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Induction of Anti-Tumor Immunity in a Immune Checkpoint Inhibitor Resistant Murine Lung Cancer

By

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B.S., Emory University, 2016

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Advisor: Dr. Periasamy Selvaraj, Ph.D.

An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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2017

Abstract

FDA approved immune checkpoint inhibitors (ICIs), such as monoclonal antibodies for PD-1 and CTLA-4, have shown efficacy in abrogating tumor growth, as well as in improving survival and prognosis in a variety of preclinical and clinical studies. However, only a small fraction of the patient population is responsive to immune checkpoint blockade. One of the reasons suggested to account for this selective efficacy of immune checkpoint blockade therapy is the lack of pre-existing anti-tumor immunity in these patients.

In this study, we hope to generate a de novo, T cell mediated anti-tumor immune response in an ICI resistant murine Lewis Lung Carcinoma model through Tumor Membrane Vesicle (TMV) based vaccines. We further enhance the TMV mediated anti-tumor immune response by “stepping on the gas” using various vaccine adjuvants such as TLR agonists to induce DC maturation and enhance activation of tumor specific T cells. Lastly, we combine TMVs, adjuvants and ICI therapy to create “TMV+ Adjuvant+ ICI” vaccines to induce protection against tumor challenge and/or abrogation of existing tumors in vivo. Our results in the LL/2 model suggest that this tumor model is resistant to both checkpoint blockade and/or TMV vaccines adjuvated with common synthetic adjuvants.

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“A truly great mentor is hard to find, difficult to part with and impossible to forget.”

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knowledge. More importantly, thank you both for grooming Nanditha and I into self-confident, conscientious and responsible citizens of the world: You both are our greatest strength. Thank you to my sister, Nanditha, for being the light that brightens my day. Amumma, and the rest of my family in India, thank you for your blessings and continued support.

Lastly, I would like to dedicate this thesis to my grandfather, my dearest Appuppa, who instilled in me the love for science and reading, and taught me the importance of serving the community. Thank you for being my greatest inspiration.

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Introduction

"A disease that, starting from an insignificant injury, can attack a person in perfect health, in the full vigor of early maturity, and in some insidious, mysterious way, within a few months, destroy life, is surely a subject, important enough to demand our best thought and continued study [11] ."

- Dr. William D. Coley, commenting about the importance of studying "malignant disease" in *II. Contribution to the Knowledge of Sarcoma , 1891*

In the late 1800s, as a young surgeon, William D. Coley, was deeply distraught by the death of one of his earliest patients, a young girl, to metastatic sarcoma [11]. Instilled with a vengeance to find better therapy, and inspired by historical accounts of cancer patients being "cured" of their disease after a concomitant infection of erysipelas, a bacterial infection that produces a skin rash, Coley set up an unconventional treatment paradigm for "malignant disease": he administered his cancer patients with a cocktail of bacterial products – "Coley's Toxins" - and observed over half of his patients' diseases regress, successfully treating many cancer cases during his lifetime[12]. His courageous "continued study" into "malignant disease", allowed him to hypothesize about the immune system's ability to combat cancer, and inspired the field of cancer immunotherapy which has today evolved from the realm of alternative medicine to main-stream therapy by providing potent treatment options for cancer patients that have failed and/or are resistant to standard of care therapies.

Cancer immunotherapy harnesses the power of the host's immune system and strategically directs it towards selectively attacking and eliminating transformed, neoplastic cells. Over the years, a wide array of immunotherapy approaches have shown to improve cancer treatment paradigms - ranging from using monoclonal antibodies such as a HERceptin [13] to curb unrestrained, self-sufficient growth signaling[14] that drives HER-2 positive breast cancer growth, to employing Chimeric Antigen Receptor T cells, or CAR-T Cells to mount potent cytolytic response against lymphoma and multiple myeloma[15, 16], to using antibodies to overcome immunosuppressive properties of immune checkpoints such as PD-L1/ PD-1 and CTLA-4.[17-19]

Tumor Associated Antigens in Cancer Immunotherapy

Another branch of active immunotherapy involves the use of therapeutic cancer vaccines – carriers of tumor associates antigens – to mount long lasting, adaptive, cell-mediated anti-tumor immunity in vivo. [20-26]Cancer vaccines rely on boosting the immune system's ability to recognize the tumor through delivering tumor associated, or tumor specific antigen to Antigen Presenting Cells of the immune system [20-26].

During the step wise mutagenesis and genetic instability that underlies carcinogenesis, genetic and epigenetic changes occur, giving rise to specific antigenic signatures associated to tumors that may not be present in healthy, non-transformed cells. [14, 27]Such antigens mark the tumor's carcinogenic phenotype and are referred to as Tumor Associated Antigens, or TAAs.[28-32] Tumor associated antigens are of different types – some are novel proteins, neoantigens, that arise on neoplasms such as the NY-ESO protein and MAGE in melanoma.[33] Others may be altered self-peptides such as the mutant receptor tyrosine kinase BCR-ABL that propagates aberrant growth signaling

in chronic myelogenous leukemia (CML).[34] TAAs can also be overexpressed endogenous proteins such as HER-2, the human epidermal growth factor receptor 2, whose amplification characterizes HER-2 positive breast cancer. [35]TAAs also come in the form of oncofetal antigens, which are also known as carcinoembryonic antigens or CEAs, which are expressed upon a shift towards de-differentiation of tumors, which is reflective of alternative gene expression profiles adopted by tumors. [36]To add to the TAA antigen repository, are also those antigens that have not been identified yet, adding diversity to the stockpile of Tumor Associated Antigens that can be used to prime the immune system to fight against cancer.

Thus, the step-wise mutagenesis that propagates the indefinite self-renewal and immortalization of cancer cells, functions as a silver lining by providing us with a resource to use against neoplastic propagation.[28-31] Therapeutic cancer vaccines use these TAAs to prime, boost, and generate an immune response against TAA expressing cancers. The presence of TAAs also allows vaccine based immunotherapy to be highly specific by eradicating only tumors cells harboring TAAs whilst sparing healthy cells – a favorable property that traditional cancer treatment approaches like chemotherapy fail to achieve.

The Cancer Immunity Cycle: The dynamics of an ideal anti-tumor response following vaccination with a TAA- carrying cancer vaccine

The ultimate aim of a therapeutic TAA based cancer vaccine is to induce the proliferation and activation of tumor antigen specific T cells- CD 4 and CD8 effector cells that immuno-survey and target cancer cells by inducing an anti-tumor cytokine milieu, antibodies against the tumor or by directly abrogating the tumor through cell lysis. Additionally, a successful vaccine may also activate Natural Killer cells, players of the

innate immune system that use their Fc receptors to engulf and lyse antibody opsonized tumor cells, and those that may downregulate the expression of MHC I from their surfaces.

The dynamics of an optimal immune response directed against a tumor are well summarized in Chen and Mellman's 2013 review article outlining the Cancer-Immunity Cycle, which is briefly described in Figure 1:

