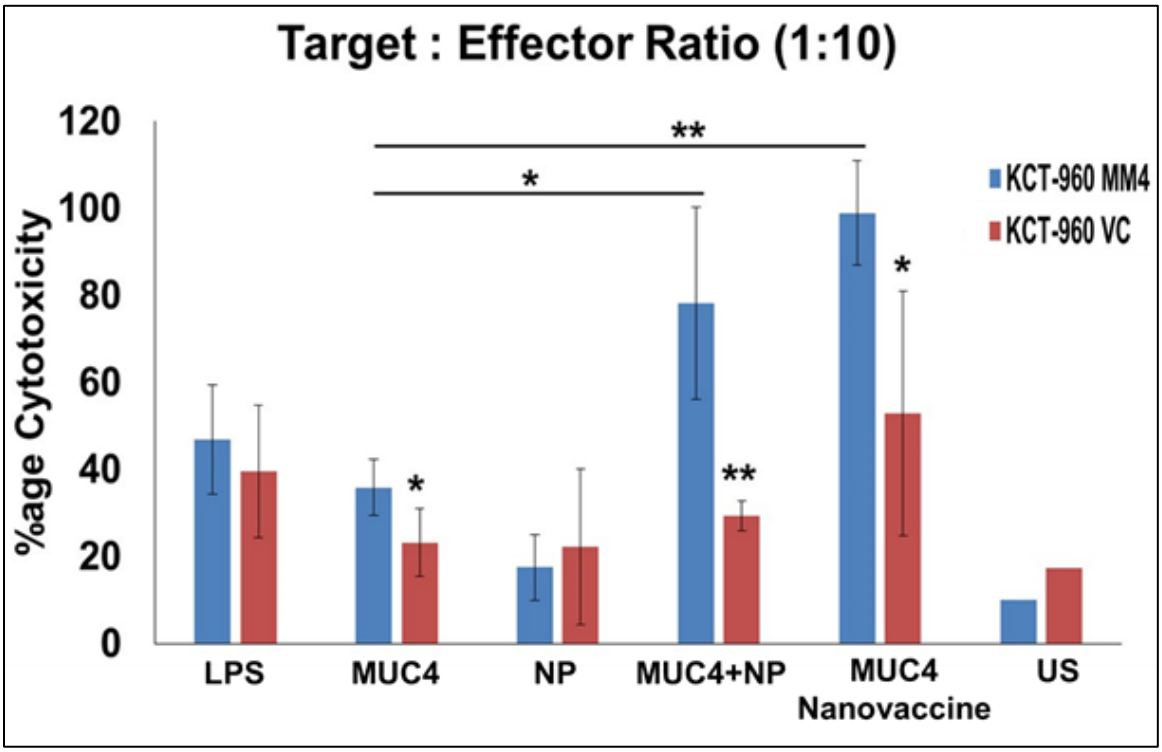
haplotypes could lead to limited activation of cytotoxic T cells and recognition of tumor cells by CTLs. Another reason for the limited success was due to the immune suppression exhibited by the PD-L1 expressed by tumor cells on CTLs. We propose that IFNγ secreted by effector T-cells induced PD-L1 immunoinhibitory ligand expression on miniMUC4 tumor cell surface as an immunosuppressive response and thus induced inactivation of CTLs that resulted in reduced tumor volume but no complete regression.

To evaluate and elucidate the full potential of MUC4 nanovaccine as a potent immunotherapy strategy for PC, we need to study MUC4 nanovaccine in a syngeneic PC mice model. Further to fully replicate clinical settings, we are required to study the nanovaccine in human MUC4 transgenic genetically engineered PC mice model. Our data suggest that combining checkpoint blockade therapy with MUC4 nanovaccine could potentially levitate the immunosuppression exhibited by PC tumor, which needs to be investigated in the future.

**Figure 1: MUC4 nanovaccine pulsed DCs activated CTLs in an antigen-specific manner and induced cytotoxic killing of miniMUC4-expressing murine pancreatic cancer cell lines.**

Spontaneous pancreatic cancer mouse model (KPC)-derived pancreatic cell line (KCT-960) was transfected with the Mini-MUC4 construct and was used for LDH cytotoxicity assay. The assay showed the specific killing of Mini-MUC4 expressing cells compared to vector control **(A)**.

# Figure 1


Target : Effector Ratio (1:10)
%age Cytotoxicity
120
100
80
60
40
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**
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*
KCT-960 MM4
KCT-960 VC
LPS
MUC4
NP
MUC4+NP
MUC4 Nanovaccine
US

**Figure 2: Schematic representation of tumor implantation and treatment by MUC4 nanovaccine. MUC4 nanovaccine-immunized mice showed a decrease in tumor growth.**

Immunization of mice was done as indicated. Mice were injected with $1x10^6$ cells of miniMUC4 or Vector expressing into right and left flanks of mixed background mice (n=5), respectively. Immunization of tumor-bearing mice to analyze the efficacy of the MUC4 nanovaccine **(A)**. Tumors were harvested at day 23. MUC4 nanovaccine-treated mice (inverted green triangle) showed tumor size reduction (below the red median line) in mini-MUC4 tumors with respect to contralateral vector control tumor, in comparison to unimmunized control mice (orange sphere) **(B).** Representative photograph of tumors isolated from Control and MUC4 nanovaccine immunized mice group **(C)**.

