Analyzing Childhood Recall Antigens in Patients with Pancreatic Cancer
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Analysis of T cells to tetanus toxoid antigens in patients with pancreatic cancer treated with gemcitabine

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1.0 OBJECTIVES

Treating PDAC patients with GEM and one TT booster

Validate cytotoxic capacity of TT-specific memory T cells in blood of PDAC patients

Validate MDSC cytokines in blood of PDAC patients

Primary Outcomes: The primary outcome is the relative change in CD4 and CD8 T cell responses before and after the TT booster.

2.0 SCIENTIFIC BACKGROUND

Scientific Validity. Patients with pancreatic ductal adenocarcinoma have a five-year survival of only 4%. Gemcitabine is an FDA-approved drug for advanced pancreatic cancer that improves median survival by less than six months as a result of chemoresistance[1-3], underscoring the need for additional and/or new approaches. One such approach could be *Listeria monocytogenes*-based cancer immunotherapy. However, poor immunogenicity of tumor cells and strong immune suppression induced by myeloid-derived suppressor cells (MDSC), are major problems in cancer immunotherapy[4-6]. In this proposal we will utilize the unique and synergistic capabilities of the attenuated Listeria bacteria and Gemcitabine (GEM) to overcome these problems and develop an effective and safe second line treatment for patients with PDAC. Our laboratory discovered that Listeria infects tumor cells and kills them through the generation of high levels of reactive oxygen species (ROS)[7]. Moreover, Listeria selectively survives and multiplies in tumors and metastases but not in healthy tissues because of the strong immune suppression in the tumor microenvironment (TME) that is absent in healthy tissues[6,8]. To overcome the poor immunogenicity of tumor cells, we will use Listeria to deliver highly immunogenic recall antigens (RA), such as tetanus toxoid (TT), measle virus (MV), and poliovirus (PV) (already cloned into Listeria) selectively into tumor cells through in vivo infection, thereby converting poorly immunogenic tumor cells into a highly sensitive target for RA-specific T cells. Most individuals received human childhood vaccinations to these RA. We expect that vaccination of cancer patients with Listeria-RA will infect tumor cells and simultaneously reactivate the memory T cells to these antigens, which now will destroy the infected tumor cells presenting high levels of RA.

It is known that GEM reduces the MDSC population[9] and therefore also decreases immune suppression. We found strong synergistic effects of Listeria-TT and GEM on late stage pancreatic cancer (Panc-02), resulting in a strong eradication of the cancer, in correlation with a 50% reduction in the MDSC population and improved cytotoxic T cell responses to TT. One of the main reasons for GEM resistance is the production of cytidine deaminase (CDA) in tumor cells and macrophages, an enzyme that inactivates GEM[10]. It has been shown that high levels of ROS reduce CDA resulting in improved GEM sensitivity[11,12]. We expect that the strong effect we observed of Listeria-TT and GEM on the tumor and metastases in the Panc-02 model was the combination of improved T cell responses to the highly immunogenic RA through GEM, and improved GEM sensitivity of the tumor cells (through reduction in CDA levels) by Listeria-induced ROS **(Fig 1).** Our ultimate goal is to translate this combination therapy into a clinical trial. However, little is known about the tumor-killing capacities of RA-specific T cells in humans. Therefore, in this IRB protocol we will first analyze T cells in the blood of PDAC patients, that have been treated with GEM and boosted with the human TT childhood vaccine, for their tumor killing capacities through the production of Perforin, Granzyme B, Tbet, and IFNγ.

