Tumor-targeted delivery of Tetanus toxoid by Listeria improves immunotherapy against ovarian cancer in mice

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Running title: Listeria-Tetanus and gemcitabine reduces ovarian cancer

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ABSTRACT

Ovarian cancer is known for its poor neoantigen expression and strong immunosuppression. Here, we utilized an attenuated non-pathogenic bacterium *Listeria monocytogenes* delivering a highly immunogenic Tetanus Toxoid protein (Listeria-TT), as a neoantigen surrogate, inside tumors through infection in a metastatic mouse ovarian cancer model (Id8p53-/-Luc). Gemcitabine (GEM) was added to reduce immune suppression. Listeria-TT+GEM treatments resulted in tumors expressing TT and reactivation of pre-existing CD4 and CD8 memory T cells to TT (generated early in life), which were then attracted to the TT-expressing tumors and producing granzyme B and perforin. This correlated with a strong reduction in the ovary tumors and metastases, and a significant improvement of the survival time compared to all control groups. Moreover, two treatment cycles with Listeria-TT+GEM doubled the survival time compared to untreated mice. Checkpoint inhibitors have little effect on ovarian cancer partly because of low neoantigen expression. We evaluated if delivery of TT by Listeria to the TME improved the effect of anti-PD1 antibodies. We found that Listeria-TT+GEM+PD1 was significantly more effective (efficacy and survival) than PD1 or Listeria-TT+GEM alone, and that more treatment cycles with Listeria-TT+GEM+PD1 significantly increased the survival time compared to Listeria-TT+GEM alone. In summary, the results of this study suggest that our approach may benefit ovarian cancer patients.

INTRODUCTION

Globally, 225,500 new cases of ovarian cancer are diagnosed each year, with 140,200 cancer-specific deaths (1-3). Ovarian cancer is a disease subdivided in different histological subtypes that have different risk factors which require different treatments (4). Newly diagnosed cancer is treated with cytoreductive surgery and platinum-based chemotherapy. In recurrent cancer, chemotherapy, anti-angiogenic agents and poly ADP-ribose polymerase inhibitors are used. High

grade-serous carcinoma (HGSC) is the most common histological subtype of ovarian cancer, that initially is highly responsive to platinum-based chemotherapy, but frequently relapses and become increasingly chemoresistant (5). It has been reported that these relapses are caused through the development of cancer stem cells (CSC) with a capacity of unlimited self-renewal and proliferative capacity (6). This underlines the desperate need for new alternative approaches.

While checkpoint inhibitors have shown impressive results for melanoma and lung cancer, this is less so for ovarian cancer (7). This is partly because ovarian cancer has low levels of neoantigen expression (8). We hypothesized that ovarian cancer patients could benefit from Listeria-TT. In a previous study, we have shown that Listeria delivers highly immunogenic recall antigens, like tetanus toxoid (TT), to the tumor microenvironment (TME) and inside tumor cells of the poorly immunogenic pancreatic cancer, as a neoantigen surrogate. Low doses of Gemcitabine (GEM) were added to reduce immune suppression in the TME. We found that high levels of TT in the TME attracted TT-specific memory CD4 T cells (generated earlier in life) to the TME, producing perforin and granzyme B, resulting in a strong reduction in tumors and metastases and significant longer survival. Based on these results we tested in the current study whether Listeria-TT+GEM was effective against metastatic ovarian cancer. We also hypothesized that delivery of neoantigen surrogate TT to the TME, may improve the efficacy of checkpoint inhibitors in mouse models of ovarian cancer.

Here, we show that Listeria-TT+GEM is highly effective against ovarian cancer metastases and tumors in the Id8p53-/-Luc model (efficacy and survival), and that two treatment cycles doubled the survival time compared to untreated mice. This correlated with a significant increase in both CD4 and CD8 T cells producing perforin and granzyme B. Most interestingly, Listeria-TT+GEM+anti-PD1 antibodies was significantly more effective than anti-PD1 or Listeria-TT+GEM alone (efficacy and survival), and more treatment cycles with LM-TT+GEM+PD1 significantly further improved the survival of mice compared to those that received Listeria-TT+GEM alone. Low dose anti-PD1 was more effective than high dose anti-PD1. Potential

mechanism(s) of improvement will be discussed. In conclusion, these results are promising for those ovarian cancer patients with low neoantigen expression.

Results

Listeria-TT infection of tumor cells *in vitro* and in vivo

In previous studies we have shown that Listeria infects human and mouse pancreatic and breast tumor cells (9-12). Here, we show that Listeria also infects mouse and chemoresistant human ovarian cancer cells *in vitro*. The infection rate of Id8p53-/- (mouse) or Hey (human) tumor cells was determined by culturing the tumor cells with *Listeria*-TT for 1hr, followed by lysis in water, and plating the lysate on agar. Next day, the number of CFU of Listeria-TT was counted **(Supplemental Figure 1A).**

Previously, we showed that Listeria destroys human (MCF7) and mouse (4T1) breast tumor cells through the activation of the NADPH-oxidase pathway, resulting in mitochondrial reactive oxygen species and subsequent tumor cell death (9). Here, we demonstrated that *Listeria*-TT also efficiently kills Id8 and Hey tumor cells *in vitro* **(Supplemental Figure 1BC)**. For this purpose, tumor cells were incubated for 2 hrs with Listeria-TT, followed by gentamicin and cultured overnight. Dead and alive tumor cells were counted based on trypan blue staining.

The Id8p53-/- Luc model

p53 is mutated in 80% of high grade serous ovarian cancers. Therefore, we used an ovarian cancer model with deletions in p53 and expressing Luciferase (Id8p53-/- Luc model)(13, 14). Expression of Luciferase allowed us to analyze real-time effect(s) of therapy in live mice on the ovarian cancer. We generated two models, i.e. the peritoneal model and the ovary model. Using the peritoneal model, tumor cells were directly injected into the peritoneal cavity resulting metastatic tumors all over the peritoneal cavity (on the surface of liver, diaphragm, pancreas, and GI) like high serous ovarian cancer **(Supplemental Figure 2A).** However, no tumor cells were

found inside the ovaries **(Supplemental Figure 2B).** Therefore, we generated an ovary model in which we injected tumor cells directly into the bursa of the ovaries, resulting in big ovarian tumors (and later metastasized to the pc as well) **(Supplemental Figure 2CD).**

Accumulation of Listeria-TT in the TME

Before testing Listeria-TT as immunotherapy against ovarian cancer we first determined whether Listeria-TT accumulated in the TME. Briefly, $2x10^6$ Id8p53-/-tumor cells were injected ip, and four weeks later, ascites was drained, followed by the ip injection of one high dose of Listeria-TT. At different time points (day 1 and 3 after injection of Listeria-TT), bacteria were isolated from all metastases and normal tissues, and the number colony forming units (CFU) per gram tissue was calculated. We found that Listeria-TT predominantly accumulated in the metastases, with the highest number in the metastases of the diaphragm, GI and liver **(Figure 1A).**

***Listeria*-TT+GEM attracts CD4 and CD8 T cells to the TME producing perforin and Granzyme B**

Here, we confirmed that Listeria delivered TT to the ovary tumors. For this purpose, we treated the mice with a complete cycle of Listeria-TT+GEM **(Supplemental Figure 3A)** and analyzed the tumors by immunohistochemistry (IHC) for the expression of TT. As shown in **Figure 1BC,** TT was expressed in 50% and 80% of the tumor area of mice that received Listeria-TT or Listeria-TT+GEM, respectively. Ovarian tumors are immunologically cold with hardly T cells present in the TME of mice and human (15). After a complete treatment cycle of the Id8p53-/-Luc (ovary model) with Listeria-TT+GEM we found that the number of both CD4 and CD8 T cells was significantly increased compared to the saline group **(Figure 2AB and Supplemental Figure 4AB).** This is in contrast compared to the pancreatic tumors of KPC mice, in which we observed CD4 T cells only (12). Also, in the GEM-treated mice the number of CD4 and CD8 T cells was significantly increased compared to the saline group. This is not surprisingly since GEM eliminates the immune

suppressive myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages in the TME and in blood (12), which affect T cell trafficking (16). To show that these T cells were functional, we also analyzed the production of perforin and granzyme B. We found that the production of perforin and granzyme B was significantly increased in mice treated with LM-TT+GEM, compared to all control groups **(Figure 2CD and Supplemental Figure 4CD).** Of note, MDSC and TAM strongly inhibits T cell function through different pathways (12, 17). We have shown earlier that GEM eliminate these MDSC and TAM (12).

Listeria-TT+GEM reduces ovarian cancer in Id8p53-/-Luc mice (peritoneal model) and improves survival

After evaluating the effect of *Listeria*-TT+GEM on immune responses in the TME, we also analyzed the therapeutic effect on ovarian cancer in the Id8p53-/- Luc mice. For this purpose, C57BL/6 mice were pre-vaccinated twice at young age (6-8 weeks of age) with the TTvacc (the same vaccine as humans get) one week apart prior tumor development to generate the TT-specific memory T cells. One week after the last vaccination, Id8p53-/-Luc tumor cells were injected intraperitoneally (peritoneal model). After the presence of metastases were confirmed by H&E and IVIS (**Figure 3AB),** the mice were treated with *Listeria*-TT+GEM as outlined in **Supplemental Figure 3A,** and the region of interest (ROI) of the luciferase signal (measurement for tumor growth) was determined by IVIS before (day 0 of treatment), during (day 10) and after treatment (day 20). On day 20 (2 days after the last treatment), the IVIS signal was significantly lower in the Listeria-TT+GEM compared to all other groups **(Figure 3BC).** However, GEM or Listeria-TT alone did have a significant effect on the metastases as well when compared to the saline group. After the last treatment, the mice were monitored without any further treatment in a survival study. Confirming the IVIS data, mice treated with Listeria-TT+GEM lived significantly longer than the mice in all other treatment groups **(Figure 3D)**.

In a previous study we showed that TT was expressed in about 80% of the tumor after a complete treatment cycle (12). A similar result we observed in the ovary tumors **(Figure 1BC).** This indicates that 20% of the tumor do not express Tetanus, which will grow back because only TT-positive tumor cells will be destroyed by the TT-specific memory T cells. To address this problem, we followed up with a second treatment cycle aiming to deliver TT to the TT-negative tumor cells. As shown in **Figure 3E,** we increased the survival time by 50% after two treatment cycles compared to untreated mice, and by 23% compared to one treatment cycle.

Listeria-TT+GEM reduces ovarian cancer in Id8p53-/-Luc mice (ovary model) and improves survival

A similar treatment protocol of Listeria-TT+GEM has been evaluated in the ovary model **(Supplemental Figure 3A).** Tumors developed 11 days after injection of tumor cells into the ovaries. Tumor growth was determined by tumor weight (at the end of treatment) **(Figure 4A)** or by IVIS (days 27, 37, and 47) **(Figure 4B).** The IVIS signal was significantly reduced in the Listeria-TT+GEM group compared to the saline group, and reduced 2-fold over time (before vs after treatment) within the group that received Listeria-TT+GEM **(Figure 4C).** However, little effect of GEM or Listeria-TT was observed on the primary tumors. Survival data confirmed the results by tumor weight and IVIS signal, i.e. mice treated with Listeria-TT+GEM lived significantly longer than all other groups **(Figure 4D).**

Listeria-TT+GEM+anti-PD1 is significantly more effective than anti-PD1 or Listeria-TT+GEM alone (peritoneal model)

In mice (7, 8) and patients (NCT02580058, NCT02718417, NCT02674061) anti-PD1 has no effect on HGSC. This is partly because HGSC has poor neoantigen expression (18). One of our hypotheses is that delivery of TT as neoantigen surrogate to the TME may improve the effect of checkpoint inhibitors. To prove our hypothesis, we delivered neoantigen surrogate TT by Listeria

to the TME. More specifically, we combined Listeria-TT+GEM with anti-PD1 antibodies and compared their effect with Listeria-TT+GEM or anti-PD1 alone treatments. Prior to these experiments, we first tested two different doses of anti-PD1. A high dose of 130 μg/dose and a low dose 50 μg/dose was tested, because in the literature higher doses (130 μg/dose) were mostly used (19, 20). However, one report described Listeria-E7 combined with a low dose of anti-PD1 (21). Therefore, we tested both doses. For a schematic view of the treatment protocol see **Supplemental Figure 3B.** It appeared that the low dose anti-PD1 (50 μg/dose), was more effective than the high dose **(Figure 5AB),** which was confirmed by a survival study **(Figure 5C).** Subsequently, we tested whether GEM was required for optimal treatment. As shown in **Figure 5DE,** Listeria-TT+GEM+anti-PD1 was significantly more effective than Listeria-TT+anti-PD1. We then tested Listeria-TT+GEM+anti-PD1 in comparison to Listeria-TT+GEM or anti-PD1 alone or the isotype control. Anti-PD1 alone showed little effect on the metastases in the Id8p53-/- model **(Figure 5FG)** compared to the saline group. However, Listeria-TT+GEM+anti-PD1 was significantly more effective than anti-PD1 or Listeria-TT-GEM alone **(Figure 5FG).** As expected, the isotype control for PD1 + Listeria-TT+GEM showed similar results as Listeria-TT+GEM alone **(Figure 5FG).** Also, in a survival study we showed that Listeria-TT+GEM+anti-PD1 was significantly more effective than all control groups **(Figure 5H).**

To determine if we were able to further increase the survival time we administered three treatment cycles with Listeria-TT+GEM+anti-PD1. As shown in **Figure 6AB,** Listeria-TT+GEM+anti-PD1 is significantly more effective than anti-PD1 or Listeria-TT+GEM alone or the saline group. This was confirmed by survival data **(Figure 6C).** Also, the survival time of mice that received three treatment cycles with LM-TT+GEM+anti-PD1 was significantly longer than those who received one treatment cycle or saline **(Figure 6D).**

Mechanistic mode of action of LM-TT+GEM plus anti-PD1

Our results raised the question whether TT improved the effect of anti-PD1 or whether anti-PD1 improved the effect of Listeria-TT+GEM? To answer this question, we repeated the experiment but now we compared the efficacy of Listeria-TT+GEM+anti-PD1 (TT present in Listeria construct) vs LM+GEM+anti-PD1 (TT absent in Listeria construct). We found that the IVIS signal was 6-fold lower in the Listeria-TT+GEM+anti-PD1 compared to Listeria+GEM+anti-PD1 on day 37 of treatment, while this difference became smaller after the last treatment (day 47) **(Figure 5l**).

It has been reported that Listeria upregulates PD-L1 on the surface of mouse DC in the spleen of the TC-1 model (21) which may improve the effect of anti-PD1. We determined by IHC if Listeria also upregulated PD-L1 in the spleen of the Id8p53-/-Luc model after a full treatment cycle. While the number of PD-L1-positive cells in spleens of mice treated with GEM or LM-TT+GEM, was significantly higher (the number of PD-L1-positive cells were counted per square millimeter of spleen area) than the saline group ($p<0.0001$), this was less robust (and not significant) although increased for Listeria-TT **(Figure 7AB).** Moreover, the saline group was almost completely negative for PD-L1 expression. In the same mice we also determined the effect of Listeria-TT and/or GEM on PD-L1 expression in the ovary tumors by IHC. Also, here the number of PD-L1-positive cells per square millimeter of tumor area was counted in all four treatment groups. Only GEM significantly upregulated PD-L1 in the primary tumors ($p=0.0002$) **(Figure 7CD).**

Why are low doses more effective than high doses of anti-PD1 antibodies *in vivo*

As shown in **Figure 5,** low doses of anti-PD1 antibodies were more effective (efficacy (ROI) and survival) than high doses, when combined with Listeria-TT+GEM. To answer this question, we performed pathological analysis on normal tissues (liver, lungs, kidneys and gastrointestinus (GI)) of H&E-stained tissue sections of mice that received high (130 ug/dose) or low doses (50 ug/dose) of anti-PD1. Minimal effects of the high or low doses anti-PD1 antibodies was observed on spleen,

liver, kidneys, lungs and intestines **(Supplemental Table 1).** In more detail, no pathological differences were observed between the high and low doses of anti-PD1.

