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14. ABSTRACT Pancreatic ductal adenocarcinoma (PDA) is a lethal disease that is notoriously resistant to standard therapies including chemotherapy. This is due in part to a tumor microenvironment that impedes drug delivery. However, leukocytes actively infiltrate tumors. Within the tumor microenvironment, macrophages are the dominant leukocyte and are involved in orchestrating an environment conducive to tumor growth. To reverse pro-tumor activity by leukocytes in PDA, we are investigating <i>Listeria monocytogenes</i> , a facultative intracellular bacterium that infects macrophages and activates them with anti-tumor activity while stimulating productive anti-tumor adaptive immunity. In our studies, we are using the <i>Kras</i> ^{G12D} ; <i>Trp53</i> ^{R172H} ; <i>Pdx-1Cre</i> (KPC) mouse model of PDA. Preliminary findings show that macrophages adoptively transferred to KPC mice selectively traffick to tumors with fibrosis. We are now examining the anti-tumor impact of adoptively transferred macrophages activated with <i>Listeria</i> by-products. We have also explored the capacity of <i>Listeria</i> vaccines to induce anti-tumor T cell immunity in the KPC model. We have found that <i>Listeria</i> vaccines produce little impact on late-stage tumors with an absence of T cell infiltration into tumor tissue but do appear to have non-antigen specific anti-stromal effects. We are now investigating the anti-stromal activity observed and the mechanism by which T cells fail to infiltrate tumors.					
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Table of Contents

	<u>Page</u>
Introduction.....	1
Body.....	2
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusion.....	9
References.....	11
Appendices.....	12
Supporting Data.....	13

INTRODUCTION: Pancreatic ductal adenocarcinoma (PDA) is a devastating disease that is notoriously resistant to standard treatments with chemotherapy and radiation therapy. However, immune-based therapies are a promising approach for the treatment of PDA due to the ability of the immune system to decisively direct cancer biology. Whereas macrophages present within tumors frequently predict a poor prognosis (1), the infiltration of tumors by effector CD8⁺ T cells is commonly associated with a more favorable prognosis (2). However, in PDA, T cells are rarely observed to infiltrate tumor lesions with macrophages representing the dominant leukocyte found within the tumor microenvironment (3). Macrophages can promote cancer growth and spread while also contributing to tumor resistance to conventional forms of cancer therapy including chemotherapy and radiation. In addition, macrophages can suppress T cell immunosurveillance (4). However, the behavior of macrophages is dependent on their surrounding microenvironment such that under the appropriate conditions, macrophages can be persuaded to attack growing tumors and to stimulate productive anti-tumor adaptive immunity (3). It is for this reason that *Listeria monocytogenes*, a bacteria that infects macrophages and induces them with anti-tumor activity, is an attractive and innovative vaccine for the treatment of PDA. *Listeria* vaccines are well-recognized for their ability to elicit potent adaptive immune responses and they can induce macrophages with the ability to kill tumor cells (5, 6). In early cancer clinical trials, *Listeria* vaccines have demonstrated safety as well as promising therapeutic results (7). It is the priority of this research award from the Department of Defense to use a clinically relevant genetically-engineered mouse model of PDA to explore the role of *Listeria* vaccines and *Listeria* by-products as therapeutics for the treatment of PDA. Our studies to date have investigated 1) the capacity of *Listeria* by-products, in particular Listeriolysin O, to activate macrophages with anti-tumor activity for adoptive transfer into mice with established PDA lesions, and 2) the capacity of a *Listeria* vaccine, engineered to induce tumor antigen-specific T cell immunity, to impact the biology of spontaneously arising PDA lesions in genetically-engineered mice.

BODY: Over the past year, effort has been directed toward investigating both Aims One and Two of the project. This Project uses the *Kras*^{G12D}, *Trp53*^{R172H}, *Pdx-1Cre* (KPC) mouse model of pancreatic ductal adenocarcinoma (PDA) to investigate *Listeria* by-products and *Listeria* vaccines for the treatment of PDA. The KPC mouse model is an attractive system for studying immunotherapy for PDA based on its ability to faithfully reproduce the salient features of human PDA and its demonstrated ability to inform the clinical development of novel treatment approaches for PDA (3, 8). Here, we provide an annual report of our accomplishments, difficulties, and next steps as they relate to each Task of the Statement of Work for this Project.

Task 1: Obtain regulatory review and approval processes for Animal use approvals.

IACUC approval was granted on 07/16/2012. ACURO approval was granted on 08/02/2012. At the start of this Discovery Award on 09-30-2013, my laboratory was in the process of moving to the Smilow Center for Translational Research at the University of Pennsylvania. This move took place on Sept 27, 2012 and involved setting up the laboratory as well as mouse colony in the new facility. This move delayed our progress on this project by approximately 3 months. For example, our brightfield and fluorescent microscopes did not arrive in the laboratory until late December 2012 and biosafety cabinets did not arrive until 1.5 months after the official move. Finally, it was necessary to expand our breeding colony to accommodate the studies outlined in the Statement of Work. Despite these delays, we have been quite productive with our efforts on this Project as is outlined below.

Task 2: Perform studies as described in Aim One. Aim One is to determine the ability of live *Listeria monocytogenes* and *Listeria* by-products to convert the innate immune response associated with PDA from tumor-promoting to tumor-suppressing.

For these studies, our first priority was to examine the ability of *Listeria* by-products to prime bone marrow derived macrophages (BMDM) *in vitro* for anti-tumor activity upon adoptive transfer *in vivo* and to evaluate the ability of *Listeria* by-products to directly activate innate anti-tumor immunity *in vivo*. To do this, we established a procedure for expanding BMDM from bone marrow cells (Figure 1A). Briefly, bone marrow cells from C57BL/6 mice were harvested and cultured in the presence of M-CSF (CSF-1) for 7 days. To confirm a macrophage phenotype of the cultured cells, we performed flow cytometry for the mature macrophage markers F4/80 and CD115 (CSF-1R) and the immature myeloid cell marker Ly6C. Our findings demonstrate that bone marrow cells cultured in the presence of M-CSF become F4/80⁺, CD115⁺, Ly6C^{neg} cells which is consistent with a mature macrophage phenotype (Figure 1B).

Having established an approach to generate BMDM for adoptive transfer, we next adoptively transferred BMDM labeled with a fluorescent carbocyanine dye (DiI) into KPC mice with ultrasound confirmed PDA tumors. By immunofluorescent imaging, we evaluated the trafficking of DiI-labeled BMDM to tumor lesions 72 hours after adoptive

transfer. Our findings demonstrate that BMDM preferentially traffick to distinct areas within tumors (Figure 1C). Using immunohistochemistry, we determined that KPC mice develop two distinct types of tumors classified histologically into stromal (marked by an extensive leukocyte infiltrate with extensive fibrosis) and non-stromal (marked by a minimal leukocyte infiltrate with little stroma) tumors. In our experiments, we found that BMDM traffick to stromal but not non-stromal tumors. Using a panel of KPC tumor cell lines that we have developed in the laboratory, we found that some tumors express chemokines such as MCP-1 (CCL2) and RANTES (CCL5) which are associated with macrophage recruitment to tumors (9) whereas others do not express either chemokine (Figure 1E). This finding has provided insight into our on-going studies with this Project by illustrating that stromal tumors are more likely to be impacted than non-stromal tumors by adoptive transfer of BMDM activated with anti-tumor properties.

We next studied the impact of *Listeria* by-products on BMDM. As a read-out of anti-tumor activity, we evaluated the ability of *Listeria* by-products to induce BMDM to secrete cytokines such as IL-12 and TNF- α which are commonly associated with an anti-tumor phenotype (10). To do this, we first manufactured the *Listeria* by-product Listeriolysin O (LLO). We produced 52.8 mg of LLO protein, assessed purity using SDS-PAGE and examined endotoxin levels using a Limulus Amebocyte Lysate (LAL) gel clotting test which revealed endotoxin levels of <0.02 EU/mg (Figure 2A). We next examined the potential of LLO to activate BMDM macrophages *in vitro* 24 hours after stimulation (Figure 2B). We evaluated LLO at 3 concentrations (1, 10, and 100 ug/mL) with and without IFN- γ priming for 18 hours and compared results to stimulation with heat killed *L. monocytogenes* (HKLM) and lipopolysaccharide (LPS). Our findings demonstrate that LLO did not induce BMDM to produce cytokines. Moreover, increasing doses of LLO were found to inhibit the capacity of IFN- γ to drive the production of TNF- α by BMDM. In contrast, HKLM effectively stimulated BMDM to produce cytokines with similar magnitude as observed with LPS. The inability of LLO to activate BMDM was unexpected but upon evaluation of the cell cultures it became clear that LLO was toxic to the cells. Using a crystal violet blue viability assay, we found that LLO induced cell death most likely due to the pore forming capacity of LLO. To neutralize this toxic behavior of LLO, we are now exploring the use of cholesterol which has previously been shown to reduce the pore forming capacity of LLO without compromising its immunostimulatory capacity (11). These studies will be critical for determining LLO for activating BMDM *ex vivo* for subsequent adoptive transfer into KPC mice.

While undertaking *in vitro* studies with LLO, we also initiated *in vivo* studies to evaluate the anti-tumor activity of LLO. However, our preliminary findings revealed that LLO was toxic when administered to KPC mice producing a rapid demise of animals within hours which was associated with a robust serum cytokine response marked by IL-6. Based on our *in vitro* findings, we suspect that this outcome is related to the toxicity of LLO in the absence of cholesterol neutralization. To circumvent this issue, we are now manufacturing a detoxified form of LLO (dtLLO) which lacks direct toxic effects (12). For *in vivo* studies, we plan to use dtLLO to evaluate the anti-tumor activity of LLO. In the meantime, we have begun to evaluate the impact of live *Listeria* on the innate immune response in tumor-bearing KPC mice. Our preliminary findings demonstrate

that administration of *Listeria* vaccines produces a rapid serum cytokine response within 18 hours in KPC mice that is associated with the production of IFN- γ , IL-6, and MCP-1 (CCL2). This finding is consistent with the activation of innate immunity. Based on our previous experience with CD40 agonists which activate macrophages to rapidly infiltrate tumor lesions within 18 hours, degrade tumor-associated fibrosis, and eliminate tumor cells (3), we are now examining the impact of live *Listeria* vaccines on tumor biology and tumor-associated fibrosis at 18 hours after treatment. These studies will provide insight into the capacity of *Listeria* vaccines to modulate the tumor microenvironment in PDA.