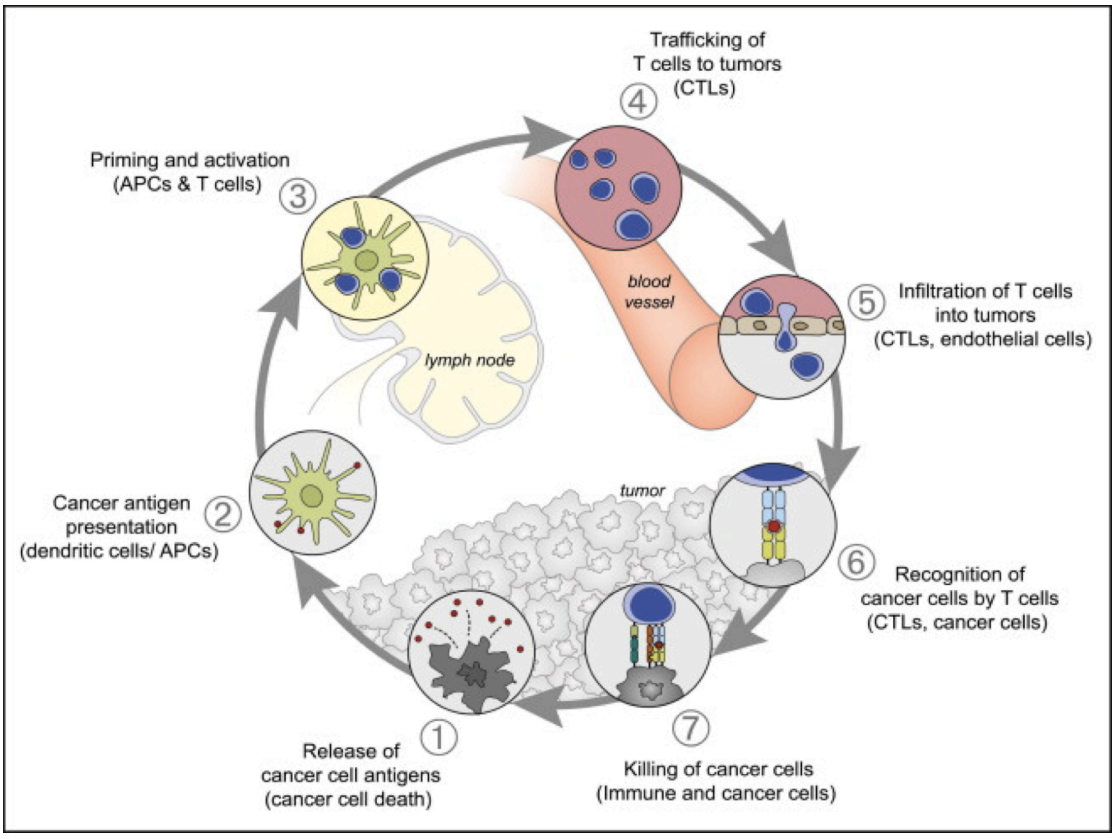


Figure 1: **The Cancer-Immunity Cycle** – step-wise induction of an anti-tumor immune response - as described by Chen and Mellman. Image sourced from [5, 6]

The cancer-immunity cycle is triggered by the presence of tumor antigens, either in the form of a TAA based cancer vaccine, or released upon necrotic tumor cell death in the immuno-tumor interactosome. These antigens are picked up by sentinel scavengers of the immune system, antigen presenting cells or APCs, such as macrophages, B cells and dendritic cells that are key initiators of the adaptive immune response. [6, 22, 28, 29, 31, 37-39] Tumor antigens are processed and converted into immunogenic peptide fragments, when APCs are activated by exogenous or endogenous immune response triggering signals such as the release of proinflammatory cytokines, the presence of a pathogen or trauma in vivo. APCs present processed antigen on their surfaces on specialized proteins called Major Histocompatibility Complexes, or MHCs, and migrate to the lymph nodes where they sojourn with naive T cells to activate them. [6, 22, 28, 29, 31, 37-39]

Upon entering the T cell zone of the lymph nodes, APCs that are matured and loaded with tumor antigen and costimulatory molecules, synapse with naive T cells harboring T-Cell Receptors (TCRs) complementary to the presented peptides on APCs. APCs activate T cells by providing them with three signals necessary for immunogenic activation: first, an antigen specific signal the form of the MHC – TAA peptide complex; second, co-stimulatory signals such as B7-1 (CD80) and B7-2 (CD86) that ligate with CD28 receptors on T cells to provide an essential growth signal, and third, a cytokine signal that polarizes and instructs the type of effector function that a naive T cell will take on. [6, 22, 28, 29, 31, 37-39]

MHC I – TAA peptide complexes on APCs along with appropriate co-stimulation activate CD8 T cells to become effector CD8 T cells or Cytotoxic T Lymphocytes (CTLs). Once activated, CTLs play the most important role in the anti-tumor response, since they have

the “license to kill”. CTLs infiltrate tumor microenvironments where they detect cells bearing tumor antigens they have been trained to recognize, and lyse the cell in one of two ways: by engaging the FAS ligand on tumor cells and causing them to apoptose or by releasing lytic enzyme such as perforin and granzyme B onto tumor cells that causes them to lyse [40].

MHC II–peptide complexes and costimulatory signals on APCs ligate with TCRs on Helper CD4 T cells, which turn into various types of effector T helper cells depending on the cytokine milieus crafted by DCs during antigen presentation. Effector CD4 T cells, the most prominent types being Th1, Th2, Th4 and Th17 cells, exhibit a wide range of effector functions. [6, 22, 28, 29, 31, 37-39, 41-46]

The type of CD4 Helper T cell response that is important for anti-tumor immunity is the Th1 response, which promotes the cell mediated arm of the adaptive immune system. Th1 cells form when naive CD4 T cells get activated by APCs in the presence of IL-12 in the environment. Once activated, CD4 Th1 cells secrete IFN-γ, which stimulates APCs like macrophages to increase phagocytosis and upregulate their lytic functions. More importantly, Th1 effectors induce proliferation of CD8 T cells, promote their cytolytic activity and induce formation of CD8 memory cells. Thus, Th1 CD4 T cells promote a cell mediated anti-tumor response. [6, 22, 28, 29, 31, 37-39, 41-46]

A Th2 response forms when a navie CD4 T cell is activated when the cytokines IL-4 and IL-2 secreted by DCs. Th2 effector cells release the cytokines IL-4, IL-5, IL-6 and IL-10 to guide their effector functions. Th2 responses are also beneficial, but are less favorable for anti-tumor immunity.

The tumor immunity cycle, once again is initiated when tumor antigen from lysed cells are released, and amplify the immune response by being picked up and presented yet again by antigen presenting cells, effectively creating a self-sustaining cycle of anti-tumor immunity. [47]Furthermore, every time this cycle is reinitiated, T cells expand rapidly in vivo, mobilizing an army of T cells specific to the tumor that infiltrate tumor tissues and directly and indirectly help abrogate tumor growth. Generating this cancer-Immunity cycle is the ultimate goal of any therapeutic (or prophylactic) cancer antigen based vaccine. [47]

Purpose and Hypothesis

The Cancer-Immunity cycle as described by Chen and Mellman[47] involves a step wise, systematic induction of an immune response against tumors that are susceptible to potential immune recognition by virtue of their modified antigenic profile, and fortuitous existence of tumor reactive T cells that have slipped through the restraints of central and peripheral tolerance.

We aim to enhance and "step on the gas" of the cancer immunity cycle by three interventions: Firstly, by increasing availability of cancer antigens to antigen presenting cells through TAA harboring Tumor Membrane Vesicle (TMV) constructs; Secondly, by enhancing the antigen presentation capacity of dendritic cells by using synthetic, commercially available immunostimulatory vaccine adjuvants such as TLR agonists, and finally, by "releasing the breaks" of immunosuppressive mechanisms in the immuno-tumor microenvironment by using immune checkpoint inhibitors- monoclonal antibodies against PD-1 and CTLA-4, which are both inhibitory receptors found on T cells.

We hypothesize that the unmodified, non-adjuvanted form of our Tumor Membrane Vesicle vaccine generates a suboptimal, antigen specific, anti-tumor immune response, which may be enhanced by using adjuvants to increase the immunogenicity and uptake of our vaccines by APCs. Furthermore, when combined with checkpoint blockade using anti-PD-1 and anti-CTLA-4 monoclonal antibodies, the vaccine effect may be enhanced further by preventing T-cell anergy mediated by PD-1/ PDL-1 or PDL-2 interactions at the tumor site, and CTLA-4/ CD80 or CD86 interactions at the site of T cell activation.

Intervention #1: Delivering Tumor Associated Antigens through Tumor Membrane Vesicles for the initiation of the Cancer-Immunity Cycle

Cancer vaccinologists have developed various strategies for capturing and delivering TAAs to antigen presenting cells, which kick start the cancer-immunity cycle. Peptide and protein vaccines [such as HER-2 peptide and protein vaccines for breast cancer[48], and gp100 peptide vaccines for melanoma[49]], DNA vaccines[50], recombinant peptide and DNA constructs[51], tumor cell lysates[52], irradiated whole cells[53], liposomes and nanoparticles encoding TAAs and directly pulsing DCs with tumor antigens[54] encompass just some of the strategies for delivering tumor antigen to the immune system.

