Title: A new immunotherapeutic concept for pancreatic cancer using childhood vaccine recall antigens and Listeria

Benson Chellakkan Selvanesan[1$], Dinesh Chandra[1$], Wilber Quispe-Tintaya[1$], Arthee Jahangir[1], Ankur Patel[1], Kiran Meena[1], Rodrigo Alberto Alves Da Silva[1], Steven K Libutti[2], Ziqiang Yuan[2], Amanda Beck[3], Lydia Tesfa[4], Wade Koba[5], Jennifer Chuy[6], John C. McAuliffe[7], Rojin Jafari[8], David Entenberg[8,9,10], Yarong Wang[8,9,10], John Condeelis[8,9,10,11], and Claudia Gravekamp[1*].

[$]These authors equally contributed to this manuscript

Running title: Cancer treatment through childhood vaccine recall antigens

[1]*Albert Einstein College of Medicine, Department of Microbiology and Immunology, 1300 Morris Park Avenue, Bronx, NY 10461, USA*

[2]*Rutgers University, Cancer Institute of New Jersey, 195 Little Albany St, New Brunswick, New Jersey 08901, USA*

[3]*Albert Einstein College of Medicine, Michael F. Price Center, 1301 Morris Park Avenue, Room 158, Bronx, NY 10461*

[4]*Albert Einstein College of Medicine, Jack and Pearl Resnick Campus, 1300 Morris Park Avenue, Chanin Building, Room 309, Bronx, NY 10461*

[5]*Albert Einstein College of Medicine, Department of Radiology, MRRC, 1300 Morris Park Avenue, Bronx, NY 10461, USA*

[6]*Montefiore/Einstein Center for Cancer Care, Department of Medical Oncology, 1695 Eastchester Road, 2nd Floor, Bronx, NY 10461, USA*

[7]*Montefiore Medical Center, Department of Surgery, 1521 Jarrett Place, 2nd Floor, Bronx, NY 10461, USA*

[8]*Albert Einstein College of Medicine, Michael F. Price Center, Department of Anatomy and Structural Biology, 1301 Morris Park Avenue.*

[9]*Albert Einstein College of Medicine, Michael F. Price Center, Gruss-Lipper Biophotonics Center, 1301 Morris Park Avenue.*

[10]*Albert Einstein College of Medicine, Michael F. Price Center, Integrated Imaging Program, 1301 Morris Park Avenue.*

[11]*Albert Einstein College of Medicine, Michael F. Price Center, Department of Surgery, 1301 Morris Park Avenue.*

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* To whom correspondence should be addressed
Claudia Gravekamp
Albert Einstein College of Medicine
Department of Microbiology and Immunology
1300 Morris Park Avenue
Forchheimer Bldg, Room 407A
Bronx, NY 10461
Email: claudia.gravekamp@einstein.yu.edu
Phone: 718-430-4048(office)/4067 (lab)/Fax: 718-430-8711

Abstract

Background: Pancreatic ductal adenocarcinoma (PDAC) is extremely difficult to cure. Cancer immunotherapy has shown promising results. However, PDAC is poorly immunogenic with few effective neoantigens present, while immune suppression by myeloid-derived-suppressor-cells (MDSC) and tumor-associated-macrophages (TAM) prevents T cell activation in the tumor microenvironment (TME). To address these problems, we developed a novel concept of immunotherapy which includes the generation of memory T cells to a highly immunogenic antigen like tetanus toxoid (TT) before tumor development and reactivation of these memory T cells after tumor development in parallel with delivering TT antigens as an alternative for neoantigens into the tumor cells by an attenuated bacterium *Listeria monocytogenes* through *in vivo* infection. When combined with low doses of Gemcitabine (reduces immune suppression by MDSC and TAM) these reactivated TT-specific memory T cells are now able to destroy the Listeria-TT-infected tumor cells.

Methods: Before tumor development, memory T cells were generated to a highly immunogenic recall antigen tetanus toxoid (TT) through repeated immunizations with the childhood TT vaccine (like in humans; most individuals have been exposed to during childhood vaccinations). After tumor development, the same mice (Panc-02 or KPC model) received one immunization with a high dose of Listeria-TT to deliver TT inside tumor cells, followed by multiple low doses of Listeria-TT to reactivate the memory T cells to TT. Gemcitabine was administered to reduce immune suppression. CD4, CD8 T cells, MDSC and TAM were analyzed by ELISPOT and/or flow cytometry.

Results: Abundant CD4 and CD8 T cell responses producing Interferon-γ, Perforin, Granzyme B, and were observed in the spleens by ELISPOT and flow cytometry. A

significant reduction was observed in tumors and metastases of the Panc-02 and KPC mice, with advanced pancreatic cancer. GEM significantly reduced the percentage of MDSC and TAM in blood and/or primary tumors, respectively.

Conclusions: Our data demonstrate that delivery of recall antigen Tetanus Toxoid (TT) into tumor cells with help of attenuated Listeria through *in vivo* infection, in parallel with reactivating existing memory T cells to TT and low doses of GEM, could be a new type of immunotherapy to treat pancreatic cancer. This novel treatment modality of Listeria-TT and GEM warrants further investigation in the clinical setting.

Introduction

Patients with PDAC have a five-year survival of only 6%. Modern systemic therapies, such as Gemcitabine (GEM), provide some survival benefits[1-4], while Gemcitabine and Abraxane or FOLFIRINOX further modestly improve survival[5, 6]. This underscores the need for additional and innovative approaches. Cancer immunotherapy with checkpoint inhibitors has shown promising results, however, less so for PDAC[7]. PDAC is poorly immunogenic because of the low mutational load and the few effective neoantigens present[8, 9], and immune suppression, particularly by MDSC and TAM, prevents T cell activation in the TME[10-12]. Moreover, evidence suggests that naïve T cells are more prone to undergo apoptosis or are less efficiently activated than memory T cells in the TME of cancer patients and tumor-bearing mice[13, 14].

To address these problems we have developed a novel treatment modality using an attenuated bacterium *Listeria monocytogenes*[15] (Listeria) to deliver a highly immunogenic recall antigen such as tetanus toxoid (TT)(as an alternative to neoantigens) selectively into tumor cells, and to reactivate existing memory T cells

against TT (generated during childhood vaccinations), which in turn destroy the now highly immunogenic infected tumor cells **(SI Fig 1).** These memory T cells circulate in blood for life and can be reactivated at any age. Such an approach avoids the need for naïve T cells during cancer treatment. To further improve T cell responses against TT, Listeria-TT was combined with low doses of GEM (to reduce immune suppressive MDSC and TAM populations).

We have shown that Listeria attracts and infects MDSC[10] (most likely through C3bi and C1q recepors[16-18]), which are present in large numbers in patients and mice with cancer[11, 19]. However, the same MDSC are selectively attracted by the primary tumor through the production of cytokines and factors[11, 20], and deliver Listeria as a Trojan horse to the TME[10, 19]. Once at the tumor site, Listeria spreads from MDSC into tumor cells through the cell-to-cell spread mechanism specific for Listeria[21]. In the TME, Listeria is protected from immune clearance through strong immune suppression, but is rapidly killed in healthy tissues that lack immune suppression. Listeria can also infect tumor cells directly[22]. It is through this combination of the selective attraction of MDSC by bacteria and cancer, and the strong immune suppression in the TME but not in normal tissues, why Listeria is able to selectively enter the TME and to multiply and survive, but not in normal tissues[10, 19, 23, 24]. Based on these results we now use Listeria as a platform for the selective delivery of anticancer agents to the TME[19, 23, 25].

In the current study, we used two mouse models of pancreatic cancer, i.e. a syngeneic Panc-02[26] and a transgenic KPC model[27, 28]. The Listeria-TT was administered through intraperitoneal (i.p.) injection, and uptake by tumor cells *in vivo* was verified by intravital imaging. Here, we demonstrate that the combination of Listeria-TT+GEM robustly reduced pancreatic cancer at early and advanced stages in correlation with a strong increase in CD4 and CD8 T cell responses in both Panc-02 and KPC mice.

Our data provide mechanistic insight into the synergistic effects between Listeria-TT and GEM for the treatment of PDAC in mice and ultimately in patients.

Materials and Methods

Animal care

C57BL/6 mice aged 3 months were obtained from Charles River and KPC mice (*LSL-p53*$^{R172/+}$*; LSL*^{KrasG12D}*; Pdx1-Cre*)[27] were generated in the laboratories of Dr. Steven K Libutti, Department of Surgery, and Dr. Claudia Gravekamp, Department of Microbiology and Immunology, Albert Einstein College of Medicine. C57Bl/6N-Tg(Cfms-gal4-vp16)-(UAS-eCFP)[29] were maintained in the laboratory of Dr. John Condeelis. All mice were housed in the animal husbandry facility Albert Einstein College of Medicine according to the Association and Accreditation of Laboratory Animal Care (AACAC) guidelines, and kept under Bsl-2 condition as required for Listeria treatments. All mice were genotyped by Transnetyx (Cordova, TN).

Cell lines

The Panc-02 cell line (Kras-G12D mutant allele) was derived from a methylcholanthrene-induced ductal adenocarcinoma growing in a C57BL/6 female mouse[30]. Panc-02 cells were cultured in McCoy's5A medium supplemented with 10% FBS, Glutamine (2 mM), non-essential amino acids, sodium pyruvate (1 mM), and Pen/Strep (100 U/ml). The Panc-02 cell line expressing Dendra-2 was developed in the laboratory of Dr. Gravekamp. Briefly, Panc-02 cells were transfected with pCDNA3.1-Dendra-2, and positive cells were selected using neomycin and FACS sorting.

Listeria and Listeria-TT

In this study, an attenuated *Listeria monocytogenes* (Listeria) was used as the vehicle for the delivery of TT to the TME and inside tumor cells. The Listeria plasmid pGG-34, expresses the positive regulatory factor A (prfA) and Listeriolysin O (LLO)[31]. prfA regulates the expression of other virulence genes, and is required for survival *in vivo* and *in vitro*. The background strain XFL-7 lacks the prFA gene, and retains the plasmid *in vitro* and *in vivo*[31]. The coding region for the C-terminal part of the LLO (cytolytic domain that binds cholesterol in the membranes) protein in the plasmid has been deleted, but Listeria is still able to escape the vacuole[31]. Mutations have been introduced into the prfA gene and the remaining LLO (expressed by the pGG34 vector), which further reduced the pathogenicity of the Listeria[31]. Listeria-TT was developed in our laboratory as outlined in the results section.

Infection of tumor cells *in vitro*

The infectivity rate of the cell lines was assessed *in vitro*, as described previously[22]. Briefly, 10^6 cells/ml were infected with 10^7 of Listeria, for 1 hr at 37°C in culture medium as described above. After the incubation with gentamicin (50 μg/ml) for 1 hr (killing all extracellular Listeria bacteria), cells were washed with PBS, lysed in water, and serial dilutions were plated onto LB agar to determine the infection rate the next day.

Evaluation of Cell Death

As described previously[22], tumor cell death induced by Listeria or Listeria-TT was determined *in vitro* as follows. $3x10^3$ Panc-02 cells plated in 96 well plates were infected with 10^7 CFU of Listeria or Listeria-TT (per well), for 3 hrs at 37°C. Gentamycin (50

μg/ml) was added until live and dead cells were counted using Trypan blue staining on the next day.

Biodistribution of Listeria-TT

C57Bl/6 mice were injected with $2x10^6$ tumor cells as described above, and 14 days later injected once with a high dose 10^7 CFU of Listeria-TT. Mice were euthanized at various time points after injection of the Listeria-TT, and metastases, tumors and normal tissues were dissected, weighted, and analyzed for the number of CFU of Listeria-TT as described previously[23].

Dose-limiting toxicity

C57Bl6 mice were injected i.p. with various doses of Listeria-TT, and analyzed for survival over the next 20 days as described previously[23].

Tumor development

Panc-02 tumor cells were injected into the mammary fat pad (10^5) or orthotopically in the pancreas (10^6) of C57Bl/6 mice. When injected into the mammary fad pat, tumor cells metastasize to different organs (predominantly in the pancreas and liver, and less abundantly in the mesenchymal lymph nodes along the GI and in the diaphragm)[19], while orthotopically injected tumor cells (only used for IVMI studies) result in a pancreatic tumor but rarely metastasize[32]. In KPC mice, multiple primary tumors develop spontaneously in the pancreas, which metastasizes to the liver and lungs[23, 27].

Treatments

<u>Panc-02 mice.</u> C57Bl/6 mice were immunized i.m. twice with the “human” TT (TTvac: 0.3 μg/50 μL) vaccine (one week apart) to generate the memory T cells to TT.