# Figure 2

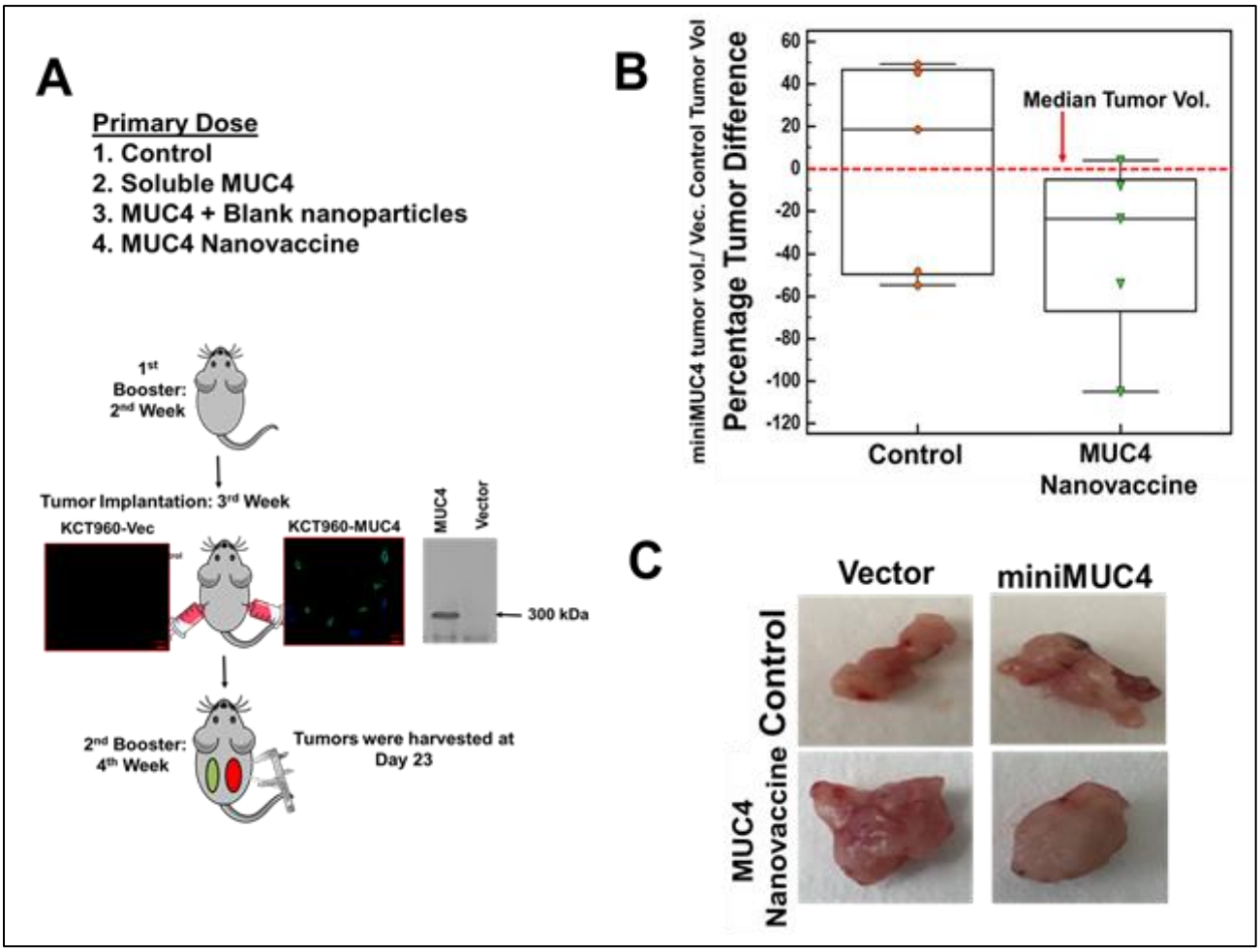

A
Primary Dose
1. Control
2. Soluble MUC4
3. MUC4 + Blank nanoparticles
4. MUC4 Nanovaccine
1st Booster: 2nd Week
Tumor Implantation: 3rd Week
KCT960-Vec
KCT960-MUC4
MUC4
Vector
300 kDa
2nd Booster: 4th Week
Tumors were harvested at Day 23
B
miniMUC4 tumor vol./ Vec. Control Tumor Vol
Percentage Tumor Difference
Median Tumor Vol.
Control
MUC4 Nanovaccine
C
Vector
miniMUC4
Control
MUC4 Nanovaccine

**Figure 3: MUC4 nanovaccine enhanced immune infiltration and corresponding necrosis in the miniMUC4 tumor.**

The miniMUC4- or vector-expressing KCT-960 cells were implanted into contralateral right and left flank of the same mouse, respectively. These mice were pre-immunized and received second booster post-tumor implantation, with MUC4 free protein mixed with empty CPTEG: CPH 20:80 nanoparticles (MUC4+NP) in PBS, MUC4 free protein in PBS and MUC4 nanovaccine in PBS as indicated in **Figure 2A**. Mice immunized with the MUC4 nanovaccine formulation demonstrated increased infiltration of immune cells **(A)**. Pathological analysis of tumor tissues showed that encapsulated MUC4 induced greater necrosis in MUC4-expressing tumors compared to all other treatment groups **(B)**.