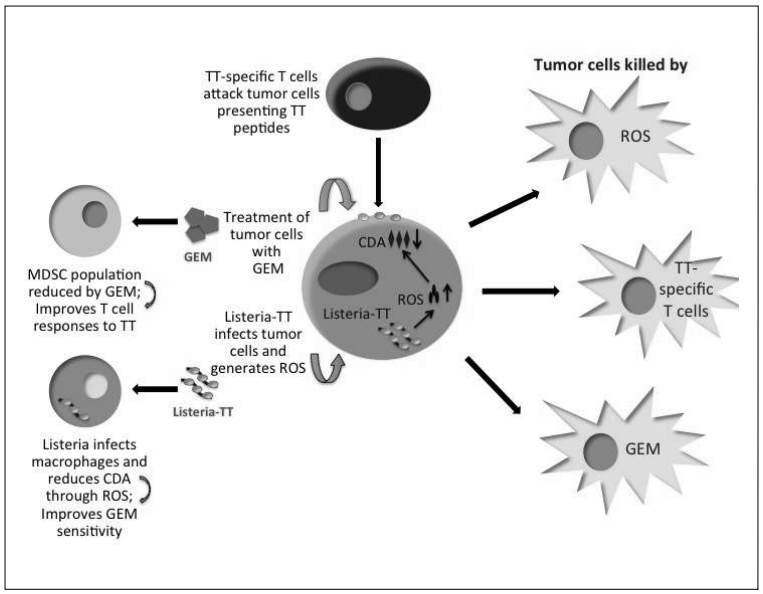

Fig 1: Potential synergistic effects of Listeria-TT and GEM on pancreatic cancer. Listeria delivers TT into tumor cells through infection, resulting in highly immunogenic tumor cells, and reactivates memory T cells to TT through infection of DC (not shown). Simultaneously, Listeria induces high levels of ROS in tumor cells and macrophages, which improves GEM sensitivity through reduction of CDA. GEM reduces the MDSC population, resulting in improved T cell responses. These synergistic effects will lead to tumor cell kill by TT-specific T cells, GEM, and by Listeria-induced ROS.

3.0 PATIENT ELIGIBILITY CRITERIA

Inclusion Criteria

1. Histologically or cytologically confirmed adenocarcinoma of the pancreas
2. Patients is a candidate for gemcitabine chemotherapy (adjuvant, metastatic, locally advanced, borderline resectable settings all permitted)
3. Patients at least 18 years of age
4. ECOG performance status 0-2
5. Consent to donate 12 tubes of peripheral blood of 10 mL each
6. Adequate organ function as defined as
   -neutrophil count ≥ 1200
   -platelets ≥ 75,000
   -hemoglobin ≥ 8.0
   -bilirubin ≤ 2.0
   -creatinine ≤2.0 or calculated GFR ≥ 30
7. Ability to understand and willingness to sign a written informed consent document
8. Prior chemotherapy permitted, as long as 60 days have lapsed since last dose. Prior radiation therapy permitted, as long as 28 days lapsed since last treatment.
9. Patients may receive other concurrent chemotherapy, immunotherapy, or radiotherapy

Exclusion Criteria

1. Patients never been immunized with tetanus toxoid (TT). Patients with a history of adverse reaction to tetanus vaccine (with the exception of self-limited fever or local tissue reaction
2. Patients may not be receiving any investigational agents
3. Pregnant women
4. Patients with HIV

4.0 REGISTRATION PROCEDURE

All patients will be registered with study coordinator Jyoti Pooja at the Montefiore-Einstein Center for Cancer Care.
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5.0 TREATMENT PLAN AND STUDY DESIGN

Our final goal is to move this combination therapy of Listeria-RA and GEM into a clinical trial in the near future together with Dr. Chuy. The constructs we have developed contain multiple immunodominant epitopes for both mouse and human T cells[14-22]. We have chosen for TT as the recall antigen in the PDAC patients, because it's the most extensively studied childhood antigen in humans. A map of major human CD4 T cell epitopes has been published which covers ~80% of all TT-vaccinated donors including their HLA haplotype **(Table 1)**[14]. This implies that most patients can be used for this study. In previous studies of PDAC patients, only CD4 T cell responses to TT have been analyzed by measuring proliferation or IFNγ production[13]. However, we are more interested in evaluating their tumor-killing capacity by determining the production of Perforin, Granzyme B, or the expression of Tbet (in addition to IFNγ, IL-2 and TNFα). We have shown in the Panc-02 model that the combination Listeria-TT and GEM strongly improved the production of Perforin, Granzyme B, Tbet, CD69 and IFNγ, by both CD4 and CD8 T cells, compared to the control groups. We expect that one TT booster in PDAC patients treated with GEM, will induce the production of Perforin, Granzyme B, Tbet, IFNγ, IL-2 and TNFα in both CD4 T and CD8 T cells. Dr. Jennifer Chuy (Co-PI) will provide the TT booster to the PDAC patients and the blood samples. She will recruit twenty-five PDAC patients at the Montefiore Medical Center for this pilot study. Power analysis has been performed to determine the sample size of the human blood samples needed (see statistical analysis below).