Another possibility is that high doses of anti-PD1 reduce the viability of Listeria-TT. We therefore incubated Listeria-TT overnight with serial dilutions of high and low doses of anti-PD1 and plated them on LB agar. No significant difference in the number of CFU of Listeria-TT was observed between of high (130, 13, 1.3 and 0 μg/200 μL) or low (50, 5, 0.5 and 0 μg/200μL) doses of anti-PD1 antibodies **(Figure 7E),** indicating that anti-PD1 had no effect on the viability of Listeria-TT *in vitro.*

We also tested whether high doses of anti-PD1 affected the T cell function of TT-specific T cells *in vitro.* For this purpose, mice were immunized twice with TTvacc one week apart. One week after the last immunization, 200,000 spleen cells were restimulated with Listeria-TT in an ELISPOT for 2 hrs, followed by gentamycin treatment during the restimulation period (to kill all extracellular Listeria), in the presence of serial dilutions of high and low doses of anti-PD1 antibodies as described above. We found a dose-response effect of anti-PD. Higher doses of anti-PD1 correlated with less production of IFNγ by spleen cells **(Figure 7FG).**

Discussion

Previous studies from our lab showed that immunotherapy with Listeria-TT and GEM was highly effective against the poorly immunogenic pancreatic ductal adenocarcinoma (PDAC)(12). Delivery of TT to the TME using an attenuated Listeria resulted in the attraction of CD4 T cells to the tumors, the production of perforin and Granzyme B, both involved in tumor cell destruction, and a strong and significant effect on advanced pancreatic cancer (efficacy and survival) in various mouse tumor models. In the study presented here we tested whether Listeria-TT+GEM was also effective against ovarian cancer. We generated two Id8p53-/-Luc models. One model developing predominantly metastases in the peritoneal cavity (peritoneal model), and the other

model developing predominantly a tumor in the ovaries (ovary model). The effect of the immunotherapy on the cancer was analyzed by IVIS since the tumor cells expressed Luciferase, and the survival by a Kaplan-Meier curve. We found that 80-90% of the ovarian tumors expressed TT, which attracted both CD4 and CD8 T cells to the TME, producing perforin and granzyme B. This is in contrast to the pancreatic tumors, which attracted CD4 T cells only (12). A strong reduction was observed in the primary ovary tumors, and inhibition of the growth of metastases, resulting in great improvement in survival time compared to all control groups. However, 10-20% of the tumor and metastases was left after the therapy. These tumor cells don't express TT and will thus not be eliminated by the TT-specific T cells. We hypothesized that delivering more TT to these remaining TT-negative tumor cells by a second treatment cycle of Listeria-TT+GEM, will lead to longer survival. Two treatment cycles doubled (50%) the survival time compared to untreated mice (saline), and an increase in survival time of 24% when compared to one treatment cycle. In summary, we were able to strongly improve the survival time, but not to completely eliminate the cancer. These results encourage combining our therapy with other therapies in order to further improve the survival time.

One such therapy could be checkpoint inhibitors. Checkpoint inhibitors have been extensively studied in ovarian cancer patients. For instance, PD1 inhibitors (pembrolizumab, dostarlimab) were not effective, partly because ovarian cancer is poorly immunogenic with low neoantigen expression and poor T cell infiltration (22). They found in a phase 3 trial with pembrolizumab(anti-PD1) as single-agent in recurrent ovarian cancer an overall response rate of 8% only. Avelumab (anti-PDL1) plus chemo or avelumab alone significantly improved progression-free survival or overall survival versus chemo alone in the platinum resistant/refractory ovarian cancer in a phase 2 trial (NCT02718417). In another study they examined nivolumab alone (anti-PD1) vs Ipilimumab(anti-CTLA4)+Nivolumab in recurrent ovarian cancer (NCT02498600). Patients treated with Nivolumab alone demonstrated an overall response rate of only 12.2% while the combo showed a response rate of 31.4%. However, the

progression free survival in the nivolumab alone group was 2 months and in the combo group was only 3.9 months which is clinically disappointing as the relatively improved response rate observed in the combination therapy group, must be balanced by the lack of benefit for the majority of patients enrolled as well as limited duration of progression-free survival (PFS) observed in the study. These trials demonstrate that checkpoint inhibitors (PD1 or CTLA4) alone were not effective in patients with ovarian cancer. As mentioned above, such failure is partly because ovarian cancer is poorly immunogenic. Also, in our mouse models we found that anti-PD1 alone is not very effective. We hypothesized that if we deliver our own neoantigen surrogate (TT) to the TME, it may improve the effect of checkpoint inhibitors.

We first tested two different doses of anti-PD1 antibodies. It appeared that the low dose (50 μg/dose) was more effective than the high dose (130 μg/dose) anti-PD1. We then repeated the experiments with low doses of anti-PD1 and found that Listeria-TT+GEM+anti-PD1 was significantly more effective than anti-PD1 or Listeria-TT+GEM alone, as shown by IVIS and ROI counts quantified, and supported by survival data. From a clinical perspective, the high vs low doses of anti-PD1 is an important issue because of the immune related adverse (irAE), often described for PD1 inhibitors. This includes autoimmune-like/inflammatory side effects, causing collateral damage to normal organ systems and tissues such as skin, GI, hepatic, pulmonary, mucocutaneous, and endocrine systems)(23). Therefore, we investigated why the low doses were more effective than the high doses, i.e. we evaluated the effect of high and low doses anti-PD1 on the Listeria viability (growth), immune function, and on potential toxicities on organs (H&E staining of the tumors). Very mild toxicities were observed in the tissues (liver, spleen, lungs, kidneys and GI) with both high and low doses of anti-PD1 antibodies. Also, no effect of the high or low doses of anti-PD1 was observed on the viability of Listeria. However, we found a dose-response effect of anti-PD1 antibodies on spleen cells of TT-vaccinated mice, infected with Listeria-TT in the presence of various concentration of anti-PD1 antibodies. The higher the anti-PD1 dose, the more inhibition of the production of IFNγ in the spleen was observed. This reflected

what we observed *in vivo*, i.e. higher doses were less effective against the tumors and metastases *in vivo*. This may be the result of reduced T cell function, but it is also possible that high doses of anti-PD1 may have inhibited the infection rate of antigen-presenting cells by Listeria in the ELISPOT, resulting in decreased antigen presentation and decreased production of IFNγ. This is currently under investigation.

Without GEM, the Listeria-TT+anti-PD1 therapy was less effective. This might not be surprising since anti-PD1 blocks the interaction between PD1 and PD-L1 unleashing the TCR signaling pathway required for stimulation of the T cells (24), while GEM eliminates the immune suppressive MDSC and TAM, thereby reducing the immune suppressive cytokines like IL-6, IL-10, and TGFβ in the TME (12). Thus, both treatments (anti-PD1 and GEM) activate the T cells at different levels.

We also compared one vs more treatment cycles with Listeria-TT+GEM+anti-PD1. We found that three treatment cycles improved the survival time by 48% compared to untreated mice, and by 26% compared to one treatment cycle. These results are clinically relevant and warrant further evaluation in patients with ovarian cancer.

Others have studied checkpoint inhibitors in combination with CAR T cells in mice. In one study, human Muc16-specific CAR T cells combined with anti-PD-L1 in NSG mice bearing OVCAR3 tumors expressing Muc16, PD-1 and Luc (25). The tumor cells (OVCAR3) were transduced with muc16 tumor antigens and PD1 and Luc *in vitro*. Results were highly promising, i.e. the survival time was significantly improved compared to the control mice (25). In another study they combined CAR T cells against mesothelin with anti-PD1 (26). In this study, SKOV3 and OVCAR4 human tumor cells were transduced *in vitro* with mesothelin antigen-GFP-Luc. Their CAR T cells, when combined with anti-PD-L1, were able to control the growth of SKOV3 and OVCAR4 tumors and significantly improved the survival time (26). These results support our data because overexpression of antigens by the tumor made that anti-PD1 or anti-PD-L1 became

effective. However, while these studies are highly promising, it needs to be noted that the antigens were delivered into the tumor cells *in vitro* through transduction, which is impossible to do with patients. This is completely different from our study, where we deliver the strong antigens (TT) *in vivo* in the tumors through Listeria infection.

Mechanistically, we were wondering whether delivery of TT improved the effect of anti-PD1 or whether anti-PD1 improved the effect of Listeria-TT+GEM? To answer this question, we compared the effect of LM-TT+GEM+anti-PD1 (with TT in the Listeria construct) vs LM+GEM+anti-PD1 (no TT in the Listeria construct) by IVIS and found that the tumors of LM-TT+GEM+anti-PD1 on day 10 of treatment exhibited 6x less ROI signal than LM+GEM+anti-PD1, suggesting that TT improved the effect of anti-PD1. However, on day 20 this difference was 3x less in the LM+GEM+anti-PD1 group compared to the LM-TT+GEM+anti-PD1 group. A potential explanation for the smaller difference in ROI signal on day 20 between the two groups could be that the number of TT-positive tumor cells in the group of LM-TT+GEM+anti-PD1 at that time point was decreased because they were eliminated by the TT-specific T cells, and thus were less TT-positive tumor cells present to destroy.

It has been reported that Listeria upregulates PD-L1 on the surface of mouse DC of the TC-1 model (21) which may also improve the anti-PD1 effect. We found that PD-L1 was significantly upregulated in the spleen of GEM-, and LM-TT+GEM-treated mice, while Listeria-TT upregulated PD-L1 in the spleen but not significantly. In the ovary tumors, PD-L1 expression was significantly increased of GEM-treated mice, but not of Listeria-TT- or Listeria-TT+GEM-treated mice. Also, others reported that GEM or Listeria upregulates PD-L1 in tumors or spleen (21, 27). This may explain why Listeria-TT+GEM+anti-PD1 was more effective (efficacy and survival) than Listeria-TT+anti-PD1 alone. GEM has been linked to chemoresistance, inability of tumor penetration and activation. We have shown that low doses of GEM stimulate immune responses resulting in higher T cell infiltration in tumors, producing more perforin and granzyme B (12).

Upregulation of PD-L1 leads to inhibition of T cell responses if not blocked by PD1, but if blocked by anti-PD1, apparently it leads to a stronger effect on the ovarian cancer.

In summary, the results of this study suggest that patients with HGSC may benefit from our approach. Currently, we are preparing for a Phase 1 clinical trial of Listeria-TT+GEM in patients with advanced PDAC. If successful, we will follow up with HGSC patients.

Methods

Animals

C57BL/6 mice (females only) of 6-8 weeks were obtained from Charles River. These mice were used to generate mice with orthotopic Id8p53-/- Luc tumors in the ovaries and mice with metastases in the peritoneal cavity.

Cell lines

The Id8p53-/- Luc cell line is a high grade serous ovarian carcinoma (HGSC) model when injected into the peritoneal cavity. This cell line was developed (from the original Id8 model) and kindly provided by Dr. Mc Neish (13), Centre for Molecular Oncology, Barts Cancer Institute, Queen Mary University of London, and Dr. Janet Sawicki, Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania (14). The HEY tumor cell line was derived from human HGSC(28) and was kindly provided by Dr. Susan Horwitz, Albert Einstein College of Medicine, Bronx. This cell line was used to determine infection and destruction by Listeria-TT *in vitro.*

Mouse models

In this study two mouse models were used, i.e. a peritoneal model and an ovary model. One model consisted of injection of the Id8p53-/-Luc cells in the peritoneal cavity, which resulted in

metastases in the peritoneal cavity but no tumors in the ovaries or uterus **(Supplemental Figure 2AB)**, while the other model consisted of injection of Id8p53-/-Luc cells in the ovaries resulting in primary tumor in the ovaries **(Supplemental Figure 2CD)**. In both cases, tumor growth was measured by In Vivo analysis of Small animals (IVIS) as we described earlier (29).

Peritoneal model

Metastases were generated in immune competent C57BL/6 mice by injection of 1-2x10^6 Id8p53-/-Luc cells/200uL DPBS into the peritoneal cavity. 10-14 days after the injection, when metastases were developed, the immunotherapeutic treatments were started. Metastases were visible in the portal vein area of the liver, the diaphragm, along the GI and in the pancreas (no tumors were developed in the ovaries).

Ovary model

Orthotopic tumors were generated in the ovaries by injection of 2x10^6 Id8p53-/- tumor cells in the ovaries of immune competent C57BL/6 mice. Briefly, mice were anesthetized with ketamine (Mylan Institutional LLC/xylazine (Akorn Animal Health)(respectively, 100 mg and 10mg/kg, ip)(12), the hair was removed at the location above the hindleg, and the skin was sterilized with betadine, followed by 70% alcohol. The animal was covered with gauze sponge surrounding the incision site. A 1 cm incision was made in the abdominal skin above the right hindleg to allow visualization of the ovaries and uterus. Carefully, the uterus was uplifted by forceps and 2x10^6 Id8p53-/-Luc tumor cells mixed 1:1 with Matrigel (VitroGel Hydrogel Matrix, MidSci, Scranton, PA; cat#VHM01S) in 50 ul volume were injected into the bursa of the ovary. The ovary was then replaced within the abdominal cavity, and both skin and muscle layers were closed with sutures. Following recovery of the surgery, mice were monitored and weighed daily. The ovary tumor was visible by IVIS within 10-14 days. The tumor growth was measured by IVIS before, during and after treatment.

Listeria and *Listeria*-TT

In this study, an attenuated *Listeria monocytogenes* (*Listeria*) was used as the vehicle for delivery of $TT_{856-1313}$ (30) to the TME and inside tumor cells. The *Listeria* plasmid pGG-34, expressing the non-cytolytic truncated form of Listeriolysin O (LLO) under control of the hemolysin promoter *(Phly)* and the positive regulatory factor A (*prfA*), with mutations to further reduce pathogenicity, have been described elsewhere (31). *Listeria*-$TT_{856-1313}$ was developed in our laboratory as described previously (12). The $TT_{856-1313}$ fragment contains both mouse and human immunodominant T cell epitopes (32, 33).

Biodistribution of *Listeria*-TT

C57BL/6 mice were injected intraperitoneally with $2x10^6$ Id8p53-/- mouse ovarian tumor cells as described above. To visualize big metastases in the peritoneal cavity we injected with a single large dose 10^7 CFU of *Listeria*-TT ip, 4 weeks after tumor cell injection. At this time point the mice developed visible metastases by eye and ascites. The ascites was drained from live mice before injection of the Listeria-TT. Mice were euthanized at various time points after the injection (as indicated in the figure), and metastases and normal tissues were dissected, weighed, and analyzed for CFU of *Listeria*-TT, as described previously (10).

Infection of tumor cells *in vitro*

The *in vitro* infectivity of Id8p53-/- (mouse) or Hey tumor (human) ovarian cancer cells was assessed as described previously (9). Briefly, 10^6 cells/ml were infected with 10^7 CFU of *Listeria* or *Listeria* -TT for 1hr at 37°C in culture medium, then incubated with gentamicin (50 μg/ml) for 1 hr to kill extracellular *Listeria*. Finally, cells were washed with phosphate-buffered saline (PBS), lysed in water, and serial dilutions were plated onto LB agar to quantify the infection rate the next day.

Infection of tumor cells *in vitro* in the presence of various doses of anti-PD1 antibodies

We also tested the effect of high and low doses of anti-PD1 antibodies on the infection rate of Id8p53-/- cells by Listeria-TT. Briefly, the infection was performed as described above but now in microtiter wells (200 μL) in the presence of serial dilutions of high or low doses of anti-PD1 (low dose 50, 5, and 0.5 μg/well and high dose 130, 13, and 1.3 μg/well). After 1hr culture, Gentamicin was added (50 μg/mL) for 1 hr, and subsequently the bacteria were isolated as described above.

Evaluation of cell death

As described previously (9), tumor cell killing by *Listeria* or *Listeria*-TT was determined *in vitro* as follows. Id8p53-/- or Hey tumor cells ($3x10^3$) plated in 96 well plates were infected with 10^7 CFU/well of *Listeria* or *Listeria*-TT for 2hrs at 37°C, then gentamicin (50 μg/ml) was added. Live and dead cells were counted the next day using Trypan blue staining.