# Figure 3

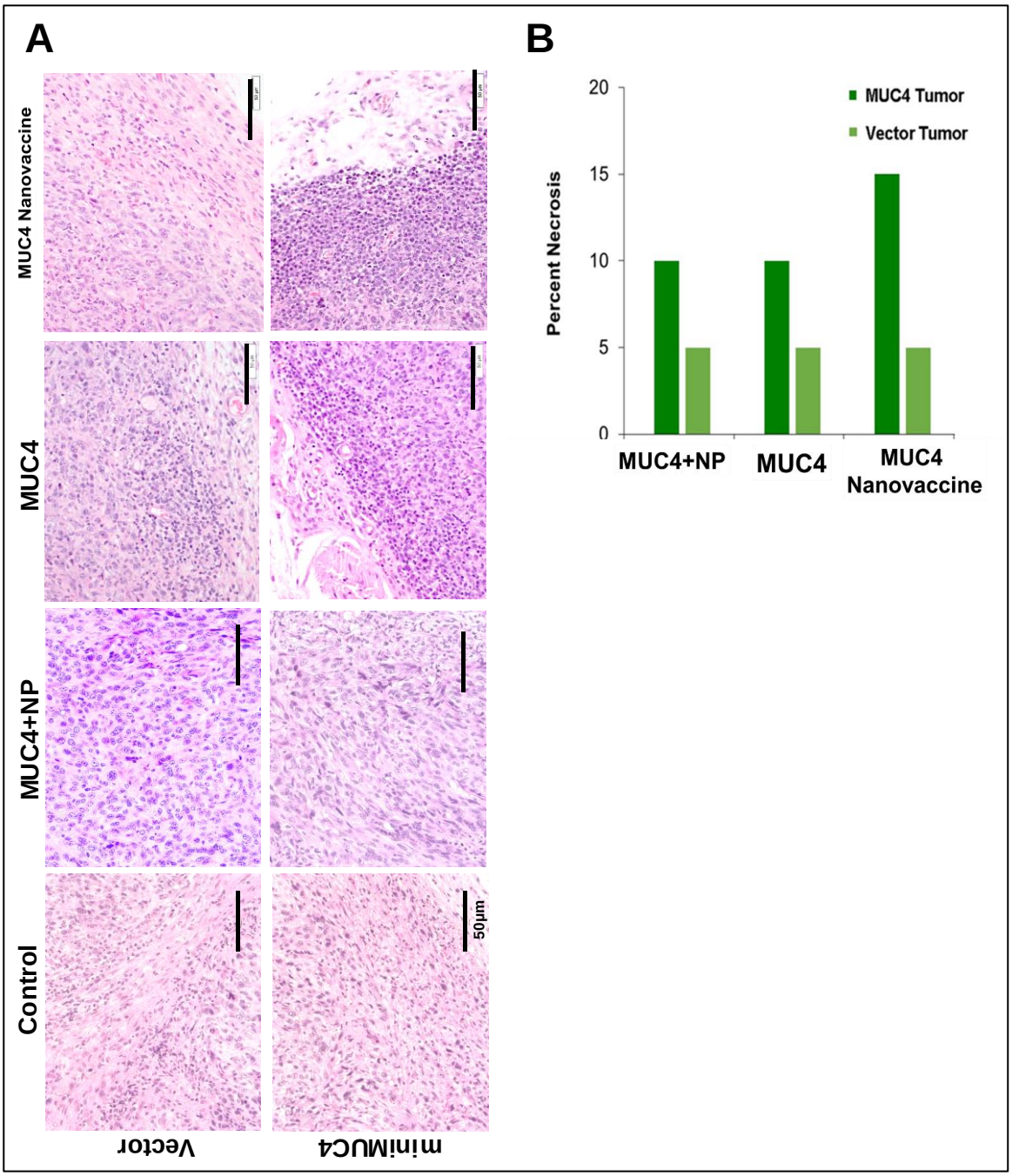

A
B
MUC4 Nanovaccine
MUC4
MUC4+NP
Control
Vector
miniMUC4
50μm
Percent Necrosis
20
15
10
5
0
MUC4 Tumor
Vector Tumor
MUC4+NP
MUC4
MUC4 Nanovaccine

**Figure 4: $CD4^+$ and $CD8^+$ T cell populations in miniMUC4 or Vector expressing mouse pancreatic ductal (PDAC) tumors.**

Mice were pre-immunized and received second booster post-tumor implantation, with MUC4 free protein mixed with empty CPTEG: CPH 20:80 nanoparticles (MUC4+NP) in PBS, MUC4 free protein in PBS and MUC4 nanovaccine in PBS as indicated in **Figure 2A**.Tumor tissues were stained with CD8 **(A and B)** CD4 **(C and D**) T cells surface marker antibodies and were subjected to Immunofluorescence (IF) analysis by EVOS microscope. Quantification of infiltrating $CD8^+$ and $CD4^+$T cells in miniMUC4 or Vector expressing PDAC tumors in MUC4 nanovaccine- or miniMUC4+NP-immunized mice ($p<0.01$ by Tukey's t-Test) **(B and D)**.