In summary, we have established an approach to activate BMDM *ex vivo* for adoptive transfer into KPC mice. We have found that adoptively transferred BMDM preferentially traffick to tumors marked by extensive desmoplasia. We have evaluated two *Listeria* by-products (LLO and HKLM) for their capacity to activate BMDM *ex vivo*. Based on these studies, we have had to modify our approach with LLO due to its inherent cellular toxicity. Given this set-back, we initiated studies with live *Listeria* vaccines to evaluate the capacity of *Listeria* to convert innate immunity associated with PDA from tumor-promoting to tumor-suppressing. In addition, we began manufacturing a detoxified form of LLO. These studies are currently underway and will progress over the course of the next year.

Task 3: Perform studies as described in Aim Two. Aim Two is to examine the ability of live engineered *Listeria* vaccines to induce productive anti-tumor immunity capable of targeting tumor cells and the stromal microenvironment of PDA.

For these studies, our first priority was to examine the expression of Her2 within tumor tissues in the KPC model. Using immunofluorescence imaging, we have determined that Her2 expression within tumors is heterogeneous with expression ranging from high to low, even within the same lesion (Figure 3A). In our analysis of tumor cell lines developed from PDA lesions in KPC mice, we have similarly observed that Her2 expression ranges from high to low in tumor cells (Figure 3B).

We next manufactured Lm-LLO-Her2 and Lm-LLO-E7 *Listeria* vaccines. Lm-LLO-Her2 is an engineered *Listeria* vaccine which expresses multiple regions of the Her2 protein fused to the *Listerial* virulence factor, LLO (Figure 4A). We have selected this vaccine construct because it incorporates both extracellular and intracellular regions of the Her2 protein which may allow for a broader anti-Her2 immune response. While this vaccine uses human Her2, sequence homology is 88% to mouse Her2. In addition, we have compared mouse and human Her2 sequences using computer algorithms (SYFPEITHI and BIMAS) and determined that all potential mouse MHC class I (H-2^b) epitopes are maintained in the Her2 chimeric vaccine. Lm-LLO-E7 serves as a control vaccine and expresses the human papilloma virus protein E7 fused to LLO.

To evaluate the impact of *Listeria* vaccine delivery on PDA growth in KPC mice, tumor-bearing KPC mice were block randomized into three treatment groups (PBS

control, Lm-LLO-E7, and Lm-LLO-Her2). Ultrasonography was performed at baseline prior to treatment and 14 days after beginning therapy (Figure 4B). In our analysis, we unexpectedly found that treatment with Lm-LLO-E7 or Lm-LLO-Her2 produced no impact on tumor growth when compared to PBS control treatment (Figure 4A). To understand this finding, we examined serum from mice at day +14 after therapy. We found that *Listeria* vaccine therapy produced an increase in serum cytokines associated with activation of innate immunity including IL-12, G-CSF, RANTES, and MIP-1 β . However, no serum changes consistent with the production of T cell cytokines (e.g. IL-2, IL-4, IL-5, IL-13, IL-17, IFN- γ) were observed (Figure 5B). This finding prompted an investigation of the impact of treatment on the tumor microenvironment.

By histology, we observed little difference in the architecture of tumors arising in mice receiving control PBS compared to *Listeria* vaccines with the exception that in mice treated with *Listeria* vaccines (Figure 6C), islands of tumor cells were found surrounded by regions of necrosis (Figure 6D). In addition, we observed a decrease in collagen deposition, detected by Masson's trichrome staining, in focal regions of the tumor microenvironment from mice treated with *Listeria* vaccines but not PBS control (Figure 6C). Finally, we detected a marked increase in CD31⁺ blood vessels along the periphery of tumors in mice treated with *Listeria* vaccines. This finding coupled with the necrosis observed centrally within tumors suggests that *Listeria* vaccine therapy, despite producing a marked necrosis, may not impact tumor growth as a result of compensatory tumor proliferation at the tumor margin facilitated by increased angiogenesis.

We next examined cell infiltration into tumors in response to treatment by first focusing our analysis on T cell subsets. By immunohistochemistry, we observed a lack of a T cell infiltrate in each of the treatment groups (Figure 7A). However, in the *Listeria* vaccine groups only, we did observe an increase in CD3⁺4⁺ and CD3⁺8⁺ T cells at the tumor margins (Figure 7B). Because the lack of T cell infiltrate could represent an absence of tumor antigen, we also evaluated for Her2 expression in tumors and determined that Her2 expression was maintained despite treatment with either Lm-LLO-E7 or Lm-LLO-Her2 (Figure 7C). This finding demonstrates that antigen-loss was not the mechanism responsible for a lack of T cell infiltration into tumors.

In our analysis of the tumor microenvironment, we have found no change in F4/80⁺ macrophage infiltration at 14 days after treatment with *Listeria* vaccine therapy. However, this finding can be deceiving as we have previously found that macrophage phenotype can shift within tumors in response to therapies such as an agonist CD40 antibody (3). In addition, macrophage infiltration may occur early after treatment and no longer be apparent at 14 days after treatment. Therefore, we are now investigating the impact of *Listeria* vaccine therapy on macrophage phenotype within tumors and exploring the impact of therapy at one day after treatment as described in Task 2 of the Statement of Work. To do this, we have already initiated studies in PBS control mice in which the phenotype of F4/80⁺ macrophages is examined using three color immunofluorescence imaging. In PBS control mice, we have found that F4/80⁺ macrophages display a unique signature, marked by activation of the STAT3 and NF-kB

signaling pathways, that is commonly associated with a pro-tumor phenotype (Figure 8) (13). We hypothesize that *Listeria* vaccine treatment may alter macrophage activation status which may be detected by examining for expression and nuclear translocation of the NF- κ B p65 protein which has been associated with an anti-tumor phenotype (13). Studies are underway to examine this hypothesis.

We have also initiated studies to understand the impact of *Listeria* vaccination on the tumor microenvironment. To do this, we have developed three and four color immunofluorescence imaging to detect changes in extracellular matrix (ECM) proteins and the presence of ECM-producing cells, namely α -SMA⁺ myofibroblasts (Figure 9). Our future studies will examine day +1 and day +14 after *Listeria* vaccine treatment to understand the impact of therapy on myofibroblasts and ECM proteins produced within the tumor microenvironment.

Our finding that *Listeria* vaccine therapy is unable to induce a productive T cell immune response in the KPC model suggests the presence of immunosuppressive mechanisms. To address this question, we propose to conduct a parallel study of *Listeria* vaccine therapy in a subcutaneous tumor model using Her2 expressing tumor cell lines that we have previously developed in the laboratory from KPC mice harboring spontaneously arising PDA tumors. This study would evaluate the ability of *Listeria* vaccine therapy to induce a productive T cell mediated anti-Her2 immune response capable of targeting Her2 expressing PDA cell lines. If T cell immunity can be generated with *Listeria* vaccine therapy in a subcutaneous tumor model, this would suggest the presence of immunosuppressive mechanisms present within KPC mice that either inhibit the development of Her2 immunity or restrain the infiltration of T cells into the tumor microenvironment. We also are currently evaluating Her2 peptides that may serve as tools to evaluate the development of a Her2 specific T cell immune response in KPC mice treated with *Listeria* vaccines. Finally, in separate on-going studies in the laboratory funded under a distinct mechanism, we have determined that CD40 agonists in combination with chemotherapy can induce T cell immunity in the KPC model. However, we have found that this T cell immune response that is induced with therapy is restrained by immunosuppressive cell subsets that inhibit T cell trafficking into tumor tissues. We believe that a similar mechanism may be occurring in our *Listeria* vaccine studies. Understanding this mechanism of T cell immune privilege orchestrated by PDA tumors will be critical for advancing T cell immunotherapy, including *Listeria* vaccines, for PDA.

KEY RESEARCH ACCOMPLISHMENTS: The following research accomplishments have emanated from this research over the past year:

1. Two manuscripts were submitted, accepted and are currently *in press* regarding macrophage targeted therapies for the treatment of cancer. These manuscripts were supported, in part, by the Department of Defense Discovery Award (see Reportable Outcomes section).

2. Summary of milestones related to Aim One investigating the ability of *Listeria* by-products to convert innate immunity from pro- to anti-tumor.

- a. We have established a process to label bone marrow derived macrophages for injection into KPC mice and demonstrated that they traffick to tumor tissues.
- b. We have identified a potential resistance mechanism to macrophage adoptive cell therapy which involves decreased tumor-derived chemokine production leading to reduced macrophage trafficking to tumor lesions.
- c. We have manufactured Listeriolysin O (LLO) and are currently manufacturing a detoxified form of LLO.
- d. We have determined that LLO is toxic to macrophages and are examining LLO formulated with cholesterol as a method of neutralizing this toxicity.
- e. We have preliminarily characterized the early serum cytokine response (day +1) induced with *Listeria* vaccination.

3. Summary of milestones related to Aim Two investigating the ability of live engineered *Listeria* vaccines to induce productive anti-tumor immunity.