Protocol for Listeria-TT+GEM treatment of Id8p53-/-Luc mice (efficacy and survival studies)

A detailed rationale for this treatment protocol and schematic view of all treatments are shown in **Supplemental Figure 3A.** To generate memory T cells to TT, C57BL/6 mice were immunized twice intramuscularly with the TTvacc on days 0 and 7 (the same used for childhood vaccinations, 0.3 μg/50 μL). Subsequently, Id8p53-/-Luc tumor cells were injected intraperitoneally (1-$2x10^6$/200 μL, as indicated in the legends) or orthotopically ($2x10^6$/50 uL) in the bursa of the ovaries on day 10-14 (as indicated in the legends), and after we confirmed the presence of metastases or tumor by IVIS, we started the treatments. High doses of *Listeria*-TT (10^7 CFU, 3 doses total) were injected ip on days 28, 34 and 43. Low doses of GEM (Fresenius kabi oncology Ltd) were injected on days 29, 32, 35, 38, 41, and 44 (1.2 mg/mouse, every 3 days) (six doses in

total). Concomitantly, low doses of *Listeria*-TT were administered on days 30, 31, 33, 36, 37, 39, 40, and 42 (10^4 CFU, 8 doses in total). Growth of tumors and metastases were analyzed by IVIS before (Day 27=day 0 of treatment), during (Day 37=day 10 of treatment) and after treatment (Day 43=day 20 of treatment). Subsequently, all mice were monitored in a survival study (Kaplan-Meier curve). At the end of treatments mice were monitored without any further treatment until they succumbed spontaneously or were terminated upon appearance of severe pre-morbid symptoms requiring euthanasia as specified by our approved animal use protocol. Results were analyzed by a Kaplan-Meier Plot.

Protocol for Listeria-TT+GEM+anti-PD1 treatment of Id8p53-/-Luc mice (efficacy and survival studies)

In the Id8p53-/-Luc model, mice were immunized twice with Tetanus vaccine (TTvac) to generate memory T cells (days 0 and 7). Subsequently, Id8p53-/-Luc tumor cells were injected intraperitoneally or orthotopically in the ovaries (day 14) and metastases and tumors allowed to develop to an advanced stage detectable by IVIS and H&E staining. Three high doses of Listeria-TT were administered on days 28, 34, an 41, combined with four low doses of Gemcitabine on days 29, 32, 37, and 41, and four low dose of Listeria-TT on days 30, 33, 38, and 42, followed by anti-PD1 antibodies on days 31, 35, 39 and 43. Tumors and metastases were monitored by intravital imaging of small animals in vivo (IVIS) before (day 27), during (day 37) and after the therapy (Day 47). At the end of treatments, we continued monitoring until death. LM-TT=Listeria-TT. A schematic view of the treatment protocol has been shown in **Supplemental Figure 3B.**

In Vivo Imaging System (IVIS)

C57BL/6 mice were injected with $2x10^6$ Id8p53-/-Luc cells (in 50 µl DMEM) into the peritoneal cavity or ovaries as described above. Ip injection results in metastases in the peritoneal cavity

(Supplemental Figure 2AB), while intra-ovary injections result in a primary tumor in the ovaries **(Supplemental Figure 2CD).** Metastases and primary tumors were visible by IVIS by 10-14 days after injection. The luciferase activity was measured using an intensified charged-coupled device video camera of the In Vivo Imaging System (IVIS 100; Xenogen). The Id8p53-/-Luc tumor-bearing mice were injected ip with 200 µl of D-luciferin sodium salt (Synchem OHG; catalogue number BC218) dissolved in phosphate-buffered saline (100 mg/kg of body weight). Luciferine distributed systemically for 3 min while the animals were anesthetized (mixture of isoflurane and oxygen). Anesthetized animals were placed in the camera chamber, and a bioluminescent signal was acquired for 5 min. Bioluminescence measurements produced by the IVIS 100 system are expressed as a pseudocolor on a gray background, with red denoting the highest intensity and blue the lowest. To quantify the luminescence, we outlined a region of interest (ROI) and analyzed it by use of the Living Image (version 2.11;Xenogen) and Igor Pro (version 4.02A; WaveMetrics) software.

Immunohistochemistry (IHC) and H&E staining

Tumors were dissected from pancreas and immediately fixed with buffered formalin, and the tissue was embedded in paraffin. Sections (5 µm) were sliced and placed on slides, then deparaffinized at 60ºC for 1 hr, followed by xylene, an ethanol gradient (100-70%), water, and PBS. Slides were then incubated for 30 min in 3% hydrogen peroxide followed by boiling in citrate buffer for 20 min. Once the slides were cooled, washed, and blocked with 5% goat serum, the sections were incubated with primary antibodies such as anti-CD4 (1:100 dilution), anti-CD8α (1:400 dilution), anti-Perforin (1:300 dilution), anti-Granzyme (1:200 dilution), anti-PD-L1 (dilution 1:100), anti-TT (1:50 dilution), or anti-MHC class II (Invitrogen cat#14-5321-82)(1:100 dilution), followed by incubation with secondary antibody (mouse anti-goat IgG-HRP), and SignalStain® Boost IHC Detection Reagent (Cell Signaling Technology, cat# 8114S). Subsequently, the slides were incubated with 3,3'-diaminobenzidine (DAB)(Vector Laboratories, cat# SK-4100),

counterstained with hematoxylin, dehydrated through an ethanol gradient (70-100%) and xylene, and mounted with Permount. The slides were scanned with a 3D Histech P250 High Capacity Slide Scanner to acquire images and quantification data. Secondary antibodies without primary antibodies were used as negative control. In addition, sections were stained with H&E as we described previously. Briefly, tumors and metastases were fixed in 10% formaldehyde for 48 h, and then kept in 70% ethanol until use. Sections of 1 mm thick were stained with hematoxylin/eosin and analyzed to confirm the presence of tumors and metastases.

ELISPOT in the presence of various doses of anti-PD1 antibodies

Spleen cells were isolated from C57BL/6 mice that received TT vaccinations twice, one week apart for ELISPOT (BD Biosciences, cat# 551083) analysis, as described previously (34). To immune responses, $2x10^5$ spleen cells were incubated with Listeria-TT (1:5). To measure the effect of anti-PD1 antibodies on immune function of spleen cells and T cells (production of IFNγ), anti-PD1 (Biolegend cat#135247) was added to the spleen cells (low dose 50, 5, and 0.5 μg/well and high dose 130, 13, and 1.3 μg/well). The frequency of IFNγ-producing spleen cells was measured 48 hrs later using an ELISPOT reader (CTL Immunospot S4 analyzer).

Statistics

To statistically compare the effects of *Listeria*-TT+GEM with or without anti-PD1 antibodies on the growth of metastases and tumors or on immune responses in the mouse models, a non-parametric Mann-Whitney was applied using Prism. For the invitro studies (ELISPOT, infection rate and viability of Listeria) the unpaired t test was used. The type of statistical test was determined by prism. *p<0.05, **<0.01, ***<0.001, ****<0.0001. Values of p<0.05 were considered statistically significant. To statistically compare the effects of *Listeria*-TT+GEM on IHC and efficacy Mann-Whitney (two-tailed) test was used. To statistically compare the effects of *Listeria*-

TT+GEM with or without anti-PD1 antibodies in a survival study a Kaplan-Meier curve was generated using Mantel-Cox . ****p<0.0001.

Legends

Figure 1: Listeria-TT accumulates and delivers TT in the TME. (A) Biodistribution of Listeria-TT in the Id8p53-/- model. Listeria-TT accumulated in the metastatic tumors of the Id8p53-/-Luc (peritoneal model). n=3 mice per time point. From each tissue 3 pieces were analyzed in triplicate. The number of CFU of Listeria-TT was determined per gram tissue or per mL blood. **(B)** Listeria delivers TT to the TME (ovary model) as shown by IHC, and quantified **(C).** 10 fields in each group were counted and averaged. n=3 mice per group. Statistical significance was determined by using Mann-Whitney, p<0.05 is significant. Error bars represent standard error of the mean (SEM). LM-TT=Listeria-TT. GEM=Gemcitabine**.**

Figure 2: CD4 and CD8 T cells are attracted to and activated in the ovary tumors by Listeria-TT+GEM treatment in the Id8p53-/- Luc model. CD4 **(A)** and CD8 **(B)** T cells are attracted to the TME by Listeria-TT+GEM treatment in Id8p53-/- ovary model. Listeria-TT+GEM increased the production of Granzyme B **(C)** and Perforin **(D)** in the Id8p53-/- ovary tumors**.** 20 fields per group were counted and the results were averaged. The average number of positive cells were calculated per square millimeter tumor area. n=3 mice per group. Statistical significance was determined by using Mann-Whitney, p<0.05 is significant. All groups in **fig A-D** were compared to the Saline group. We also compared in **fig A:** LM-TT vs GEM p=0.0143, LM-TT+GEM vs GEM p=0.8774, LM-TT+GEM vs LM-TT p=0.0094. **In fig B:** LM-TT vs GEM p=0.3483, LM-TT+GEM vs GEM p=0.3700, LM-TT+GEM vs LM-TT p=0.1046. **In fig C:** LM-TT vs GEM p=0.0281, LM-TT+GEM vs GEM p=0.0304, LM-TT+GEM vs LM-TT p=0.0023. **In fig D:** LM-TT vs GEM p=0.0538, LM-TT+GEM vs GEM p<0.0001, LM-TT+GEM vs LM-TT p<0.0001. Error bars represent SEM**.**

Figure 3: Listeria-TT+GEM significantly inhibits growth of metastatic ovarian cancer and improves survival time compared to all control groups in the Id8p53-/- Luc peritoneal model. At the start of treatment, metastatic tumors are visible in the peritoneal cavity (in diaphragm, liver and along GI) by H&E staining **(A)** and by IVIS **(B).** Listeria-TT+GEM significantly inhibits the growth of metastatic tumors in the peritoneal cavity (pc) as shown by IVIS **(B)** and by ROI counts quantified **(C).** All groups were compared to the Saline group at day 20 of treatment. Listeria-TT+GEM significantly improves the survival of ID8p53-/- Luc mice compared to all control groups **(D).** The data was obtained in two independent experiments with 5 mice per group. Two treatment cycles of Listeria-TT+GEM significantly improved the survival time compared to one treatment cycle or saline **(E).** n=5 mice per group. Statistical significance was determined for efficacy by using Mann-Whitney, $p<0.05$ is significant, and for survival by using Mantel-Cox, $p<0.05$ is significant. Error bars represent SEM.

Figure 4: Listeria-TT+GEM significantly reduces ovary tumors and improves the survival time of the Id8p53-/- Luc ovary model. Listeria-TT+GEM significantly reduces the weight of the ovary tumors **(A),** and the ROI counts by IVIS compared to the saline group **(BC).** All groups were compared to the Saline group at day of treatment. Listeria-TT+GEM significantly improves the survival time of Id8p53-/-Luc mice compared to all control groups **(D).** n=5 mice per group. Statistical significance was determined for efficacy by using Mann-Whitney, $p<0.05$ is significant, and for survival by using Mantel-Cox, $p<0.05$ is significant. Error bars represent SEM.

Figure 5: Synergistic effect of Listeria-TT+GEM +anti-PD1 antibodies on metastatic ovarian cancer. Low dose anti-PD1 is more effective than high dose anti-PD1 when combined with Listeria-TT+GEM as shown by IVIS **(AB)** and in a survival curve **(C).** n=5 mice per group. GEM is required for an optimal effect of Listeria-TT+GEM+anti-PD1 in the ID8p53-/- model **(DE).** n=4

mice per group. Mice treated with Listeria-TT+GEM+anti-PD1 has significantly less metastatic tumors compared to all other control groups, as shown by IVIS **(FG)** and significantly live longer as shown by the survival curve **(H).** In fig G**,** all groups were compared to LM-TT+GEM at day 30 of treatment. n=5 mice per group. Listeria alone and anti-PD1 was less effective than Listeria-TT and anti-PD1 on day 10 of treatment **(I).** n=5 mice per group. Statistical significance was determined for efficacy by using Mann-Whitney, $p<0.05$ is significant, and for survival by using Mantel-Cox, $p<0.05$ is significant. Error bars represent SEM.

Figure 6: Three treatment cycles of Listeria-TT+GEM+anti-PD1 significantly further improves survival compared to one treatment cycle. Metastatic tumors were generated in the Id8p53-/-Luc pc model. Three treatment cycles with Listeria-TT+GEM+anti-PD1 significantly inhibited tumor growth compared to Saline and anti-PD1 control groups, and close to significance compared to LM-TT+GEM group as shown by IVIS **(A),** and ROI counts quantified **(B).** Mice treated with LM-TT+GEM+anti-PD1 significantly lived longer than all control groups **(C).** We also compared three with one treatment cycle, and found that mice receiving three treatment cycles significantly live longer compared to one treatment cycle. In this fig C, mice with one and mice with three treatment cycles were performed as two independent experiments. LM-TT+GEM+anti-PD1 one cycle: 1x10E6 tumor cells injected, start treatment 11 days after tumor cell injection. LM-TT+GEM+anti-PD1 three cycles: 2x10E6 tumor cells injected, start treatment 14 days after tumor cell injection. Statistical significance was determined for efficacy by using Mann-Whitney, $p<0.05$ is significant, and for survival by using Mantel-Cox, $p<0.05$ is significant. Error bars represent SEM.

Figure 7: Mechanisms of anti-PD1 antibodies *in vitro* and *in vivo*. GEM but not LM-TT significantly upregulates PD-L1 *in vivo.* PD-L1 expression measured by IHC in spleens **(A),** and quantified **(B),** as well as in ovary tumors **(C),** and quantified **(D)** of Id8p53-/- mice treated with Listeria-TT+GEM. All groups were compared to the Saline group. In fig A-D, n=3 mice per group,

and 4 sections with 10 fields per section were analyzed. The results were averaged and analyzed by Mann-Whitney, $p<0.05$ is significant. Anti-PD1 does not affect the viability of Listeria-TT *in vitro* **(E).** High doses of anti-PD1 reduces the response to Listeria-TT (production of IFNγ) in the spleen of TT-vaccinated mice *in vitro* in an ELISPOT **(FG).** All groups were compared to tumor cells or immune cells lacking anti-PD1 (with Listeria-TT infection). Fig E-G shows the average of two independent experiments, and the results were analyzed by Mann-Whitney, $p<0.05$ is significant. Error bars represent SEM.

Authors Contribution

LRS, LG, OK, SA, EL performed experiments, analyzed the data and prepared the manuscript. KL, DYSK and NN contributed intellectually to the manuscript, while CG conceptualized and supervised the project, revised the manuscript, and provided the resource for this project.

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Conflict of interest: C.G. is the inventor of the Listeria-Recall Antigen Technology, which was developed in her laboratory, and is described in a patent application 11213577 (granted in the

United States, China and Japan). The patent is licensed to Loki Therapeutics. C.G. is a stockholder of Loki Therapeutics and is an employee of Albert Einstein College of Medicine. All other authors declare that they have no competing interests.