# Figure 4

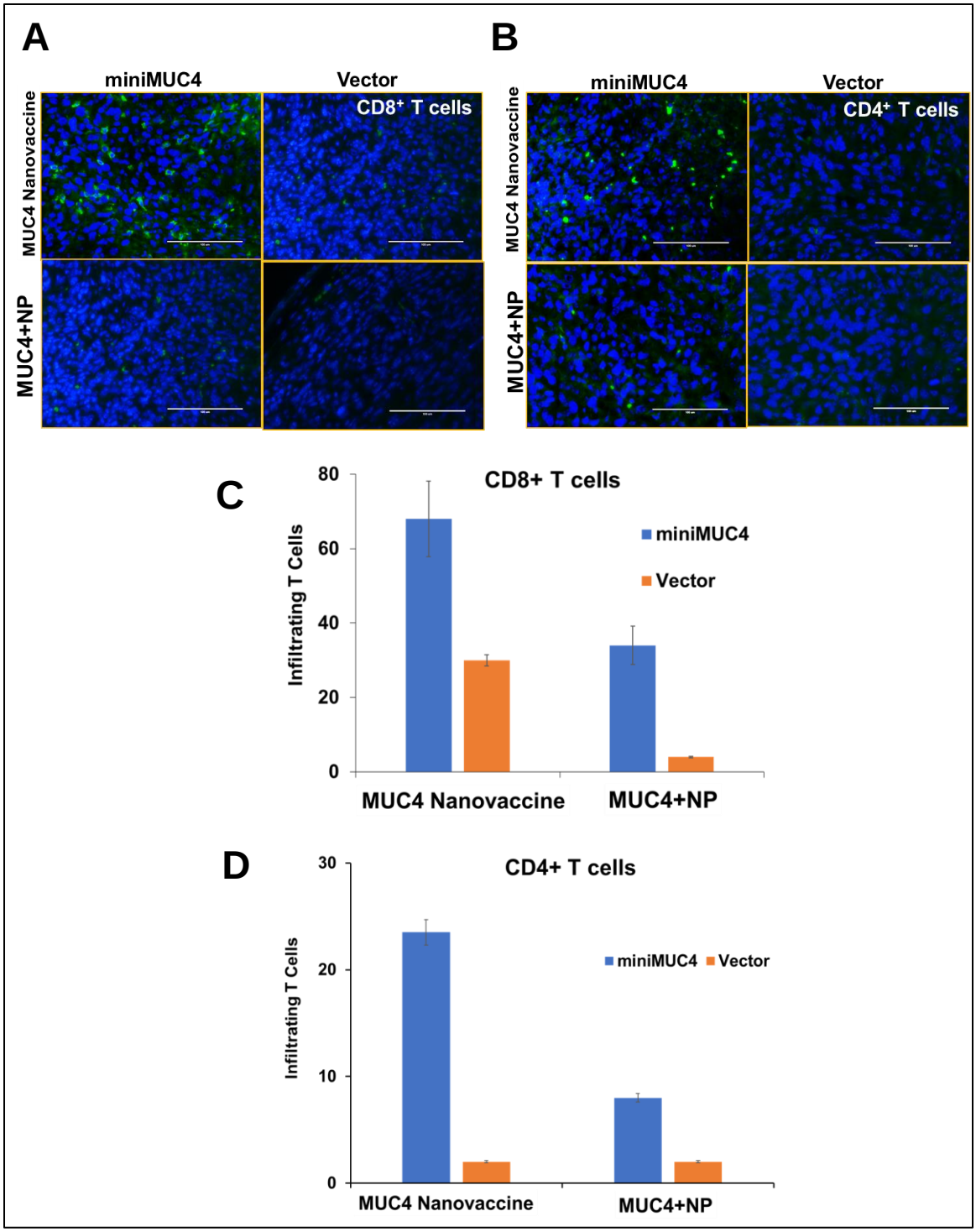
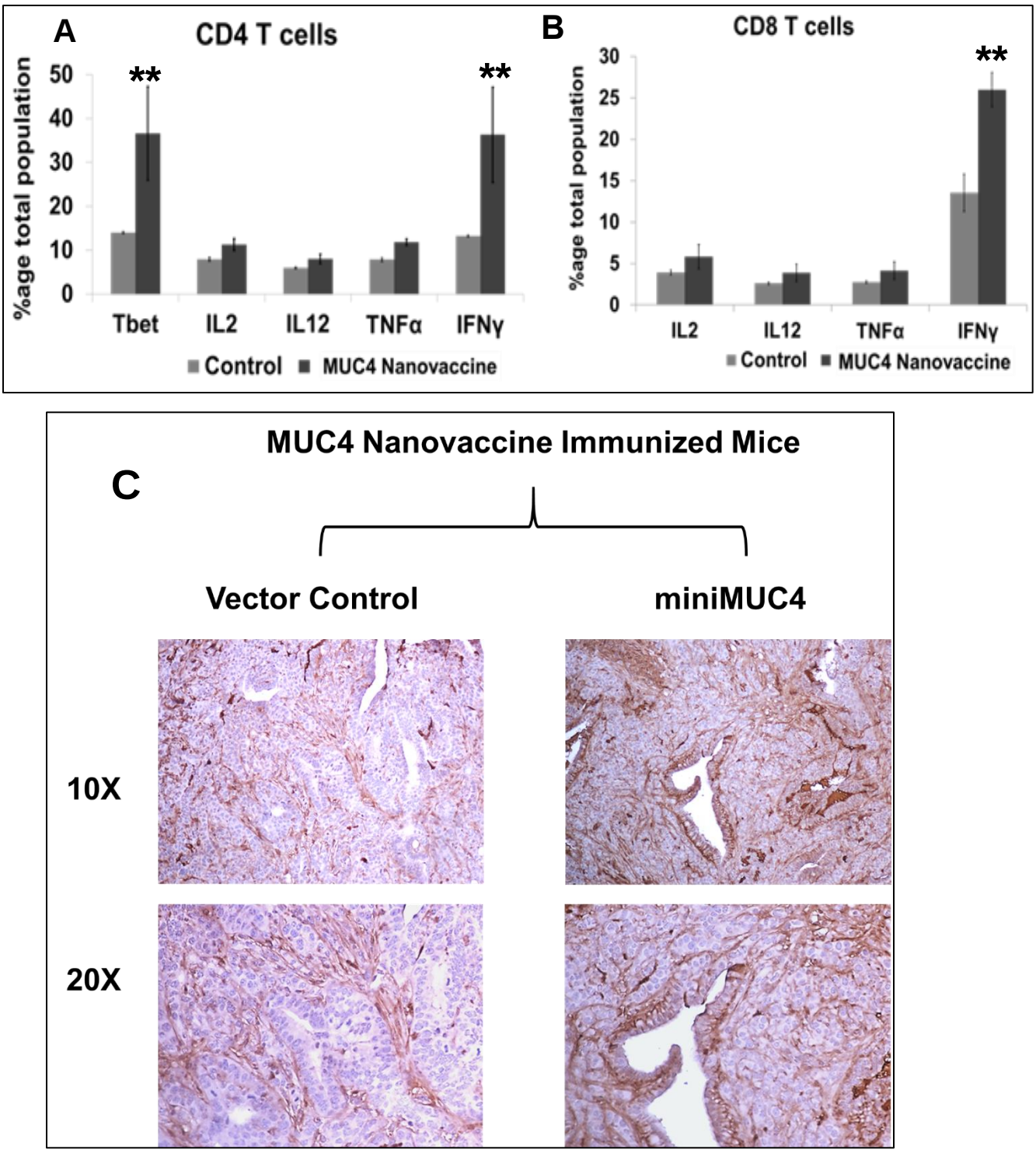

A
miniMUC4
Vector
CD8⁺ T cells
MUC4 Nanovaccine
MUC4+NP
B
miniMUC4
Vector
CD4⁺ T cells
MUC4 Nanovaccine
MUC4+NP
C
CD8+ T cells
Infiltrating T Cells
80
60
40
20
0
miniMUC4
Vector
MUC4 Nanovaccine
MUC4+NP
D
CD4+ T cells
Infiltrating T Cells
30
20
10
0
miniMUC4
Vector
MUC4 Nanovaccine
MUC4+NP

**Figure 5: MUC4 nanovaccine immunized mice activates T-cells in Th1 phenotype that induces the corresponding expression of PD-L1 by miniMUC4 tumor cells.**

Mice pre-immunized with MUC4 nanovaccine showed that MUC4-delivered through nanoparticles induced Th1 immune responses of both $CD4^+$ and $CD8^+$ T-cells in immunized mice compared to unimmunized control (** signifies $p<0.01$ by Tumey's t-Test) **(A & B)**. IHC analysis for PD-L1 on MiniMUC4 tumors and vector control tumors treated with MUC4 nanovaccine showed an upregulation in PD-L1 expression only in MiniMUC4 tumors, indicating an immunosuppressive response by MiniMUC4 tumors **(C)**.