Subsequently, Panc-02 tumor cells (10^5/100 μL) were injected in mammary fat pad. When tumors were 3 mm (3-5 days after tumor cell injection) or 10 mm (10-14 days after tumor cell injection), one high dose of Listeria-TT (LM-TT) (10^7 CFU) was injected i.p. Three days later, the GEM treatment was started and continued for two weeks (every 3 days 1.2 mg/mouse) (6 doses in total). Concomitantly, low doses of Listeria-TT were administered daily for 2 weeks (14 doses in total). Tumor weight was determined and the number of metastases by the naked eye, as we have described previously[19]. KPC mice. KPC mice received the same treatment after verification of the presence of tumors and metastases by PET (3.5-8 months of age). A detailed rationale for this treatment protocol and schematic view of all treatments are shown in **Fig 1.**

Intravital multiphoton imaging (IVMI)

Panc-02 tumor cells (10^6) expressing Dendra-2 were injected into the pancreas of transgenic mice [C57Bl/6N-Tg(Cfms-gal4-vp16)-(UAS-eCFP)][29] in which the macrophages were labeled with cyan fluorescent protein (CFP). Three weeks later, when a pancreatic tumor developed, Listeria-TT-Alexa-680 was injected i.p. Listeria-TT was labeled with an Alexa-680 dye (Listeria-TT was incubated with a rabbit anti-Listeria polyclonal antiserum (BD, Sparks, MD, cat# 223021, dilution 1:200), and anti-rabbit IgG-Alexi-680 (ThermoFisher, Rockford, IL; cat# A-21109, dilution 1:250)), on the same day as the imaging. Four hours later (when Listeria-TT had accumulated in the TME and infected tumor cells), mice were anesthetized using isofluorane (#029405, Henry Schein, Inc.). Pancreatic tumors were exposed and externalized through a small incision (~0.7 cm) through the skin and peritoneal wall and stabilized for imaging using previously published protocols[33]. Multiphoton intravital microscopy was performed using a custom-built two-laser multiphoton microscope[34]. Tumors were imaged with a femtosecond laser set to 880nm for excitation of the CFP and the green form of Dendra-2, and an optical

parametric oscillator (OPO) set to 1240nm for excitation of the Alexa 680 dye. The animal was placed in a heated chamber maintained at physiological temperature during the course of imaging. Mice were monitored for heart rate, breath rate, pulse distension, breath distension and oxygen saturation using a pulse oximeter (Kent Scientific, MoseSTAT). Z-stacks of up to 45 μm of depth were acquired with a 1 μm slice interval.

PETscan

Positron Emission Tomography (PET) was performed as described previously[35]. Briefly, the effect of the Listeria-TT+GEM treatment on the tumor and metastases was monitored by microPET (Siemens Imaging System) before and after treatment. For this purpose, the mice were injected with positron emitter Fluorine-18 (^{18}F)-labeled deoxyglucose (^{18}F-FDG) and the uptake of ^{18}F-FDG in the tumors and metastases was quantified by microPET as described previously[19]. Increased FDG uptake reflects increased metabolic activity of cancer cells, expressed as SUVmax.

Flow cytometry

Immune cells from spleens, blood or metastases of treated and control mice were isolated as described previously[36]. To identify CD8 T cells anti-CD3 and CD8 antibodies, for CD4 T cells anti-CD3 and anti-CD4 antibodies, for MDSC anti-CD11b and anti-Gr1 antibodies, and for TAM anti-CD11b and anti F4/80 antibodies were used. To detect the production of intracellular cytokines, the cells were incubated with GolgiPlug (1 ug/ml) for 6 hrs, and then treated with cytofix/cytoperm (BD Pharmingen) according to manufacturer's instructions, for staining with antibodies to intracellular cytokines and IFNγ, Granzyme B, Perforin, IL-6, IL-10, TNFα, and other markers such as MARCO (inhibits T cell responses) CD69 (activation marker for T cells), CD80 (co-stimulatory molecule) were used. Appropriate isotype controls were used for each sample. 50,000-

100,000 cells were acquired by scanning using a Fluorescence Activated Cell Sorter (Beckton and Dickinson; LSR-II, special order system), and analyzed using FlowJo 7.6 software. Cell debris and dead cells were excluded from the analysis based on scatter signals and use of Fixable Blue or Green Live/Dead Cell Stain Kit (Invitrogen). All antibodies were purchased from BD Pharmingen or eBiosciences.

ELISPOT

Spleen cells were isolated from treated Panc-02 and KPC mice and control mice for analysis by ELISPOT as described previously[37]. To detect T cell responses to TT, 10^5 spleen cells were incubated with purified TT protein (5 μg/ml). After 72 hrs, the frequency of IFNγ-producing spleen cells was measured (Pharmingen, San Diego, CA) using an ELISPOT reader (CTL Immunospot S4 analyzer, Cellular Technology, Ltd, Cleveland, OH). To determine the frequency of IFNγ-producing CD4 and CD8 T cells, spleen cells were depleted for CD4 and CD8 T cells using magnetic bead depletion techniques according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA). All antibodies were purchased from BD Pharmingen.

Western Blotting

Expression of TT protein in tumor cells was analyzed by western blotting. Briefly, cells were lysed in RIPA buffer containing protease inhibitors, and proteins were separated on 4-12% gradient SDS-polyacrylamide gel, and then electro-transferred to PVDF filters membrane. Membranes were incubated with rabbit anti-Myc IgG antibody (Cell Signaling Technology, Danvers, MA), followed by horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibodies (Santa Cruz, Dallas, TX) and detection was obtained using a chemiluminescence detection kit (Thermo scientific, Rockford, IL). Antibody to beta-actin was used to ensure equal loading.

Statistical analysis

To statistically analyze the effects of Listeria-TT+GEM on the growth of metastases and tumors or on immune responses in both mouse models Panc-02 and KPC, the Mann-Whitney was used. *$p<0.05$, **<0.01, ***<0.001, ****<0.0001. Values $p<0.05$ were considered statistically significant.

Results

Generation and characterization of Listeria-TT

The Listeria-TT vaccine was developed as follows. The $TT_{856-1313}$ (52 kDa) was cloned as a fusion-protein with a truncated Listeriolysin O (LLO)(48 kDa) in the Listeria vector (pGG34) under the control of the LLO promoter (P), and a myc sequence for detection of the TT protein was included **(Fig 2a).** Secretion of LLO-$TT_{856-1313}$ protein by the Listeria-based vaccine was detected in the culture medium by western blotting using anti-myc antibodies **(Fig 2b).** Infection of Panc-02 tumor cells with Listeria-$TT_{856-1313}$ resulted in the expression of TT protein in the tumor cells **(Fig 2c).**

To test whether Listeria-TT remained functional as a delivery platform for anticancer agents, we determined the infection rate of pancreatic tumor cells with Listeria-TT in comparison to Listeria alone. For this purpose, Panc-02 tumor cells were cultured with Listeria or Listeria-TT, lysed in water and plated on agar, as described previously[19]. Both abundantly infected the Panc-02 cells **(Fig 2d).** We also determined whether Listeria-TT was able to kill Panc-02 cells, as described previously[22]. As shown in **Fig 2e,** Listeria-TT abundantly killed the Panc-02 cells.

TT is highly immunogenic and may lead to faster elimination of Listeria-TT than Listeria alone and consequently may prevent accumulation in the TME. One high dose of Listeria-TT was injected i.p. into Panc-02 mice and the number of CFU of Listeria-TT was measured in all tissues (tumors, metastases, and normal tissues) at different time

points. As shown in **Fig 2f,** Listeria-TT robustly accumulated in tumors and metastases, but hardly in normal tissues. Interestingly, when injected intravenously Listeria hardly reached the tumors and metastases **(SI Fig 2).** This was also found by others in a murine colon carcinoma model CT26[38]. It is most likely that the effect of immune suppression in the peritoneal cavity generated through tumors and metastases is responsible for the preferential colonization of Listeria in the TME, and that immune suppression in blood is less potent.

To confirm that Listeria was taken up by tumor cells within the TME of live mice, we injected Listeria, labeled *ex vivo* with Alexa-680 dye, into the peritoneal cavity of transgenic mice whose macrophages express cyan fluorescent protein [C57Bl/6N-Tg(Cfms-gal4-vp16)-(UAS-eCFP)][29] and which were bearing orthotopic Dendra-2 labeled pancreatic tumors (Panc-02-Dendra). We then imaged these tumors using high-resolution intravital multiphoton microscopy (IVMI), and showed individual Listeria inside tumor cells and macrophages (**Fig 2g**).

Generation of memory CD4 and CD8 T cells to TT before tumor development and reactivating these memory T cells after tumor development

First, we generated TT-specific memory CD4 and CD8 T cell responses through two immunizations with the human TT vaccine (TTvac) before tumor development **(Fig 3a).** Subsequently, the Panc-02 tumor cells were injected in the mammary fat pad of mice with the existing memory T cells to TT**.** Once the Panc-02 tumors were 10 mm in diameter, the treatment was started with one high dose of Listeria-TT injected followed by multiple low doses of Gemcitabine, concomitantly with low doses of Listeria-TT over a period of two weeks as outlined in **Fig 1.**

To evaluate recall of the memory T cell responses to TT after tumor development, spleen cells were isolated from the tumor-bearing mice (after the complete treatment

cycle), restimulated with TT protein *in vitro*, and then analyzed for memory T cell responses producing IFNγ by ELISPOT. Depletion of CD4 and CD8 T cells from the total spleen population showed significant reduction in the production of IFNγ of mice that received Listeria-TT+GEM or Listeria-TT alone (Mann-Whitney $p<0.05$ and $p<0.001$), indicating CD4 and CD8 memory T cell responses to the TT protein, while no T cell responses were observed in the treatment groups that received GEM or saline only **(Fig 3b).**

We also analyzed T cell responses directly *in vivo* in mice with the Panc-02 tumor-bearing mice after a full treatment cycle with TTvac, Listeria-TT+GEM, by flow cytometry, without restimulation *in vitro*. Our goal was to analyze if a complete treatment cycle with Listeria-TT+GEM could reduce the immune suppression in the TME and activate T cells *in vivo*. T cell responses (CD69, Granzyme B, Perforin, and IFNγ) were strongly improved in the spleen (pooled) of mice treated with Listeria-TT+GEM compared to all control groups **(Table 1a and SI Fig 3ab).** Not only did both CD8 and CD4 T cells produce high levels of Granzyme B, Perforin, and/or IFNγ, but CD4 T cells also demonstrated an even stronger response than the CD8 T cells. Moreover, Listeria-TT or GEM alone also increased the production of Perforin in CD4 and CD8 T cells compared to the saline treatments.

Similar results were found in the spleens of KPC mice by flow cytometry (which received the same treatment as the Panc-02 mice). CD4 and CD8 T cell responses were robustly improved in mice that received Listeria-TT+GEM compared to all control groups, i.e. the percentage of CD4 and CD8 T cells producing Granzyme B, Perforin, and/or IFNγ, or expressing CD69, was strongly increased in mice that received Listeria-TT+GEM compared to all control groups **(Table 1b).**

Finally, in a pilot study using RNAseq we found that CD3/4/8/28, Granzymes and Perforin were abundantly upregulated in pancreatic tumors of KPC mice that received

TTvac, Listeria-TT+GEM compared to untreated KPC mice **(SI Fig 4a).** These results support the *in vivo* and *in vitro* analysis of the T cell responses to Listeria-TT+GEM **(Fig 3b, Table 1ab).**

Listeria-TT and GEM alter the TME

Panc-02 tumor bearing mice received a full treatment cycle with Listeria-TT+GEM as described in **Fig 1.** Here, we show that GEM reduced the MDSC population in blood by 80-90% and in primary tumors by 50% compared to the saline group **(Fig 3c),** and the TAM population by 67% in primary tumors compared to the saline group **(Fig 3c)**.

Moreover, mice treated with Listeria-TT+GEM showed a decrease in the percentage of MDSC **(Table 2a and SI Fig 5a)** and TAM **(Table 2b and SI Fig 5b)** secreting IL-10, IL-6 or expressing MARCO (all involved in T cell inhibition) in the pancreatic metastases, but increased percentages of MDSC and TAM producing TNFα (involved in tumor cell killing) and/or CD80 (involved in T cell activation), compared to the controls. A similar effect on cytokines was shown by Listeria-TT alone but less abundantly.

Reducing early and advanced pancreatic cancer in Panc-02 and KPC mice by Listeria-TT and GEM

Here we tested Listeria-TT+GEM therapeutically in Panc-02 mice with early and advanced pancreatic cancer as outlined in **Fig 1**. We found an almost complete elimination of early stage pancreatic cancer **(Fig 4a),** and a significant reduction in primary (85%; p=0.0001) and metastatic tumor burden (89%; p=0.0001) of advanced pancreatic cancer compared to the saline group in the Panc-02 model **(Fig 4b and d).**

We also tested Listeria-TT+GEM therapeutically in KPC mice with advanced pancreatic cancer (3.5-8 months old). A significant reduction in the SUVmax of PDAC

tumors (SUVmax 1.9 to 0.4) and liver metastases (SUVmax 2.1 to 0.3) was observed in 5 out of the 9 mice (these 5 mice were 3-5.5 months of age at the start of treatment) **(Fig 4c and e).** The PDAC tumors reduced by 80% (p=0.0020) and the liver metastases by 87% (p=0.03). Finally, 4 out of the 9 mice died during or shortly after treatment and could not be rescued because their extensive tumor burden prior treatment initiation (KPC mice were 8 months at the start of treatment).