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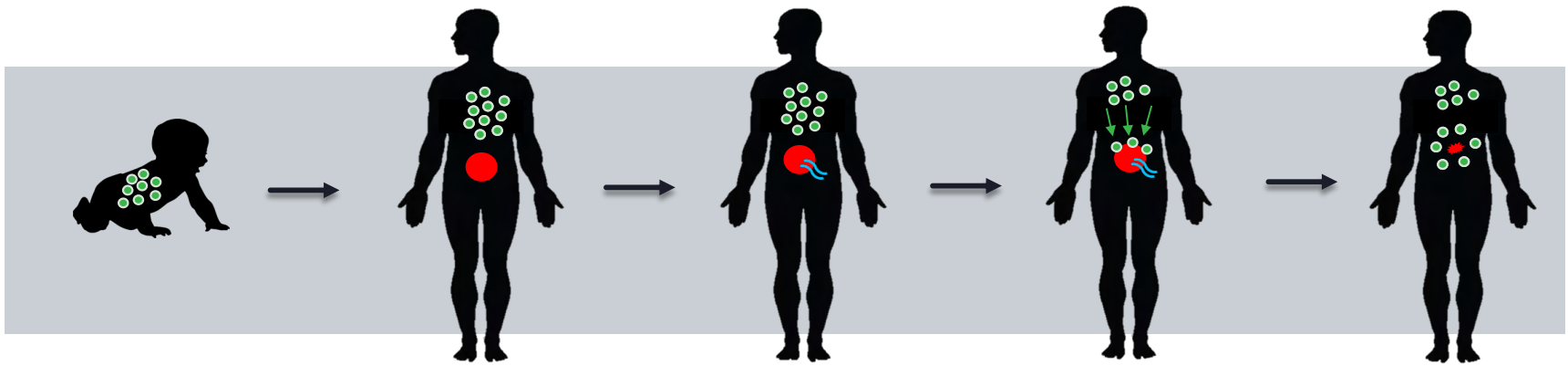
Graphical abstract



CHILDHOOD VACCINATION

Generates memory T cells to Tetanus

CANCER DIAGNOSIS

Patient develops a tumor and metastases later in life

TREAT PATIENT WITH LISTERIA-TETANUS

Listeria-Tetanus infects tumors cells through Myeloid Mediated Delivery(MMD)

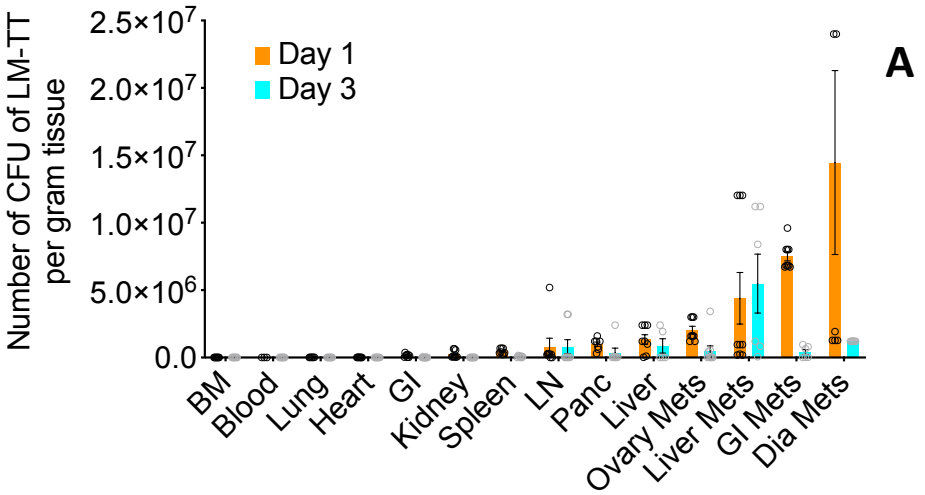
REACTIVATE AND REDIRECT MEMORY T-CELLS

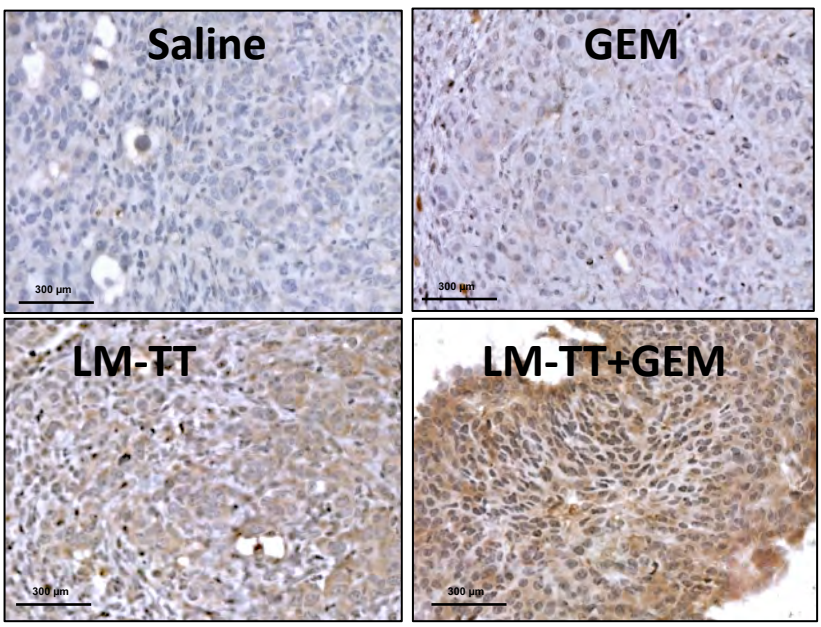
Listeria-Tetanus reactivates pre-existing memory T cells to Tetanus

CANCER DESTRUCTION

Memory T cells to Tetanus kill infected tumor cells

Figure 1


A
Number of CFU of LM-TT per gram tissue
2.5×10^7
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0.0
Day 1
Day 3
BM
Blood
Lung
Heart
GI
Kidney
Spleen
LN
Panc
Liver
Ovary Mets
Liver Mets
GI Mets
Dia Mets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GEM
LM-TT
LM-TT+GEM
300 µm

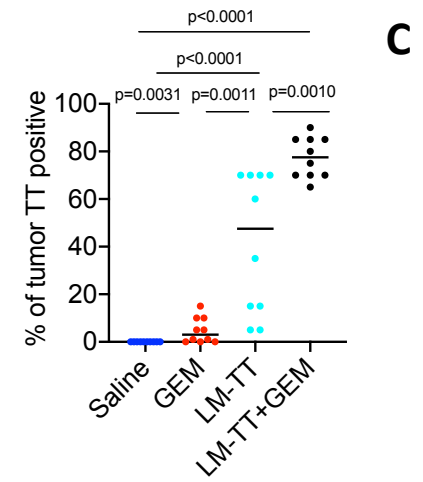
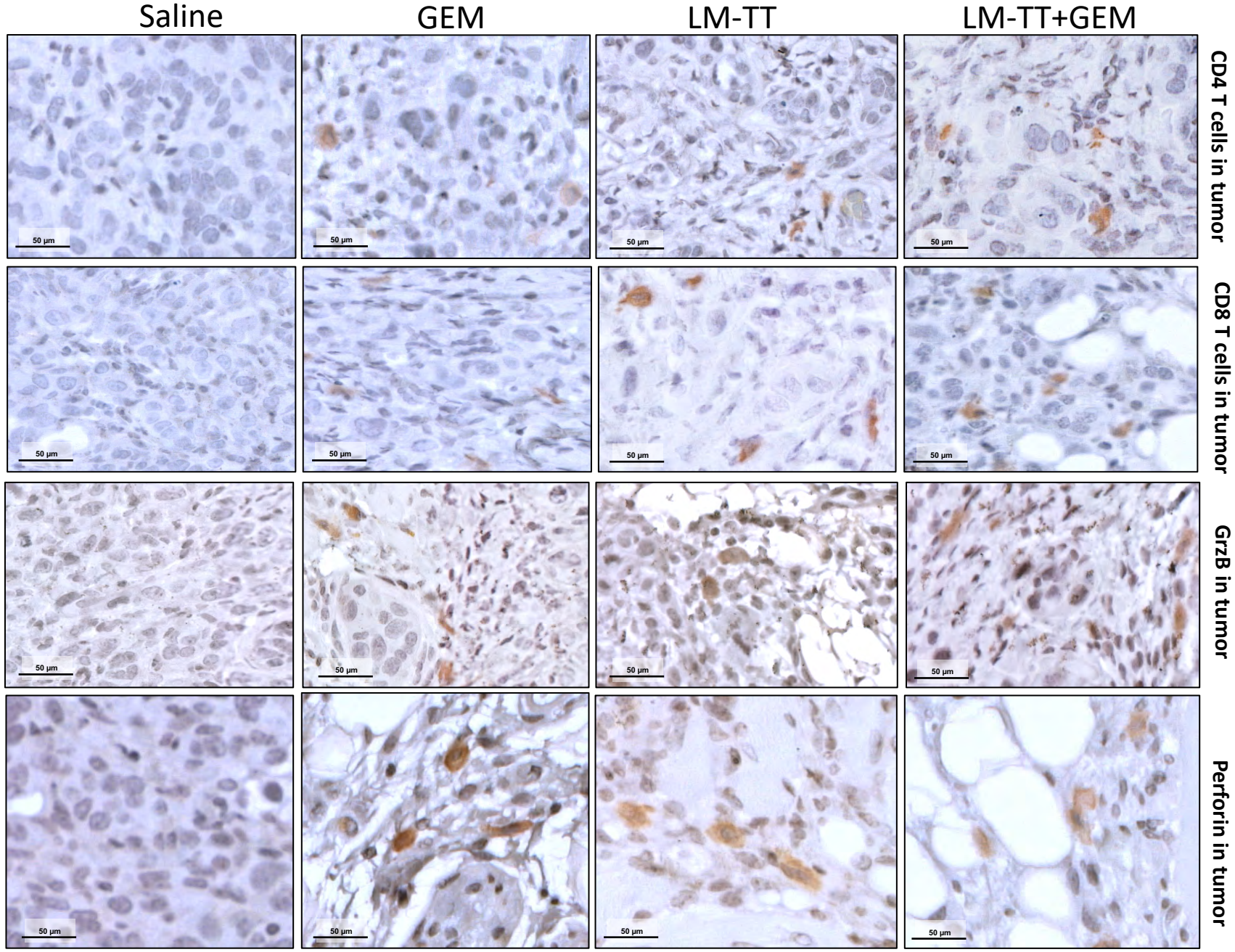

B
C
p<0.0001
p<0.0001
p=0.0031
p=0.0011
p=0.0010
% of tumor TT positive
100
80
60
40
20
0
Saline
GEM
LM-TT
LM-TT+GEM

Figure 2


Saline
GEM
LM-TT
LM-TT+GEM
CD4 T cells in tumor
CD8 T cells in tumor
GrzB in tumor
Perforin in tumor
50 µm

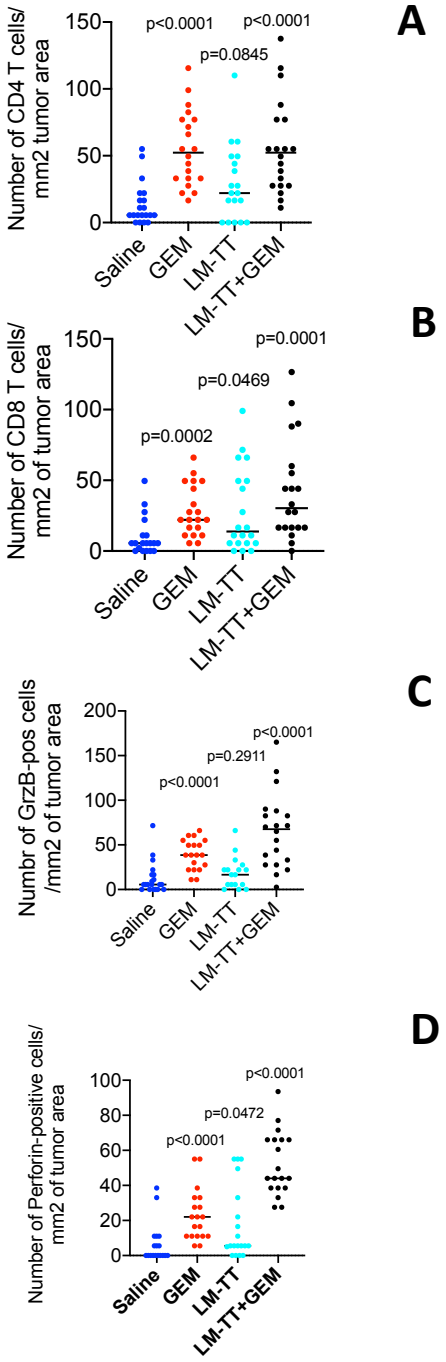
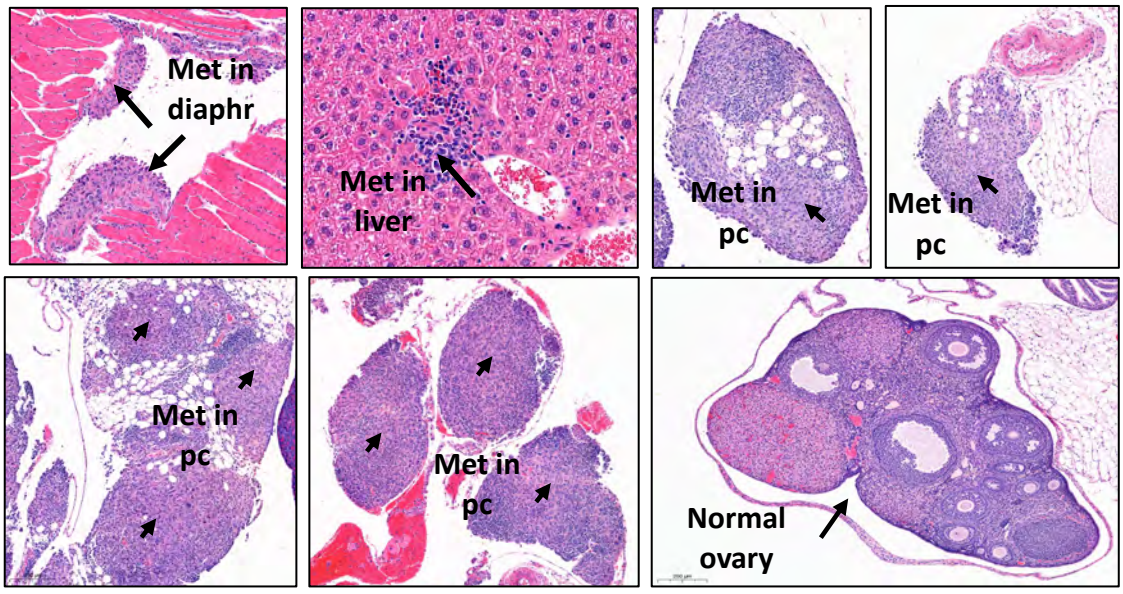
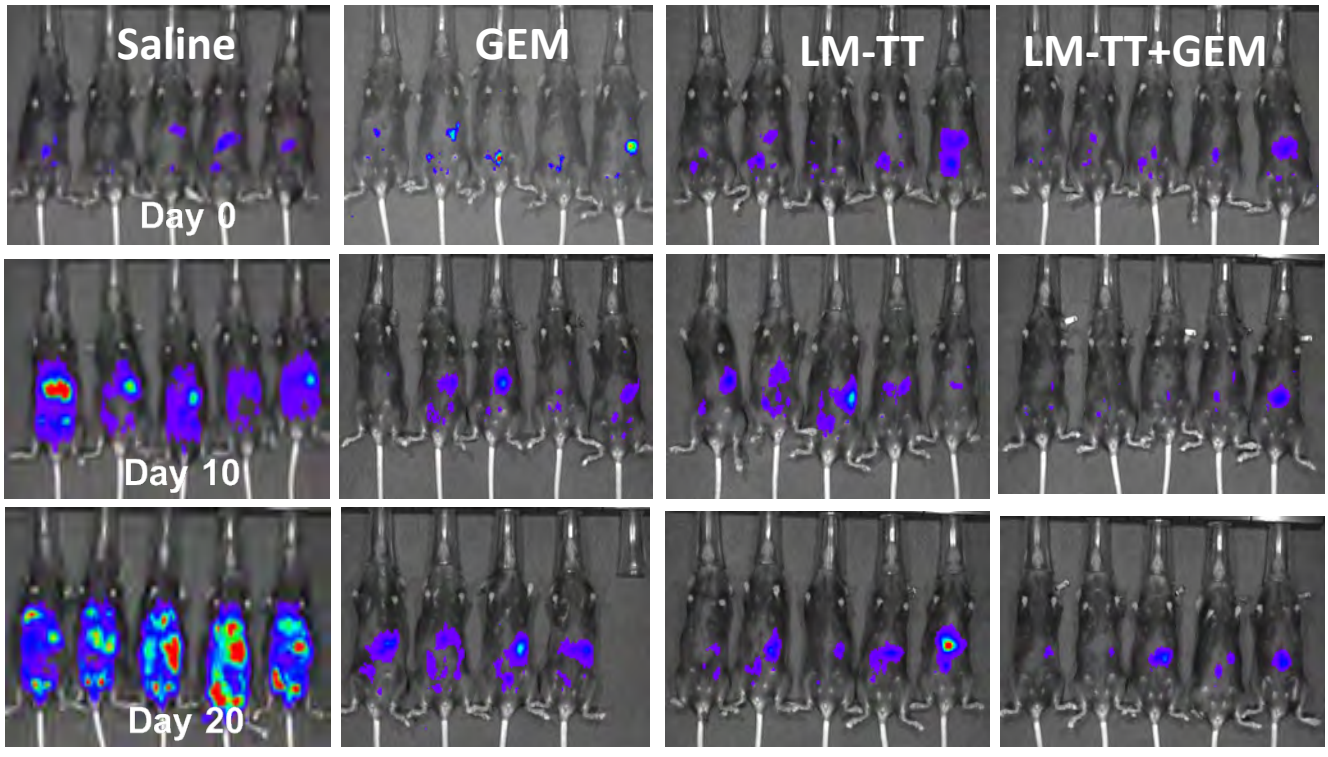
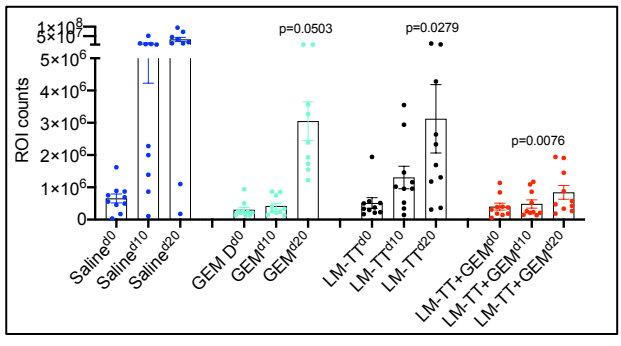