Our laboratory's strategy for generating an anti-tumor immune response involves using Tumor Membrane Vesicles (TMVs), biocompatible lipid bilayer constructs generated from tumor tissue or cultured cell pellets, and serve as tumor antigen delivery platforms[55]. `TMVs capture the entire antigenic profile of the tumor: all surface anchored proteins – including tumor associated antigens and neoantigens that would have arisen during carcinogenesis – are delivered through TMVs, which are optimal in size for uptake by antigen presenting cells such as dendritic cells, the key players involved in generating the adaptive immune response.

TMVs are lipid bilayer plasma membrane vesicles prepared from homogenizing tumor tissue or cultured cancer cell pellets. This homogenate is then ultra-centrifuged through a 41% sucrose gradient that allows TMVs to accumulate in an interface between the sucrose and the tumor homogenate supernatant, whilst pelleting the cellular debris. [56-60]TMVs are about 300 to 500 nm in size [55]- and are heterogeneous in shape and size

(electron microscope images). This particulate antigen delivery scaffold exists as a cloudy suspension in the injection medium, and can be pelleted down to doses or amounts needed. They are easy to prepare and store – once prepared through the process described above, they can be aliquoted and frozen, or stored at 4 C for short-term usage. TMVs are convenient for storage and transport as a therapeutic in the future.

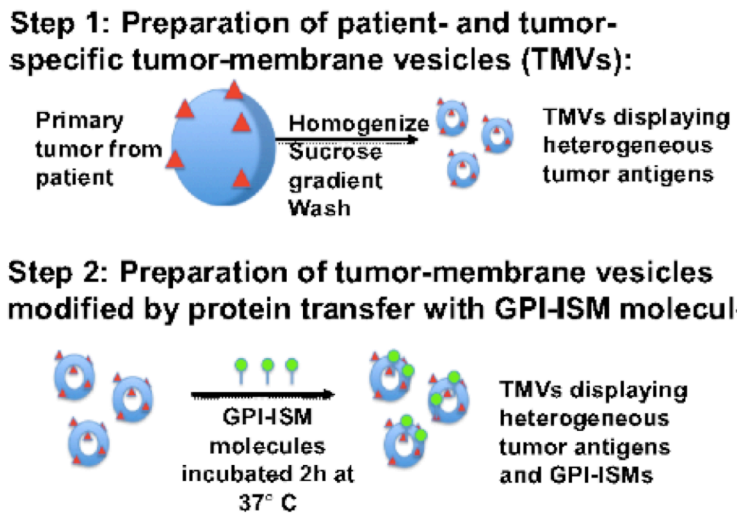
Preparing TMVs from tumor tissue allows us to firstly, generate a vaccine construct that is personalized, and unique to the patient's tumor. Secondly, TMVs created from whole tumor tissue allow us to target the entirety of the tumor – not just a few clones or selective populations that are sensitive to the chemotherapy or antigen vaccine paradigm. Creating a vaccine made out of whole tumor tissue allows us capture both the intra and inter clonal heterogeneity of tumors [61] effectively compiling an accurate and comprehensive antigenic signature of the tumors to deliver to the immune system to launch an attack against. In addition, TMVs can also harness cytosolic antigens that are presented on MHC molecules by tumors, and capture antigens on stromal cells in the tumor microenvironment, such as cancer associated fibroblasts, tumor associated macrophages[62] and myeloid derived suppressor cells[63] adding richness to the antigen repository to which the immune response will be mounted. This provides TMVs with the potential of targeting multiple, if not all possible genetic [64] and epigenetic [62] clones that may arise in the neoplastic site(s). Unlike peptide and DNA vaccines that can only prime a response against the selected antigen in the vaccine, and thus, may be rendered ineffective once the tumor clone harboring the vaccine TAA is abrogated or that TAA becomes mutated in vivo[65], TMV vaccines have the power to generate immunity against multiple, varied and a wide-ranging array of antigens expressed on tumors. This provides TMVs the unique advantage of adapting and customizing the

immune response in vivo according to the changing landscape of the tumor microenvironment.

Furthermore, TMVs can also be used as vaccine for cancers that do not have identified TAAs such as triple negative breast cancer in humans or the murine Lewis Lung Carcinoma, for which traditional cancer vaccine approaches such as peptide vaccines or monoclonal antibody based therapies cannot be employed since they are contingent upon knowledge of the tumor antigen. Because of its all-inclusive, multi-antigen harnessing capacities, TMVs can theoretically be employed without knowledge of the tumor's antigenic profile, and also activate immune responses against not just one, but all possible antigens represented by tumors.

Additionally, TMVs' particulate nature and size and prolonged persistence at vaccination site, optimize it for uptake by APCs. In our previous studies, TMVs have also shown to induce a depot effect at the site of vaccination – creating a site of high antigen concentration and prolonged persistence where approximately 50% of TMVs persist up to 6 days after vaccination.[66] Unlike MelanA/MART-1 peptide vaccines, that have a half life of a mere 22 seconds in vivo before being cleaved by endoproteases[67], TMV based vaccines provide a durable, long lasting and secure antigen carrying system. Thus, TMV's physical properties prevent premature antigen clearance before APCs have a chance to uptake them.

Furthermore, TMVs can also be modified to express a variety of immunostimulatory molecules such as the cytokine IL-12 to promotes T cell activation and proliferation in



GPI-ISM: GPI linked Immunostimulatory Molecule, such as GPI- IL-12

Figure 2: Protein Transfer of GPI-proteins onto TMVs
Figure created by, and used in this thesis with permission from Dr. Periasamy Selvaraj and Dr. Ramireddy Bommireddy, Emory University

vivo. [68]In the past, we have used the GPI anchored forms of the cytokine IL-12 to decorate TMVs, allowing us to create a biologically adjuvanted TMV vaccine that can generate potent anti-tumor immunity in vivo [55, 57, 59, 69]. Glycosyl Phosphatidylinositol is a gyloclipid anchor whose intracellular domain encoding signal sequence CD59 cDNA, can be recombinantly linked to IL-12 to convert it into a form that is amenable for incorporation into cell membranes. GPI-proteins spontaneously intercalate into lipid bilayers during a process called Protein Transfer that entails incubation of GPI-anchored proteins with the target cell or membrane vesicle of interest for two hours at 37°C. GPI-linked forms of proteins can be incorporated into cell membranes of live cancer cells [56] and membrane vesicle constructs [55-57, 69-72], both being strategies that our lab has used in the past.

Lastly, TMVs are biologically vacuous – unlike irradiated tumor cell based vaccines that continue to be biologically active in vivo and have shown to cause immunosuppression through the secretion of factors such as TGF-b upon vaccination[73], TMVs are devoid of cellular machinery, making them optimal carriers of tumor antigens.

Thus, Tumor Membrane Vesicles form optimal scaffolds for introducing and delivering tumor associated antigen to the immuno-cancer interactosome, and contribute to the initiation of the cancer immunity cycle.

Intervention #2: Enhancing APC maturation and antigen presentation by co-administration of TMVs with vaccine adjuvants

Through this study, we hope to enhance the antigen-specific immune response generated by TMVs by co-administering our TMV constructs with synthetic, commercially available immunostimulatory vaccine adjuvants such as alum and MF59 and TLR agonists. Through this intervention, we hope to *"adjuvare*", or to help, the immune response by enhancing the maturation of dendritic cells (via TLR agonists such as MPL-A and CpG); by inducing increased antigen uptake via the depot effect (created by alum based adjuvants), or by attracting immune cells towards the vaccination site via chemokine inducing emulsions (such as MF59).

A wide variety of adjuvants were surveyed, and seven – *Alum/Alhydrogel, MPL-A, CpG, Poly I:C, Imiquimod, Saponin/Quil A and AddaVax/MF59* - were chosen for use in this study based on the following criteria: FDA approval status [*Alum/Alhydrogel, MPL-A, Imiquimod, MF59/AddaVax]*, ability to induce a Th1 response [*MPL-A, CpG, Poly I:C, imiquimod, AddaVax, Saponin/QuilA*] and novelty in co-administration with cancer vaccines [MF59]. The adjuvants being used in this study can be broadly classified into

three categories according to their mechanism of action: DC maturation and T cell activation stimulating TLR agonists *MPL-A, CpG, Poly I:C and Imiquimod;* depot effect inducing *Alum/Alhydrogel* and the immune cell homing agent *MF59* [2, 3], [9]. The mechanism of action of *Saponin/Quil A* is unknown. This information is summarized in Table 1.