## Figure 5


A
CD4 T cells
%age total population
50
40
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10
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**
**
Tbet
IL2
IL12
TNFα
IFNγ
Control
MUC4 Nanovaccine
B
CD8 T cells
%age total population
30
25
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**
IL2
IL12
TNFα
IFNγ
Control
MUC4 Nanovaccine
C
MUC4 Nanovaccine Immunized Mice
Vector Control
miniMUC4
10X
20X

# REFERENCES

[1] K. Banerjee, S. Kumar, K.A. Ross, S. Gautam, B. Poelaert, M.W. Nasser, A. Aithal, R. Bhatia, M.J. Wannemuehler, B. Narasimhan, J.C. Solheim, S.K. Batra, M. Jain, Emerging trends in the immunotherapy of pancreatic cancer, Cancer Lett, 417 (2018) 35-46.

[2] S. Gasser, L.H.K. Lim, F.S.G. Cheung, The role of the tumour microenvironment in immunotherapy, Endocr Relat Cancer, 24 (2017) T283-T295.

[3] L.G. Melstrom, M.D. Salazar, D.J. Diamond, The pancreatic cancer microenvironment: A true double agent, J Surg Oncol, 116 (2017) 7-15.

[4] L.H. Butterfield, Cancer vaccines, BMJ, 350 (2015) h988.

[5] M. Tagliamonte, A. Petrizzo, M.L. Tornesello, F.M. Buonaguro, L. Buonaguro, Antigen-specific vaccines for cancer treatment, Hum Vaccin Immunother, 10 (2014) 3332-3346.

[6] A.H. Banday, S. Jeelani, V.J. Hruby, Cancer vaccine adjuvants--recent clinical progress and future perspectives, Immunopharmacol Immunotoxicol, 37 (2015) 1-11.

[7] C. Gouttefangeas, H.G. Rammensee, Personalized cancer vaccines: adjuvants are important, too, Cancer Immunol Immunother, (2018).

[8] J. Chen, C.C. Jiang, L. Jin, X.D. Zhang, Regulation of PD-L1: a novel role of pro-survival signalling in cancer, Ann Oncol, 27 (2016) 409-416.

[9] L.M. Francisco, P.T. Sage, A.H. Sharpe, The PD-1 pathway in tolerance and autoimmunity, Immunol Rev, 236 (2010) 219-242.

[10] P. Loke, J.P. Allison, PD-L1 and PD-L2 are differentially regulated by Th1 and Th2 cells, Proc Natl Acad Sci U S A, 100 (2003) 5336-5341.

[11] K. Abiko, N. Matsumura, J. Hamanishi, N. Horikawa, R. Murakami, K. Yamaguchi, Y. Yoshioka, T. Baba, I. Konishi, M. Mandai, IFN-gamma from lymphocytes induces PD-L1

expression and promotes progression of ovarian cancer, Br J Cancer, 112 (2015) 1501-1509.

[12] B.T. Fife, K.E. Pauken, The role of the PD-1 pathway in autoimmunity and peripheral tolerance, Ann N Y Acad Sci, 1217 (2011) 45-59.

[13] G.J. Freeman, A.J. Long, Y. Iwai, K. Bourque, T. Chernova, H. Nishimura, L.J. Fitz, N. Malenkovich, T. Okazaki, M.C. Byrne, H.F. Horton, L. Fouser, L. Carter, V. Ling, M.R. Bowman, B.M. Carreno, M. Collins, C.R. Wood, T. Honjo, Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation, J Exp Med, 192 (2000) 1027-1034.

[14] F. Shi, M. Shi, Z. Zeng, R.Z. Qi, Z.W. Liu, J.Y. Zhang, Y.P. Yang, P. Tien, F.S. Wang, PD-1 and PD-L1 upregulation promotes CD8(+) T-cell apoptosis and postoperative recurrence in hepatocellular carcinoma patients, Int J Cancer, 128 (2011) 887-896.

[15] S. Amarnath, C.W. Mangus, J.C. Wang, F. Wei, A. He, V. Kapoor, J.E. Foley, P.R. Massey, T.C. Felizardo, J.L. Riley, B.L. Levine, C.H. June, J.A. Medin, D.H. Fowler, The PDL1-PD1 axis converts human TH1 cells into regulatory T cells, Sci Transl Med, 3 (2011) 111ra120.

[16] K.C. Soares, A.A. Rucki, A.A. Wu, K. Olino, Q. Xiao, Y. Chai, A. Wamwea, E. Bigelow, E. Lutz, L. Liu, S. Yao, R.A. Anders, D. Laheru, C.L. Wolfgang, B.H. Edil, R.D. Schulick, E.M. Jaffee, L. Zheng, PD-1/PD-L1 blockade together with vaccine therapy facilitates effector T-cell infiltration into pancreatic tumors, J Immunother, 38 (2015) 1-11.

[17] E.R. Lutz, A.A. Wu, E. Bigelow, R. Sharma, G. Mo, K. Soares, S. Solt, A. Dorman, A. Wamwea, A. Yager, D. Laheru, C.L. Wolfgang, J. Wang, R.H. Hruban, R.A. Anders, E.M. Jaffee, L. Zheng, Immunotherapy converts nonimmunogenic pancreatic tumors into immunogenic foci of immune regulation, Cancer Immunol Res, 2 (2014) 616-631.

[18] K. Mehla, J. Tremayne, J.A. Grunkemeyer, K.A. O'Connell, M.M. Steele, T.C. Caffrey, X. Zhu, F. Yu, P.K. Singh, B.C. Schultes, R. Madiyalakan, C.F. Nicodemus, M.A.

Hollingsworth, Combination of mAb-AR20.5, anti-PD-L1 and PolyICLC inhibits tumor progression and prolongs survival of MUC1.Tg mice challenged with pancreatic tumors, Cancer Immunol Immunother, 67 (2018) 445-457.