- a. We have identified that tumor cell expression of the Her2 tumor antigen is variable within tumors and ranges from absent to high expression.
- b. We have manufactured the *Listeria* vaccines Lm-LLO-E7 (control vaccine) and Lm-LLO-Her2.
- c. We have treated cohorts of mice with PBS, Lm-LLO-E7, and Lm-LLO-Her2 and determined that treatment has little impact on tumor growth.
- d. We have characterized the systemic cytokine response induced with treatment and observed an absence of a productive T cell response.
- e. We have examined the tumor microenvironment of vaccinated mice and determined a lack of T cell infiltration induced with *Listeria* vaccine treatment which is not due to Her-2 antigen loss.
- f. We have identified tumor-associated stromal effects, including areas of necrosis and decreased collagen deposition, induced by *Listeria* vaccines. Conversely, we have also observed increased vascularity at tumor margins in tumors treated with *Listeria* vaccines which may explain the lack of treatment response associated with therapy.

REPORTABLE OUTCOMES:

1. Manuscript accepted at *Oncoimmunology* and currently in press. Manuscript is entitled "Harnessing the anti-tumor potential of macrophages for cancer immunotherapy". This Discovery Award from the Department of Defense was acknowledged.

citation: Long, K.L. and G.L. Beatty. Harnessing the anti-tumor potential of macrophages for cancer immunotherapy. *Oncoimmunology* 2013 *in press*.

2. Manuscript accepted at *Oncoimmunology* and currently in press. Manuscript is entitled "Macrophage immunotherapy for the treatment of pancreatic ductal adenocarcinoma". This Discovery Award from the Department of Defense was acknowledged.

citation: Beatty, G.L. Macrophage immunotherapy for the treatment of pancreatic ductal adenocarcinoma. *Oncoimmunology* 2013 *in press*.

CONCLUSION:

Summary of findings. The immune reaction associated with cancer is an innovative therapeutic target for the treatment of PDA because of 1) its inherent lack of genetic instability and 2) its decisive ability to impact tumor biology. In PDA, macrophages are the dominant tumor-infiltrating leukocyte and for this reason, we have directed our effort toward inducing macrophages with anti-tumor properties. We have found that tumor infiltrating macrophages are a critical determinant of the tumor microenvironment in PDA such that they can be harnessed to disrupt the stromal reaction that is a hallmark of PDA and to induce tumor regression (3). However, we have also found that macrophages can suppress the development of productive anti-tumor T cell immunity (14). Over the past year, we have explored the capacity of *Listeria* vaccines to induce productive anti-tumor immunity in a clinically relevant mouse of PDA. Our findings demonstrate a potential impact of *Listeria* vaccines on tumor biology with increased necrosis and a decrease in extracellular matrix proteins. However, these findings are also associated with increased vascularity along tumor margins and rapid tumor outgrowth. Unexpectedly, we observed an absence of T cell infiltration into tumor tissue after *Listeria* vaccination despite an abundance of Her2 tumor antigen expression within tumors. This finding demonstrates the impressive ability of PDA to establish a site of T cell immune privilege.

Similar to our findings with *Listeria* vaccination in KPC mice, we have recently found that vaccination with chemotherapy and an agonist CD40 antibody can drive a productive T cell immune response against tumors implanted subcutaneously into KPC mice but not against PDA lesions arising spontaneously within the KPC model (unpublished data). This finding also supports the concept of PDA as a site of T cell immune privilege. However, it is unclear at this time whether the inability of *Listeria* vaccines to produce T cell infiltration into PDA lesions in the KPC model is a result of locoregional immune suppression as is implied by our CD40 chemoimmunotherapy studies or systemic immune suppression that impedes the development of a productive Her2-specific T cell immune response. To address the role of immunosuppression in regulating *Listeria* vaccine efficacy, further studies will be necessary to understand the capacity of *Listeria* vaccines to induce Her2-specific T cell immunity. These studies can be performed by evaluating the T cell response induced with *Listeria* vaccination using *in vitro* cytotoxicity assays. In addition, it will be critical to understand the capacity of *Listeria* vaccines to induce T cell immunity capable of targeting Her-2-positive PDA cells *in vivo*. To do this, we propose to study the impact of *Listeria* vaccination in a subcutaneous implantation model using the Her-2-positive PDA cell lines that we have previously developed within our laboratory.

Our finding that *Listeria* vaccination can impact the stromal microenvironment by inducing necrosis with a loss of collagen deposition and enhanced vascularity is an intriguing finding. A loss of collagen with increased vascularity is an observation that has also been seen with sonic hedgehog inhibitors in PDA (8, 15). This finding may suggest the need to combine *Listeria* vaccines in PDA with anti-angiogenic approaches. To this end, over the course of the next year we will evaluate the impact of *Listeria*

vaccination designed to induce T cell immunity against CD105, a vascular endothelial protein expressed by tumor vasculature.

Macrophages are a promising therapeutic target for PDA. In our preliminary studies, we have found that macrophages selectively traffick to PDA lesions associated with fibrosis. This finding provides key insight into our studies investigating the adoptive transfer of macrophages activated *ex vivo* with *Listeria* by-products. Because of this finding, over the course of the next year we will begin our studies with macrophages activated with *Listeria* by-products by examining first the early impact of macrophage adoptive therapy at 1-3 days after transfer. We will use immunohistochemistry and immunofluorescence imaging to examine the impact of adoptive transfer on tumor biology and the extracellular matrix. In addition, we have now developed two *in vitro* assays that evaluate macrophage tumoricidal capacity and collagen degradation ability, respectively. We will use these assays to evaluate macrophages that are activated using *Listeria* by-products. We expect that this data will help guide the frequency with which macrophages will need to be administered to KPC mice to achieve an anti-tumor effect measurable by ultrasonography.

"So what section". The use of T cell immunotherapy in PDA has demonstrated modest to little effect in patients. Using a clinically relevant mouse model of PDA, our findings exploring *Listeria* vaccines are providing important insights into the immunobiology of PDA. Our results suggest that PDA is a site of T cell immune privilege. It will be critical to understand this barrier to productive T cell immunosurveillance in order to realize the potential of the adaptive immune system as a target for cancer therapy in PDA.

Macrophages can be decisive mediators of anti-tumor immunity. Because *L. monocytogenes* is macrophage tropic, *Listeria* vaccines are an exciting therapeutic approach for unleashing productive anti-tumor activity by macrophages. However, our results are demonstrating that the capacity of macrophages to mediate anti-tumor activity may be dependent on tumor biology such that tumor cell production of macrophage-recruiting chemokines may be essential. Understanding this biology using *Listeria* as a tool to activate macrophages with anti-tumor properties may offer improved insight into which tumors are most susceptible to macrophage-directed immunotherapy.

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DOD Discovery Award (W81XWH-12-1-0411)
Principal Investigator: Gregory L. Beatty
Listeria Vaccines for Pancreatic Cancer

APPENDICES:

Appendix 1: NIH Biosketch (Gregory L. Beatty, Principal Investigator)

Appendix 1

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME Gregory L. Beatty		POSITION TITLE Assistant Professor of Medicine	
eRA COMMONS USER NAME GBEATTY			
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)			
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University of Pennsylvania, Philadelphia, PA	Ph.D.	2000	Immunology
University of Pennsylvania, Philadelphia, PA	M.D.	2004	Medicine
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Resident in Medicine, Hospital of the University of Pennsylvania, Philadelphia, PA			Internal Medicine
Clinical Fellow, Hospital of the University of Pennsylvania, Philadelphia, PA			Hematology/Oncology

A. Personal Statement.

Dr. Beatty's research uses a genetically engineered mouse model of cancer to study macrophage biology within the tumor microenvironment. In his studies, he has applied advanced imaging strategies to this model. By doing so, Dr. Beatty has developed a research platform within his laboratory that allows for the study of basic immune biology within the tumor microenvironment as well as the rapid screening of novel immunotherapeutic strategies, including cell and gene therapies, for the treatment of cancer. Dr. Beatty's research approach has led to the discovery that macrophages can be recruited to degrade the stromal matrix that is a critical scaffold for growing tumors arising within the pancreas. He has successfully translated these findings to the clinic in a Phase I clinical study. Based on these findings Dr. Beatty's laboratory is using preclinical models of cancer to advance our understanding of the role of macrophages in regulating tumor biology with the primary goal to inform the development of novel immunotherapeutics for translation to the clinical setting.

B. Positions and Honors**Positions and Employment**

2004-2006	Residency in Medicine, Hospital of the University of Pennsylvania, Philadelphia, PA
2006-2010	Fellow in Hematology/Oncology Division, Hospital of the University of Pennsylvania, Philadelphia, PA
2010-2012	Instructor in Hematology/Oncology Division, Hospital of the University of Pennsylvania, Philadelphia, PA
2012-	Assistant Professor of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA

Other Experience and Professional Memberships

2008- Associate member, American Society of Clinical Oncology
2008- Active member, American Association for Cancer Research

Medical License History

2007 Board certified in Internal Medicine
2010 Board certified in Medical Oncology

Honors:

1993 GTE Academic All-American, Bucknell University
1994 GTE Academic All-American, Bucknell University
1994 Tau Beta Pi Honor Society, Bucknell University
1995 Summa Cum Laude, Bucknell University
1995 Phi Beta Kappa Award, Bucknell University
1995 The President's Award for superior academic achievement, Bucknell University
1995 Sigma Xi, The Scientific Research Society, Bucknell University
1995 Alpha Chi Sigma Fraternity Prize, Bucknell University
2000 Rose Meadow Levinson Memorial Prize for meritorious research in cancer, University of Pennsylvania
2004 The William Osler Society of Fellows in Medicine, University of Pennsylvania
2007 Amgen Hematology and Oncology Fellowship Award
2008 ECOG Young Investigator Symposium Awardee
2009 ASCO Cancer Foundation Young Investigator Award
2010 ASCO Cancer Foundation Merit Award
2012 Damon Runyon-Rachleff Innovation Award
2012 Burroughs Wellcome Career Development Award Finalist
2012 W.W. Smith Charitable Trust Medical Research Award
2013 AAAS Martin and Rose Wachtel Cancer Research Award Finalist
2013 Doris Duke Charitable Foundation Clinical Scientist Development Award