Table 1: The properties of vaccine adjuvants used in this study

Adjuvant Name	**Mechanism of Action**	**Expected Immune Response**	**FDA Approved?**
AddaVax	Immune cell homing to site of vaccination through chemokine induction	Th1 and Th2 [2, 3]	Yes
Alhydrogel	Depot Effect	Th2[8]	Yes
MPL	TLR4 Agonist	Th1 and Th2 [9]	Yes
Imiquimod	TLR 7/8 agonist	Th1[9]	Yes
CpG	TLR 9 agonist	Th1[9]	No
Poly I:C	TLR 3 agonist	Th1[9]	No
Quil A/ saponin	?	Th1/ CTL	No

TLR Agonists as Adjuvants: Initiating the adaptive immune response through activation of the innate immune system

The key to activating an adaptive immune response is mediated by the innate immune system sentinels, the Antigen Presenting Cells, whose maturation is an essential pre-requisite for the activation of antigen specific T cells. [74-76] Among APCs are dendritic cells, which express both MHC I and MHC II molecules on their surface giving them the unique ability to activate both CD4 and CD8 T cells, as well as cross present tumor antigen to further activate CTLs. [28, 31, 32]

Dendritic cells are professional antigen presenting cells eponymous with their appearance – they are highly spiculated, with dendrite like cytoplasmic structures extending from their cell bodies. [28, 31, 32]They are highly specialized sentinels of the innate immune system and are key mediators, if not the most important players, in generating optimal effector T cell responses. DCs' primary function is to scout for potentially pathogenic or foreign antigens, process and present it to naive T cells in the lymphoid organs. [28, 31, 32, 77, 78]

Four types of DCs – monocyte derived, myeloid and plasmacytoid DCs - strategically occupy sites of interface between the body and the environment.[79] Tissue resident DCs reside in physiological niches where exposure to foreign (pathogenic) antigen is most likely, such as the skin, mucous membranes and epithelial tissue of the lungs and the intestinal tract. In their immature state, DCs are phagocytosis machines. [28, 31, 32, 77, 78] They constitutively and actively sample the immune environment around them through phagocytosis and macropinocytosis. Even though DCs encounter potentially pathogenic or foreign antigen all the time, it is not until these antigen are encountered by DCs in the appropriate "context", that an immune response is mounted. [3, 28, 31, 32, 37, 74, 75, 78-87]

The cue for mounting an immune response is received by dendritic cells that have encountered antigen in the context of an exogenous or endogenous "danger signal". [3, 28, 31, 32, 37, 74, 75, 78-87] Danger signals may be delivered in the form of endogenous DAMPs, Danger Associated Molecular Patterns, or exogenous PAMPs, Pathogen Associated Molecular Patterns, both of which activate inflammation pathways through the induction of NFkB signalling[88] in non-infectious or infectious contexts, respectively. Reactive Oxygen Species that are released during cardiovascular trauma

or injury; DNA or RNA from necrotic or damaged cells/tissue, heat shock proteins and HMGB1 proteins [89] are examples of some DAMPs that induce inflammation. PAMPs are pathogen associated moieties such as single or double stranded RNA or DNA of viral origin, and endotoxins such as LPS from gram-negative bacteria and thus, are pathogenic “danger” signals for immune activation. [28, 31, 32, 74, 75, 77, 78], Furthermore, DC maturation signals can also be mediated by the presence of cytokines such as GM-CSF, IL-12 and IL-2 , IL-1, GM-CSF and TNF-α [3, 28, 31, 32, 37, 74, 75, 78-87] in the microenvironment.

Upon ligation of DAMPs and PAMPs with their receptors on antigen presenting cells, or stimulated by the presence of the appropriate cytokine in situ, downstream inflammatory cascades are activated that lead to[85], activation and maturation of DCs. Upon activation, DCs stop phagocytizing, and maximize their T cell activating potential by upregulating MHC Class I and II molecules as well as loading more processed antigen on these MHC on their surface[86]. Activated/mature DCs also increase expression of co-stimulatory molecules for T cells such as CD 80 and CD86. Mature DCs further upregulate adhesion molecules such as ICAM-1, ICAM-2, LFA-1 and LFA-3 in preparation for the impending immunological synapse with the naive T cells in the lymph nodes [3, 28, 31, 32, 37, 74, 75, 78-87]. All these phenotypic changes occur as activated, mature DCs begin their journey towards the lymph nodes, where they sojourn with naive T cells and activate them in an MHC restricted, antigen specific manner. [3, 28, 31, 32, 37, 74, 75, 78-87]

It is these mature, antigen presenting DCs that allow for appropriate activation of T cells by being bearers of all three signals needed for T cell activation: signal one in the form of the antigen being presented; signal 2 in the form of costimulatory molecules like CD80

and cD86 (B7.1 and B7.2), and signal 3 in the form of a cytokine signal during T cell activation that informs and polarizes the effector function of the T cells.[37, 74, 90-92]

Thus, DC maturation is an essential prerequisite for T cell activation, making it a topic of interest in cancer vaccinology.[80] DC immunotherapies such as the pioneer, FDA approved Sipuleucel-T vaccine for castration-resistant prostate cancer [93], were game changing in their novel ex vivo DC maturation process in which DCs extracted from patients were matured using a fusion protein consisting of a prostate cancer antigen and DC stimulating cytokine GM-CSF. Sipuleucel-T's preclinical success did translate beneficially in clinical trails but only marginal improvement in overall survival of a mere 4 months[94]. Others have used cytokine cocktails injected in vivo, such as IL-2, IL-12, IL-15 and TNF-a,[95-98] to induce DC maturation, but have only achieved enhancement of immune response at the cost of inducing systemic toxicity.[95-98]

TLR Agonists enhance DC maturation to activate naive T cells

As mentioned before, the key signal needed to initiate and activate an adaptive immune response is received and mediated by cells of the innate immune system, such as dendritic cells. [87]One of the mechanisms by which dendritic cells mature and can take on adaptive immune activating, antigen presenting capacity is by receiving a "danger signal" through evolutionarily conserved receptors called Pattern Recognition Receptors. [1, 91] A well characterized set of PRRs are Toll Like Receptors, or TLRs, whose primary function is to detect the presence of PAMPs, pathogenic motifs [1, 91]. In contrast to many PRRs that stimulate maturation of Th2 promoting DCs, the majority of TLRs promote activation and maturation of dendritic cells that promote a Th1 polarization of the CD4 Helper T cell response, thereby promoting the activation and

enhancement of cell mediated immunity[87]. It is this ability of TLR-ligand interaction that allows the maturation of DCs, and polarization of the favorable Th1 response that makes TLR agonists effective adjuvants in the process of generating a T cell mediated anti-tumor response.[74-76, 99-101] In this study, we hope to use various TLR agonists in conjunction with out TMVs to mount a Th1 mediated T cell response, thereby directly, and indirectly promoting TAA specific, CD4 and CD8 T cell mediated anti-tumor immunity.

TLRs are evolutionarily conserved transmembrane receptors that detect the presence of pathogenic structural units essential for pathogenic survival and propagation. [74]TLRs detect a wide range of pathogenic motifs such as lipoproteins present in cell walls of bacteria, viral DNA and RNA that that is essential for the virus' ability to hijack cellular machinery of the host cell and flagellin, proteins that make up the flagella of bacteria, the tail like extensions that confer bacteria the ability to swim.[1] Thus, evolution has primed our innate immune system to conserve TLRs as a sensor and detector of threat to the lines of security.