Treating PDAC patients with gemcitabine and one TT booster

Gemcitabine will be delivered as is standard of care. However, doses may be modified by the treating physician based on patient tolerance. Patients diagnosed with PDAC will be treated with Gemcitabine and boosted once with the human childhood vaccine to TT by Dr. Chuy as outlined in **Fig 2.** Gemcitabine will be administered on days 1, 8, 15 every 28 days, and one booster with the human TT childhood vaccine will be administered on day 8 (there must be 2 hrs between the TT booster and the Gemcitabine treatment). Blood will be drawn just before each Gemcitabine treatment, except on day 8 at least 2 hrs will be needed between the blood draw and Gemcitabine treatment because the TT booster needs to be given just after the blood draw but 2 hrs before the Gemcitabine treatment **(Fig 2).** Three tubes of 10 mls each with heparinized blood will be needed for the isolation of peripheral blood mononuclear cells (PBMC). Two tubes will be used to analyze the T cells and one tube for analyzing the MDSC. The memory T cells and MDSC will be analyzed in the laboratory of Dr. Gravekamp.

Validate cytotoxic capacity of TT-specific memory T cells in blood of PDAC patients

Table 1: Immunodominant epitopes within the TT protein matching HLA-DR

Peptide 1-700	Peptide 701-1300
TT (73-99)	TT (830-844)
TT (141-171)	TT (916-932)•
TT (257-599)	TT (947-967)•
TT (593-599)	TT (1273-1284)•
TT (616-631)	Ref [31]
TT (640-651)	
TT (652-663)	

(position aa of epitope within TT protein)
•epitopes are present in Listeria-TT

Blood cells from PDAC patients treated with GEM and the TT vaccine will be isolated by Ficoll-Hypaque (Bio-One Inc, Longwood, FL) as described previously[23], and then analyzed for memory T cell responses to the TT as described below by ELISPOT and by flow cytometry. *ELISPOT.* 200,000 blood leukocytes will be restimulated with the TT peptides (10 µg/200 µl) **(see Table 1)**[14], with purified protein of our cloned TT fragment (100 µg/200µl)(aa856-1313), and with the human TT protein vaccine (dilution 1:100), and will be subsequently cultured for 72 hrs and analyzed for CD4 and CD8 T cell producing IFNγ, IL-2, and TNFα using an ELISPOT reader and magnetic beads technique. ELISPOT kits will be purchased from BD BioSciences.

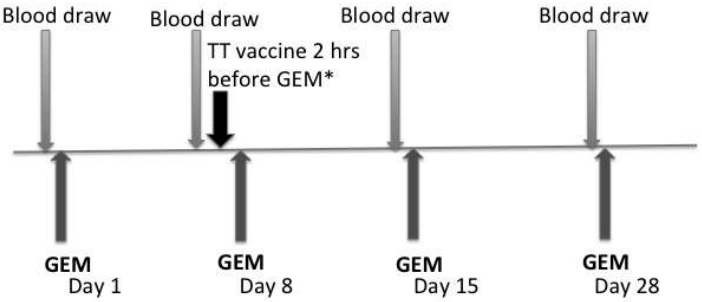
F*low cytometry.* The TT-specific T cells will also be analyzed by flow cytometry. Briefly, the T cells will be restimulated with TT as described above. Memory T cells with high potential for antigen-driven stimulation and associated with robust protective immunity[24] ($CD62L^{hi}CCR7^{hi}CD127^{hi}$CD3CD4 or CD8 T cells) will be analyzed for the production of Perforin, Granzyme B, IL-2, IFNγ, and TNFα, or the expression of Tbet and CD69. A 2-fold increase in the percentage of

activated T cells following the TT booster will be considered as a positive response. All antibodies will be purchased from BD Biosciences or Abcam.