C. Selected peer-reviewed publications or manuscripts in press (in chronological order)

1. **Beatty, G.L.**, and Y. Paterson. IFN-gamma can promote tumor evasion of the immune system *in vivo* by down-regulating cellular levels of an endogenous tumor antigen. *J. Immunol.* 2000; 165:5502-5508.
2. **Beatty, G.L.**, and Y. Paterson. Regulation of tumor growth by IFN-gamma in cancer immunotherapy. *Immunologic Research.* 2001; 24: 201-210.
3. **Beatty, G.**, and Y. Paterson. IFN-gamma-dependent inhibition of tumor angiogenesis by tumor-infiltrating CD4+ T cells requires tumor responsiveness to IFN-gamma. *J. Immunol.* 2001; 166:2276-2282.
4. *Dominiecki, M.E., ***Beatty, G.L.**, Pan, Z-K, Neeson, P., and Y. Paterson. Tumor sensitivity to IFN-gamma is required for successful antigen-specific immunotherapy of a transplantable mouse tumor model for HPV-transformed tumors. *Cancer Immunol Immunother.* 2005; 54:477-488. * **Contributed equally to the work**
5. Fowler, D.H., Odom, J., Steinberg, S.M., Chow, C.K., Foley, J., Kogan, Y., Hou, J., Gea-Banacloche, J., Sportes, C., Pavletic, S., Leitman, S., Read, E.J., Carter, C., Kolstad, A., Fox, R., **Beatty, G.L.**, Vonderheide, R.H., Levine, B.L., June, C.H., Gress, R.E., and M.R. Bishop. Phase I clinical trial of costimulated, IL-4 polarized donor CD4+ T cells as augmentation of allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant.* 2006; 12:1150-1160.

6. Domchek, S.M., Recio, A., Mick, R., Clark, C.E., Carpenter, E.L., Fox, K.R., DeMichele, A., Schuchter, L.M., Leibowitz, M.S., Wexler, M.H., Vance, B.A., **Beatty, G.L.**, Veloso, E., Feldman, M.D., and R.H. Vonderheide. Telomerase-specific T-cell immunity in breast cancer: effect of vaccination on tumor immunosurveillance. *Cancer Research*. 2007; 67:10546-10555.
7. **Beatty, G.L.**, and B.J. Giantonio. Bevacizumab and oxaliplatin-based chemotherapy in metastatic colorectal cancer. *Expert Rev. Anticancer Ther*. 2008; 8:683-688.
8. **Beatty, G.L.**, and R.H. Vonderheide. Telomerase as a universal tumor antigen for cancer vaccines. *Expert Rev. Vaccines*. 2008; 7:881-7.
9. Clark, C.E., **Beatty, G.L.**, and R.H. Vonderheide. Immunosurveillance of pancreatic adenocarcinoma: insights from genetically engineered mouse models of cancer. *Cancer Letters*. 2009; 279:1-7.
10. Carpenter, E.L., Mick, R., Rech, A.J., **Beatty, G.L.**, Colligon, T.A., Rosenfeld, M.R., Kaplan, D.E., Chang, K.M., Domchek, S.M., Kanetsky, P.A., Fecher, L.A., Flaherty, K.T., Schuchter, L.M., and R.H. Vonderheide. Collapse of the CD27+ B-cell compartment associated with systemic plasmacytosis in patients with advanced melanoma and other cancers. *Clin Cancer Res*. 2009; 15:4277-87.
11. **Beatty, G.L.**, Smith, J.S., Reshef, R., Patel, K.P., Colligon, T.A., Vance, B.A., Frey, N.V., Johnson, F.B., Porter, D.L., and R.H. Vonderheide. Functional unresponsiveness and replicative senescence of myeloid leukemia antigen-specific CD8+ T cells after allogeneic stem cell transplantation. *Clin Cancer Res*. 2009; 15:4944-53.
12. **Beatty, G.L.**, Chiorean, E.G., Fishman, M.P., Saboury, B., Teitelbaum, U.R., Sun, W., Huhn, R.D., Li, D., Sharp, L.L., Torigian, D.A., O'Dwyer, P.J., and R.H. Vonderheide. CD40 agonists alter tumor stroma and show efficacy against pancreatic carcinoma in mice and humans. *Science*. 2011; 331:1612-16.
13. Rhim, A.D., Mirek, E.T., Aiello, N.M., Maitra, A., Bailey, J.M., McCallister, F., Reichert, M., **Beatty, G.L.**, Rustgi, A.K., Vonderheide, R.H., Leach, S.D., and B.Z. Stanger. EMT and dissemination precede pancreatic tumor formation. *Cell*. 2012; 148:349.
14. Bayne, L.J., **Beatty, G.L.**, Jhala, N., Clark, C.E., Rhim, A.D., Stanger, B.Z., and R.H. Vonderheide. Tumor-derived granulocyte-macrophage colony stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. *Cancer Cell*. 2012; 21:822.
15. Vonderheide, R.H., Bajor, D.L., Bayne, L.J., and **G.L. Beatty**. CD40 immunotherapy for pancreatic cancer. *Cancer Immunol Immunother*. 2013; 62:949.
16. Maus, M.V., Haas, A.R., **Beatty, G.L.**, Albelda, S.M., Levine, B.L., Liu, X., Zhao, Y., Kalos, M., and C.H. June. T cells expressing chimeric antigen receptors can cause anaphylaxis in humans. *Cancer Immunology Research*. 2013; 1:1-6.
17. **Beatty, G.L.**, Torigian, D.A., Chiorean, E.G., Saboury, B., Brothers, A., Alavi, A., Troxel, A.B., Sun, W., Teitelbaum, U.R., Vonderheide, R.H., and P. O'Dwyer. A phase I study of an agonist CD40 monoclonal antibody (CP-870,893) in combination with gemcitabine in patients with advanced pancreatic ductal adenocarcinoma. *Clin Cancer Res*. 2013; in press.
18. **Beatty, G.L.** Macrophage immunotherapy for the treatment of pancreatic ductal adenocarcinoma. *Oncoimmunology* 2013; in press.
19. Long, K.L. and **G.L. Beatty**. Harnessing the anti-tumor potential of macrophages for cancer immunotherapy. *Oncoimmunology*. 2013; in press.

D. Research support

Active Research Support

K08-CA138907

NIH

"CD40 pathway in pancreatic adenocarcinoma"

The goal of this grant is to evaluate the ability of CD40 agonists to induce anti-tumor immunity in pancreatic adenocarcinoma. This award provides salary support for the PI.

Role: Principal Investigator

Beatty, G.L. (PI)

08/01/2011 – 07/31/2016

Damon Runyon-Rachleff Innovation Award Damon Runyon Cancer Research Foundation "Targeting macrophages for cancer therapy" The goal of this grant is to investigate strategies to engineer macrophages with anti-tumor properties and to be resistant to the immunosuppressive signals produced within the tumor microenvironment. Role: Principal investigator.	Beatty, G.L. (PI) 07/01/2012 – 06/30/2015
Department of Defense Discovery Award "Listeria vaccines for pancreas cancer" The goal of this grant is to investigate the role of <i>Listeria</i> vaccines in stimulating productive innate and adaptive anti-tumor immunity in pancreas cancer. Role: Principle Investigator	Beatty, G.L. (PI) 09/30/2012 – 09/29/2014
Clinical Grant The Lustgarten Foundation and the Cancer Research Institute "Mesothelin-specific chimeric antigen receptor (CAR) T cell therapy for pancreatic cancer" The goal of this grant is to investigate the safety and feasibility of administering mesothelin-specific CAR T cells to patients with chemotherapy refractory pancreatic cancer. Role: Co-Principal Investigator	Beatty, G.L. (co-PI) 07/01/2013 - 06/30/2014
Medical Research Grant W.W. Smith Charitable Trust "Regulation of the tumor microenvironment by macrophages" The goal of this grant is to understand the mechanism by which macrophages regulate stromal fibrosis within the tumor microenvironment and to explore the ability of macrophages to remodel the tumor microenvironment for improved intravascular delivery of chemotherapy. Role: Principal Investigator	Beatty, G.L. (PI) 01/01/2013 - 12/31/2013
Clinical Scientist Development Award Doris Duke Charitable Foundation "A role for peripheral blood monocytes in regulating tumor biology in pancreatic cancer" The goal of this grant is to examine the interaction between monocytes and tumor cells in directing tumor metastasis in patients with advanced pancreatic carcinoma. Role: Principal Investigator	Beatty, G.L. (PI) 07/01/2013 - 06/30/2016

SUPPORTING DATA:

Figure 1. Adoptive transfer of bone marrow derived macrophages (BMDM) in KPC mice. (A) Bone marrow cells harvested from wild-type (WT) C57BL/6 mice were cultured *in vitro* in the presence of M-CSF for 7 days to generate BMDM. (B) BMDM were stained with antibodies recognizing F4/80, CD115, and Ly6C with comparison to a control antibody and analyzed by flow cytometry. (C) Tumor tissues from KPC mice on a C57BL/6 background were analyzed by immunofluorescence imaging 72 hours after adoptive transfer of Dil⁺ BMDM (red) and stained for EpCAM (green). Shown are areas of a tumor marked by extensive fibrosis (I, stromal) and a lack of fibrosis (II, non-stromal). (D) Hematoxylin and Eosin (H&E) staining and immunohistochemistry for F4/80 on PDA tumors from treatment naive KPC mice showing tumor regions characterized as stromal (I) and non-stromal (II). (E) A heat map showing the production (pg/mL) of cytokines and chemokines detected in serum-free tumor-conditioned media from individual PDA cell lines derived from KPC mice.