The SUVmax of tumors and metastases slightly increased or decreased in the Saline and GEM control groups, and considerably decreased (50%) in the LM-TT control group but this was statistically not significant **(Fig 4c).**

Safety of Listeria-TT and GEM

To determine the safety of Listeria-TT+GEM, a dose-limiting toxicity study was performed as described previously[23]. Briefly, increasing doses of Listeria-TT were injected in C57Bl/6 mice without cancer and the survival time was determined over the course of the next 20 days. As shown in **Fig 2h,** the LD_{50} of Listeria-TT was $2x10^8$ CFU. In our treatment studies we used 10^7 Listeria-TT, which is far below the LD_{50}.

We also analyzed potential toxicity by pathological examination, 2 days after a complete treatment cycle with Listeria-TT+GEM in the Panc-02 mice as outlined in **Fig 1.** Again, no significant pathological effects of Listeria-TT+GEM were observed (a mild increase in leucocytes was observed in the spleen, liver and lungs) **(SI Table 1a).**

Finally, we analyzed liver functions of C57Bl/6 mice that received the full treatment cycle compared to the saline group (untreated). A small increase in the aminotransferase (AST) level in plasma from Listeria-TT+GEM-treated mice compared to untreated mice (107 to 137 U/L) was observed, while alanine aminotransferase (ALT) levels in Listeria-TT+GEM-treated mice were similar compared to untreated mice (15 and 16 U/L) **(SI Table 1b).**

Discussion

Although success has been reported with cancer immunotherapy, its utility in PDAC has been underwhelming, due to the low immunogenicity of the tumor, strong immune suppression in the TME, and less efficient activation of naïve T cells in the TME[8, 9, 11-14]. We addressed these problems by using a new concept of a Listeria-based immunotherapy, which includes delivery of a highly immunogenic foreign TT antigen selectively to the TME and into tumor cells through *in vivo* infection, by reducing immune suppression with GEM, and by avoiding the need of naïve T cells during treatment through reactivation of existing memory T cells generated during childhood.

Using the childhood TT vaccine (TTvac), memory CD4 and CD8 T cells were generated to TT before tumor development and reactivated with Listeria-TT+GEM after tumor development. This resulted in strong CD4 and CD8 T cell responses (producing Perforin, Granzyme B, and IFNγ) in the spleens of tumor-bearing Panc-02 and KPC mice. Also, RNAseq analysis showed that CD3/4/8/28, Granzymes and Perforin were abundantly upregulated in pancreatic tumors of KPC mice by TTvac, Listeria-TT+GEM compared to the untreated KPC mice **(SI Fig 4a)**, in correlation with increased expression of cell death pathways such as Casp 1,3,4,6,8,12, Fas, and tumor necrosis factor (TNF) **(SI Fig 4a).** These cell death pathways are potentially activated by Perforin and Granzyme B. Moreover, RNAseq analyses demonstrated that Listeria-TT+GEM also up regulated gene pathways involved in metastatic behavior, such as Cadherins (3, 5, 6, 10, 11, 13, 15, 17), and Claudins (2, 4, 5, 6, 7, 8, 10, 20) **(SI Fig 4b).** Both genes are involved in adherence of cells and tight junctions. Low expression of these genes is associated with strong metastatic behavior of tumors, and high expression with poor metastatic behavior[39, 40].

We demonstrated that early treatment of Panc-02 mice with Listeria-TT+GEM reduced the pancreatic tumors and metastases almost completely, i.e. 9 out of 10 mice

were free of cancer, which was not possible with Listeria-TT or GEM alone. Even when the treatment was started in Panc-02 and KPC mice (12-20 weeks of age) with advanced pancreatic cancer, a strong reduction in their tumors and metastases was observed.

Listeria-based immunotherapies has also been reported by others. Keenan et al. demonstrated improved significant survival and delayed the PanIN progression in KPC mice with early PDAC (4-6 weeks of age), when immunized with Listeria (Listeria double deleted ActA and InlB, both involved in spread of Listeria)(LADD) engineered to express the Kras12GD mutation, and combined with anti-CD25 Abs and cyclophosphamide[41]. Another interesting study recently published includes 4T1 and CT26 mice (using the flank model), immunized with LADD expressing a viral antigen AH1, which was also expressed by their tumors. This stimulated $CX3CR1^{+}CD62L^{-}KLRG1^{+}PD\text{-}1^{lo}$ tumor-specific CD8 T cells migrating into the AH1-expressing tumors and changed the TME by reducing Tregs and changing M2 macrophages into M1 macrophages[38]. In addition, mouse models of cervical, breast, and skin cancer has been successfully treated with Listeria expressing E7, Her2/neu, Flk-1, CD105, Mage-b, ISG15, and HMW-MAA[42].

Of note is that the concept of Listeria used by others is different from our concept. Listeria used by others is based on the delivery of tumor-specific antigens into dendritic cells to stimulate naïve and memory T cells which are naturally expressed by the tumors, while our concept is based on colonization and changing the TME by Listeria, and using Listeria to deliver highly immunogenic recall antigens inside tumor cells through in vivo infection, and to reactivate existing memory T cells to the recall antigens now also expressed by the tumors.

Listeria has also been tested in clinical trials in patients with various cancers, including pancreatic cancer, showing safety and tumor-specific T cell responses and improved survival (for a review see Forbes et al)[43].

Of note is that Listeria does not infect all tumor cells. However, we have shown in a previous study that Listeria induces immunogenic tumor cell death through ROS and that T cells were activated to multiple (if not all) TAA expressed by tumors and metastases in a metastatic breast tumor model[14]. This resulted in a significant reduction in the number of metastases and tumors. Based on this earlier study and results from this study (Listeria-TT abundantly killing Panc-02 tumor cells **Fig 2e**), it is likely that the TAA-specific T cells will destroy the non-infected tumor cells. However, this needs to be further analyzed.

Elderly cancer patients react less efficiently to vaccines than young adults[44, 45], which is also true for cancer immunotherapy[36, 46]. The cause for this differential response is mainly due to lack of naïve T cells at older age[47]. We believe that our novel approach of reactivating memory T cells generated during childhood (when plenty naïve T cells are available), avoids the need for naïve T cells at older age, and thus potentially making this therapeutic strategy more effective for patients of an older age. However, this belief still needs to be validated.

Abbreviations: ALT: alanine aminotransferase; AST: aminotransferase; CFP: cyan fluorescent protein; CFU: colony-forming unit; CTL: cytotoxic T lymphocyte; CD: cluster differentiation; DLT: dose-limiting toxicity; 18F-FDG: Fluorine-18 (^{18}F)-labeled deoxyglucose; FBS: fetal bovine serum; Flk: Fms-like tyrosine kinase; GEM: gemcitabine; HMW-MAA: high molecular weight melanoma-associated antigen; HRP: horse-radish peroxidase; IFN: interferon; IL: interleukin; ISG: interferon-stimulated gene; IVMI: intravital multiphoton imaging; KLRG: killer cell lectin-like receptor subfamily G; KPC: Kras, p53, Cre; LADD: Listeria double deleted; LLO: Listeriolysin O; LM: Listeria monocytogenes; Mage: melanoma-associated antigen; MARCO: macrophage receptor with collagenous structure; MDSC: myeloid-derived suppressor cells; OPO: optical

parametric oscillator; PD: programmed cell death; PET: positron emission tomography; PDAC: pancreatic ductal adenocarcinoma; prfA: positive regulatory factor; SDS: sodium dodecyl sulfate; SUV: standard uptake value; TAM: tumor-associated macrophages; TME: tumor microenvironment; TT: tetanus toxoid; TNF: tumor necrosis factor.

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Conception and Design: B.C. Selvanesan, D. Chandra, W. Quispe-Tintaya, Z. Yuan, S.K. Libutti, C. Gravekamp

Development of Methodology: B.C. Selvanesan, D. Chandra, W. Quispe-Tintaya, Z. Yuan, S.K. Libutti, C. Gravekamp

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc): B.C. Selvanesan, D. Chandra, W. Quispe-Tintaya, A. Jahangir, A. Patel, K. Meena, R. Alves Da Silva, A. Beck, L. Tesfa, W. Koba, Z. Yuan, S.K. Libutti, C. Gravekamp

Analysis and interpretation of data (e.g statistical analysis, biostatistics, computational analysis): B.C. Selvanesan, D. Chandra, W. Quispe-Tintaya, A. Jahangir, A. Patel, K. Meena, R. Alves Da Silva, A. Beck, L. Tesfa, W. Koba, J. Chuy, Z. Yuan, S.K. Libutti, C. Gravekamp

Writing, Review and/or revision of the manuscript: B.C. Selvanesan, D. Chandra, W. Quispe-Tintaya, Z. Yuan, S.K. Libutti, C. Gravekamp

Administrative, technical or material support (i.e., reporting or organizing data, constructing data bases): B.C. Selvanesan, D. Chandra, W. Quispe-Tintaya, Z. Yuan, S.K. Libutti, C. Gravekamp

Study Supervision: B.C. Selvanesan, D. Chandra, W. Quispe-Tintaya, Z. Yuan, S.K. Libutti, C. Gravekamp

Ethics approval and consent to participate

All in vivo experiments were approved by the NCI/NIH Animal Care and Use Committee (ACUC).

Competing interests

Dr. Gravekamp has ownership interest in a patent application filed about the Listeria-recall antigen concept (96700/2230). No competing interests were disclosed by the other authors.

LEGENDS

Figure 1: Treatment protocol Listeria-TT+GEM for Panc-02 and KPC mice. Panc-02 model: C57Bl/6 mice were immunized two times with the human TT vaccine (TTvac) one week apart to generate the memory T cells to TT similar as in humans. Subsequently, Panc-02 tumor cells (10^5/100 µL) were injected in the mammary fat pad. When tumors were 3 mm (3-5 days after tumor cell injection[early pancreatic cancer]) or 10 mm (day 10-14 after tumor cell injection[advanced pancreatic cancer]), one high dose of Listeria-TT (10^7 CFU) was injected intraperitoneally (ip). Three days later, allowing the Listeria-TT to accumulate and multiply in the TME, the treatment with GEM was started (every 3 days 1.2 mg/mouse) and continued for 14 days to eliminate MDSC and TAM (6 doses in total). At this moment MDSC are no longer required to bring the Listeria-TT to the TME. Instead,

reducing the MDSC and TAM populations will improve T cell responses. Concomitantly, low doses of Listeria-TT were administered every day for 2 weeks (14 doses in total) to reactivate the T cells to TT improved by GEM. Of note is that multiple high doses of Listeria-TT induced T cell anergy but multiple low doses induced strong T cell responses to TT. All mice were euthanized 2 days after the last treatment, and analyzed for tumor weight and the number of metastases. KPC model: KPC mice of 3.5-8 months old (after having verified the presence of tumors and/or metastases by PET/CT) received the same treatments as the Panc-02 mice. The SUVmax of tumors and metastases was measured before compared to the SUVmax after treatment. LM-TT = Listeria-TT.

Figure 2: Development and characterization of the Listeria-TT$_{856\text{-}1313}$ construct. **(a)** Listeria-TT construct. A non-toxic fragment of the C-terminus of TT cDNA (aa position 856-1313) was cloned as a fusion protein with a truncated non-cytolytic ListeriolysinO (LLO) in the Listeria plasmid pGG34, under the control of the LLO promoter (P). A myc tag has been included for the detection of TT protein. **(b)** Detection of TT protein in culture supernatant. Anti-myc antibodies were used. **Lane 1**: neg control (medium); **Lane 2**: supernatant of Listeria-TT culture; **Lane 3**: pellet of Listeria-TT culture. **(c)** Detection of TT protein in tumor cells. **Lane 1**: Panc-02 tumor cells; **Lane 2**: Panc-02 tumor cells infected with Listeria alone; **Lane 3**: Panc-02 tumor cells infected with Listeria-TT. **(d)** Panc-02 tumor cells infected with Listeria-TT. **(e)** Panc-02 tumor cells killed by Listeria-TT. **(f)** Biodistribution of Listeria-TT in Panc-02 model. n=3 mice per time point. **(g)** Listeria-TT infecting tumor cells and macrophages (MΦ) in Panc-02-dendra-2 tumors of live mice, by intravital imaging. Tumor cells are green, macrophages blue, and Listeria-TT red. **(h)** Dose- limiting toxicity study of Listeria-TT in tumor-naive C57Bl6 mice. Survival was analyzed over the next 20 days. n=5 mice per group. In

figure d, e and f, the error bars represent SEM. Mann-Whitney Mann-Whitney *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001. LM-TT = Listeria-TT.

Figure 3: Generation and recall of memory CD4 and CD8 T cell responses to TT. **(a)** Generation of CD4 and CD8 memory T cells to TT mice before tumor development through immunizations with the human TT vaccine (TTvac). **(b)** Reactivation of CD4 and CD8 T cell responses to TT after tumor development through a complete treatment cycle with Listeria-TT+GEM. T cells producing IFNγ before and after tumor development were analyzed in a restimulation assay with TT protein by ELISPOT. Representative of 2 experiments. n=3 mice per group (pooled). **(c)** GEM reduces MDSC populations in blood and primary tumors, and TAM in primary tumors. MDSC (CD11b+Gr1+) and TAM (CD11b+F4/80+) were analyzed after a full treatment cycle with TTvac, and Listeria-TT plus GEM as described in **Fig 1.** Representative of 2 experiments. n=3 mice per group (pooled). Mann-Whitney *p<0.05, ***p<0.001 and *****p<0.0001.