TLRs derive their name from the homologous Drosophila Toll Protein, which determines dorsal ventral polarity in the developing fly embryo.[74, 77, 99, 102, 103] With an extracellular domain consisting of leucine-rich repeats, and an intracellular signaling domain called the Toll interleukin receptor, or TIR domain, TLRs act as signaling molecules - they recognize the presence of microbial structures in the host environment, dimerize and initiate an adaptive immune response suited to clear the detected pathogen. TLRs 1 through 13 have be described in the literature, each with specialized abilities to detect specific pathogens, and mount specialized downstream inflammation signaling cascades that result in the production of cytokine milieus that not just induce

maturation of APCs and activate the adaptive immune system, but also polarize and inform the type of adaptive, cell mediated response needed to combat the pathogen of interest.

TLRs populate the cell membranes of antigen presenting cells and are found embedded on both the extracellular surfaces of APCs and inside endosomes, where they can detect intracellular pathogens that are captured by phagocytosis of an affected cell by the APC. [77]Thus, through two front lines, TLRs man the host's environment, and mobilize an adaptive immune response when needed. TLRs 1, 2, 4, 5, 6 and 11 are

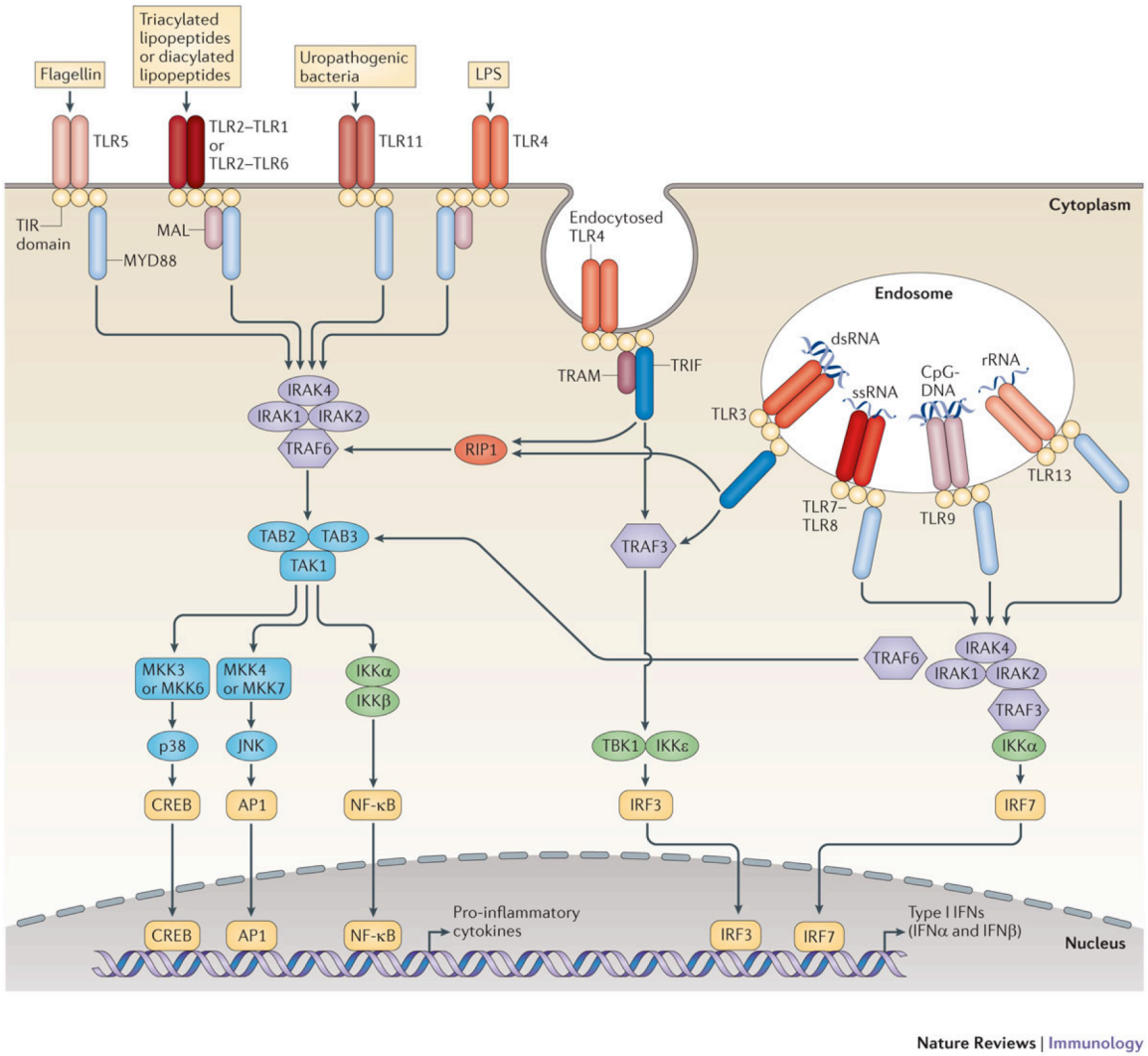


Figure 3: TLR Signaling: The figure above has been taken from [1], and depicts the intricate TLR signaling network.

present on extracellular surface of APCs. [67-69, 93-95] TLR 1, 2 and 6 detect lipopeptides found on cell walls of gram positive bacteria and zymosan from yeast, TLR 4 detects LPS from gram negative bacteria, TLR5 detects flagellin from the flagella of bacteria and TLR 11 detects bacteria that target the urogenital system. [67-69, 93-95] TLRs 3, 7, 8, 9 and 13 are found encompassed inside endosomes which fuse with phagosomes accrued by APCs. [67-69, 93-95] TLRs in the endosome are especially designed to detect the presence of intracellular pathogenic infections – TLR 3 detects viral dsRNA, TLR 7 and 8 detect viral/ foreign ssRNA, TLR 9 detects bacterial unmethylated cytosine-guanine dinucleotide CpG DNA and TLR 13 detects bacterial ribosomal RNA [76, 101].Upon TLR activation, the TIR domain associates with a variety of adaptor proteins on the inner leaflet of the cell membrane, the most important of these adaptors being MyD88, or the Myeloid differentiation primary response gene 88, that mediates activation of downstream inflammation cascades in all TLRs except TLR3 [67-69, 93-95].

MyD88, through association with other adaptor proteins such as the MyD88-adaptor-like MAL protein (also known as TIRAP), activate and translocates NFkB into the nucleus, where it acts as a transcription factor to promote the expression of genes coding pro-inflammatory cytokines such as TNF-a, IL-6 and IL-12, essential for the activation and maturation of DCs. [1, 74, 76, 92, 104]MyD88 signaling also activates MAP kinases that cause translocation of other transcription factors such as activator protein 1 (AP1) and cyclic AMP-responsive element-binding protein (CREB) transcription factors, that further promote the creation of pro-inflammatory cytokines.[1, 74, 76, 92, 104]

In addition to the activation of the inflammation promoting NFkB by all TLRs, endosomal TLRs – TLR 3, 7, 8 and 9 - also activate IFN Related Factors – transcription factors that

promote the production of Type I interferons – IFN - a and IFN - b. IFN a and b prime the immune system to take on an “anti-viral state” that activates cell mediated immunity for removal of intracellular pathogens such as a virus, or in our case, a neoplasm. [1, 74, 76, 92, 104-106]IFN-a and b, like pro-inflammatory cytokines, also promote DC maturation through upregulation of costimulatory molecules and adhesion molecules on their surface.[105, 106]

Thus, the activation of TLRs can potentiate the maturation of DCs, and thus, enhance the presentation of TAAs encapsulated in our TMV vaccine. The specific immune activation mechanisms, and a brief review of TLR agoinists’ use as adjuvants for cancer vaccines is presented below. The TLR agoinsts used as adjuvants in this study are: MPL, CpG, Poly I:C, Imiquimod and Saponin/Quil A.