Figure 2. *Ex vivo* activation of BMDM with *Listeria* by-products for macrophage adoptive cell therapy. (A) Listeriolysin O was manufactured, purified, and analyzed by SDS-PAGE. Coomassie blue staining of the gel shows detection of the LLO protein (58 kDa) with minimal degradation products. Endotoxin levels using an LAL gel clotting test revealed levels of <0.02 EU/mg. (B) *In vitro* macrophage activation schema showing stimulation of BMDM for 24-48 hours. (C) BMDM (1e5 cells) were primed with or without IFN- γ for 18 hours followed by stimulation with LPS (1 μ g/mL), LLO-1 (1 μ g/mL), LLO-2 (10 μ g/mL), LLO-3 (100 μ g/mL), or HKLM (1e8 CFU/mL). Shown is cytokine bead array (CBA) flow cytometric analysis for cytokines detected in culture supernatants 48 hours after BMDM stimulation. (D) Shown is BMDM viability determined by crystal violet blue staining 24 hours after stimulation with Media, LPS (1 μ g/mL), HKLM (1e8 CFU/mL), or LLO (1, 10, or 100 μ g/mL).

Figure 3. Heterogeneous Her-2 expression on PDA tumors in the KPC model. (A) Representative image of PDA lesion stained for IgG vs Her2/neu (green). Nuclei are stained with DAPI (blue). Red arrowhead shows an area of strong Her-2 staining whereas white arrowhead shows an area of weak/absent Her-2 staining. (B) Four PDA cell lines derived from the KPC model were stained with an anti-Her-2 or control antibody to detect surface expression of Her-2.

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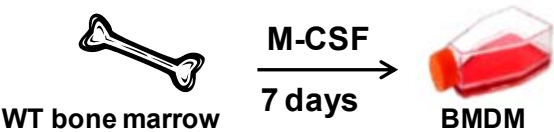
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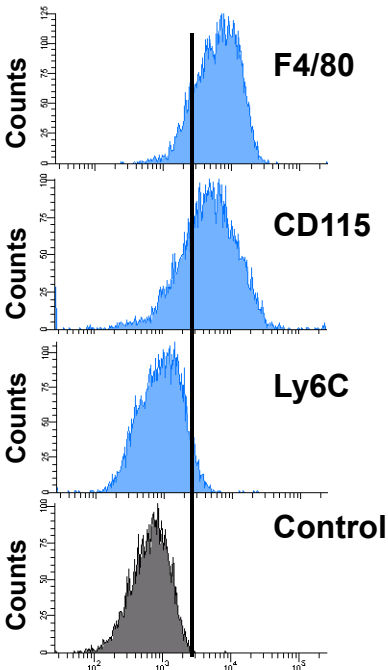
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Figure 1

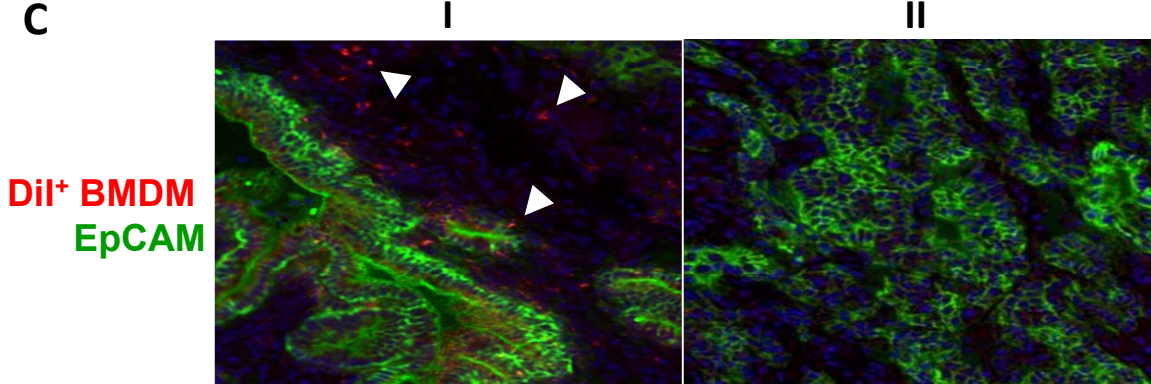
A



B



C



D

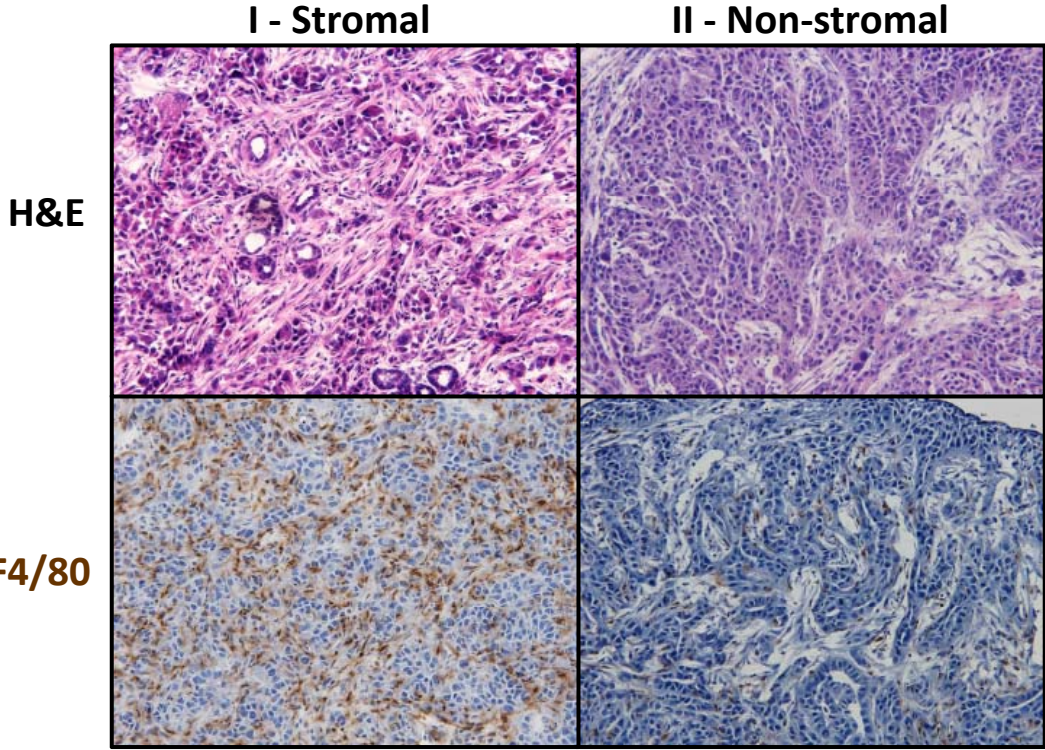


Figure 1 (cont'd)

E

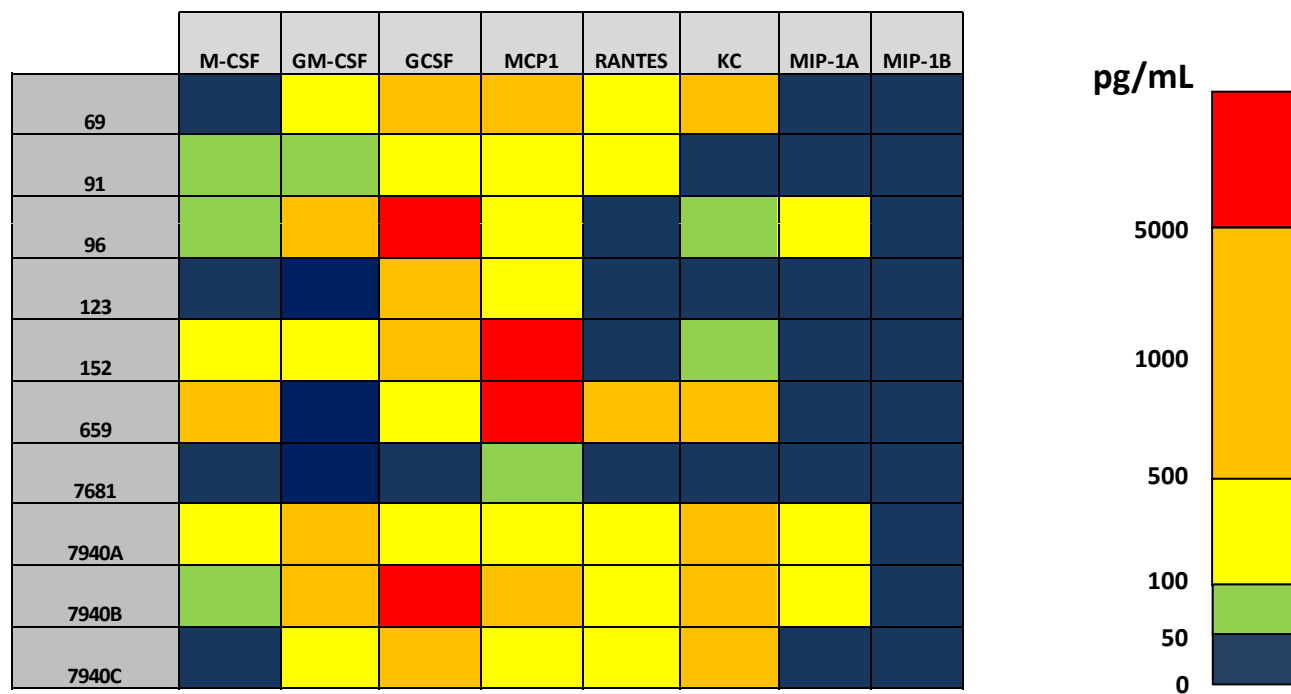
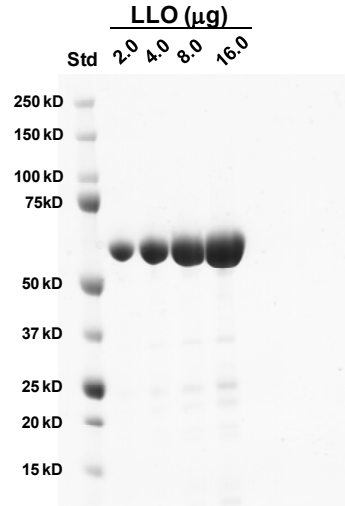