[19] D. Imai, T. Yoshizumi, S. Okano, H. Uchiyama, T. Ikegami, N. Harimoto, S. Itoh, Y. Soejima, S. Aishima, Y. Oda, Y. Maehara, The prognostic impact of programmed cell death ligand 1 and human leukocyte antigen class I in pancreatic cancer, Cancer Med, 6 (2017) 1614-1626.

[20] Y. Zhuan-Sun, F. Huang, M. Feng, X. Zhao, W. Chen, Z. Zhu, S. Zhang, Prognostic value of PD-L1 overexpression for pancreatic cancer: evidence from a meta-analysis, Onco Targets Ther, 10 (2017) 5005-5012.

[21] S. Yamaki, H. Yanagimoto, K. Tsuta, H. Ryota, M. Kon, PD-L1 expression in pancreatic ductal adenocarcinoma is a poor prognostic factor in patients with high CD8(+) tumor-infiltrating lymphocytes: highly sensitive detection using phosphor-integrated dot staining, Int J Clin Oncol, 22 (2017) 726-733.

[22] Y. Wang, J. Lin, J. Cui, T. Han, F. Jiao, Z. Meng, L. Wang, Prognostic value and clinicopathological features of PD-1/PD-L1 expression with mismatch repair status and desmoplastic stroma in Chinese patients with pancreatic cancer, Oncotarget, 8 (2017) 9354-9365.

[23] N. Moniaux, P. Chaturvedi, G.C. Varshney, J.L. Meza, J.F. Rodriguez-Sierra, J.P. Aubert, S.K. Batra, Human MUC4 mucin induces ultra-structural changes and tumorigenicity in pancreatic cancer cells, Br J Cancer, 97 (2007) 345-357.

# CHAPTER 6: SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

# 1. Summary and Conclusion

PC has an elaborate immunosuppressive microenvironment and immunotherapy has emerged as a tool to effectively target PC and its microenvironment [1]. Developing immunotherapy strategies for PC treatment comes with its own challenges that are: i) identifying a tumor-associated antigen that could be targeted, ii) identifying biodegradable adjuvants that could efficiently deliver antigens while preserving their antigenicity, iii) eliciting robust anti-tumor responses on the face of immunosuppression and iv) overcoming peripheral tolerance and immunosuppression elicited by the tumor.

Mucins are overexpressed in PC tumor cells and, due to their overexpression and aberrant glycosylation, they have emerged as potential candidates for targeted immunotherapeutic strategies for PC treatment [2]. MUC1 is one of the well-studied candidates for PC immunotherapy strategies but have attained only limited success in clinical trials. Most of these immunotherapies are MUC1 peptide-based vaccines that capture limited epitopes and excludes possible antigen epitopes present on the entirety of the protein. In addition, MUC1 is expressed at low levels in all normal tissues thus it doesn't provide tumor specificity [1-3]. Over the past several years, various aspects of MUC4 function and regulation in PC has been investigated [4-6]. In contrast to MUC1, MUC4 has restricted expression on normal tissues and is undetectable in normal pancreas [7, 8], whereas it is differentially overexpressed in PC tumor and its expression gradually increases with the disease progression [4, 8], thus MUC4 overexpression provides a spatiotemporal specificity to PC tumors that could be immunogenic. Further, MUC4 has been extensively studied and reported to be instrumental in PC pathogenesis [4], therefore we proposed that MUC4 could serve as a potential target for PC immunotherapy.

## 1.1 Pancreatic cancer patients have compromised peripheral tolerance against MUC4.

Here we focused on understanding whether the tolerance against MUC4 in PC patients is compromised. Peripheral tolerance is comprised of CD4 T helper cells and B-cells and identification of tumor-associated antigens activate B-cells to generate autoantibodies against those antigens. Thus detection of autoantibodies against MUC4 in PC patients will be an indicator of compromised peripheral tolerance in those patients. Our data demonstrated that autoantibodies are present against the recombinant MUC4β protein as well as to randomly selected MUC4 peptides derived from the entirety of MUC4 protein. Further our analysis showed that the autoantibodies to MUC4 peptides are generated in a PC-specific manner and negligible in healthy or chronic pancreatitis individuals. Additionally, IgM autoantibodies to MUC4 peptides (A2, D3 and B1) significantly correlated with PC patient overall survival. Thus suggesting that immunologically targeting MUC4 through a unique immunotherapy platform could exploit this compromised tolerance and generate efficacious anti-tumor responses potentially contributing to clinical success.

## 1.2 Encapsulation of MUC4β protein in novel amphiphilic polyanhydrides nanoparticles shifts its immunogenicity from Th2 to Th1 phenotype, and induces robust DC activation and humoral responses.

Most of the cancer vaccines developed are peptide-based due to the difficulty of encapsulating proteins while preserving their antigenicity and allowing sustained release in the circulation for activation of immune cells [9, 10]. Prior to our study, isolation and purification of MUC4 recombinant proteins and its characterization have not been achieved. Here we successfully purified the MUC4β protein from bacterial expression

system, and encapsulated the protein in novel amphiphilic polyanhydrides nanoparticles to develop MUC4 nanovaccine. Polyanhydride nanoparticles is a suitable platform due to its pH-neutral core that stabilizes protein, pathogen-mimicking properties and tunable release kinetics that enable immune-modulation [11-16]. In addition, MUC4 recombinant protein provides a wide repertoire of potential epitopes when compared to single epitope peptide-based immunotherapy strategies thus potentially eliciting a robust and sustained anti-tumor Th1 immune responses.

We demonstrated that soluble MUC4βprotein alone activates DCs in the Th2 pathway. It is well understood that Th1 immune responses have anti-tumor outcomes, therefore the protein by itself is not suitable for PC treatment. However, encapsulation of MUC4β protein shifted the overall response to Th1 phenotype. MUC4 nanovaccine robustly activated DCs and induced both surface expression of activation markers and secretion of Th1 cytokines. Further, in the immunized mice MUC4 nanovaccine induced generation of Th1 IgG2b humoral responses, which was not observed in mice immunized with soluble MUC4 or MUC4+NP groups. Thus indicating that the encapsulation of MUC4β in the nanoparticles is crucial for reprogramming the immune responses from Th2 phenotype to Th1 phenotype.