Validate MDSC cytokines in blood of PDAC patients

In previous studies we found that Listeria converts immune suppressive human and mouse MDSC into an immune-stimulating phenotype by producing high levels of IL-12[6]. Our preliminary results showed that GEM reduces the MDSC population by 50% in the Panc-02 mice. Also in humans GEM reduces the MDSC population[9]. Therefore, we will analyze the blood samples by flow cytometry for the number of MDSC (CD11b+CD33+CD14+CD15+HLA-DR-) and the production of cytokines/factors involved in T cell activation/inhibition such as arginase I, inducible nitric oxide synthetase (iNOS or NOS2), or ROS/ IL-6, IL-10, and TGFβ, IL-12, IFNγ, IL-2 and TNFα, and expression of MHC class II, and CD80/86, similar as described for the mice. All antibodies will be purchased from BD Biosciences. In addition, we will infect MDSC with Listeria in vitro to analyze the production of IL-12 and the other as cytokines described above. The MDSC will be isolated by dextran[25].

Figure 2: Schedule of GEM treatment, TT vaccination and blood draws of PDAC patients



* 2hrs between TT vaccine and GEM

6.0 SUPPORTIVE THERAPY GUIDELINES

Use of supportive therapy including antiemetic therapy will be at the discretion of the treating physician. It is suggested that patients receive a 5-HT3 antagonist, prochlorperazine, metoclopramide, or dexamethasone prior to gemcitabine.

7.0 MONITORING PATIENTS

Patients will be evaluated as is standard of care for patients receiving gemcitabine chemotherapy. It is suggested that they at least be evaluated once every cycle by the treating physician.

Imaging studies to assess response to therapy will be ordered at the discretion of the treating physician.

Refer to study calendar for details.

8.0 STUDY CALENDAR

The consent date is within four weeks of registration for study.
All blood tests and physical exam must be performed within 2 weeks of starting treatment.

	Pre-study		C1D1	D8	D15	C2D1	
	(Day -30 to start)	(Day -15 to start)					
Informed consent	X						
History and		X				X	

Physical							
Vital Signs		X				X	
PS		X				X	
AE/SAE monitoring		X	X	X	X	X	
CBC		X		X	X	X	
Chemistry-7		X				X	
LFT		X				X	
Gemcitabine dosing			X	X		X	
Tetanus booster[a]			X				
Research blood draw[b]			X	X	X	X	
CT scans[c]	X						
HIV test	X						

Index for Study Calendar
a. Tetanus booster will be given 2 hours prior to gemcitabine.
b. Blood will be collected at on Cycle 1 Days 1, 8, 15 and Cycle 2 Day 1 prior to gemcitabine. On D8, the blood will be collected prior to tetanus booster vaccine.
c. CT abdomen/pelvis with contrast as per standard of care
d. CT scans will be retrospectively analyzed by a computational program to determine aggressiveness of the cancer before and after treatment. These analyses will be performed by computational biologist Dr. Amber Simpson, of Memorial Sloan Kettering Cancer Center.

9.0 DRUG FORMULATION

Storage and stability: Gemcitabine vials are stored intact at room temperature of 20°C to 25°C (68°F to 77°F). Reconstituted vials are stable for up to 35 days and infusion solutions diluted in 0.9% sodium chloride are stable up to 7 days at 23°C when protected from light; however, the manufacturer recommends use within 24 hours for both reconstituted vials and infusion solutions. Solutions of reconstituted gemcitabine should not be refrigerated, as crystallization may occur.

Preparation: To reconstitute, 5mL of 0.9% sodium chloride injection will be added to the 200mg vial or 25mL of 0.9% sodium chloride injection to the 1g vial. Shake to dissolve. These dilutions each yield a gemcitabine concentration of 38mg/mL which includes accounting for the displacement volume of the lyophilized powder (0.26mL for the 200mg vial or 1.3mL for the 1g vial). The total volume upon reconstitution will be 5.26mL or 26.3mL, respectively. Complete withdrawal of the vial contents will provide 200mg or 1g of gemcitabine, respectively. The appropriate amount of drug may be administered as prepared or further diluted with 0.9% sodium chloride to concentrations as low as 0.1mg/mL.