Monophosphorylated Lipid A (also known as MPLA or MPL)

Monophosphorylated Lipid A is a TLR 4 agonist[107], and produces a strong Th1 and partial TH2 mediated immune response when introduced with a vaccine of interest. [108, 109]It is the detoxified, attenuated form of LPS, a major component of the cell walls of gram negative bacteria such as *Salmonella minnesota*. MPLA consists of LPS’s immunostimulatory moiety called Lipid A, that can be recognized as a PAMP by TLR 4 and initiate an immune response, whilst preventing the immunopathology associated with infection of gram negative bacteria such as fever and pyrogenic inflammation[110]. Starting from the 1960s, Ribi et.al, and Myers et.al, have modified and refined the structure of MPLA from LPS by using acid based hydrolysis techniques to systematically isolate the agents from LPS. [111, 112]

MPL mediates the activation of TLR 4 [107, 112-114]on dendritic cells and macrophages stimulating maturation of DCs both in vitro and in vivo[113], transforming them into potent antigen presenting cells. Macrophages and B cells treated with MPL also convert into potent initiators of humoral immune response by stimulating the production of IgG1 (Th2 mediated) and IgG2a (TH1 mediated) antibodies[113]. Furthermore, cytokine production –IFN-γ, IL-4 and IL-5 – also increased, even though it did not disrupt or skew the existing Th1/Th2 balance in the microenvironment.[113] Lastly, De Becker et al also showed that MPL primed dendritic cells were most likely to migrate to T cell zones, and thus, enhance priming and activation of naive T cells to their effector forms.[113]

As mentioned before, MPL is currently FDA approved for use in human vaccines as ASO4 (GlaxoSmithKline) in conjunction with alum. Furthermore, it has also been successfully used in three other GlaxoSmithKline vaccines for hepatitis B – Fendrix, Energix and HBVaxPro[115]. In addition to being validated as a safe and efficient for prophylactic vaccination against diseases, MPL has also shown favorable indications as a vaccine adjuvant for cancer, owing to the TH1 response that is generated.

Shariat et.al, [116] successfully generated a potent anti-HER-2 CTL response through vaccination with liposomes carrying a HER-2 peptide adjuvanted with MPL. Splenocytes harvested from mice immunized with MPL and HER-2 peptide expressing liposomes produced higher levels of IFN-γ upon in vitro stimulation. CD8 T cells from these mice produced higher levels of IFN-γ and also induced antigen specific CTL mediated cytoxicity of Her-2 expressing TUBO cells in vitro[116]. Furthermore, immunized mice grew significantly smaller tumors, and had significantly prolonged survival rates when compared to controls[116]. Thus, MPL is able to induce a shift to a Th1 mediated

immune response and potent CTL responses, successfully exhibiting anti-tumor immunity.

In addition to several clinical trials being conducted involving AS04, MPLA is also currently being tested in a Phase I clinical trial for melanoma, where it is being combined with a NY-ESO-1 peptide vaccine to enhance its effectiveness[117].

Poly IC

Poly IC, or polyinosinic:polycytidylic acid is a TLR3 agonist and mimics double stranded viral RNA. [118]It is made up of two mismatched ribonucleotide analogs, poly inosinic acid and poly cytidylic acid, and is organized in a double helix like DNA. [75, 118]Upon ligation with the endosomal TLR 3, Poly I:C induces a strong Th1 response that is associated with the induction of Type I Interferon – IFN a and IFN b- and the induction of an anti-viral state that promotes the initiation of a cell mediate adaptive response through DC maturation.[75, 119] Poly IC also induces NFKb signaling, but in a MyD88 independent manner, inducing proinflammatory cytokines such as TNF-a and IFN-γ. [75, 119]It is suggested that Poly I:C is the most potent producer of the Th1 response of the TLR agonisits, and induces cross presentation of antigen via maturation of DCs.[75, 119]

When co-administered with a breast cancer vaccine that consisted of a HER-2 peptide guided to APC uptake receptors with the help of an aDEC-205 antibody, Poly IC was shown to generate a potent CD8 mediated anti-tumor activity in vivo, enhancing survival and reducing tumor size in vivo significantly.[120] Other pre-clinical cell based vaccine studies in lymphoma, lung and thymoma models have shown Poly IC to induce potent CD8 mediate tumor cell lysis in vivo.[118-121]

Poly I:C is not currently approved by the FDA but it is being tested in a number of clinical trials in conjunction with cancer vaccines namely the NY-ESO-1/gp100 peptide vaccine for melanoma, with MUC 1 vaccines for prostate cancer and for MUC1 (Mucin1) Peptide Vaccine for Non-small Cell Lung Cancer.[122]

Imiquimod

Imiquimod is a TLR 7 and TLR 8 agonist that mimics single stranded RNA to mount an anti-viral immune response that leads to the induction of a potent Th1 response in vivo mediated by induction of IL-12 by engagement of TLR 7 and induction of Type I interferon by engagement of TLR 8. [9, 123-128]Endosomal TLR7 and 8 also signal through MyD88 for the induction of NFKb.[124] Like most IFN inducing TLRs, both TLR 7 and 8 are located inside endosomes and once activated induce DC maturation.[9, 123-128] Imiquimod is especially known to activate and induce maturation of plasmacytoid DCs, that are adept at cross presentation, making imiquimod a favourable adjuvant for cancer vaccines.[125] Imiquimod is also known to trigger Natural Killer Cell and B Cell maturation.[124-126] It has also shown to enhance and expand CD4+ memory T cell populations.[126]

Imiquimod is one of the earliest FDA approved vaccine adjuvants, and is marketed as a 5% topical ointment Aldara that is widely used to treat genital warts and other skin malignancies.[9, 128] It is also used as an adjuvant therapy in melanoma and pre-cancerous skin malignancies. [9, 128]It is currently also in clinical trials for use as a complementary therapy as a topical agent for metastatic breast cancer, although results have shown a marginal response.[129]

CpG

CpG sequences are unmethylated cytosine –phosphate- guanine nucleotide sequences that mimic bacterial DNA and trigger the endosomal TLR 9. [130-134]As with all other

endosomal TLRs, CpG induces signalling via the IFN genes to promote IFNa and IFNb secretion in addition to NFKb mediated TH1 cytokines, leading to DC maturation in vivo. [131]CpG is not currently FDA approved, but a variety of preclinical cancer vaccine studies have indicated the potential CpG could have as a cancer vaccine adjuvant. [130, 131]CpG combined with a tumor lysate based vaccine in the B16 melanoma model, induced heightened CD4 and Cd8 effector activity along with inhibiting tumor growth in vivo and enhancing survival significantly. [132]In clinical trials, in addition to being used extensively to adjuvant malaria, influenza and Epstein Barr vaccines, CpG has also been combined with TAA based vaccines for various stages of melanoma, lung, pancreatic, colorectal and cervical cancers[130, 131]. Although preclinical studies have indicated potent tumor abrogation, clinical trials have shown delay in progression but not complete tumor abrogation in vivo.[130, 131]

In addition to TLR agonists, also aim to use the FDA approved adjuvants alum, which induces a depot effect and creates a slow release depot of the vaccine adjuvant[113]; and MF59, that enhances vaccine antigen uptake by attracting immune cells to the site of vaccination[135]. Quil A/Saponin also exhibits and adjuvant function in vivo. These adjuvants are discussed further below:

Alhydrogel (Alum)

Aluminum-salt (aluminum phosphate and aluminum hydroxide) based vaccine adjuvants are the oldest and the “safest” in their class, being the first vaccine adjuvants to be approved the FDA[136-138]. Aluminum based adjuvants, such as Alhydrogel, enhance the TH2 response of the immune system, favoring the production of a robust antibody mediated humoral response, which is long lasting and antigen specific.[137-139]

Alhydrogel, is a proprietary aluminum hydroxide colloidal gel that is manufactured by Brennan Bertag in Denmark. Alhydrogel mediates enhancement of the immune response via the depot effect [139-141] and activation of the NLRP3 inflammosome,[136, 139] Alhydrogel's physical gel like properties are ideal for adsorption of the vaccine of interest, thereby creating a emulsified depot of antigen at the vaccination site. [90, 138, 139, 141, 142]This depot effect allows the antigen encapsulated in the vaccine to be tethered at the vaccination site in a concentrated manner for a longer period of time – thereby forming firstly, a slow release antigen depot where antigen presenting cells such as DCs can optimally uptake antigens of interest. Secondly, Hem et al have also shown that delivering antigens adsorbed in alhydrogel increases the amount of vaccine phagocytosed by DCs [141]. Alhydrogel –vaccine constructs are lager than vaccine constructs alone preventing rapid clearance from the vaccination site. Because the alhydrogel allows the vaccine construct to bulk up and aggregate to the optimal size for phagocytosis, DCs phagocytose alhydrogel–vaccine constructs instead of pinocytosing them, uptaking more antigen, and thereby enhancing their presentation capacity [141]. Thus, with the use of alhydrogel, More dendritic cells encounter antigen, and uptake more vaccine construct when compared to the administration of vaccine alone. The gel format of the aluminum based adjuvant alhydrogel also overcomes problems faced by production of aluminum adjuvanted vaccines that had to be precipitated with the aluminum salt which rendered many protein based vaccines susceptible to modification in their charge and structural properties.[139]