Figure 4: Listeria-TT and GEM robustly reduce early and advanced pancreatic cancer in Panc-02 and KPC mice. **(a)** Early pancreatic cancer (Panc-02). C57Bl/6 mice received a full treatment cycle with TTvac, Listeria-TT+GEM as described in **Fig 1**; the combination therapy was started 3 days after tumor cell injection. **(b)** Advanced pancreatic cancer (Panc-02). C57Bl/6 mice received a full treatment cycle with TTvac, Listeria-TT+GEM as described in **Fig 1**; the combination therapy was started 10-14 days after tumor cell injection. **(c)** Advanced pancreatic cancer (KPC). KPC mice received a full treatment cycle with TTvac, Listeria-TT+GEM as described in **Fig 1**; the combination therapy was started at the age of 3-5.5 months, after tumors and metastases were verified through PETscan. Tumors and metastases were measured (SUVmax) before and after the treatment period. **(d)** Example of pancreatic tumors in the different

treatment groups of the Panc-02 mice. **(e)** Example of the pancreatic tumors in the different treatment groups of the KPC mice. In **abc**, n=5 mice per group (average of two experiments)(controls Saline, GEM, and LM-TT in **c**, n=2-3 mice). Mann-Whitney Mann-Whitney *p <0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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Figure 1

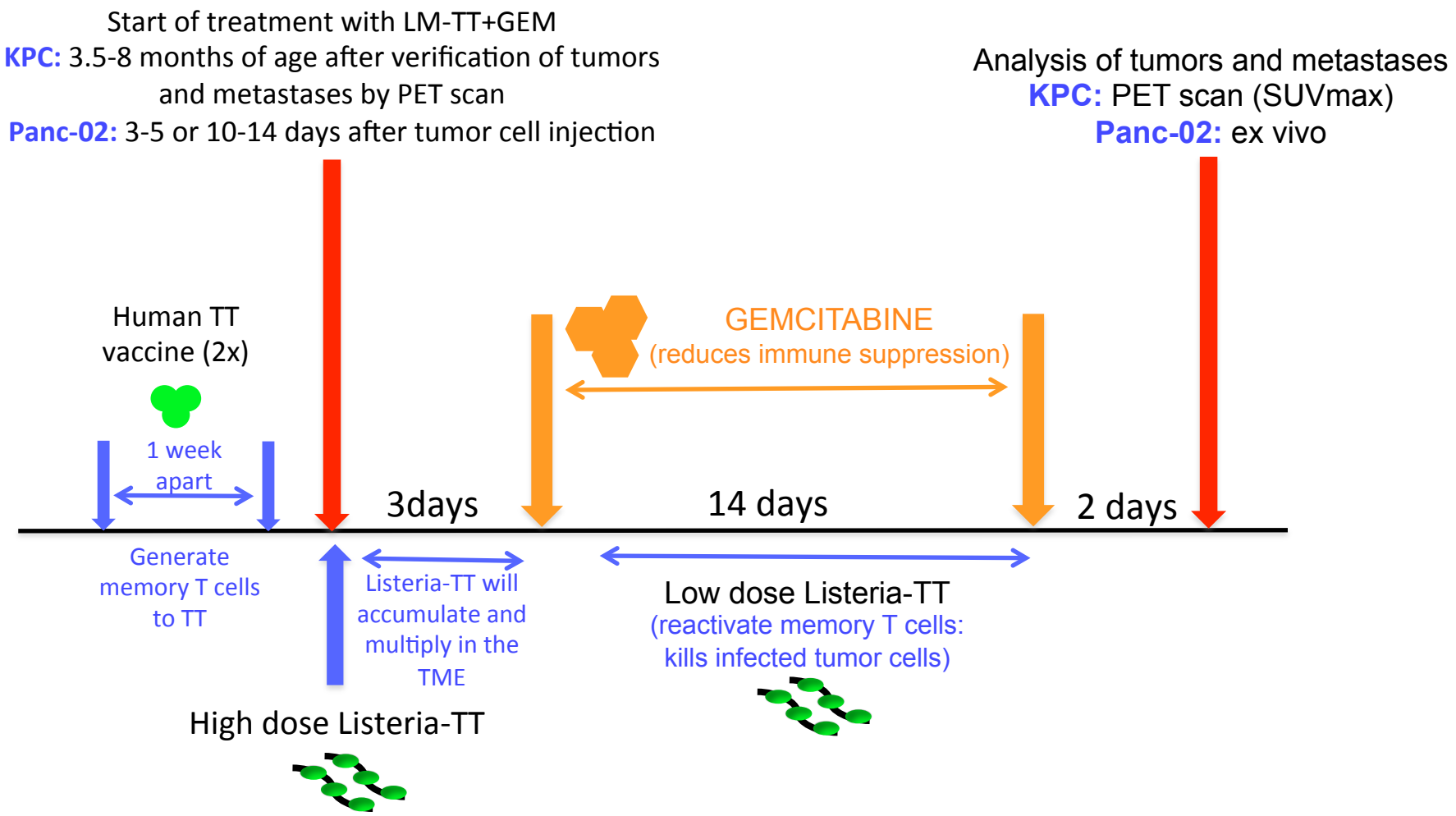

Start of treatment with LM-TT+GEM
KPC: 3.5-8 months of age after verification of tumors and metastases by PET scan
Panc-02: 3-5 or 10-14 days after tumor cell injection
Analysis of tumors and metastases
KPC: PET scan (SUVmax)
Panc-02: ex vivo
Human TT vaccine (2x)
1 week apart
GEMCITABINE
(reduces immune suppression)
3days
14 days
2 days
Generate memory T cells to TT
Listeria-TT will accumulate and multiply in the TME
Low dose Listeria-TT
(reactivate memory T cells: kills infected tumor cells)
High dose Listeria-TT

Figure 2

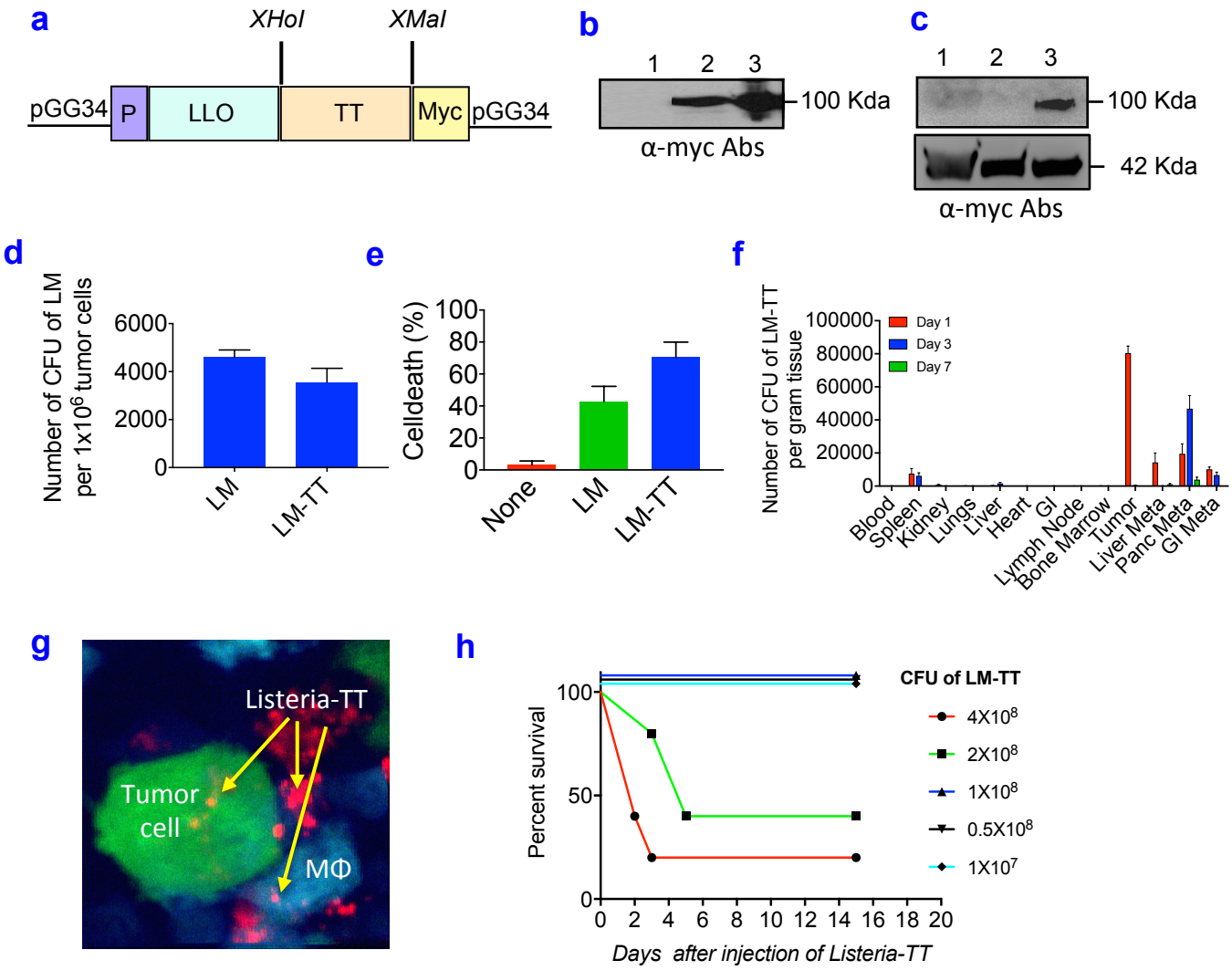
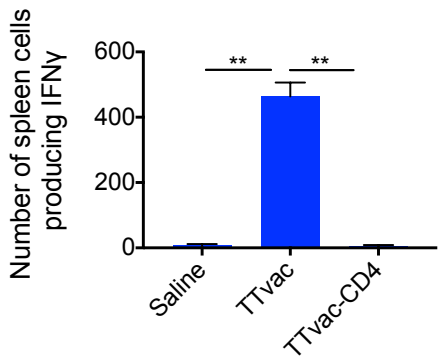
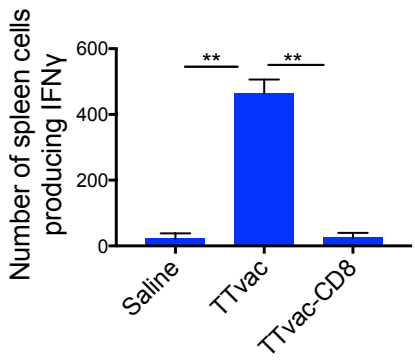

a
XHoI
XMaI
pGG34
P
LLO
TT
Myc
pGG34
b
1 2 3
100 Kda
α-myc Abs
c
1 2 3
100 Kda
42 Kda
α-myc Abs
d
Number of CFU of LM per 1x10^6 tumor cells
6000
4000
2000
0
LM
LM-TT
e
Celldeath (%)
100
80
60
40
20
0
None
LM
LM-TT
f
Number of CFU of LM-TT per gram tissue
Day 1
Day 3
Day 7
100000
80000
60000
40000
20000
0
Blood
Spleen
Kidney
Lungs
Liver
Heart
GI
Lymph Node
Bone Marrow
Tumor
Liver Meta
Panc Meta
GI Meta
g
Listeria-TT
Tumor cell
MΦ
h
Percent survival
100
50
0
0 2 4 6 8 10 12 14 16 18 20
Days after injection of Listeria-TT
CFU of LM-TT
4X10^8
2X10^8
1X10^8
0.5X10^8
1X10^7

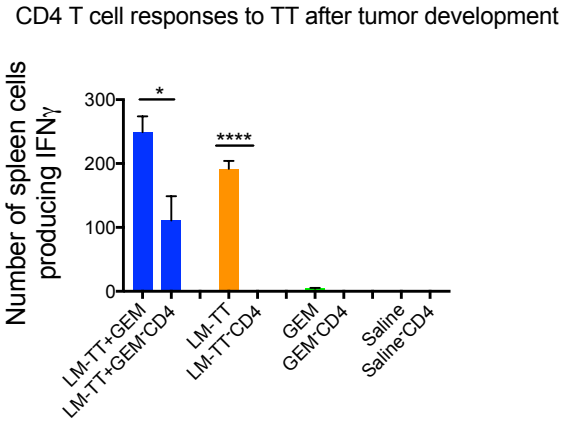
Figure 3

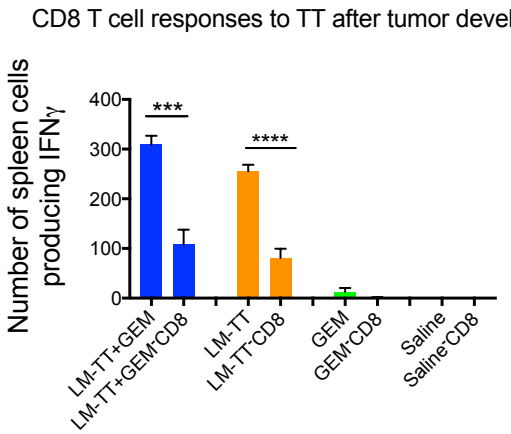
a


CD4 T cell responses to TT before tumor development
Number of spleen cells producing IFNγ
600
400
200
0
**
**
Saline
TTvac
TTvac-CD4