A
Number of CD4 T cells/ mm2 tumor area
p<0.0001
p<0.0001
p=0.0845
B
Number of CD8 T cells/ mm2 of tumor area
p=0.0001
p=0.0469
p=0.0002
C
Numbr of GrzB-pos cells /mm2 of tumor area
p<0.0001
p=0.2911
p<0.0001
D
Number of Perforin-positive cells/ mm2 of tumor area
p<0.0001
p=0.0472
p<0.0001
Saline
GEM
LM-TT
LM-TT+GEM

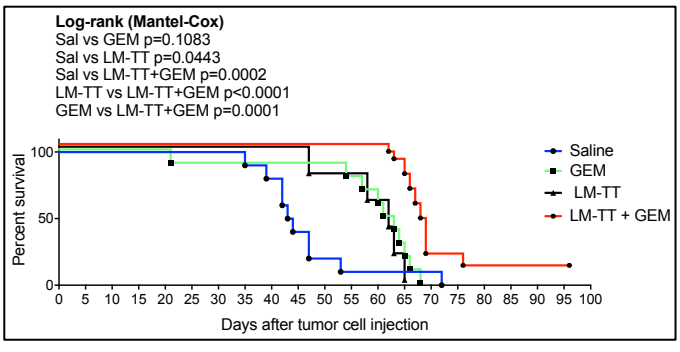
Figure 3



Mets present at start of treatment (day 0)







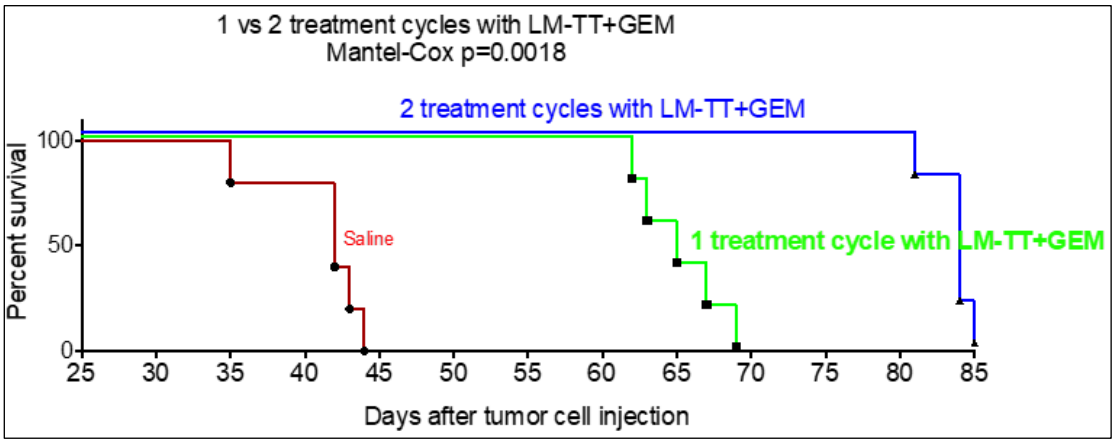
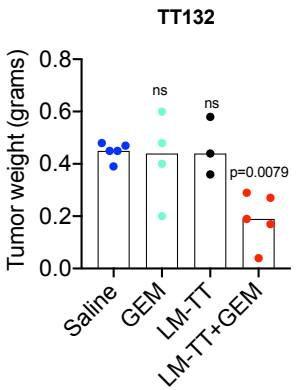
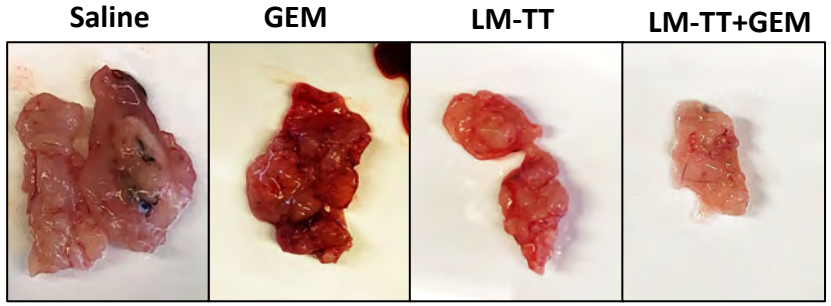
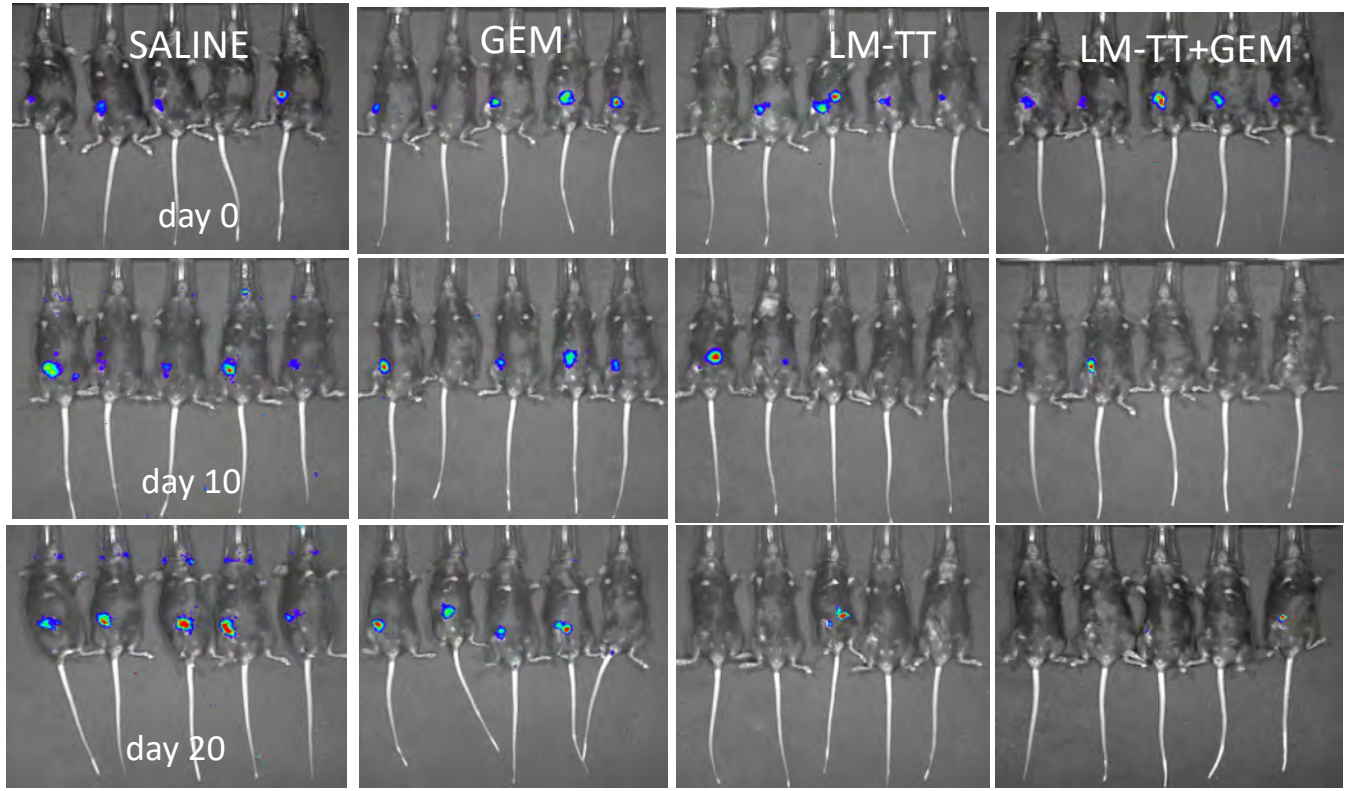