Alum salts also enhance the immune response through activation of the NLRP3 inflammosome complex.[8, 139] The nucleotide-binding domain leucine-rich repeat-containing protein or NLRP3 Inflammosome is an oligomeric group of proteins that take part in an activation cascade that leads to the induction of inflammatory cytokines IL-1B,

IL-18 and IL-33 by macrophages and dendritic cells [8, 139, 140], that allow enhanced the production of the TH2 helper T cell response.[143, 144]

Alum is widely used as vaccine adjuvant in many veterinary vaccines [139], human vaccines for DPT (diphteria, pertussis and tetanus), polio and HPV. Since alum based adjuvants generate antibody-mediated responses that do contribute significantly in abrogating tumor growth, much success has not been garnered in using alum as a cancer vaccine by itself. However, alum is widely used today in the HPV vaccine Cervavix [144] in the form of ASO4, Glaxo Smith Kline's proprietary adjuvant system which is a combination of the adjuvants alum and MPL, a TLR4 agonist. The adjuvancy of ASO4 has been reported to generate a long lasting Th1 (generated by MPL) and TH2 generated by alum) responses, generating a robust antibody titers even after 8 year post immunization as reported in this 2009 study.[136]

That said, alum has been successfully used to enhance antibody therapies against cancers that are indeed responsive to antibody-mediated clearing of the tumor such as Racotumomab, an anti-idiotype antibody gnerated agaisnt NeuGcGM3 ganglioside antigen found in non-small cell lung cancer[145-147]. The Racotumomab–alum treatment protocol has currently passed Phase II/III double-blind clinical trials, and improved overall survival and preogression-free survival in patients significantly when compared to the control groups.[145-147].

MF59/ AddaVax

MF59 is a oil-in-water emulsion that is owned by Novartis, and is marketed as AddaVax[148] by invivogen. AddaVax was acquired from Invivogen for this study, and will be used interchangeably with MF59 for the purposes of simplicity.

AddaVax or MF59 is an squalene oil and water emulsion based adjuvant and is marketed as Invivogen as a potent TH1 response inducer, although literature review suggests that the MF59 promotes both TH1 and TH2 responses.[135] AddaVax / MF59 is a emulsion of two solutions - Sorbitan trioleate in squalene oil and Tween 80 in sodium citrate buffer which are emulsified to produce droplets of approximately 160 nm in size[148]. Sorbitan trioleate and Tween 80 are detergents that help emulsify the squalene oil into a suspension with the citrate buffer. Squalene is the biological precursor to cholesterol and is an endogenous, biosafe and biologically degradable oil; and is also found in shark liver oil. It is considered to be the “active ingredient” in this emulsion, although many studies have suggested that squalene by itself cannot mount an immune response, unless it is emulsified by the surfactants that accompany it [149].

MF59 is FDA approved in the USA for use in the Fluad influenza vaccine in 2015, although it has been successfully validated as a safe and efficient vaccine adjuvant in Europe starting from 1997[150]. In Europe, MF59 is currently formulated in vaccines for HIV, influenza and H1N1 vaccines. AddaVax/ MF59 has been described to elicit both Th1 and Th2 responses[135, 148, 150-152], although some studies have suggested a TH1 polarization is favoured.[151]

The mechanism of action of MF59 is not yet fully unraveled: in addition to increasing uptake of antigen through the its particulate nature and depot effect[142, 149], many studies have implicated its role in recruiting lymphocytes and granulocytes to the site of vaccination[3, 142, 149] whose degranulation at the site of vaccination and chemokine secretion attracts even more immune cells[90]. Studies have also attributed its adjuvancy to its structural makeup as an emulsion[149] as well to the creation of self-

amplifying local zone of gene activation that recruits immune cells to the site of injection, primes them to activate APCs in peripheral tissues such as lymph notes for increased T cell and B cell activation and antibody production in vivo, that further help amplify the immune response[153].

Other studies have also shown in vivo that upon treatment with MF59, macrophages at the site of vaccination release a variety of chemokines such as CXCL-8[142] that home in granulocytes[3, 142]; CCL2, 3 and 4 that attract activated T cells and monocytes[142]. Co culture with MF59 has also been shown to encourage the maturation of monocytes (immature precursor dendritic cells) into dendritic cells, further activate and mature them by increasing endocytic activity and expression of maturation markers on their surface such as CD80 and CD86 [142]. MF59 may, thus, exhibit its adjuvant effects by being a potent promoter of immune cell infiltration to the vaccination site.

MF59 has traditionally not been used as a cancer vaccine adjuvant because of its tendency to promote a TH2 response.. In the few preclinical studies that we have come across, MF59 has shown to abrogate tumor growth, and prolong survival in a prophylactic vaccination model in MUC1+B16 melonoma[152]. The protein vaccine encoding a MUC1-heat shock fusion protein was adjuvanted with the adjuvants CpG and MF59 and led to generation of potent CTL activity and increased IFN-γ mRNA levels in splenocytes, suggesting a Th1 polarized response[152]. Furthermore, a current preclinical study is investing combining a Mesothelin protein with MF59 as a vaccine for a mesothelin positive high-grade serous carcinoma of the ovary (HGSCs) model.[154]

Quil A/Saponin

Quil A or Saponin based adjuvants are derivatives of the bark of the Quillaja saponaria Molina tree, and are potent inducers of the Th1 response and CD8 T cell activity. [10,

155]They are lipid based compounds and have a variety of commercial uses ranging from being ingredients in cosmetics to beverages to flavoring agents.[10, 155] It has also been extensively used as a vaccine adjuvant for malaria, HIV and Lieshmaniaisis. [10, 155]

Although not a very popular adjuvant due to its toxic potential and unstable nature in vivo, Quil A has been utilized as a vaccine adjuvant for cancer in the past. In a MUC-1/ GD3 ganglioside based vaccine for metastatic melanoma, QS-21, a derivative of Saponin, in cooperation with another adjuvant keyhole limpet haemocyanin, has been shown to mount potent CTL activity, enhance IgG and IgM antibody titers, induce a DTH response upon vaccination.[156, 157] Saponin is also combined with cholesterol and phospholipids to form a liposome like structure called ISCOMATRIX which has also been used to boost immunity of a NYESO-1 protein vaccine for prostate cancer.[158] [159]Vaccination induced DC maturation through MHC I and II upregulation, Th 1 and Th2 cytokine production and chemokine induction to home in immune cells to the site of vaccination. [158]ISCOMATRIX when admixed with vaccine antigen of choice also acts as an optimal antigen delivery vehicle – with its size optimal for APC uptake. [159]

Both ISCOMATRIX and Saponin are not FDA approved. ISCOMATRIX nor Saponin were found to be in any clinical trials for cancer at this moment. The mechanism of action of Saponin, is not entirely unraveled – although it is suggested that Saponin signals via adaptor proteins downstream of MyD88, in a TLR independent manner.[158]

Thus, through using the adjuvants described above, we hypothesize to generate a robust anti-tumor immune response mediated by our "TMV+adjuvant" vaccine constructs.