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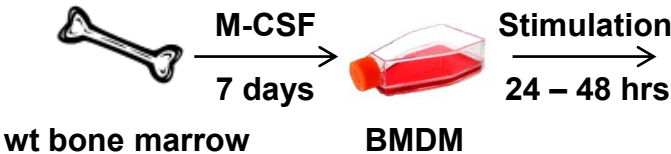
Figure 2

A

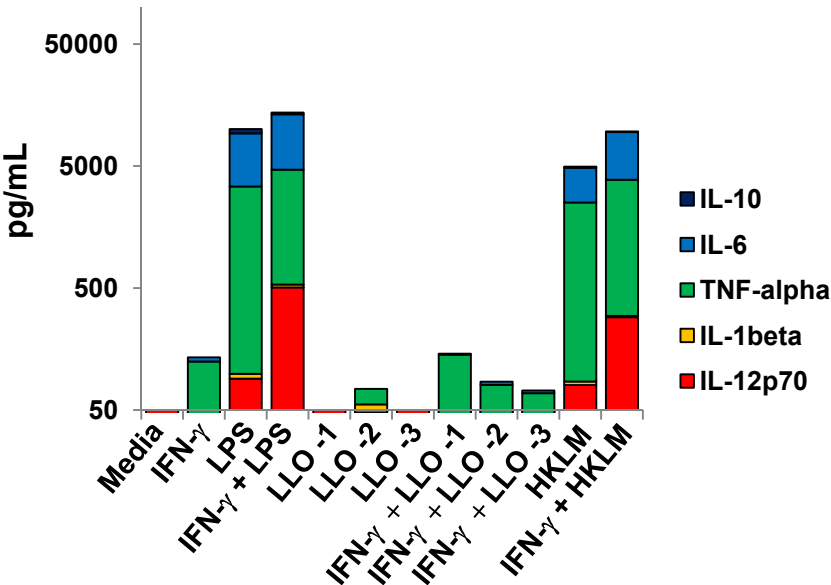


Recovery: 52.8 mg
Purity: 0.02 EU/mg

B



C



D

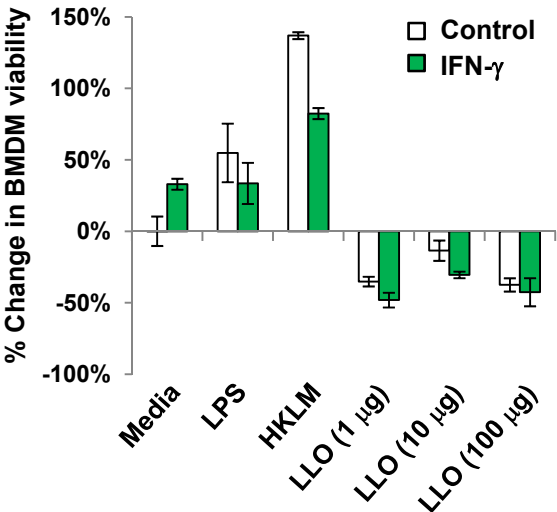
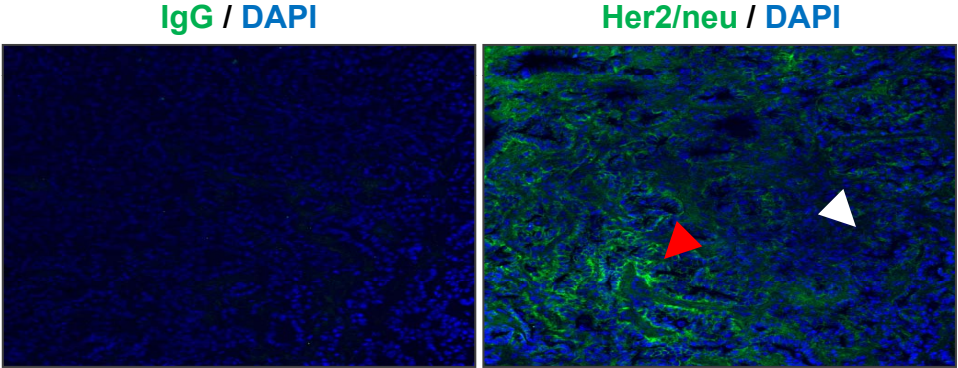


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Figure 3

A



B

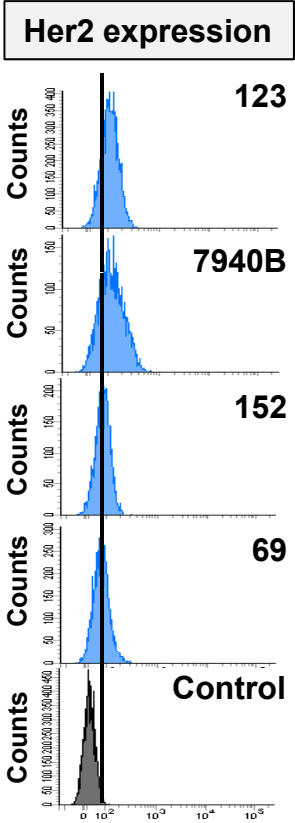
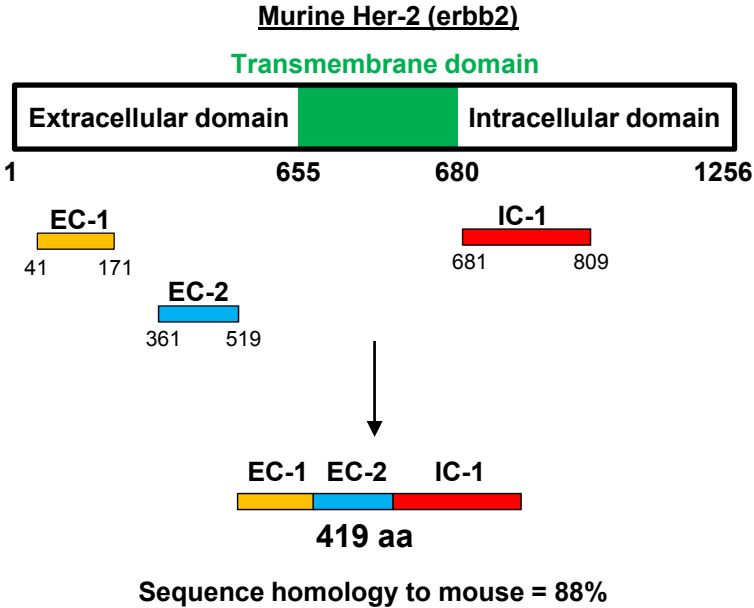


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Figure 4

A



B

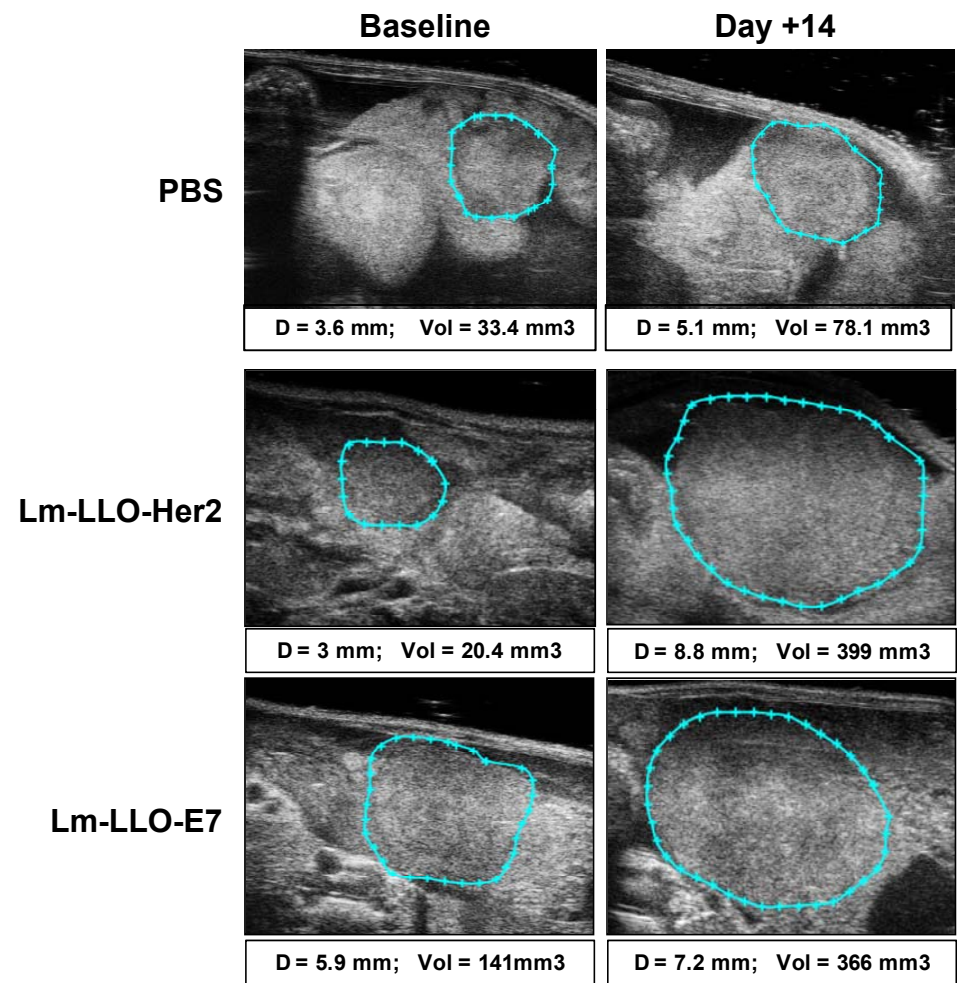


Group	Treatment	Ref
1	PBS	n/a
2	Lm-LLO-E7	J.Immunol 167:6741 (2001)
3	Lm-LLO-Her2	Clin Cancer Res 15:924 (2009)