Figure 4

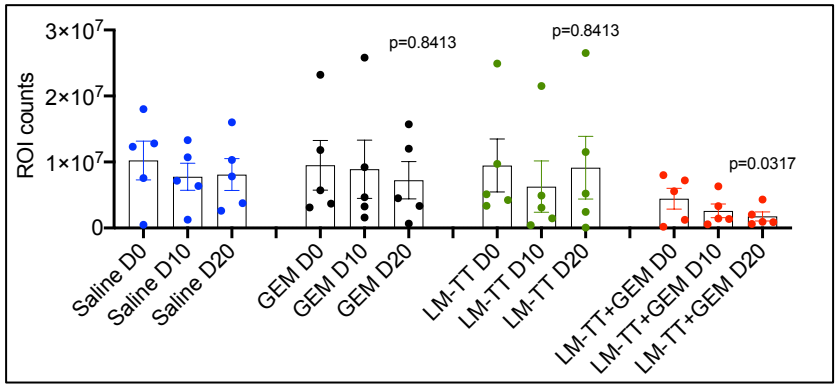




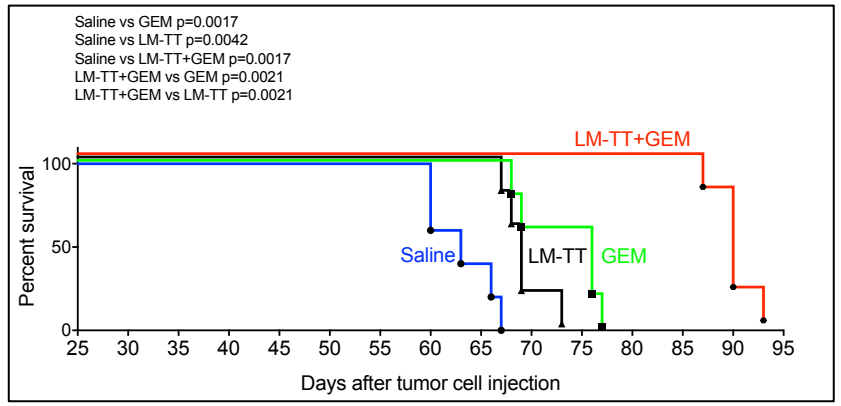
A



B



C



D

Figure 5

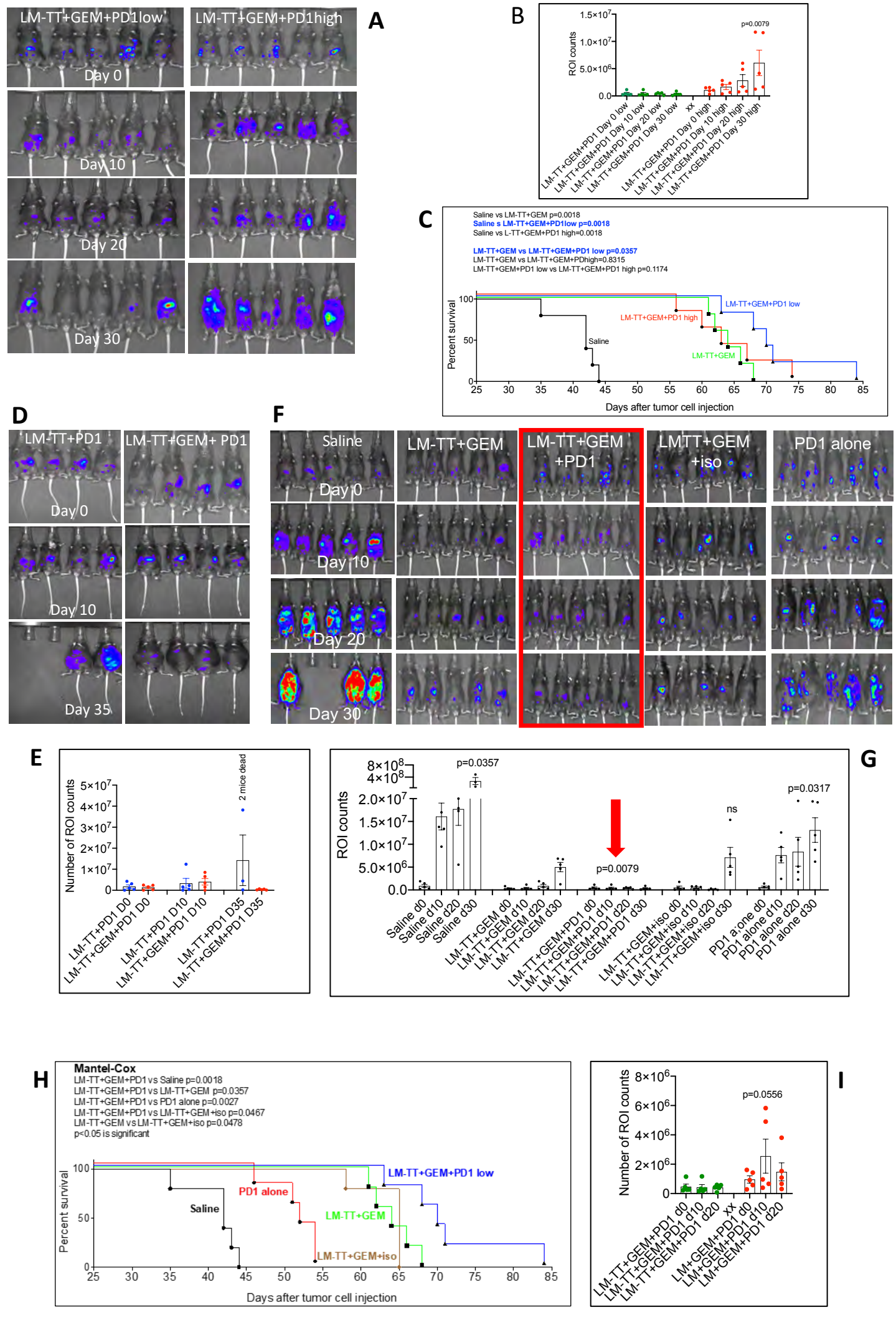
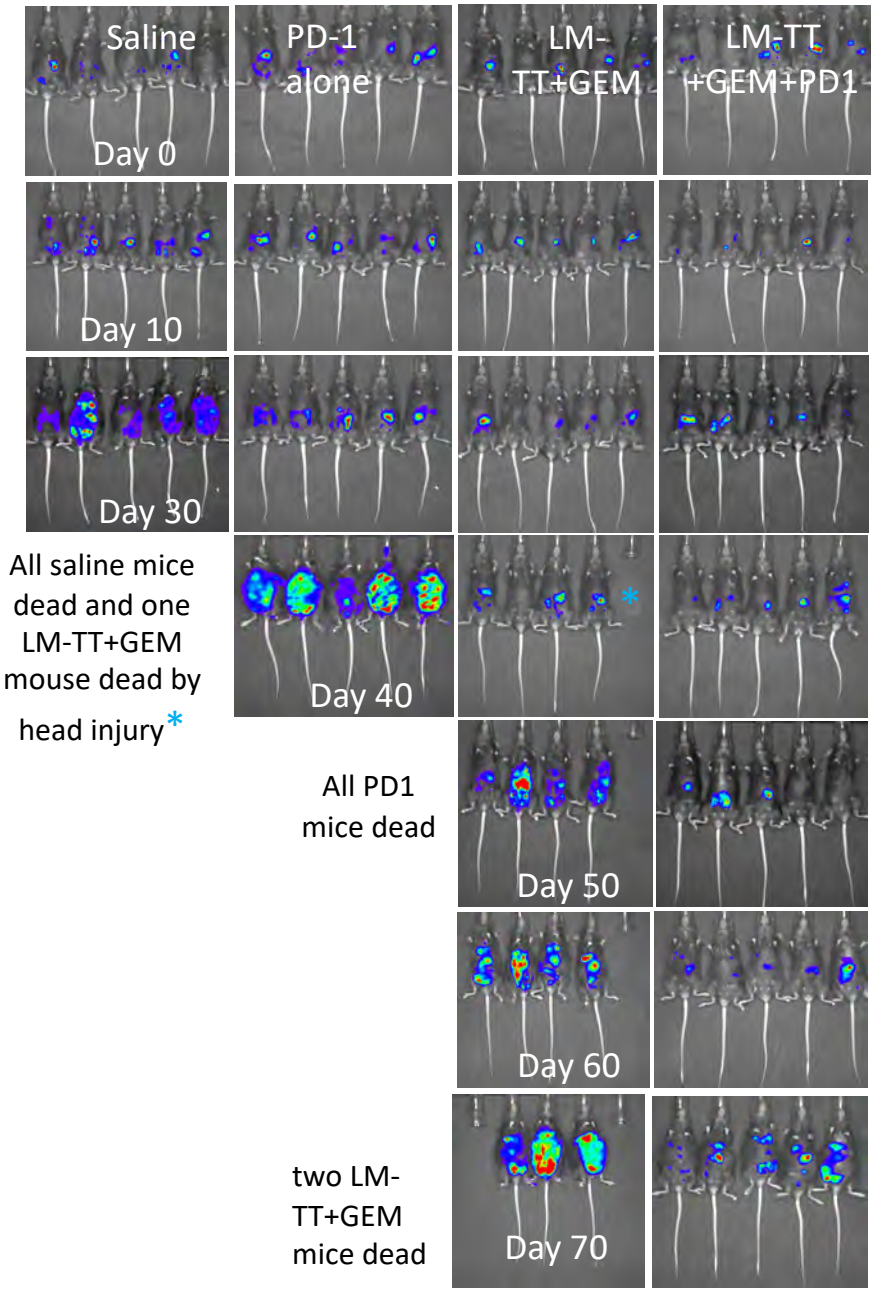
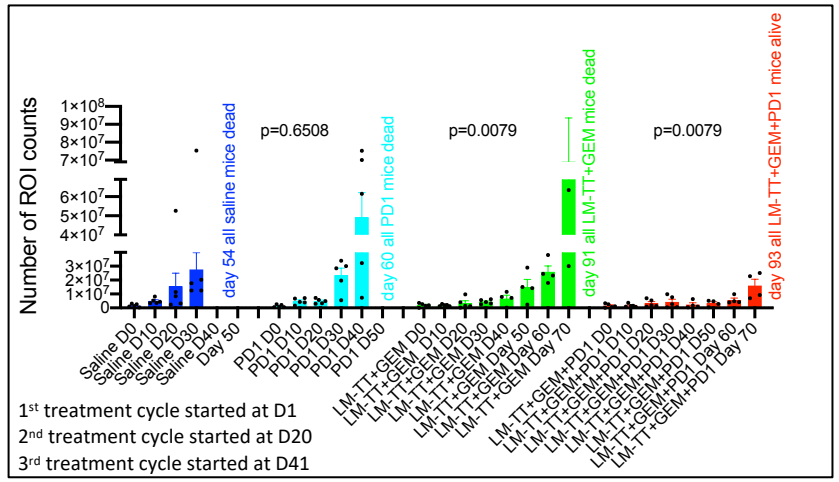
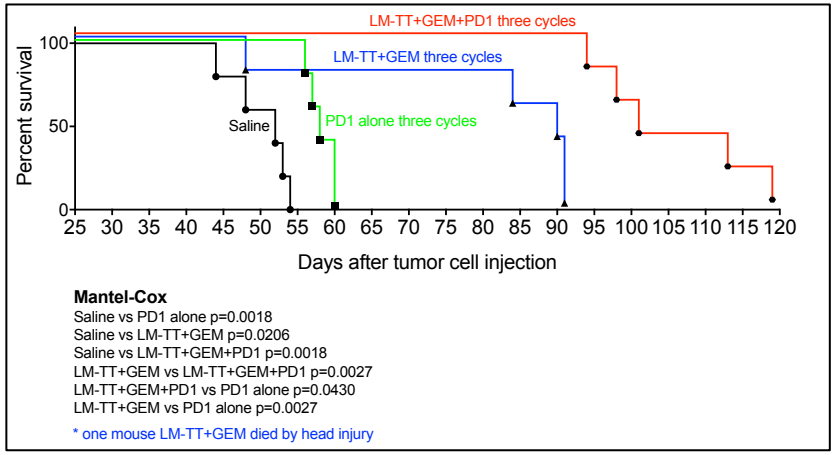

A
LM-TT+GEM+PD1low
LM-TT+GEM+PD1high
Day 0
Day 10
Day 20
Day 30
B
ROI counts
p=0.0079
LM-TT+GEM+PD1 Day 0 low
LM-TT+GEM+PD1 Day 10 low
LM-TT+GEM+PD1 Day 20 low
LM-TT+GEM+PD1 Day 30 low
xx
LM-TT+GEM+PD1 Day 0 high
LM-TT+GEM+PD1 Day 10 high
LM-TT+GEM+PD1 Day 20 high
LM-TT+GEM+PD1 Day 30 high
C
Saline vs LM-TT+GEM p=0.0018
Saline s LM-TT+GEM+PD1low p=0.0018
Saline vs L-TT+GEM+PD1 high=0.0018
LM-TT+GEM vs LM-TT+GEM+PD1 low p=0.0357
LM-TT+GEM vs LM-TT+GEM+PDhigh=0.8315
LM-TT+GEM+PD1 low vs LM-TT+GEM+PD1 high p=0.1174
Percent survival
LM-TT+GEM+PD1 low
LM-TT+GEM+PD1 high
Saline
LM-TT+GEM
Days after tumor cell injection
D
LM-TT+PD1
LM-TT+GEM+ PD1
Day 0
Day 10
Day 35
F
Saline
LM-TT+GEM
LM-TT+GEM +PD1
LMTT+GEM +iso
PD1 alone
Day 0
Day 10
Day 20
Day 30
E
Number of ROI counts
2 mice dead
LM-TT+PD1 D0
LM-TT+GEM+PD1 D0
LM-TT+PD1 D10
LM-TT+GEM+PD1 D10
LM-TT+PD1 D35
LM-TT+GEM+PD1 D35
G
ROI counts
p=0.0357
p=0.0079
ns
p=0.0317
Saline d0
Saline d10
Saline d20
Saline d30
LM-TT+GEM d0
LM-TT+GEM d10
LM-TT+GEM d20
LM-TT+GEM d30
LM-TT+GEM+PD1 d0
LM-TT+GEM+PD1 d10
LM-TT+GEM+PD1 d20
LM-TT+GEM+PD1 d30
LM-TT+GEM+iso d0
LM-TT+GEM+iso d10
LM-TT+GEM+iso d20
LM-TT+GEM+iso d30
PD1 a;one d0
PD1 alone d10
PD1 alone d20
PD1 alone d30
H
Mantel-Cox
LM-TT+GEM+PD1 vs Saline p=0.0018
LM-TT+GEM+PD1 vs LM-TT+GEM p=0.0357
LM-TT+GEM+PD1 vs PD1 alone p=0.0027
LM-TT+GEM+PD1 vs LM-TT+GEM+iso p=0.0467
LM-TT+GEM vs LM-TT+GEM+iso p=0.0478
p<0.05 is significant
Percent survival
LM-TT+GEM+PD1 low
PD1 alone
Saline
LM-TT+GEM
LM-TT+GEM+iso
Days after tumor cell injection
I
Number of ROI counts
p=0.0556
LM-TT+GEM+PD1 d0
LM-TT+GEM+PD1 d10
LM-TT+GEM+PD1 d20
xx
LM+GEM+PD1 d0
LM+GEM+PD1 d10
LM+GEM+PD1 d20

Figure 6


A
Saline
PD-1 alone
LM-TT+GEM
LM-TT +GEM+PD1
Day 0
Day 10
Day 30
Day 40
Day 50
Day 60
Day 70
All saline mice dead and one LM-TT+GEM mouse dead by head injury*
All PD1 mice dead
two LM-TT+GEM mice dead


B
Number of ROI counts
p=0.6508
p=0.0079
p=0.0079
day 54 all saline mice dead
day 60 all PD1 mice dead
day 91 all LM-TT+GEM mice dead
day 93 all LM-TT+GEM+PD1 mice alive
1st treatment cycle started at D1
2nd treatment cycle started at D20
3rd treatment cycle started at D41


C
LM-TT+GEM+PD1 three cycles
LM-TT+GEM three cycles
PD1 alone three cycles
Saline
Percent survival
Days after tumor cell injection
Mantel-Cox
Saline vs PD1 alone p=0.0018
Saline vs LM-TT+GEM p=0.0206
Saline vs LM-TT+GEM+PD1 p=0.0018
LM-TT+GEM vs LM-TT+GEM+PD1 p=0.0027
LM-TT+GEM+PD1 vs PD1 alone p=0.0430
LM-TT+GEM vs PD1 alone p=0.0027
* one mouse LM-TT+GEM died by head injury

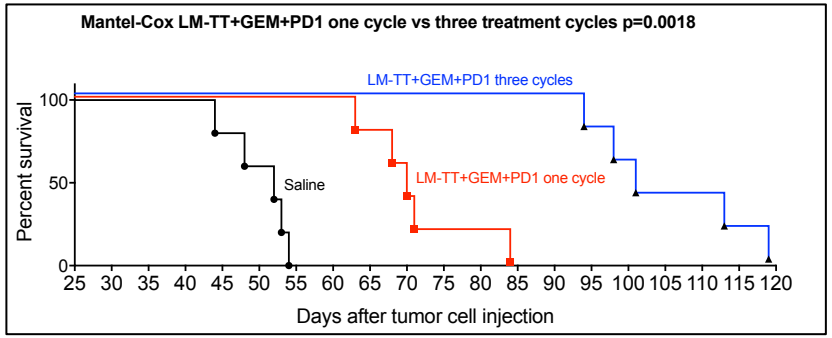
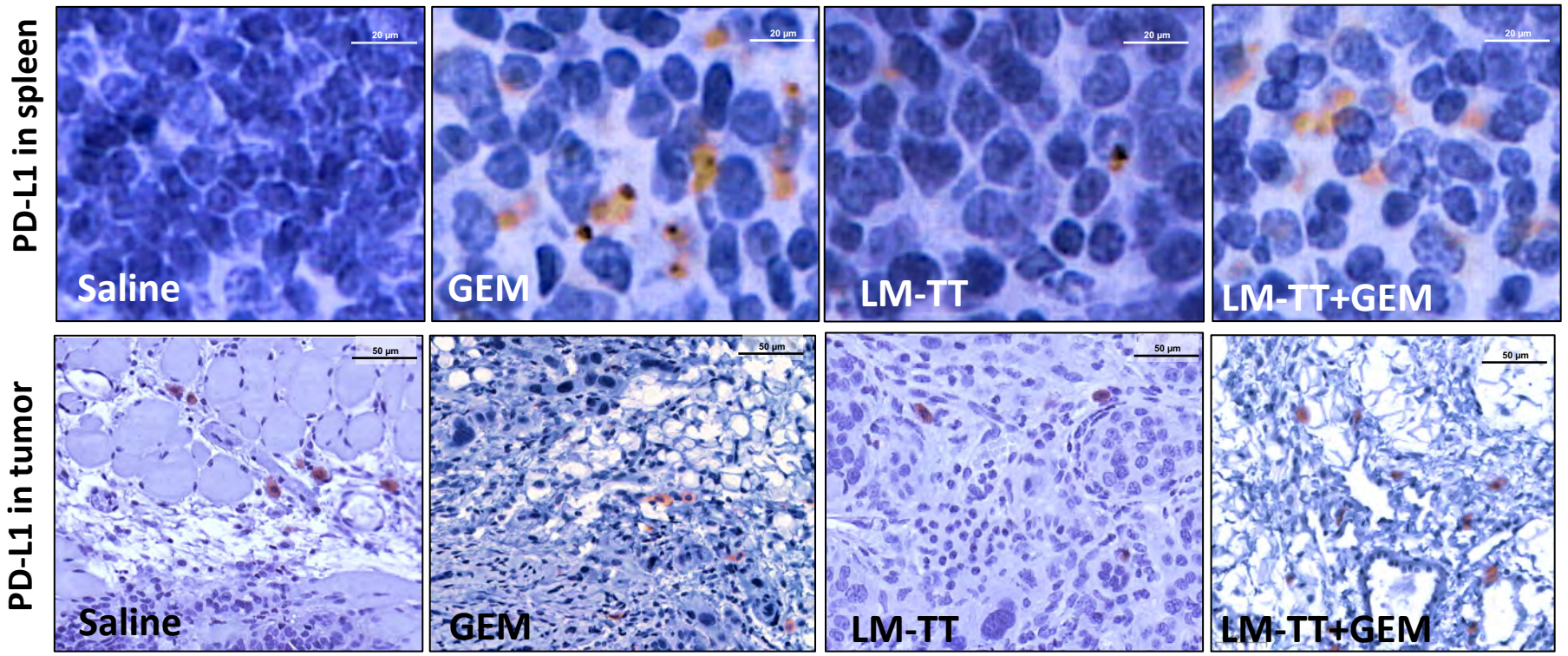
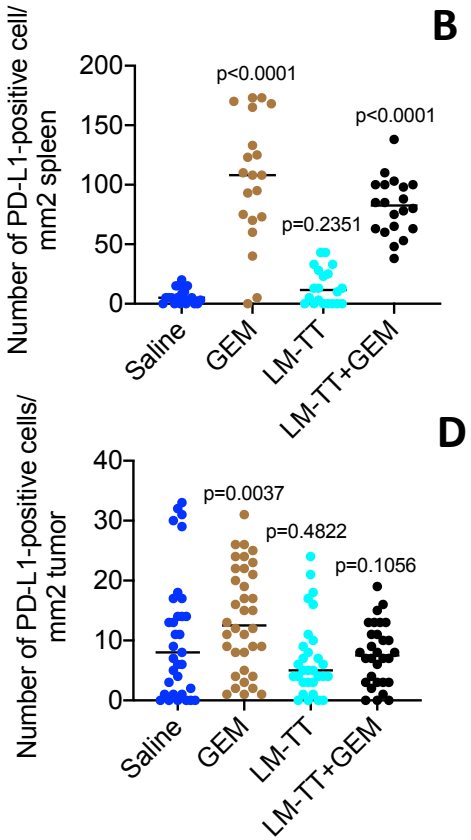
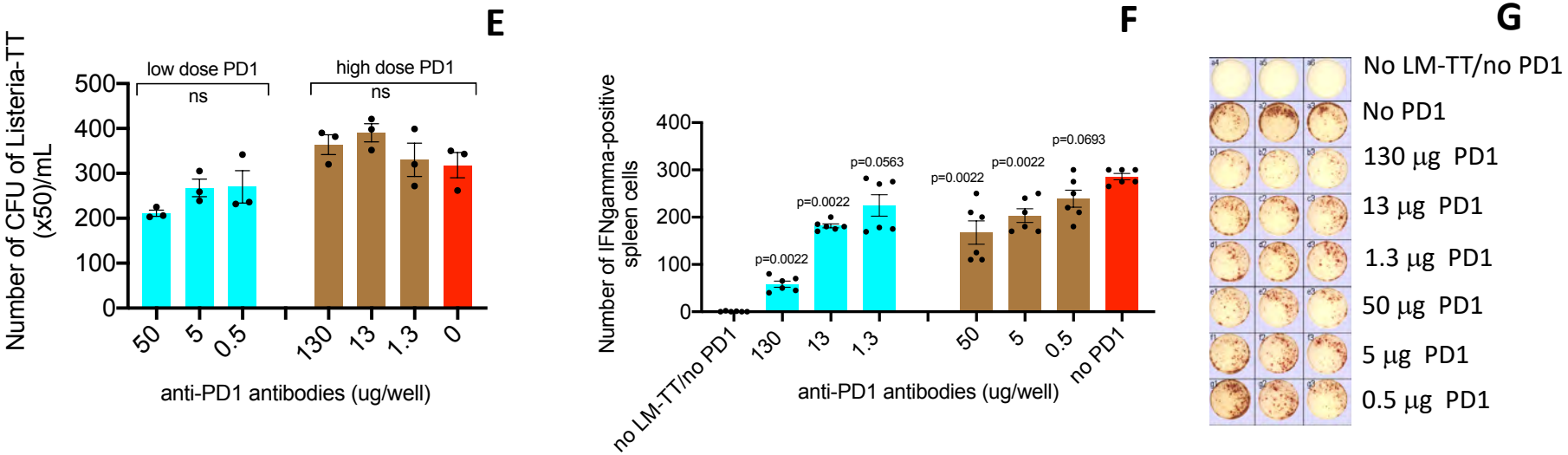