Route of administration: Gemcitabine will be administered intravenously over 30 minutes.

Tetanus vaccine

The tetanus toxoid protein vaccine that will be purchased from Grifols (see attachment for details). This vaccine also contains diphtheria. A vaccine with tetanus toxoid alone is not available anymore. This will not interfere with our study and poses no additional risk to patients.

10.0 STATISTICAL CONSIDERATIONS

The primary outcome measurement is the relative difference in CD4 counts before and after a boost. We expect 80% of patients will experience a three-fold increase. We will first diochotomize the relative change in CD4 as above or equal to 3 and otherwise. The proportion of at least 3-fold increase will be reported along with its 95% confidence interval. The actual magnitude of change will also be examined by taking a log scale of CD4 counts and report a mean of change on CD4 on its log scale along with its 95% CI. A logistic regression on diochotomized CD4 change and a linear regression on CD4 change in log-scale will also be used in order to examine how the change is affected by age and ethnicity of the patient.

With N=25 patients, we will be able to report the proportion of at least 3-fold increase with standard error not more than 3%. A paired t-test will also be applied on the change of log-scale of CD4. With N=25 patients and two-sided type I error rate not more than 5%, the study will have 80% power to detect 0.4SD change of log of CD4, a small to moderate difference. A non-parametric Wilcoxon Signed-Rank test will also be used if normality does not apply to the log-scale of CD4.

The secondary endpoint includes change in CD8 levels. Similar analyses will be performed on CD8 as we described above for CD4.

11.0 CRITERIA FOR REMOVAL OF PATIENTS FROM PROTOCOL

When a patient is removed from study, the Principal Investigator should be notified, and the reason for withdrawal noted.

- Progression of disease
- Patient desire to withdraw
- At the discretion of the principal investigator

12.0 DURATION OF STUDY

The study will last until the end of the first cycle. This will enable sufficient time for analysis of the immune response prior to and following administration of tetantus toxoid vaccine.

13.0 ADVERSE EVENT REPORTING

Reports of adverse events will be made using the Modified NCI Common Toxicity Criteria for Adverse Events version 4.0.

The following should be reported to the principal investigator or study coordinator at (718) 405-8404:

1. All unexpected grade 3 events
2. All life-threatening (Grade 4) events
3. All fatal events

A full report to the PI is expected within 10 days of the event.
All adverse events **must** be reported in routine study data submissions.

14.0 DATA MONITORING / QUALITY ASSURANCE/ RECORD RETENTION

Investigator responsibilities are set out in the ICH guideline for Good Clinical Practice (GCP) and in the US Code of Federal Regulations.

The Montefiore Medical Center, as coordinator of this study, is responsible for ensuring proper conduct of the study with regard to protocol adherence and the validity of the data recorded on the case report forms. The Data

Data Monitoring Officer (Jesus Anampa, MD.) will monitor this study. The case report forms will be monitored every 3 months against submitted support documents for accuracy, completeness, adherence to the protocol and regulatory compliance.

U.S. FDA regulations (21CFR312.62[c] require all records and documents pertaining to the conduct of this study and the distribution of investigational drug, including CRFs, consents forms, laboratory test results and medication inventory records, must be retained by the Principal Investigator for 2 years after marketing application approval. If no application is filed, these records must be kept 2 years after the investigation is discontinued and the FDA and the applicable local health authorities are notified.

15.0 DATA SAFETY AND MONITORING BOARDS

This trial will be monitored by the Albert Einstein Cancer Center Data Safety Monitoring Committee (AECC DSMC). A copy of the monitoring plan is maintainedat theCPDMU. TheDSMC as part of its function performs quarterly reviewsof Clinical Trials ComplianceAudits, monthly reviews Adverse Events Reports, and monthly reviews of internally monitored PhaseI/PhaseII trials for accrual and response. Other monitoring activities are established as necessary in a protocol specific manner.

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