### 1.3 MUC4 nanovaccine induces immune infiltration in miniMUC4 tumors in an antigen-specific pathway.

Cancer vaccines have been shown to induce effector immune cell infiltration in tumors in an antigen-specific pathway that affects the overall tumor growth [17]. Here we tried to elucidate the therapeutic potential of a MUC4 nanovaccine in eliciting robust anti-tumor responses in a PC subcutaneous tumor mouse model. Our study showed that miniMUC4 tumors in mice immunized with MUC4 nanovaccine had slower tumor growth and lower tumor volume with respect to vector control tumors on the contralateral flank of

the same mice. These data suggested a MUC4-specific immune response in these vaccinated mice. Upon further evaluation, we observed that only in MUC4 nanovaccine immunized mice there is enhanced total immune cell infiltration and also effector T cell infiltration which correlated with high levels of necrosis seen in miniMUC4 tumors. Despite high T-cell infiltration, we were unable to attain complete regression in miniMUC4 tumors. Due to the high levels of IFNγ generated by T-cells isolated from MUC4 nanovaccine-immunized mice, we hypothesized that tumor cells are expressing PD-L1 upon IFNγ induction on the cell surface and inhibiting effector T-cells, cytotoxic activity. Our IHC analysis validated our hypothesis and we observed PD-L1 surface expression on miniMUC4 tumor cells. Thus, in conclusion, MUC4 nanovaccine could potentially be exploited as a PC treatment strategy.

## 2. Future Directions

While our studies elucidated the potential of MUC4 nanovaccine as a potent strategy for treating pancreatic cancer, the fact that our *in vivo* tumor model study was done in mixed background mice limits the clinical translation of our work. To fully understand the anti-tumor potential of MUC4 nanovaccine we are required to perform our studies in a syngeneic mice model. Our lab has recently developed murine PC cell lines derived from the C57BL/6 pure background KPC mice model. We will be transfecting these cells with miniMUC4 plasmid construct and test the cytotoxic killing of this human MUC4-expressing syngeneic mouse PC cell lines by MUC4 nanovaccine-activated T-cells. This study will give us a better understanding of the potential of MUC4 nanovaccine activating T-cells in a MUC4 antigen-specific manner.

Even though our *in vitro* and subcutaneous study with human MUC4 expressing syngeneic mice model will elucidate the therapeutic potential of MUC4 nanovaccine, the

model does not recapitulate PC patient's immune system where immune cells might recognize MUC4 on tumor cells as self-antigen and are tolerant towards MUC4. Our autoantibodies study suggests that MUC4 is immunogenic in PC patients and based on serum reactivity some PC patients might respond well to MUC4 based immunotherapies. To recapitulate this clinical setting and validate our hypothesis, our lab (with the help of Dr. Satyanarayana Rachagani) has recently developed a human MUC4 transgenic mice model. We will utilize this model to primarily address two questions: i) whether MUC4 nanovaccine could break the immunological tolerance in MUC4 transgenic PC mice model and effectively kill MUC4-expressing PC tumor cells, and ii) whether MUC4 nanovaccine will lead to severe side-effects due to the non-specific recognition of MUC4 on other normal tissues. Further we have also developed a human MUC4 transgenic $Kras^{G12D}$ Pdx-1 Cre spontaneous PC mice model that expresses MUC4 only on pancreatic tumor cells. This mice model will be used to study whether MUC4 nanovaccine could either stabilize or regress human MUC4 expressing spontaneous PC tumor. A culmination of all these studies will provide us with a clearer understanding of the efficacy of MUC4 nanovaccine as a therapy strategy.

There is very limited knowledge about MUC4's contribution to the PC immunosuppressive TME. We are curious to investigate the correlation of MUC4 expression with the immune-phenotype of resected tumors, and cross-refer the observation in our MUC4 transgenic $Kras^{G12D}$ Pdx-1 Cre spontaneous PC mice model system. Further, PC tumors are fairly desmoplastic that restricts the infiltration of effector T-cells. Our *in vivo* work provided evidence that PD-L1 is expressed on miniMUC4 tumor cells as a counterattack to escape cytotoxic killing by cytotoxic $CD8^+$ T cells. Thus, based on the data that we will collect from MUC4 transgenic mice models, combining immune-checkpoint blockade agents such as anti PD-1 antibody and stroma cell depletion agents

with MUC4 nanovaccine to enhance the therapeutic efficacy of the vaccine needs to be tested.

# REFERENCES

[1] K. Banerjee, S. Kumar, K.A. Ross, S. Gautam, B. Poelaert, M.W. Nasser, A. Aithal, R. Bhatia, M.J. Wannemuehler, B. Narasimhan, J.C. Solheim, S.K. Batra, M. Jain, Emerging trends in the immunotherapy of pancreatic cancer, Cancer Lett, 417 (2018) 35-46.

[2] M.P. Torres, S. Chakraborty, J. Souchek, S.K. Batra, Mucin-based targeted pancreatic cancer therapy, Curr Pharm Des, 18 (2012) 2472-2481.

[3] R.K. Ramanathan, K.M. Lee, J. McKolanis, E. Hitbold, W. Schraut, A.J. Moser, E. Warnick, T. Whiteside, J. Osborne, H. Kim, R. Day, M. Troetschel, O.J. Finn, Phase I study of a MUC1 vaccine composed of different doses of MUC1 peptide with SB-AS2 adjuvant in resected and locally advanced pancreatic cancer, Cancer Immunol Immunother, 54 (2005) 254-264.

[4] S.K. Gautam, S. Kumar, A. Cannon, B. Hall, R. Bhatia, M.W. Nasser, S. Mahapatra, S.K. Batra, M. Jain, MUC4 mucin- a therapeutic target for pancreatic ductal adenocarcinoma, Expert Opin Ther Targets, 21 (2017) 657-669.