D
Mantel-Cox LM-TT+GEM+PD1 one cycle vs three treatment cycles p=0.0018
LM-TT+GEM+PD1 three cycles
LM-TT+GEM+PD1 one cycle
Saline
Percent survival
Days after tumor cell injection

Figure 7


PD-L1 in spleen
Saline
GEM
LM-TT
LM-TT+GEM
20 µm
PD-L1 in tumor
50 µm
A
C


B
Number of PD-L1-positive cell/ mm2 spleen
p<0.0001
p=0.2351
p<0.0001
Saline
GEM
LM-TT
LM-TT+GEM
D
Number of PD-L1-positive cells/ mm2 tumor
p=0.0037
p=0.4822
p=0.1056


E
Number of CFU of Listeria-TT (x50)/mL
low dose PD1
high dose PD1
ns
ns
anti-PD1 antibodies (ug/well)
F
Number of IFNgamma-positive spleen cells
p=0.0022
p=0.0022
p=0.0563
p=0.0022
p=0.0022
p=0.0693
no LM-TT/no PD1
no PD1
G
No LM-TT/no PD1
No PD1
130 µg PD1
13 µg PD1
1.3 µg PD1
50 µg PD1
5 µg PD1
0.5 µg PD1

SUPPLEMENTAL DATA

Tumor-targeted delivery of Tetanus toxoid by Listeria improves immunotherapy against ovarian cancer in mice

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Running title: Listeria-Tetanus and gemcitabine reduces ovarian cancer

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[2]*Montefiore Medical Center/Albert Einstein College of Medicine, Division of Gynecologic Oncology, 1695 Eastchester Road, Bronx, NY 10461, USA.*

[¶]These authors equally contributed to this *work*

Keywords: ovarian cancer, Listeria, tetanus toxoid, T cell responses

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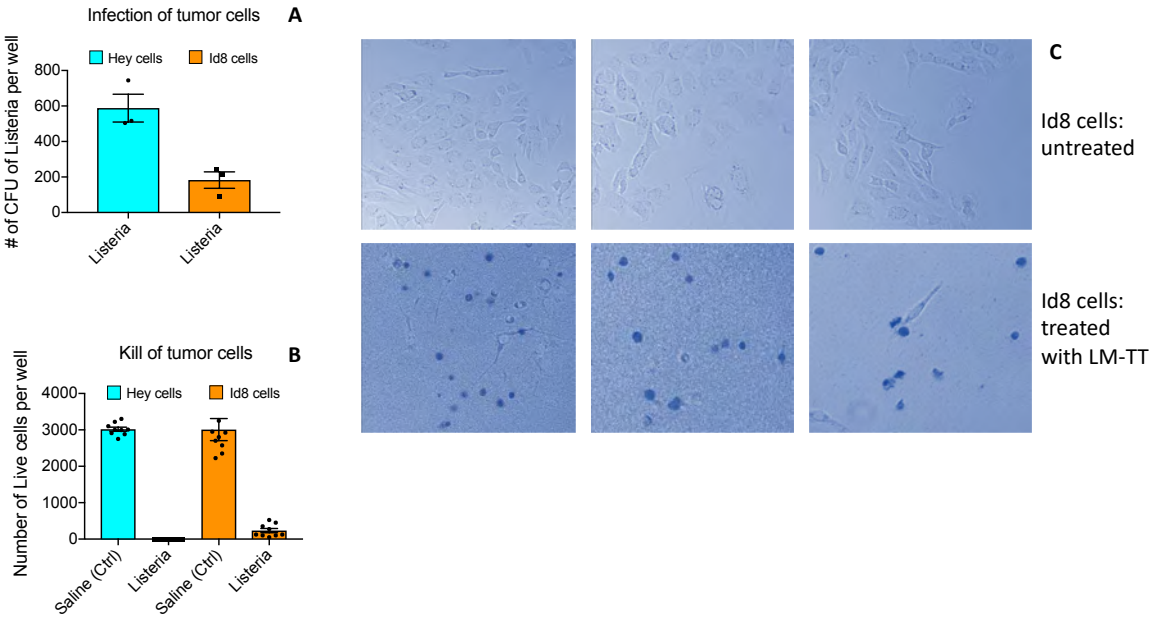
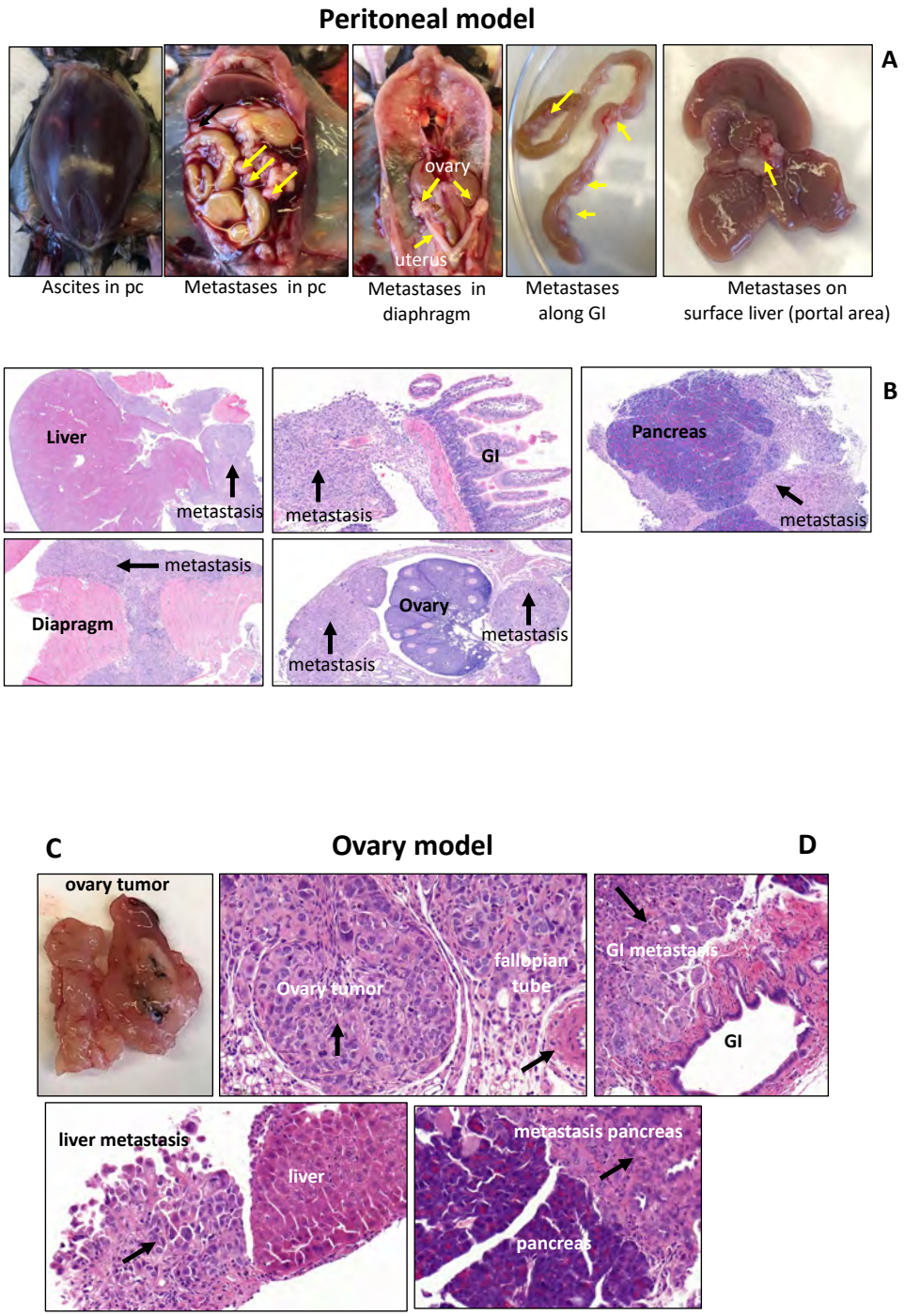
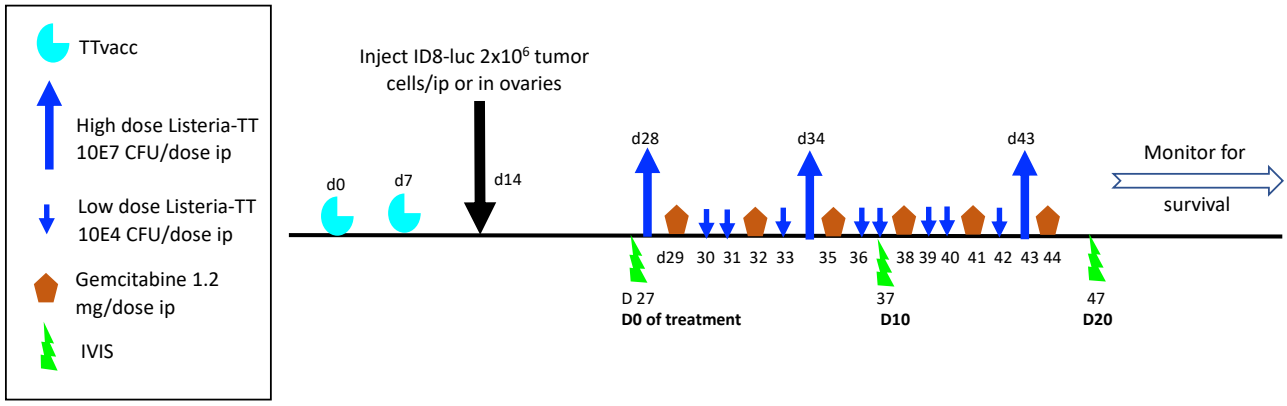
Supplemental Figures



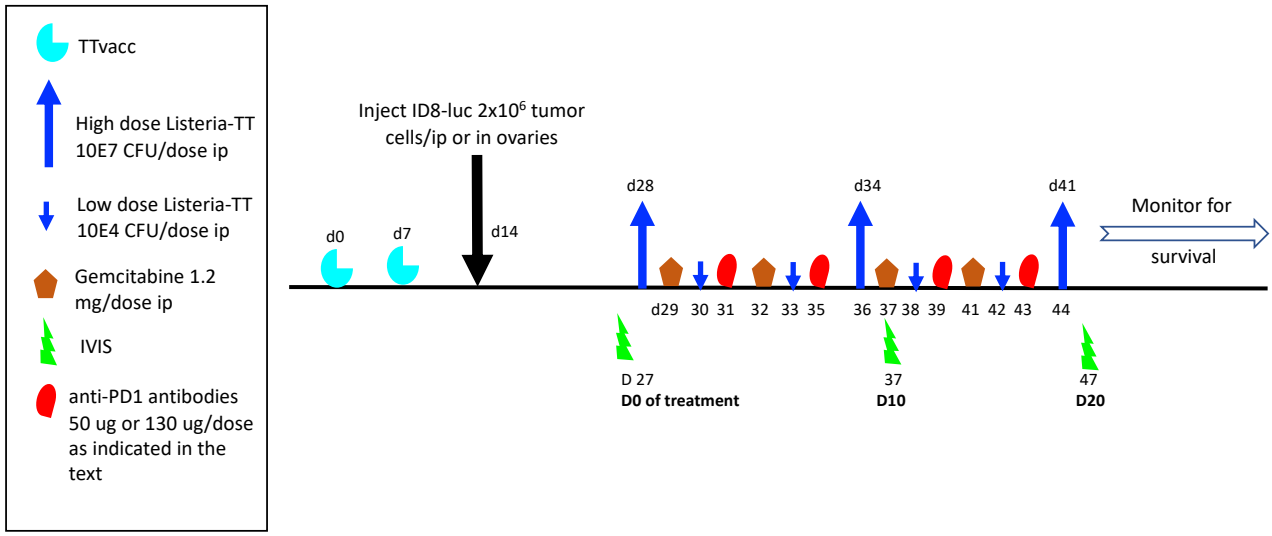
Supplemental Figure 1: Listeria-TT infecting and killing mouse and human ovarian cancer cells. Listeria-TT, infected both human chemoresistant Hey and mouse ID8p53-/- ovarian cancer cells *in vitro* with high efficiency after one hour of infection **(A).** Experiments were performed in triplicates, and repeated three times. The results presented in this figure are the average of three infection experiments. The error bars represent the SEM. We also tested the killing of Hey and Id8p53-/- tumor cells by Listeria-TT and found that both were efficiently killed by Listeria **(B).** In this experiment, tumor cells were incubated with the *Listeria* bacteria for 3 hours and then treated with gentamycin. Next day, dead and alive tumor cells were determined with trypan blue **(C).** From each sample, nine fields were blinded analyzed by two different investigators. Experiments were performed in triplicates, and repeated three times. The results presented in this figure are the average of three experiments. Mann-Whitney ****$p<0.0001$. The error bars represent SEM.