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Figure 5

A



B

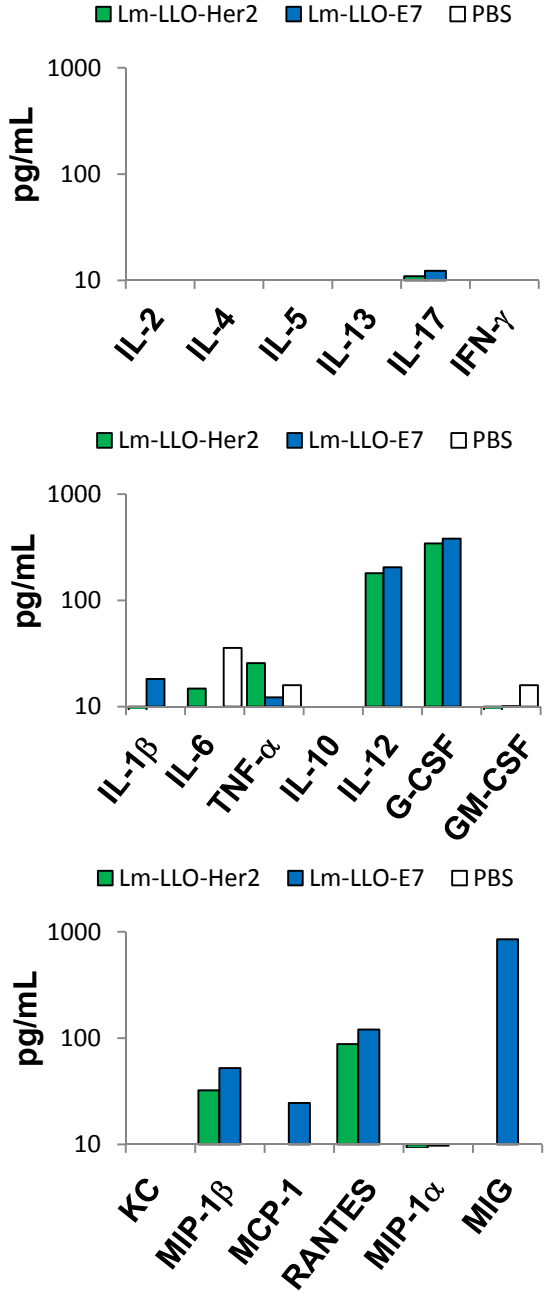


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Figure 6

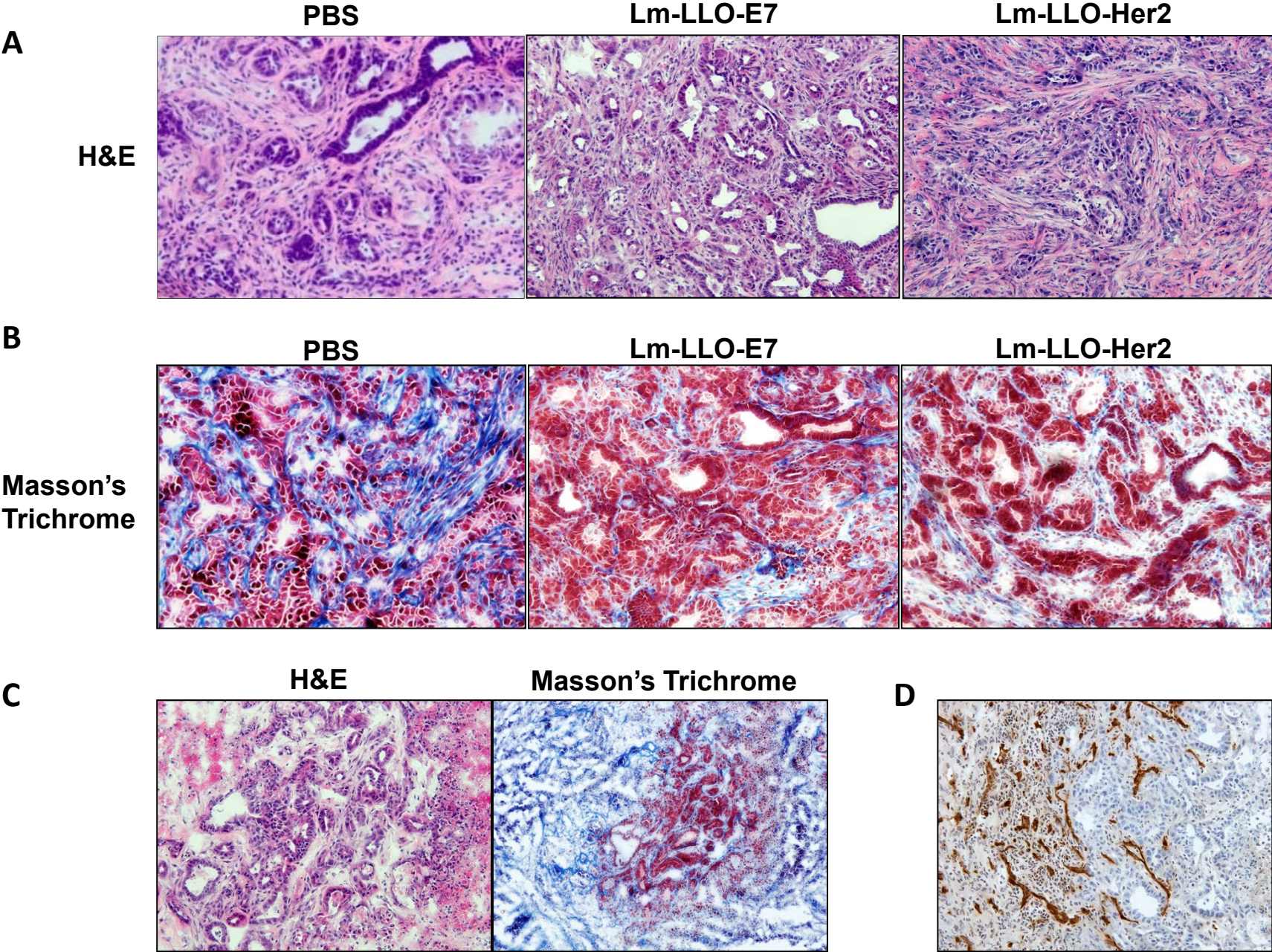
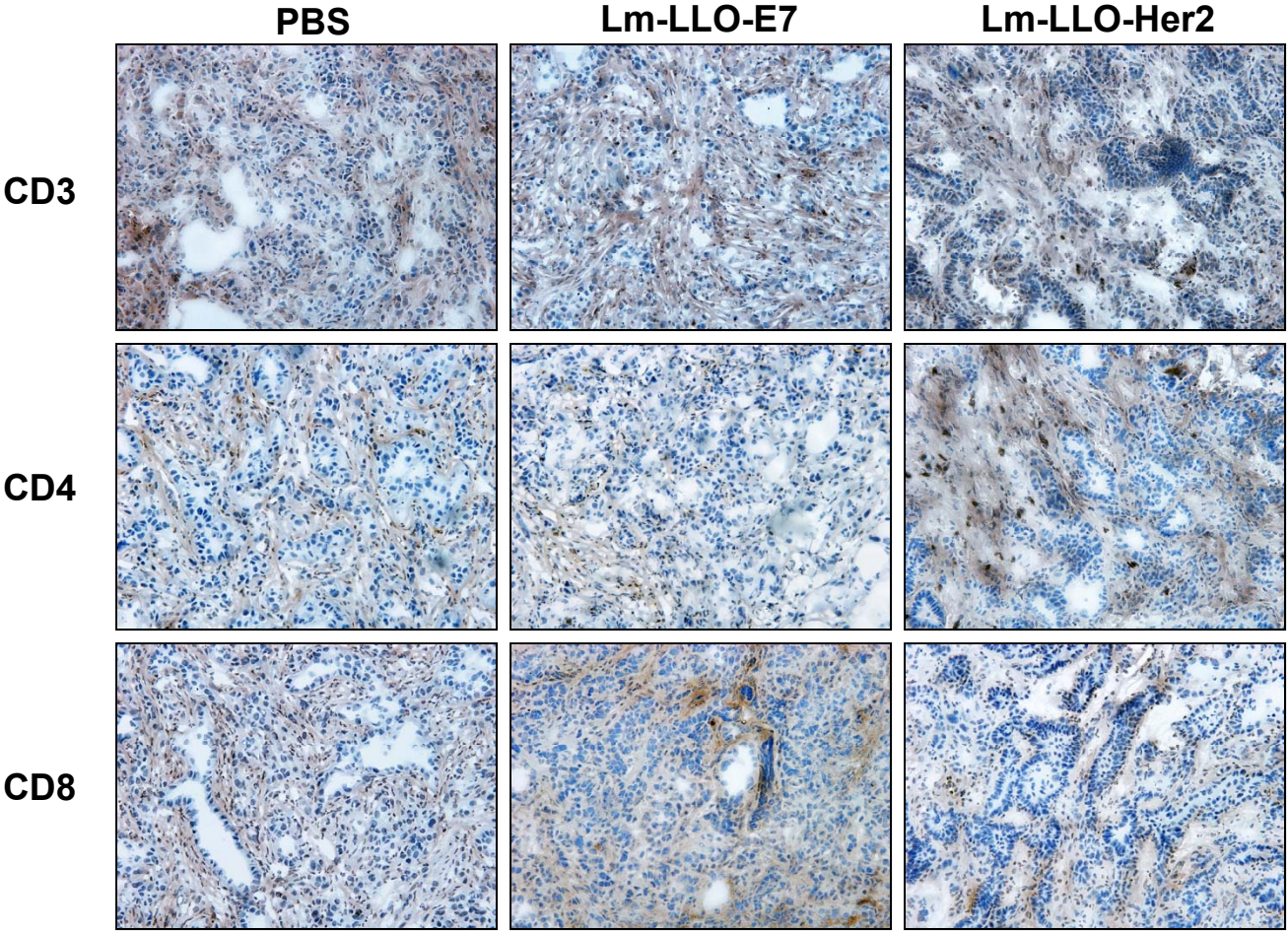