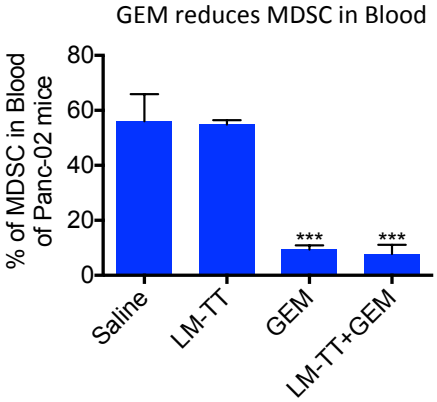

CD8 T cell responses to TT before tumor development
Number of spleen cells producing IFNγ
600
400
200
0
**
**
Saline
TTvac
TTvac-CD8

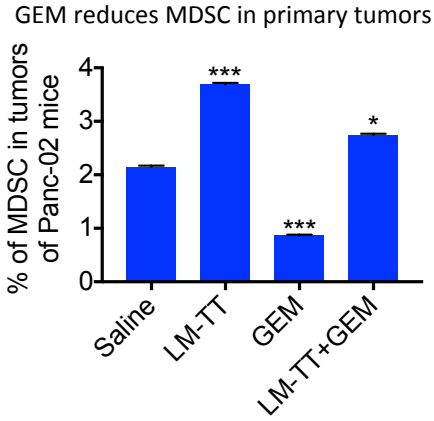
b


CD4 T cell responses to TT after tumor development
Number of spleen cells producing IFNγ
300
200
100
0
*
****
LM-TT+GEM
LM-TT+GEM+CD4
LM-TT
LM-TT+CD4
GEM
GEM+CD4
Saline
Saline+CD4


CD8 T cell responses to TT after tumor development
Number of spleen cells producing IFNγ
400
300
200
100
0
***
****
LM-TT+GEM
LM-TT+GEM+CD8
LM-TT
LM-TT+CD8
GEM
GEM+CD8
Saline
Saline+CD8

c


GEM reduces MDSC in Blood
% of MDSC in Blood of Panc-02 mice
80
60
40
20
0
***
***
Saline
LM-TT
GEM
LM-TT+GEM


GEM reduces MDSC in primary tumors
% of MDSC in tumors of Panc-02 mice
4
3
2
1
0
***
***
*
Saline
LM-TT
GEM
LM-TT+GEM

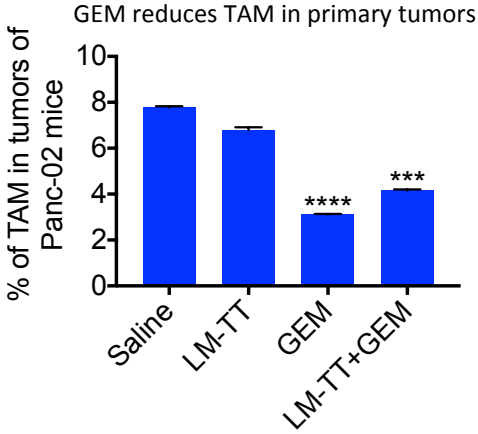
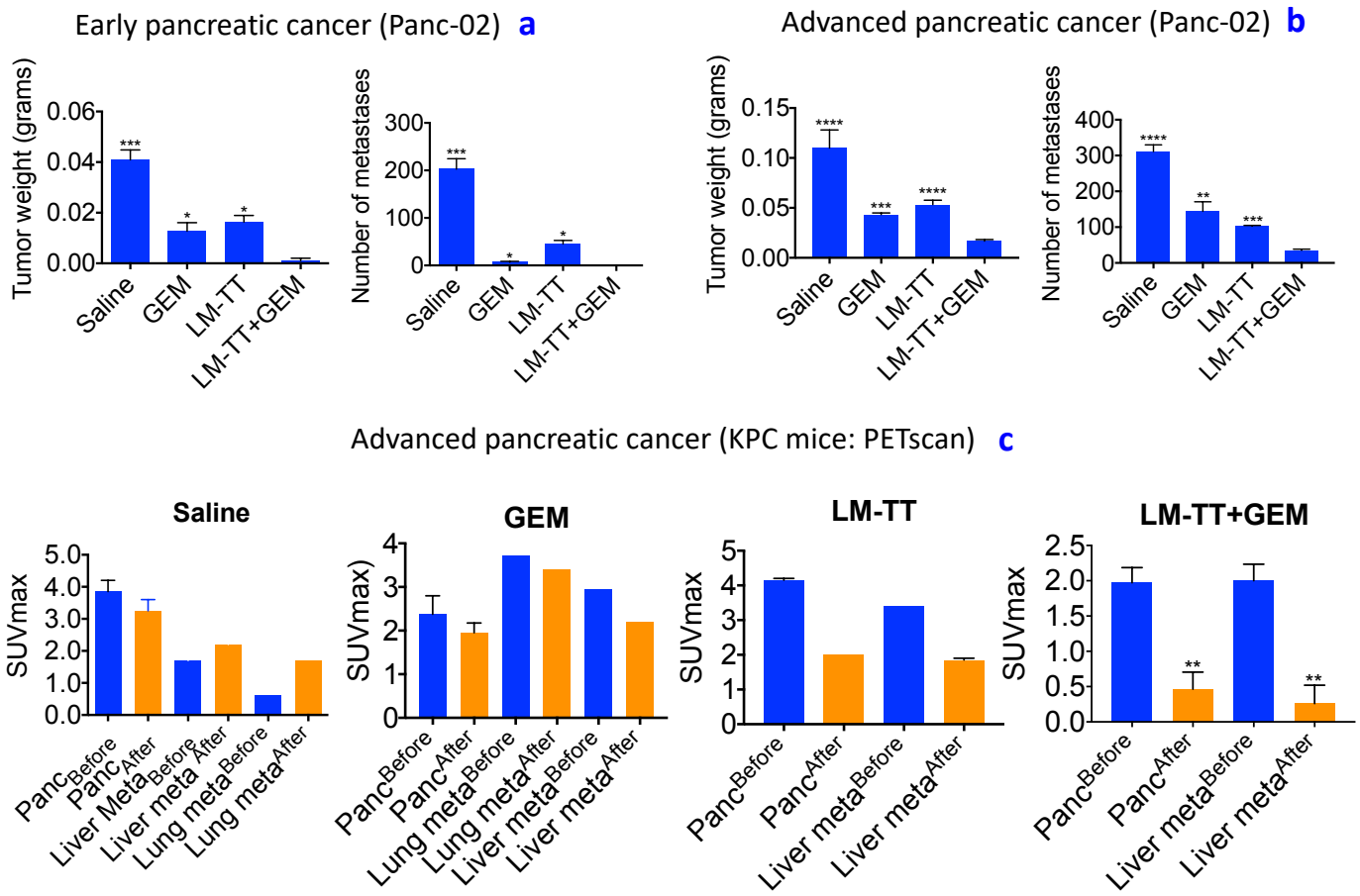

GEM reduces TAM in primary tumors
% of TAM in tumors of Panc-02 mice
10
8
6
4
2
0
****
***
Saline
LM-TT
GEM
LM-TT+GEM

Figure 4


Early pancreatic cancer (Panc-02) a
Tumor weight (grams)
0.06
0.04
0.02
0.00
***
*
*
Saline
GEM
LM-TT
LM-TT+GEM
Number of metastases
300
200
100
0
***
*
*
Advanced pancreatic cancer (Panc-02) b
Tumor weight (grams)
0.15
0.10
0.05
0.00
****
***
****
Number of metastases
400
300
200
100
0
****
**
***
Advanced pancreatic cancer (KPC mice: PETscan) c
Saline
GEM
LM-TT
LM-TT+GEM
SUVmax
SUVmax)
PancBefore
PancAfter
Liver metaBefore
Liver metaAfter
Lung metaBefore
Lung metaAfter
**

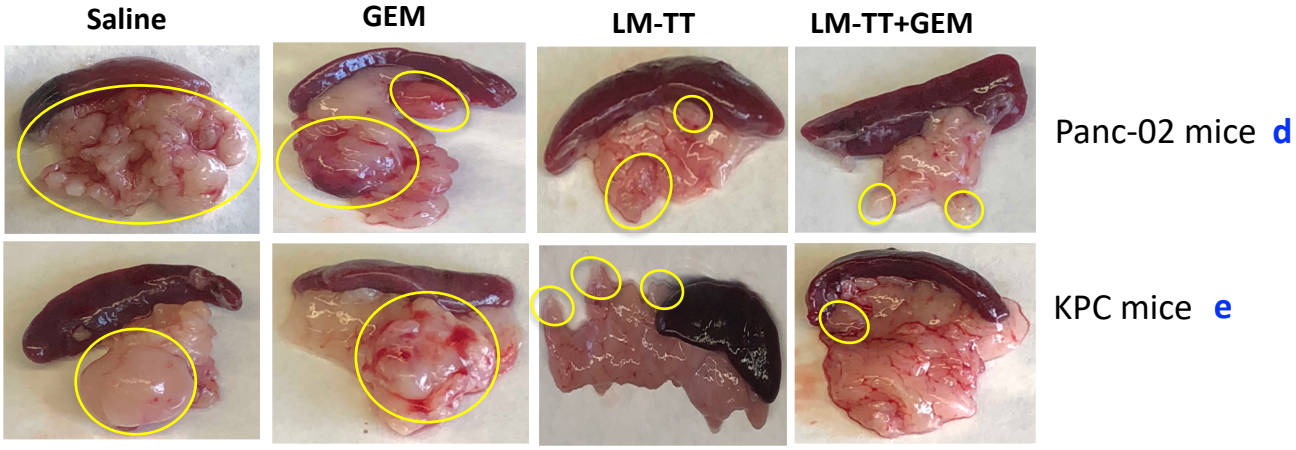

Saline
GEM
LM-TT
LM-TT+GEM
Panc-02 mice d
KPC mice e

Table 1a: Listeria-TT and GEM increases CD4 and CD8 T cell responses *in vivo* in Panc-02 mice

T cell responses*	**Saline**	**GEM**	**LM-TT**	**LM-TT+GEM**
CD3CD4CD69	15.5	14.2	14.2	28.5
CD3CD8CD69	16.7	15.5	15.5	33.2
CD3CD4Perforin	7.9	14.9	14.9	22.7
CD3CD8Perforin	2.1	4.8	4.9	6.2
CD3CD4Granzyme B	4.9	4.8	5.9	9.9
CD3CD8Granzyme B	1.2	1.1	1.2	2.4
CD3CD4IFNγ	2.6	2.7	3.0	5.0
CD3CD8IFNγ	0.5	0.4	0.4	0.9

* T cell responses are provided in percentages (Flow cytometry).
An example of gating T cells is shown in **SI Fig 3ab**
All mice were treated as outlined in **Fig 1.** LM-TT = Listeria-TT
n=3 mice per group (spleen cells were pooled). Representative of 2 experiments

Table 1b: Listeria-TT and GEM increase CD4 and CD8 T cell responses *in vivo* in KPC mice

T cell responses	Saline	GEM	LM-TT	LM-TT+GEM
CD3CD4CD69	13.5	10.01	6.59	17.34
CD3CD8CD69	12.3	17.5	18.8	23.92
CD3CD4Perforin	0.26	7.90	8.22	14.27
CD3CD8Perforin	0.25	15.60	14.73	16.70
CD3CD4Granzyme B	3.1	3.5	4.82	12.17
CD3CD8Granzyme B	1.6	9.71	9.82	12.60
CD3CD4IFNγ	0.3	0.0	0.41	0.16
CD3CD8IFNγ	0.0	1.7	1.85	3.33

*T cell responses are provided in percentages (Flow cytometry).
All mice were treated as outlined in **Fig 1.** LM-TT = Listeria-TT
n=3 mice per group (spleen cells were pooled). Representative of 2 experiments.

Table 2a: Listeria-TT and GEM reduce immune suppressive function of MDSC (CD11b+Gr1+) in metastases of Panc-02 mice

MDSC responses*	**Saline**	**LM-TT**	**GEM**	**LM-TT+GEM**
IL-10	3.46	2.44	2.76	1.45
IL-6	4.25	4.05	3.59	1.97
TNFα	3.64	8.42	8.49	11.6
MARCO	3.38	3.70	3.84	1.79
CD80	1.28	1.08	1.78	1.17

*MDSC responses are provided in percentages (Flow cytometry).
An example of gating the MDSC is shown in **SI Fig 5a.**
All mice were treated as outlined in **Fig 1.** LM-TT = Listeria-TT
n=3 mice per group (CD45+ cells were pooled). This experiment was performed once.