[5] I. Lakshmanan, P. Seshacharyulu, D. Haridas, S. Rachagani, S. Gupta, S. Joshi, C. Guda, Y. Yan, M. Jain, A.K. Ganti, M.P. Ponnusamy, S.K. Batra, Novel HER3/MUC4 oncogenic signaling aggravates the tumorigenic phenotypes of pancreatic cancer cells, Oncotarget, 6 (2015) 21085-21099.

[6] P. Seshacharyulu, M.P. Ponnusamy, S. Rachagani, I. Lakshmanan, D. Haridas, Y. Yan, A.K. Ganti, S.K. Batra, Targeting EGF-receptor(s) - STAT1 axis attenuates tumor growth and metastasis through downregulation of MUC4 mucin in human pancreatic cancer, Oncotarget, 6 (2015) 5164-5181.

[7] P. Chaturvedi, A.P. Singh, S.K. Batra, Structure, evolution, and biology of the MUC4 mucin, FASEB J, 22 (2008) 966-981.

[8] M. Andrianifahanana, N. Moniaux, B.M. Schmied, J. Ringel, H. Friess, M.A. Hollingsworth, M.W. Buchler, J.P. Aubert, S.K. Batra, Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumor marker of diagnostic significance, Clin Cancer Res, 7 (2001) 4033-4040.

[9] U. Bilati, E. Allemann, E. Doelker, Strategic approaches for overcoming peptide and protein instability within biodegradable nano- and microparticles, Eur J Pharm Biopharm, 59 (2005) 375-388.

[10] U. Bilati, E. Allemann, E. Doelker, Nanoprecipitation versus emulsion-based techniques for the encapsulation of proteins into biodegradable nanoparticles and process-related stability issues, AAPS PharmSciTech, 6 (2005) E594-604.

[11] L. Huntimer, J.H. Wilson Welder, K. Ross, B. Carrillo-Conde, L. Pruisner, C. Wang, B. Narasimhan, M.J. Wannemuehler, A.E. Ramer-Tait, Single immunization with a suboptimal antigen dose encapsulated into polyanhydride microparticles promotes high titer and avid antibody responses, J Biomed Mater Res B Appl Biomater, 101 (2013) 91-98.

[12] L.K. Petersen, A.E. Ramer-Tait, S.R. Broderick, C.S. Kong, B.D. Ulery, K. Rajan, M.J. Wannemuehler, B. Narasimhan, Activation of innate immune responses in a pathogen-mimicking manner by amphiphilic polyanhydride nanoparticle adjuvants, Biomaterials, 32 (2011) 6815-6822.

[13] K.A. Ross, H. Loyd, W. Wu, L. Huntimer, M.J. Wannemuehler, S. Carpenter, B. Narasimhan, Structural and antigenic stability of H5N1 hemagglutinin trimer upon release from polyanhydride nanoparticles, J Biomed Mater Res A, 102 (2014) 4161-4168.

[14] B.D. Ulery, H.M. Kan, B.A. Williams, B. Narasimhan, K.W. Lo, L.S. Nair, C.T. Laurencin, Facile fabrication of polyanhydride/anesthetic nanoparticles with tunable release kinetics, Adv Healthc Mater, 3 (2014) 843-847.

[15] B.D. Ulery, D. Kumar, A.E. Ramer-Tait, D.W. Metzger, M.J. Wannemuehler, B. Narasimhan, Design of a protective single-dose intranasal nanoparticle-based vaccine platform for respiratory infectious diseases, PLoS One, 6 (2011) e17642.

[16] E.I. Wafa, S.M. Geary, J.T. Goodman, B. Narasimhan, A.K. Salem, The effect of polyanhydride chemistry in particle-based cancer vaccines on the magnitude of the anti-tumor immune response, Acta Biomater, 50 (2017) 417-427.

[17] J. Wei, W. Gao, J. Wu, K. Meng, J. Zhang, J. Chen, Y. Miao, Dendritic cells expressing a combined PADRE/MUC4-derived polyepitope DNA vaccine induce multiple cytotoxic T-cell responses, Cancer Biother Radiopharm, 23 (2008) 121-128.

# BIBLIOGRAPHY OF KASTURI BANERJEE

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1. **Banerjee K.,** Kshirsagar P., Kaur S., Brand RE., Smith L., Batra SK., and Jain M. **"*Presence of MUC4 autoantibodies in the serum of pancreatic cancer patients.*"** (*Manuscript Under Preparation)*
2. **Banerjee K.[#],** Gautam S.[#], Kshirsagar P., Ross KA., Spagnol G., Sorgen P., Wannemuehler MJ., Narasimhan B., Solheim J., Kumar S., Batra SK., and Jain M. ***"Synthesis and Characterization of recombinant MUC4 nanovaccine for tailoring immune* responses"** (*Submitted)*
3. Abdalla M., Ahmad I., Rachagani S., Thompson C., **Banerjee K.,** Maurer H.S., Olive K., Britigan B., and Kumar S.. **"*Heme Oxygenase-1 inhibition sensitizes pancreatic cancer cells to gemcitabine under hypoxic conditions.*"** (Under Review)
4. **Banerjee K.,** Kumar S., Ross KA., Gautam S., Poelaert B., Nasser MW., Aithal A., Bhatia R., Wannemuehler MJ., Narasimhan B., Solheim J., Batra SK., and Jain M.. ***"Emerging trends in the immunotherapy of pancreatic cancer."*** Cancer letters 417, 35-46
5. **Banerjee K.,** Gautam S., Nasser M.W., Kshirsagar P., Ross K., Spagnol G., Rachagani S., Sorgen P., Batra S.K., Kumar S., Narasimhan B. and Jain M. ***"MUC4 Nanovaccine and Checkpoint Blockade based combination immunotherapy for Pancreatic Cancer."*** Cancer Research 77 (13 Supplement), 3678-3678
6. Macha MA., Krishn SR., Jahan R., **Banerjee K.,** Batra SK, Jain M. ***"Emerging potential of natural products for targeting mucins for therapy against inflammation and cancer."*** Cancer Treatment Reviews January 13, 2014.