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Figure 7

A



B

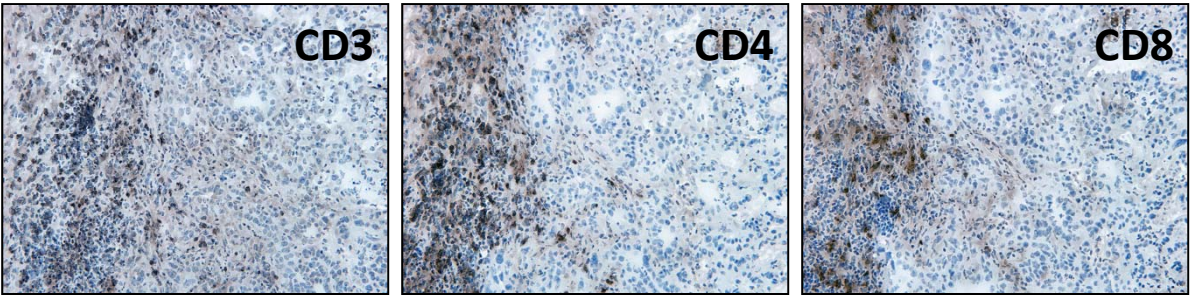


Figure 7 (cont'd)

C

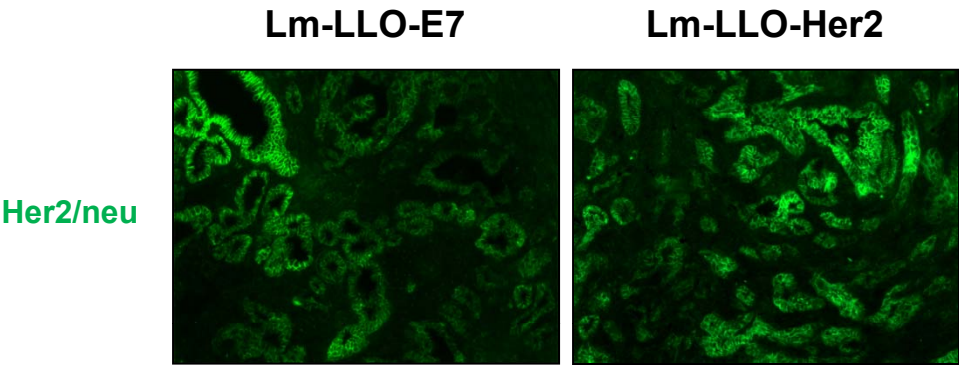
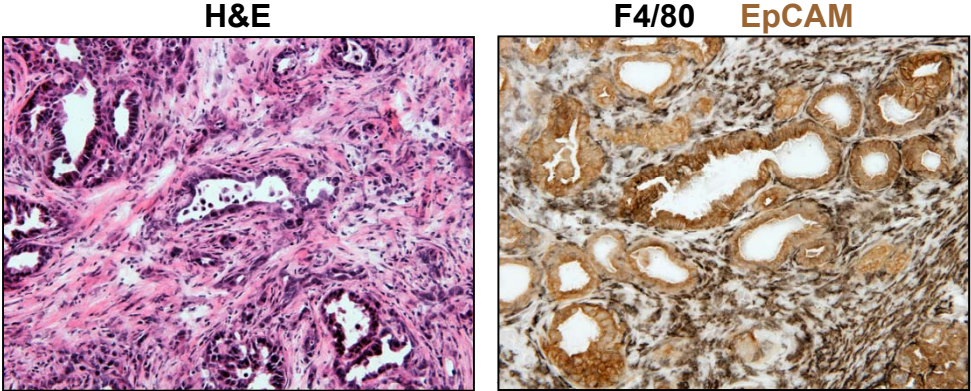


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Figure 8

A



B

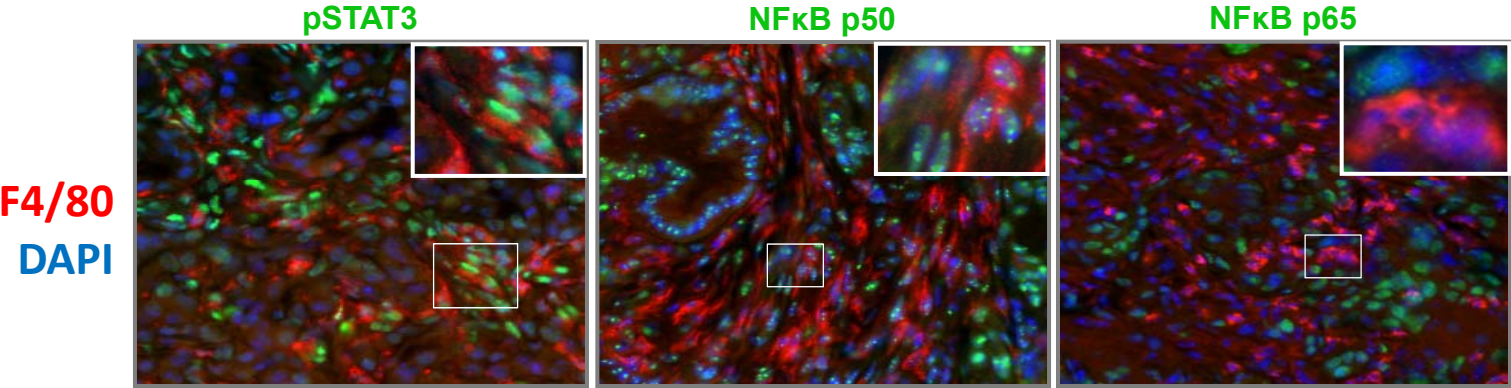


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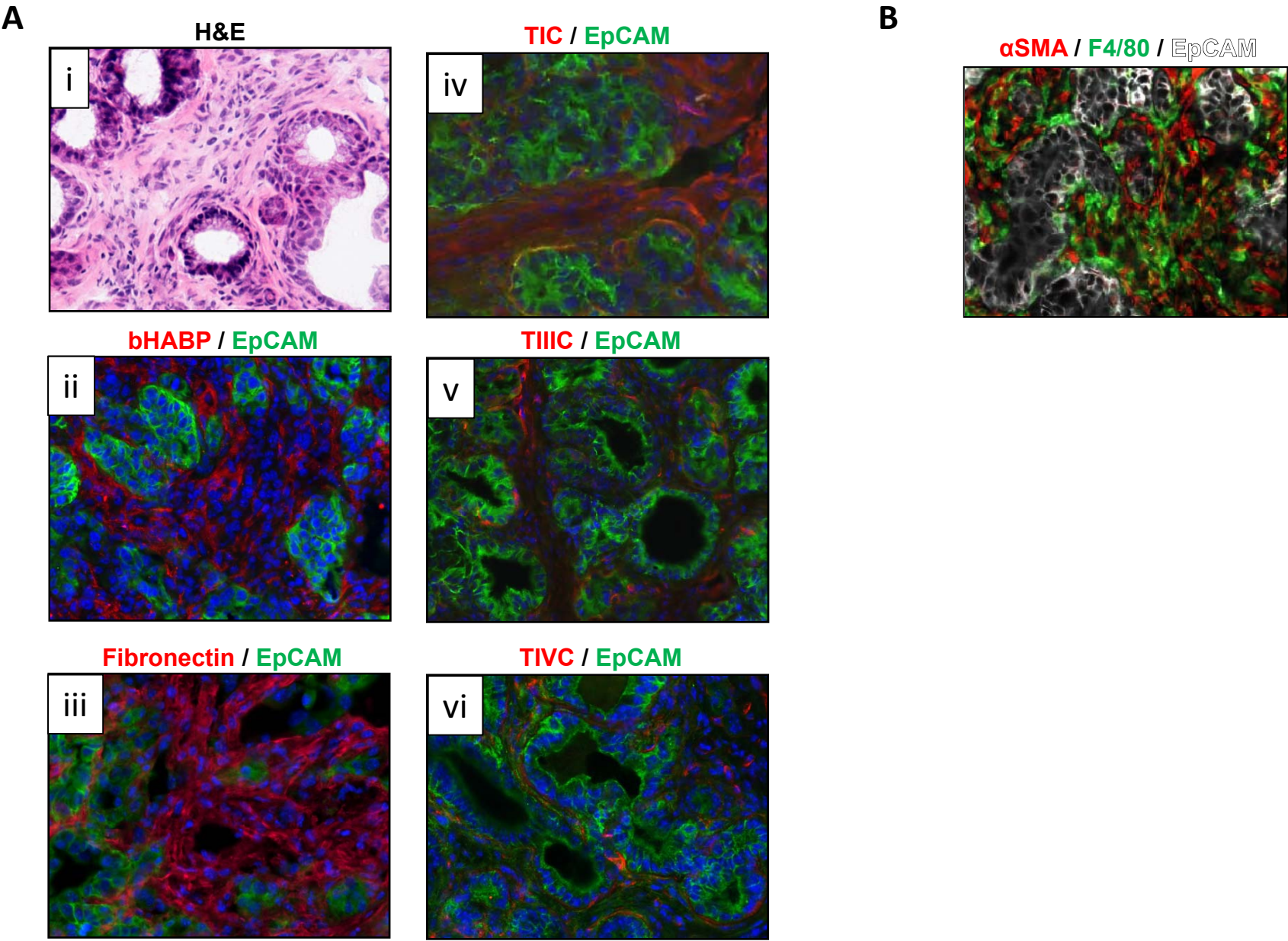


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