Table 2b: Listeria-TT and GEM reduce immune suppressive function of TAM (CD11b+F4/80+) in metastases of Panc-02 mice

TAM responses*	Saline	LM-TT	GEM	LM-TT+GEM
IL-10	1.45	0.65	1.47	0.72
IL-6	1.09	0.76	1.25	0.42
TNFα	4.78	8.73	8.85	8.04
MARCO	1.96	2.16	1.94	1.33
CD80	1.93	3.07	4.28	5.26

*TAM responses are provided in percentages (Flow cytometry).
An example of gating TAM is shown in **SI Fig 5b.**
All mice were treated as outlined in **Fig 1.** LM-TT = Listeria-TT
n=3 mice per group (CD45+ cells were pooled). This experiment was performed once.

SUPPLEMENTARY INFORMATION

Title: A new immunotherapeutic concept for pancreatic cancer using childhood vaccine recall antigens and Listeria

Benson Chellakkan Selvanesan[1$], Dinesh Chandra[1$], Wilber Quispe-Tintaya[1$], Arthee Jahangir[1], Ankur Patel[1], Kiran Meena[1], Rodrigo Alves Da Silva[1], Steven K Libutti[2], Ziqiang Yuan[2], Amanda Beck[3], Lydia Tesfa[4], Wade Koba[5], Jennifer Chuy[6], John C. McAuliffe[7], Rojin Jafari[8], David Entenberg[8,9,10], Yarong Wang[8,9,10], John Condeelis[8,9,10,11], and Claudia Gravekamp[1*].

[$]These authors equally contributed to this manuscript

Running title: Cancer treatment through childhood vaccine recall antigens

[1]*Albert Einstein College of Medicine, Department of Microbiology and Immunology, 1300 Morris Park Avenue, Bronx, NY 10461, USA*

[2]*Rutgers University, Cancer Institute of New Jersey, 195 Little Albany St, New Brunswick, New Jersey 08901, USA*

[3]*Albert Einstein College of Medicine, Michael F. Price Center, 1301 Morris Park Avenue, Room 158, Bronx, NY 10461*

[4]*Albert Einstein College of Medicine, Jack and Pearl Resnick Campus, 1300 Morris Park Avenue, Chanin Building, Room 309, Bronx, NY 10461*

[5]*Albert Einstein College of Medicine, Department of Radiology, MRRC, 1300 Morris Park Avenue, Bronx, NY 10461, USA*

[6]*Montefiore/Einstein Center for Cancer Care, Department of Medical Oncology, 1695 Eastchester Road, 2nd Floor, Bronx, NY 10461, USA*

[7]*Montefiore Medical Center, Department of Surgery, 1521 Jarrett Place, 2nd Floor, Bronx, NY 10461, USA*

[8]*Albert Einstein College of Medicine, Michael F. Price Center, Department of Anatomy and Structural Biology, 1301 Morris Park Avenue.*

[9]*Albert Einstein College of Medicine, Michael F. Price Center, Gruss-Lipper Biophotonics Center, 1301 Morris Park Avenue.*

[10]*Albert Einstein College of Medicine, Michael F. Price Center, Integrated Imaging Program, 1301 Morris Park Avenue.*

[11]*Albert Einstein College of Medicine, Michael F. Price Center, Department of Surgery, 1301 Morris Park Avenue.*

Keywords: *Listeria monocytogenes*, childhood recall antigens, pancreatic cancer, breast cancer, memory T cells

* To whom correspondence should be addressed
Claudia Gravekamp
Albert Einstein College of Medicine
Department of Microbiology and Immunology
1300 Morris Park Avenue
Forchheimer Bldg, Room 407A
Bronx, NY 10461
Email: claudia.gravekamp@einstein.yu.edu
Phone: 718-430-4048(office)/4067 (lab)/Fax: 718-430-8711

SI Figure S1: Mechanistic view of the Listeria-recall antigen concept. Memory T cells to recall antigen TT will be generated with the human TT vaccine in the Panc-02 or KPC mice at 6 weeks of age before tumor development. The TT protein will be taken up by antigen-presenting cells (APC) and presented to naïve T cells. Upon repeated TT exposures, the naïve T cells will differentiate into memory T cells, which circulate in the blood for life (also in humans after childhood vaccinations). After appearance of the primary tumor and metastases, the memory T cells will be reactivated. Briefly, mice will receive one high dose of Listeria-TT to deliver TT inside tumor cells (replication of the Listeria-TT bacteria will lead to more TT protein in the tumor cells), followed by frequent low dose immunizations with Listeria-TT for 2 weeks to reactivate the memory T cells to TT. Finally, the TT-specific memory T cells will migrate to the tumor cells, and kill the tumor cells now presenting TT epitopes. Since attenuated Listeria bacteria colonize the TME, they will also infect MDSC and macrophages (this has not been shown here). LM-TT = Listeria-TT

SI Figure S2: Intraperitoneally but not intravenously injected Listeria colonizes tumors and metastases. Mice with 4T1 tumors and metastases were injected Listeria ($0.5x10^7$ CFU) intraperitoneally or intravenously, and 10 days later the number of Listeria bacteria was determined in blood and all organs including tumors and metastases as has been done previously[1]. The results demonstrate that Listeria injected ip clearly colonizes the TME, but very poorly or not at all if injected iv.

SI Figure 3: Gating of CD4 and CD8 T cells in spleens of tumor-bearing Panc-02 mice treated with Listeria-TT and GEM combined or separately. Example of gating **(a)** the CD4 T cells and **(b)** CD8 T cells in the spleen of treated and control mice. CD3CD4-pos cells and CD3CD8-pos cells were gated followed by gating CD4 or CD8 T

cells producing IFNγ, Perforin, Granzyme B, or expressing CD69. Similar gating was used for CD4 and CD8 T cells in the spleens of KPC mice.

SI Figure 4: Gene pathways involved in immune responses, tumor cell death and metastatic behavior analyzed in pancreatic tumors of KPC mice by RNAseq

In a small pilot study we have analyzed the effect of Listeria-TT and GEM on immune responses and tumor cell death in KPC tumors by RNAseq. Treatments with Listeria-TT and GEM were administered as outlined in Fig 4 of the manuscript. Expression levels of CD3/4/8/28, Granzymes and Perforin were strongly up-regulated **(a).** Also an increase in caspase 1, 3, 6, 8, and 12, as well as in Fas and TNF was observed, in the treated compared to untreated mice. These genes are all involved in tumor cell death. In addition, gene pathways involved in metastatic behavior were analyzed by RNAseq. As shown here, Cadherins 3, 5, 6, 10, 11, 13, 17 and Claudins 2, 4, 5, 6, 7, 8, 20 were strongly upregulated by Listeria-TT and GEM compared to the untreated **(b).** Egf (stimulates proliferation of fibroblasts and endothelial cells) and Vegf, (increases permeability of blood vessels) were down-regulated by Listeria-TT and GEM. Preparation for RNAseq and analysis is outlined below. In each group n=2 mice were analyzed by RNAseq.

RNAseq

Sample preparation for RNA seq. An RNA-Seq assay for minute amounts (25 ng) of total RNA input from the metastasis and tumor tissues was used and validated by our Epigenetics Shared Facility (ESF) will be used. Total RNA isolation was performed and treated with DNase, and evaluated using the Agilent bioanalyzer. The purified high quality total RNA was submitted to the ESF for assay and analysis.

Whole-transcriptome library preparation and high-throughput RNA sequencing. ESF personnel performed rRNA-depletion using a Ribo-Zero rRNA Removal Kit (Human/Mouse/Rat) (Epicentre) and reverse transcription to synthesize ds-cDNA using Random Hexamers and OligodT. ds-cDNA was used for preparing libraries with adapters and barcodes for multiplex sequencing. Each prepared library was sequenced using the Illumina Hiseq2500. Raw FASTQ files were trimmed for adapter sequences using quart. Sequence quality on FASTQ files was performed using FastQC[2] and the reads coverage using the RSeQC package[3].

RNA-Seq data analysis. We have generated a total of 150 RNA-Seq runs. For each RNA-Seq run we obtained at least 20 million reads with >70% of them aligned to the mouse genome. We used the bowtie/tophat/cufflinks/cuffdiff software suite[4-6] by applying the tuxedo protocol.

SI Figure 5: Gating of MDSC and TAM in metastases of tumor-bearing Panc-02 mice. (a) Example of gating the MDSC cells in the metastases of treated and control mice. In all flow cytometry studies, first live cells were gated. Subsequently, CD11bGr1-pos cells (MDSC) were gated in the live cell population, followed by the gating of Gr1-pos cells producing IL-10, IL-6, TNFα, or expressing MARCO or CD80 as indicated in the figure. **(b)** Example of gating TAMs in the metastases of treated and control mice. First CD45+ cells (leukocytes) were gated to exclude tumor cells, followed by gating of CD11bF4/80-pos cells, followed by F4/80-pos cells producing IL-10, IL-6, TNFα, or expressing MARCO or CD80.