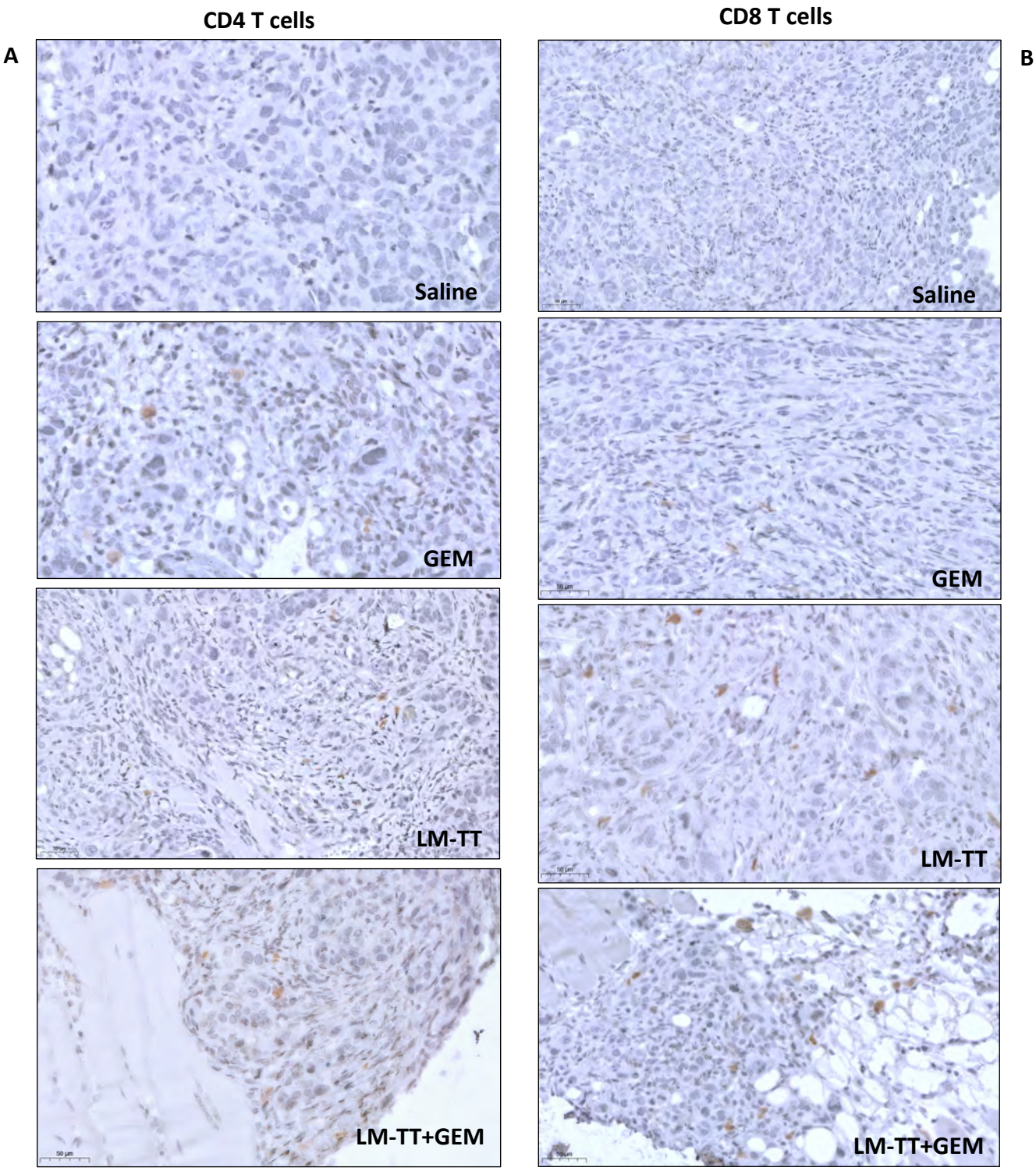
Supplemental Figure 2: Characteristics of the peritoneal and ovary Id8p53-/- models. Two ovarian cancer models were tested, i.e. the peritoneal and the ovary model. Both models were analyzed by pathological examination to confirm the cancer. In the peritoneal model, tumor cells were injected intraperitoneally resulting in metastases predominantly in the peritoneal cavity along the GI, in the diaphragm, and in the area of the portal liver. No tumor cells were detected in the ovaries. In the ovary model, tumor cells were injected into the ovaries, resulting in tumors predominantly in the ovaries. Metastases were only found 4-7 weeks later.



Supplemental Figure 3A: Treatment protocol Listeria-TT+GEM. C57BL/6 mice were immunized twice (days 0 and 7) with the Tetanus Toxoid vaccine (TTvacc) to generate memory T cells. Subsequently, Id8p53-/-Luc tumor cells were injected intraperitoneally or orthotopically in the ovaries (day 14) and metastases and tumors allowed to develop to an advanced stage detectable by IVIS and H&E staining (day 28). After an initial high ip dose of *Listeria*-TT (10^7 CFU)(day 28), Listeria-TT accumulated in the TME (within the first 4 hrs after injection). Low doses of GEM (1.2 mg/dose; 6 doses total on days 29, 32, 35, 38, 41, and 44) were then given every 3 days for 14 days in order to eliminate MDSC and TAM. At this point, MDSC are no longer required to bring *Listeria*-TT to the TME. Instead, reducing MDSC and TAM populations by GEM serves to improve T cell responses. Two additional high doses of Listeria-TT were administered to deliver more TT at the tumor site on days 34 and 43. Alternately, daily low doses of *Listeria*-TT (10^4 CFU; 10 doses total on days 30, 31, 36, 37, 39, 40, 42) to reactivate TT-specific memory T cells, improved by GEM. Tumors and metastases were monitored by intravital imaging of small animals in vivo (IVIS) before (day 27), during (day 37) and after the therapy (Day 47). At the end of treatments, we continued monitoring until death. LM-TT = *Listeria*-TT.

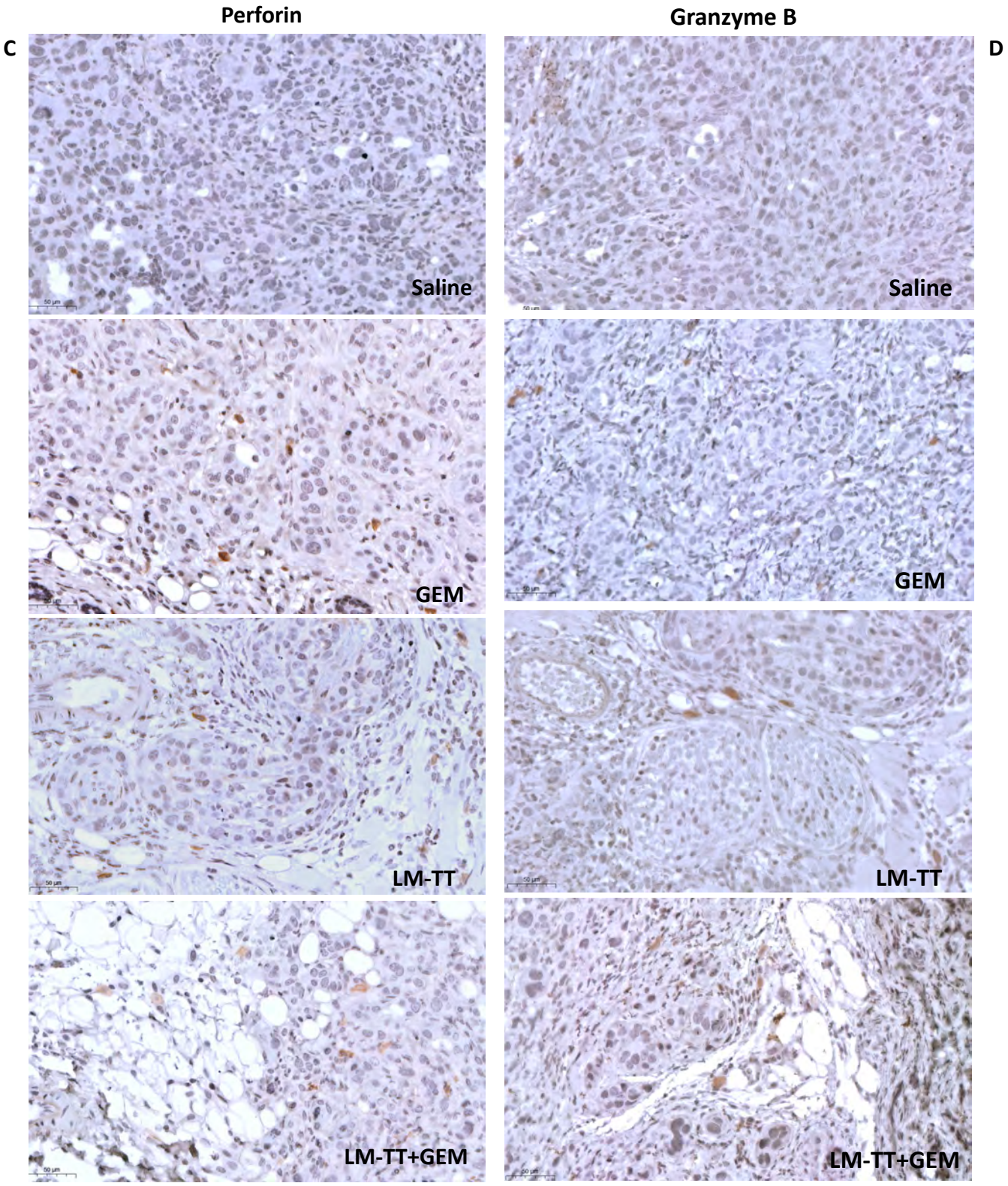


Supplemental Figure 3B: Treatment protocol Listeria-TT+GEM+anti-PD1. In the Id8p53-/-Luc model, mice were immunized twice with Tetanus vaccine (TTvac) to generate memory T cells (days 0 and 7). Subsequently, Id8p53-/-Luc tumor cells were injected intraperitoneally or orthotopically in the ovaries (day 14) and metastases and tumors allowed to develop to an advanced stage detectable by IVIS and H&E staining. Three high doses of Listeria-TT were administered on days 28, 34, an 41, combined with four low doses of Gemcitabine on days 29, 32, 37, and 41, and four low dose of Listeria-TT on days 30, 33, 38, and 42, followed by anti-PD1 antibodies on days 31, 35, 39 and 43. Tumors and metastases were monitored by intravital imaging of small animals in vivo (IVIS) before (day 27), during (day 37) and after the therapy (Day 47). At the end of treatments, we continued monitoring until death. LM-TT=Listeria-TT



Supplemental Figure 4: CD4 T cells and CD8 T cells in ovary tumors. Id8p53-/-Luc mice received treatments with Listeria-TT+GEM, or control treatments, as outlined in Fig S3. Two days

after the last treatment, tumor tissues were analyzed by immunohistochemistry (IHC) for the presence of CD4 **(A)** and CD8 **(B)** T cells. LM-TT = Listeria-TT.



Supplemental Figure 4: Perforin production and Granzyme B production in ovary tumors. Id8p53-/-Luc mice received treatments with Listeria-TT+GEM, or control treatments, as outlined

in Supplemental Figure 3A. Two days after the last treatment, tumor tissues were analyzed by IHC for the production of perforin **(C)** and granzyme B **(D).** LM-TT = Listeria-TT.

Supplemental Table 1: Minimal effect(s) of high* or low doses of anti-PD1 antibodies on spleen, lungs, kidneys, liver and small intestine of C57BL/6 mice*****

	Group Low Dose			Group High Dose		
	Animal 1	**Animal 2**	**Animal 3**	**Animal 4**	**Animal 5**	**Animal 6**
	Slides 1-2	**Slides 3-4**	**Slides 5-6**	**Slides 7-8**	**Slides 9-10**	**Slides 11-12**
Spleen						
Increased extramedullary hematopoiesis	Minimal (1)	Minimal (1)	Minimal (1)	Slight (2)	Minimal (1)	Minimal (1)
Lungs						
Hemorrhage, acute, focal	-	Minimal (1)	Minimal (1)	Minimal (1)	Slight (2)	Minimal (1)
Inflammation, interstitial	-	-	-	-	Minimal (1)	-
Granuloma, focal	-	-	-	Minimal (1)	-	-
Kidneys (HE & PAS)						
Perirenal fat, infiltration lymphocytes	-	-	-	-	Minimal (1)	-
Liver						
Extramedullary hematopoiesis	-	-	-	Minimal (1)	-	-
Necrosis, inflammation	-	-	Minimal (1)	-	-	-
Inflammation, focal	-	-	-	-	-	Minimal (1)
Small Intestine						
Serosa, granuloma	-	-	Minimal (1)	-	-	-

*high dose of anti-PD1 antibodies (130 μg/dose)

** low dose of anti-PD1 (50 μg/dose)

*** C57BL/6 mice of 6-8 weeks without cancer were treated with 4 high or low doses of anti-PD1 antibodies, each 4 days apart, as indicated in Supplemental Figure 3B. At the end of treatment, mice were euthanized and organs where subjected to pathological analysis to evaluate potential toxic effects of anti-PD1 antibodies.

KEY RESOURCES TABLE

REAGENT or RESOURCE	Company	Catalog #
Antibodies		
anti-CD3	BD Bioscience	560527
anti-CD4	BD Bioscience	552051
anti-CD8	BD Bioscience	552877
anti-TT (IHC)	Invitrogen	PA1-73168
Isotype control HRPN	BioXCell	BE0090
anti-IFNγ	BD Bioscience	564693/554411
anti-Perforin	eBioscience	17-9392-80
anti-Granzyme B	eBioscience	12-8898-82
anti-rabbit IgG-Alexa-680	Invitrogen	A21109
HRP-conjugated goat anti- rabbit IgG	Santa cruz biotechnology	sc-2004
anti-Tetanus Toxic Fragment C	Rockland	100-401-894
anti-rabbit IgG, HRP-linked Antibody	Cell signaling technology	7074S
anti-PD1 In Vivo (CD279)	BioXCell	BE0033-2
Isotype control Armenian hamster IgG	BioXCell	BE0091
Anti-PD1 In Vitro (CD279)	BioLegend	135247
Signal Stain Boost IHC	Cell Signaling Technology	8114S
Bacterial strains		
Attenuated Listeria monocytogenes	Developed by Yvonne Paterson	Ref:33
Attenuated Listeria monocytogenes expressing $TT_{856-1313}$	Developed in Gravekamp lab	
Recombinant proteins and chemicals		
Fetal Bovine Serum	Corning	35-011-CV
RPMI medium	Corning	10-040-CV
DMEM medium	Corning	10-017-CV
Trypsin EDTA solution	Gemini bio-products	400-150
Glutamine	Corning	25-005-CI
Pyruvate	Corning	25-000-CI
Penicillin/Streptomycin	Lonza	17-603E
Gentamicin	Quality biological	120-098-661
Non-essential amino acids	Corning	25-025-CI
Insulin/transferrin/sodium selenite	Gibco	41400-045
Chloroamphenicol	American bioanalytical	AB00321-00025
Ampicilline	Sigma	A9518-5G
Gemcitabine	Fresenius kabi oncology Ltd	7220131514 IND
Brain heart infusion	BD Bioscience	237500
LB agar	Fischer scientific	BP1425-500
TT vaccine (childhood vaccine)	Serum institute of India	20012753/7
VitroGel Hydrogel Matrix	MidSci	VHMO1S
D-Luciferin Sodium Salt	Synchem OHG	BC218
Formalin 10%	Fisher Chemical	SF100-4
Hydrogen peroxide 3%	Fisher Chemical	H324-500
DAB	Vector Laboratories	SK-4100

Hematoxilin	Sigma/Aldrich	1.05174-0500
Xylene (Histi-Clear II)	National Diagnostics	HS202
Permount	Fisher Chemical	SP15-100
Ethanol	Decon	2805HC

Critical Commercial Assays

ELISPOT	BD Bioscience	551083
Chemiluminescent kit	Thermo Fischer	34077
4-12% gradient SDS-polyacrylamide gel	BioRad	456-8083
PVDF membrane	BioRad	162-0177

Vectors

pGG34 (LM-LLO=Listeria vector)	Developed by Yvonne Paterson	Ref: 33
pGG34-TT	Developed in Gravekamp lab	

Cell lines

Id8p53-/-Luc cell line	Mc Neish, Barts Cancer Institute, Queen Mary University of London, and Janet Sawicki, Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA
Hey cell line	Susan Horwitz, Albert Einstein College of medicine, Bronx, NY, USA

Mouse models

Id8p53-/-Luc model	p53-/- (peritoneal model)
Id8p53-/-Luc model	p53-/- (ovary model)

Other

CTL Immunospot S4 Analyzer	
Olympus IX51 Microscope	
ZOE™ Fluorescent Cell Imager	BioRad
IVIS100 system	Living Image Version 2.11:Xenogen and Igo Pro (version 4.02A; WaveMatrics)
3D Histech P250 High Capacity Slide Scanner	