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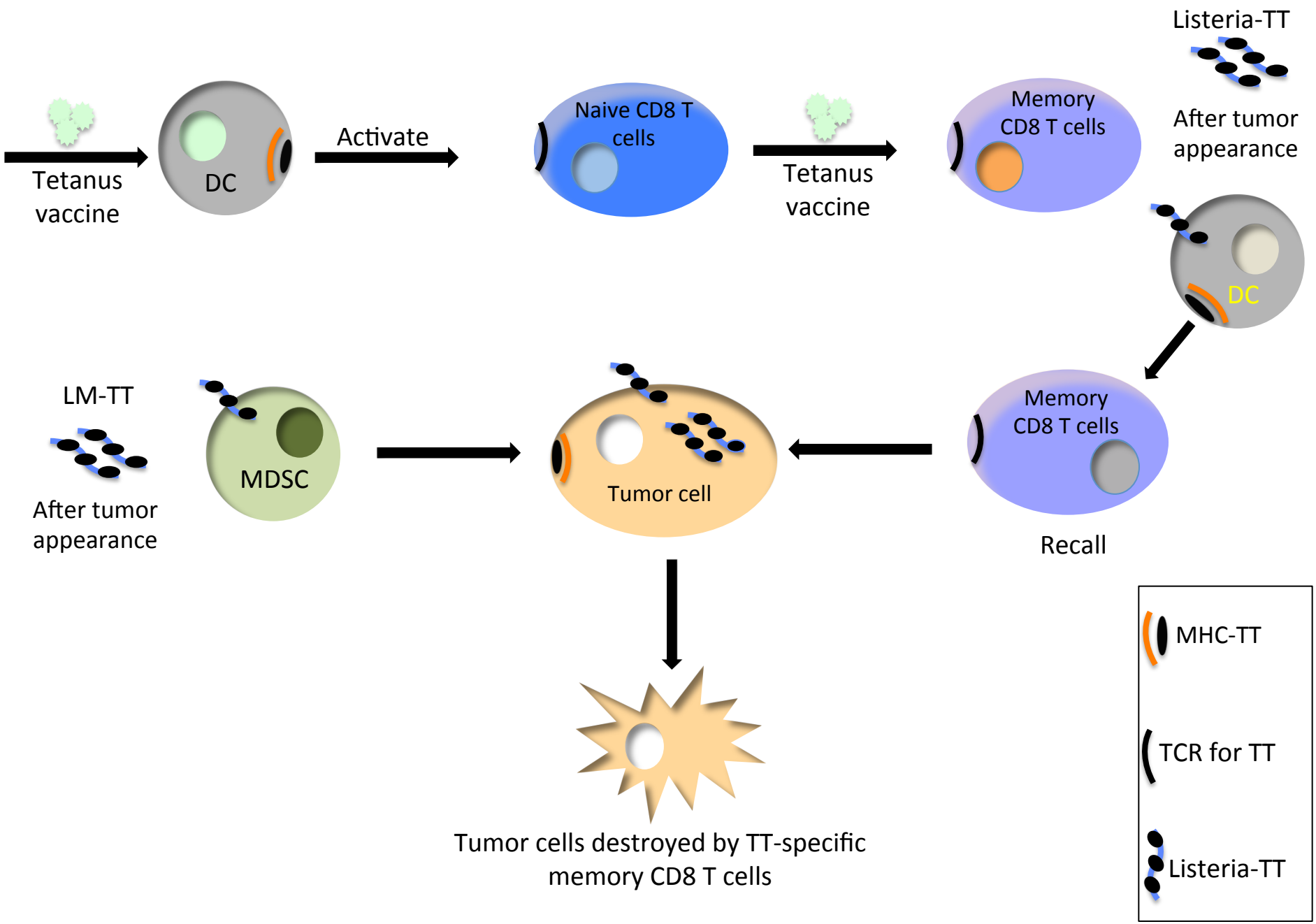
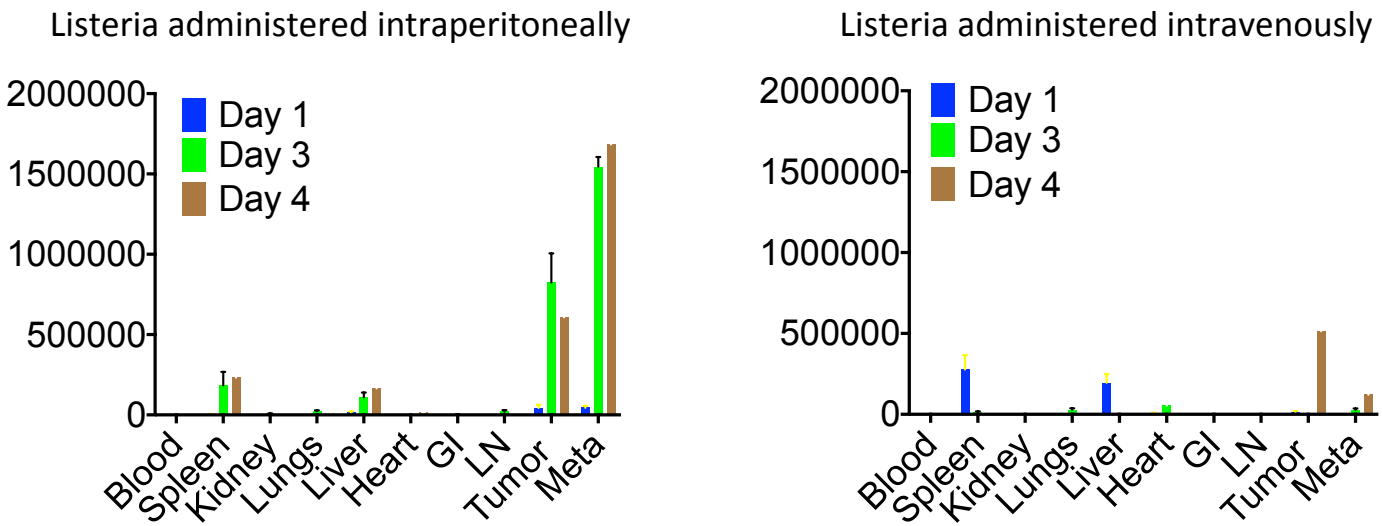
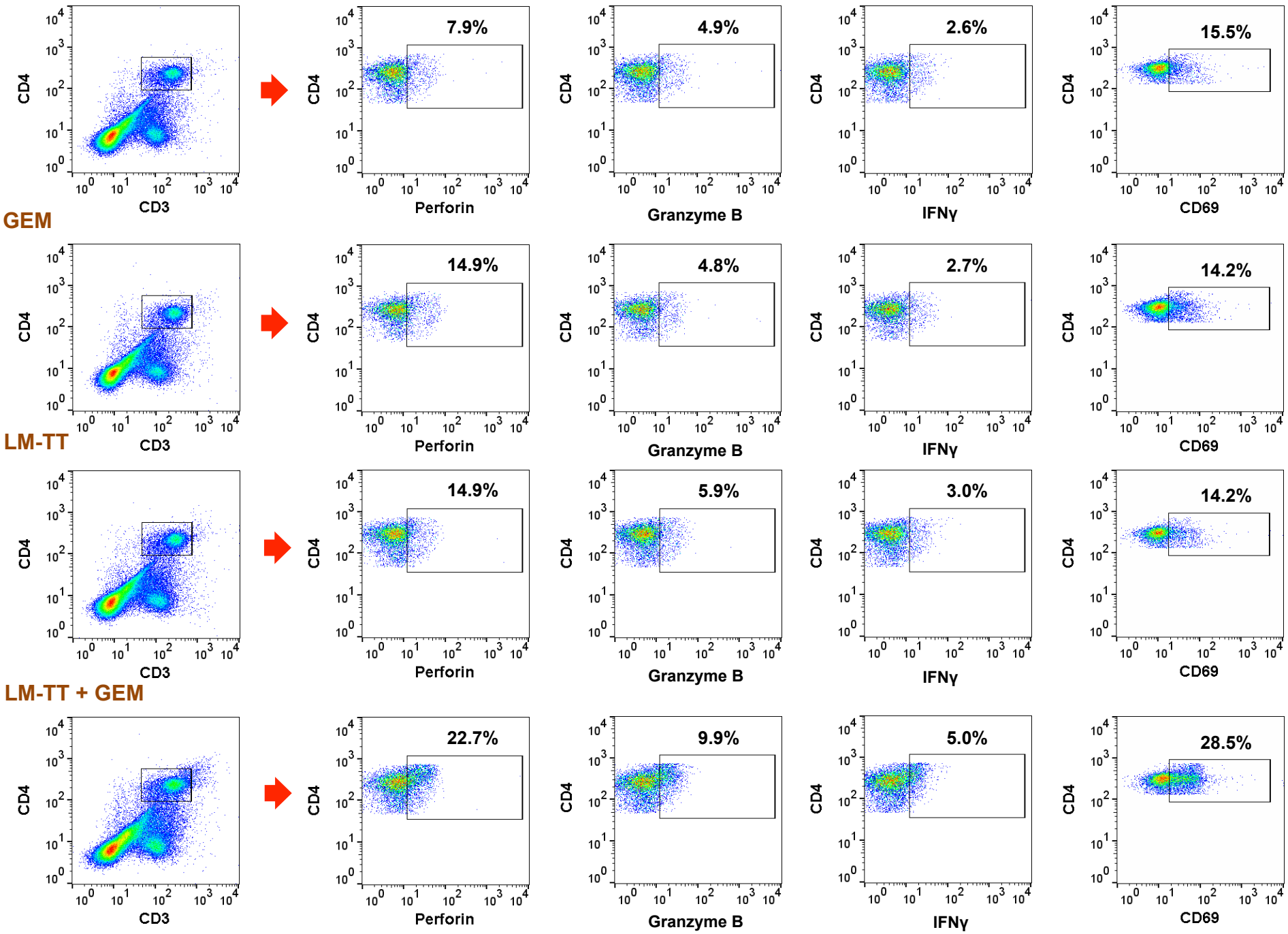
SI Figure 1


Listeria-TT
After tumor appearance
Tetanus vaccine
DC
Activate
Naive CD8 T cells
Tetanus vaccine
Memory CD8 T cells
DC
LM-TT
After tumor appearance
MDSC
Tumor cell
Memory CD8 T cells
Recall
Tumor cells destroyed by TT-specific memory CD8 T cells
MHC-TT
TCR for TT
Listeria-TT

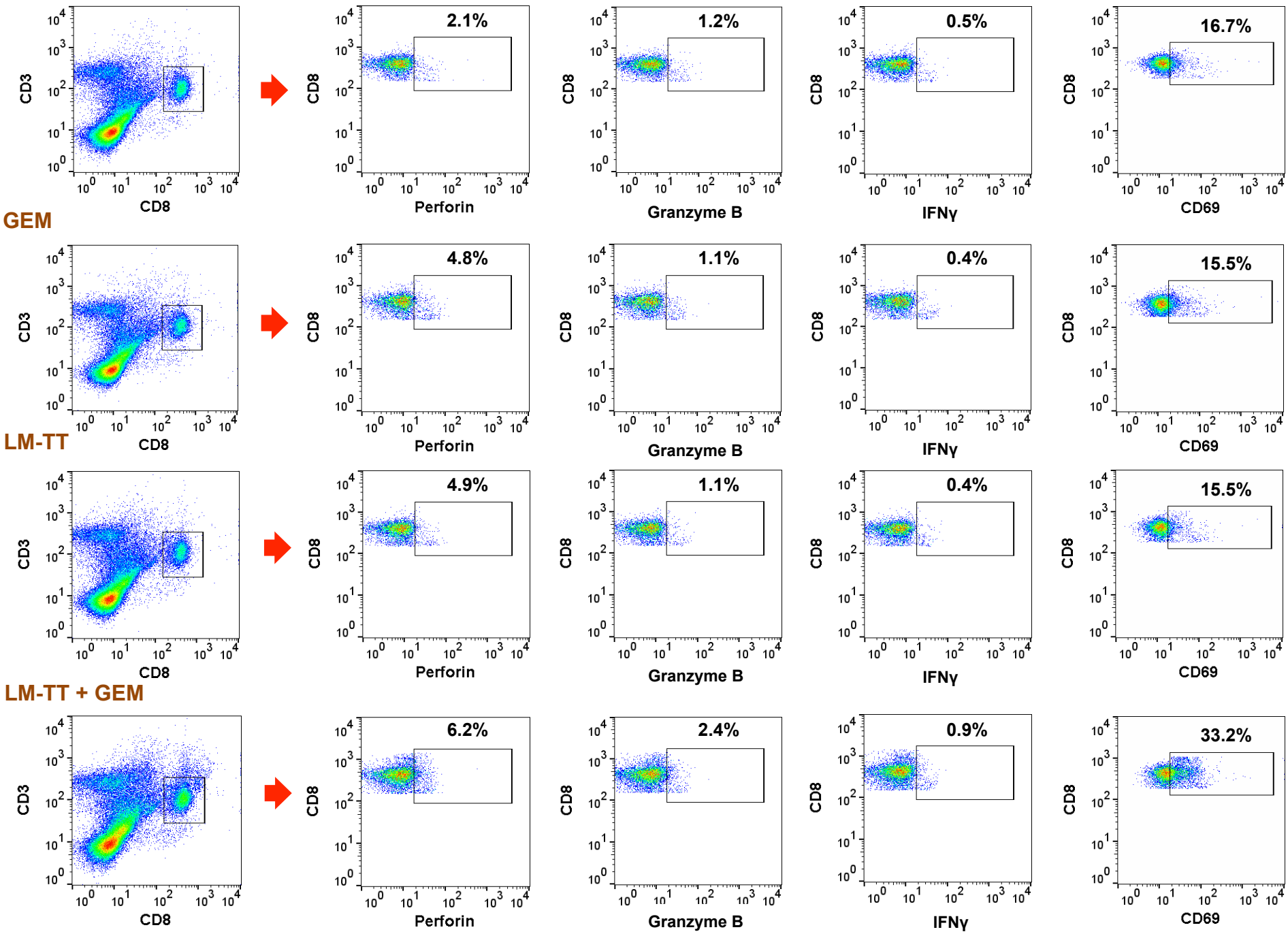
SI Figure 2


Listeria administered intraperitoneally
2000000
1500000
1000000
500000
0
Day 1
Day 3
Day 4
Blood
Spleen
Kidney
Lungs
Liver
Heart
GI
LN
Tumor
Meta
Listeria administered intravenously
2000000
1500000
1000000
500000
0
Day 1
Day 3
Day 4
Blood
Spleen
Kidney
Lungs
Liver
Heart
GI
LN
Tumor
Meta

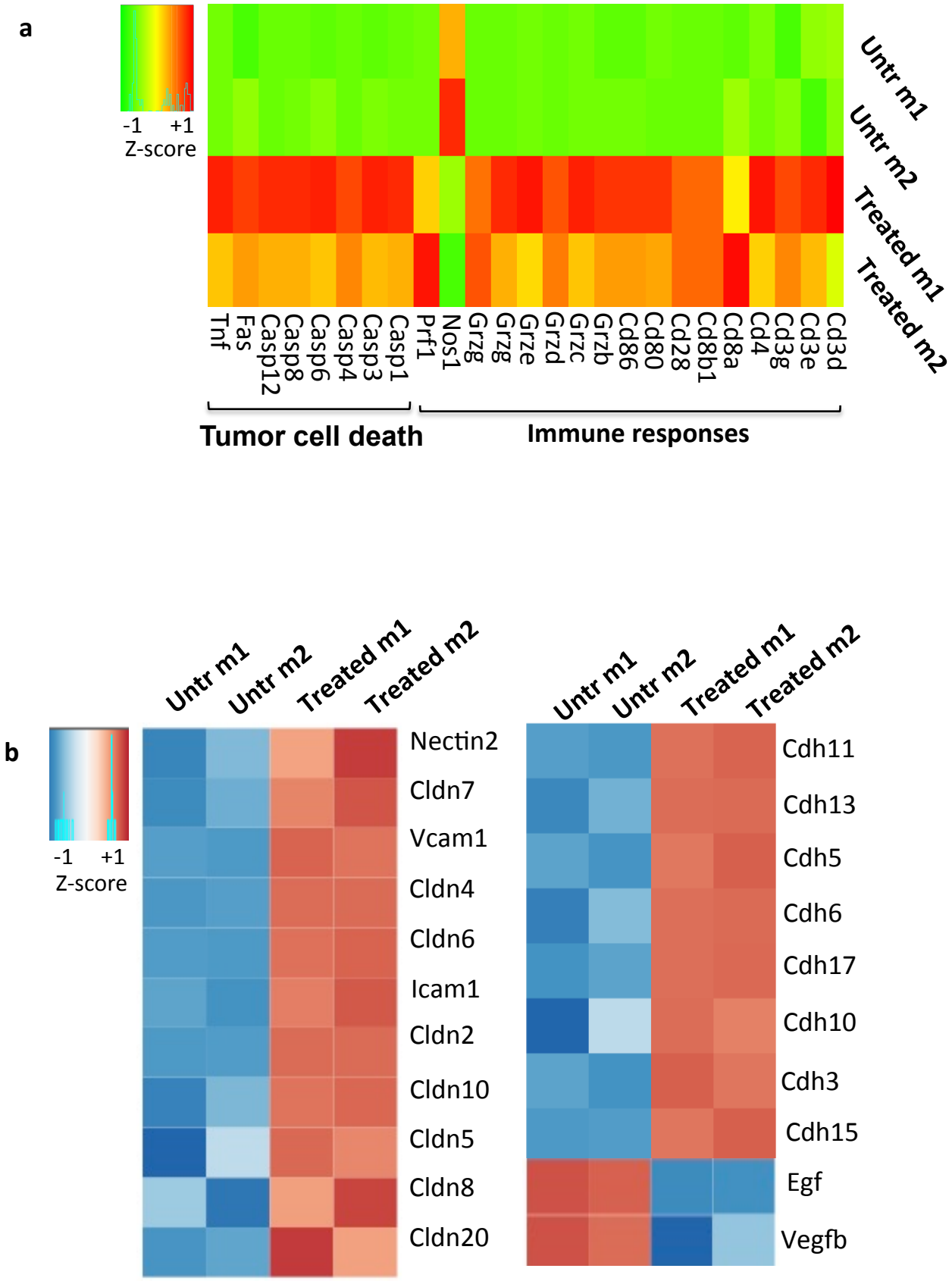
SI Figure 3a

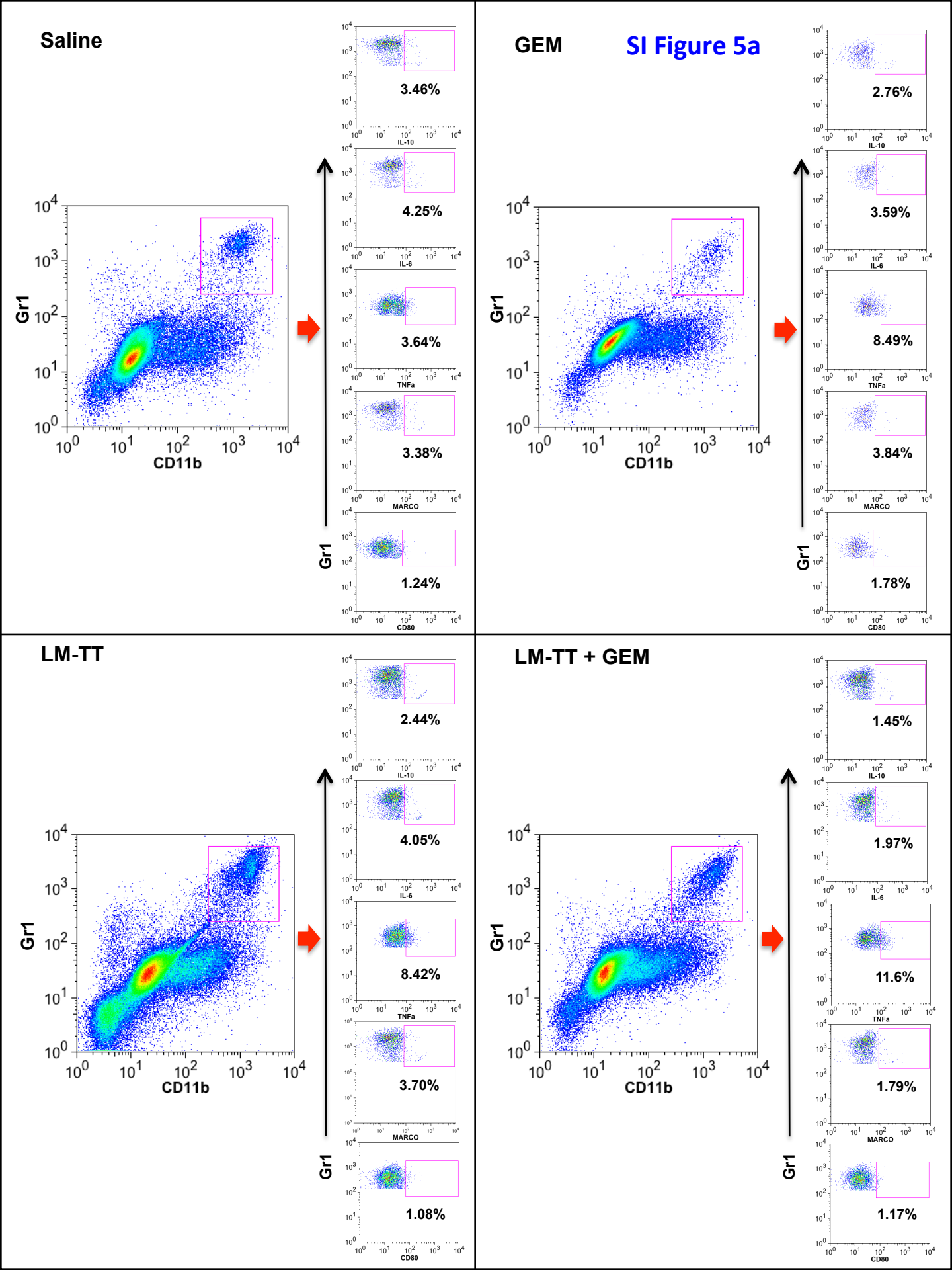

Saline
GEM
LM-TT
LM-TT + GEM
CD4
CD3
Perforin
Granzyme B
IFNγ
CD69
7.9%
4.9%
2.6%
15.5%
14.9%
4.8%
2.7%
14.2%
14.9%
5.9%
3.0%
14.2%
22.7%
9.9%
5.0%
28.5%

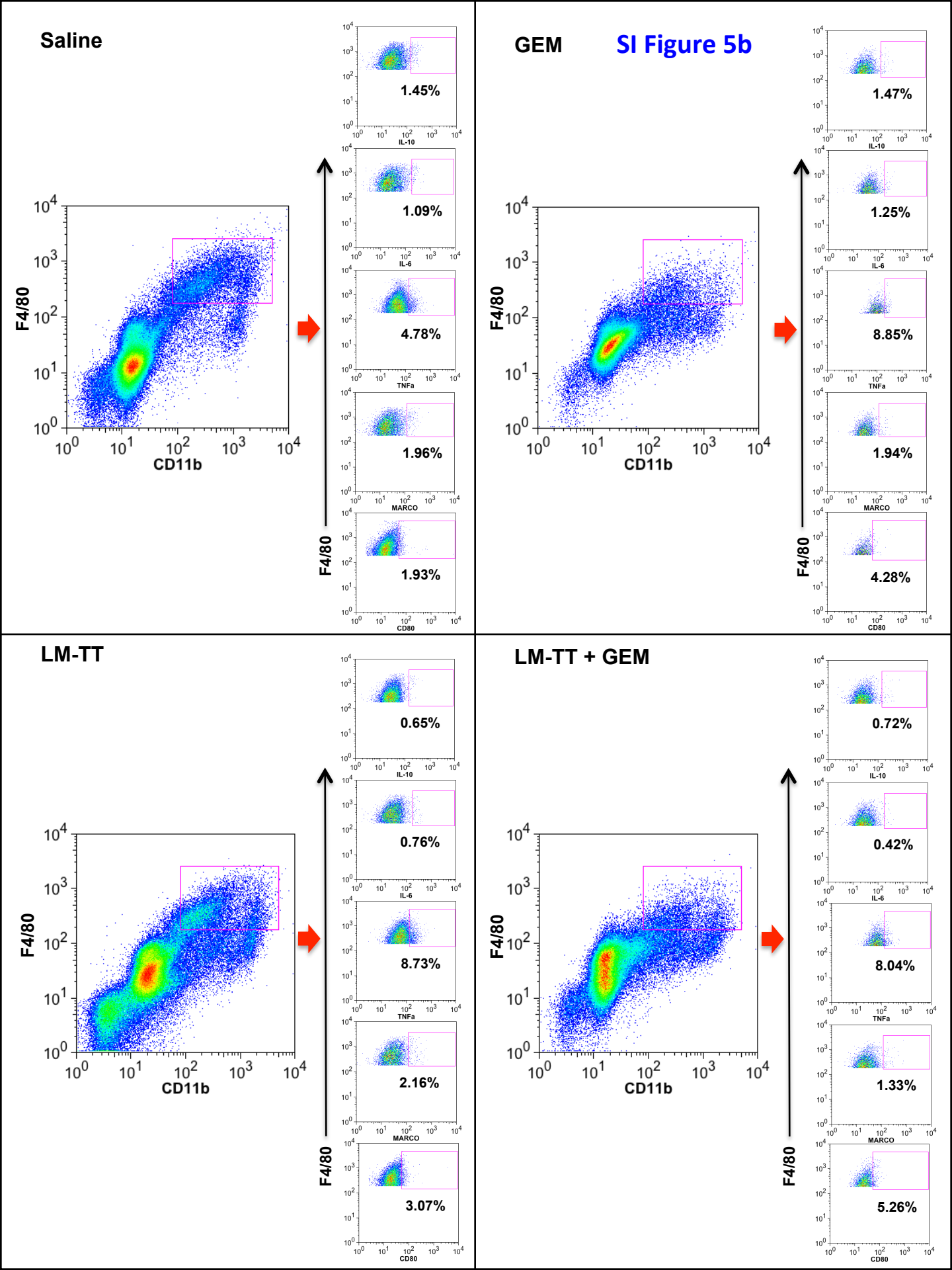
SI Figure 3b


Saline
GEM
LM-TT
LM-TT + GEM
CD3
CD8
Perforin
Granzyme B
IFNγ
CD69
2.1%
1.2%
0.5%
16.7%
4.8%
1.1%
0.4%
15.5%
4.9%
1.1%
0.4%
15.5%
6.2%
2.4%
0.9%
33.2%

SI Figure 4ab




SI Figure 5a
Saline
GEM
LM-TT
LM-TT + GEM
Gr1
CD11b
IL-10
IL-6
TNFa
MARCO
CD80
3.46%
4.25%
3.64%
3.38%
1.24%
2.76%
3.59%
8.49%
3.84%
1.78%
2.44%
4.05%
8.42%
3.70%
1.08%
1.45%
1.97%
11.6%
1.79%
1.17%


Saline
GEM
SI Figure 5b
LM-TT
LM-TT + GEM
F4/80
CD11b
IL-10
IL-6
TNFa
MARCO
CD80
1.45%
1.09%
4.78%
1.96%
1.93%
1.47%
1.25%
8.85%
1.94%
4.28%
0.65%
0.76%
8.73%
2.16%
3.07%
0.72%
0.42%
8.04%
1.33%
5.26%

SI Table 1a: Pathological examination of tissues in C57Bl/6 mice 2 days after the last treatment with Listeria-TT and Gemcitabine

Treatment	Saline			Listeria-TT+Gem		
Mouse	**1**	**2**	**3**	**1**	**2**	**3**
Liver						
Random foci of mixed leukocytes	1	1	1	0	1	1
Individual hepatocyte cell death	1	0	0	0	1	1
Extramedullary hematopoiesis	0	0	0	0	0	1
Hepatocellular cytoplasmic vacuolation	0	0	0	0	0	0
Foci of necrosis	0	0	0	0	0	0
Kidney						
Tubular ectasia, cortex	0	0	0	0	0	0
Tubular ectasia, medulla	0	0	0	0	0	1
Tubular epithelial cell cytoplasmic vacuolation, cortex	0	0	0	0	1	0
Tubular epithelial cell cytoplasmic vacuolation, medulla	0	0	0	0	0	0
Regenerative tubules, cortex	0	0	0	1-2	1-2	0
Regenerative tubules, medulla	0	0	0	0	0	0
Tubular proteinosis, cortex	0	0	0	0	0	0
Tubular proteinosis, medulla	0	0	0	0	0	1
Spleen						
Lymphoid hyperplasia, white pulp	2	2	2	0	0	0
Extramedullary hematopoiesis, red pulp	0	2	2	3	3	3
Lung						
Increased mixed leukocytes, alveolar septae	2-3	2-3	2-3	2-3	2-3	2-3
Perivascular/peribronchiolar lymphocytic to lymphohistiocytic infiltrates	1	0	0	1-2	0	0

The samples were graded on a scale of 0-5; 0=no significant findings, 1=minimal finding, 2=mild finding, 3=moderate finding, 4=marked finding, 5=severe finding. C57Bl/6 mice were treated with Listeria-TT and GEM or Saline as described in **Fig 1** of the manuscript. Two days after the last treatment mice were euthanized and analyzed for potential side effects by a Histopathological board-certified veterinary pathologist. In each group 3 mice were analyzed.

SI Table 1b: The effect of Listeria-TT and Gemcitabine on liver functions in C57Bl/6 mice

Treatment	AST (U/L)	ALT (U/L)
Saline	107	16
Listeria-TT and GEM	137	15
Normal values	50-100	25-60

C57Bl/6 mice were treated with Listeria-TT and GEM or Saline as described in Fig 1 of the manuscript. Two days after the last treatment mice were euthanized and analyzed for liver functions such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In each group 3 mice were analyzed. The plasma samples were pooled in each group.