Intervention #3: "Releasing the breaks" of the cancer immunity cycle: Preventing immunosuppression in the immuno-tumor interactosome by using immune checkpoint inhibitors - monoclonal antibodies against PD-1 and CTLA-4

In addition to having mechanisms such as TLR signaling to mount potent anti-microbial (and anti-cancer) immunity against pathogens (and cancer) during an infection, the immune system has also devised mechanisms to control and downregulate the immune response to minimize collateral damage to the body. Such mechanisms also prevent autoimmunity by promoting self-tolerance. Immune checkpoints such as the receptor-ligand pair *PD-1 (Programmed Death Receptor -1) and PD-L1/PD-L2 (Programmed Death Ligand 1 and 2, respectively*) and the receptor *CTLA-4 (Cytotoxic T-cell associated protein 4)* are major players in such processes of immune inhibition, and fine tune the immune response to be active only when needed.[160-163]

Through our cancer vaccine approach, we hope to generate an anti-tumor immune response. This anti-tumor response will exhibit its effector function maximally only if it is not subdued in vivo through immune checkpoint inhibitors such as PD-1 and CTLA-4. Through this study, we hope to combine the immunostimulatory effects of our "TMV+ adjuvant" vaccine with the anti-immunoinhibitory properties of checkpoint blockade antibodies such as anti-PD-1 (henceforth referred to as "aPD-1") and anti-CTLA-4 (henceforth referred to as "aCTLA-4") to further enhance the antitumor response by

preventing T cell anergy. Both CTLA-4 and PD -1 are physiological byproducts of T cell activation. Both are intrinsically upregulated as T cell activation and costimulation increases.[162]

Immunosuppression mediated by CTLA-4

CTLA-4 mediated immune inhibitory effects may suppress vaccine mediated T Cell activation and expansion through at least two mechanisms: firstly, by disrupting the activation of naive T cells by antigen presenting APCs[164], and then through immunosuppression induced by regulatory T cells at the site of the tumor and other peripheral sites.

CTLA-4 resides in intracellular vesicles that are transported to the surface of T cells upon activation. Once on the surface, CTLA-4 receptors outcompete CD28 and bind to costimulatory molecules CD80/CD86 (or B7.1/B7.2) on APCs with higher affinity, preventing the delivery of signal 2, the costimuatory signal essential for T cell activation. [160-162, 164-167]. T cells that receive signal one, in the form of antigen-MHC complexes presented on APCs, but without the essential co-stimulatory signals in the form of CD28 – CD80/CD86 interactions become anergic, making T cell- APC interaction vacuous [160, 162, 164-168]. T cells do not get activated, do not proliferate and also reduce production of IL-2, the growth factor cytokine essential for T cell survival and activation. [169, 170]

CTLA-4 is also constitutively expressed on a subset of T cells called Regulatory T cells that arise when CD4 T cells gain effector function under regulation of the immunosuppressive cytokine TGF-b. Regulatory T cells, which are also known as T Regs, inhibit T cell effector function in cell-cell contact mediated and cell-cell contact

independent manners.[170] T Regs, firstly, use their CTLA-4 receptors to disrupt co-stimulation delivery to T cells at the site of APC-T cell interaction. At the tumor site, they

Figure 4: aCTLA-4 antibodies can rescue T cell activation. Image sourced from [7]

infiltrate tumor tissue and negatively influence the function of effector T cells by creating an immunosuppressive cytokine milieu by secreting IL-35, TGF-b and IL-10. [171]Furthermore, T Regs also secrete lytic enzymes such as Granzyme B that are cytotoxic to T cells and APCs.[170]

Using a monoclonal antibody against CTLA-4 prevents immune suppression by blocking interaction between CTLA-4 and APCs at the site of APC-T cell activation, and by depleting T Regs through Antibody dependent Cell mediated Cytotoxicity (ADCC) through which T Regs opsonized by aCTLA-4 antiodies are engaged by Fc receptors on natural killer cells and macrophages, leading to their lysis and phagocytosis, respectively.[170, 172]

Ipilimumab, the aCTLA-4 antibody, was approved by the FDA in 2015, and is currently marketed under the trade name Yervoy by Bristol-Myers Squibb. It is approved for use for advanced stage metastatic melanoma patients who have inoperable lesions and

have failed standard of care treatments. It has shown to increase median survival by about 4 months[173], without any significant negative effects to quality of life[174]. CTLA-4 is also approved for use in non small cell lung cancer but patient response has been modest. It is currently in clinical trails for use in renal cell carcinoma, Synovial Sarcoma, prostate cancer and some solid tumors in combination with standard of care therapies.[18]

Immunosupression mediated by PD-1

Like CTLA-4, the PD-1 receptor is expressed intrinsically upon T cell activation by APCs. Upon ligation of PD-1 receptors on T cells with their ligands – PD-L1, which is found on non immune cells including tumor cells, and PDL2 found on a variety of immune cells - an inhibitory signaling cascade ensues, leaving the T cell in a state of “exhaustion”. An exhausted T cell, even if it comes in contact with further antigen and co-stimulation, does not perform its effector function. The PD-1 – PD-L1/L2 interactions leave the stimulatory signals received via the engaged TCR insignificant. Furthermore, PD-1/PD-L1 signaling directly interacts with CD80, disrupting its co-stimulatory properties. PD-1/PD-L1 signaling also reduces IFN-γ and IL-2 production by effector T cells, reducing their capacity as tumor infiltrating lymphoctyes to lyse tumor cells, and produce a TH1 cytokine milieu at the tumor site. Many dendritic cells also express the ligand PD-L1 that can exhaust T cells. DCs are also known to express the receptor PD-1, which plays a role in suppressing CD8 T cell effector function.[175]

Additionally, the PD-1- PD-L1/PD-L2 signaling pathway is also manipulated by tumor cells as a method of evading immune surveillance. Many tumors upregulate PD-L1 on their surface, and as a result prime tolerance at the tumor site. Infiltrating T lymphocytes are rendered ineffective upon activation of this checkpoint. Additionally, PD-1 is highly

expressed in T Regs too. [4, 176-179]Lastly, the effector activity of T cells itself can promote PD-1/PD-L1 mediated immune suppression in the following manner: IFN-γ released by activated T cells and NK cells activates STAT signaling in tumor cells, which leads to the upregulation of MHC I and MHC II through which they are forced to present their TAAs, and thus, be susceptible to immune recognition, but also upregulate PD-L1 on their surface. This double edged effect of IFN-γ on tumor cells, further promotes immune dysregulation at the peripheral tumor site.[180, 181]

Figure 5: Immunosuppression mediated by PD-1 and PD-L1/PD-2 interactions
Retracted due to copyright restrictions[4].

Accessible at http://www.onclive.com/publications/contemporary-oncology/2014/february-2014/immune-checkpoint-blockade-in-cancer-inhibiting-ctla-4-and-pd-1pd-l1-with-monoclonal-antibodies

In this study, we hope to use an aPD-1 antibody to abrogate the effects of immunosupression mediated by PD-1 – PD-L1/PD-L2 signaling pathway and will ensure that the immune stimulation generated by our anti-cancer vaccine is not subdued in vivo. Furthermore, using aPD-1 antibody instead of the aPD-L1 antibody comes with the benefit of ensuring that a PD-1/PD-L2 interaction mediated rescue of this inhibitory pathway does not develop.[176]

The aPD-1antibody was approved by FDA for use in advanced stage metastatic melanoma in 2014, and is currently marketed as Nivolumab/ brand name Opdivo by Bristol Myers Squibb, and as Pembrolizumab under the brand name Keytruda by Merk pharmaceuticals. It is also approved for use in non-small cell lung cancer, bladder cancer, Hodgkin's Lymphoma, head and neck squamous cell carcinoma, in addition to being tested in several other cancer models and in combination with standard of care

therapies in clinical trials.[19, 160, 182-185] Anti-PD-1 inhibitors Nivolumab and pembrolizumab have shown good efficacy in treating advanced melanoma with a response rate of 38%. [182]

For non small cell lung cancer, the KEYNOTE clinical trials which combined anti-PD-1 antibodies with standard of care therapies such as paclitaxel, carboplatin and docetaxel found improved progression free survival by about 1.5 months, with a significant overall survival benefit. [183] aPD-1 therapy has also been used for the treatment of breast cancer [184]and is currently being tested in clinical trials for a variety of cancers.

Furthermore CTLA-4 and PD-1 checkpoint blockade has been combined to produce enhanced efficacy when compared to the either treatment alone, in both preclinical models as well as in clinical trials.[4, 162, 163, 179] In a B16 melanoma model combination ICI (immune checkpoint inhibitor) therapy, has shown to expand Tumor infiltrating lymphocyte population while abrogating T Regs in the tumor microenvironment.[178] Combination checkpoint blockade has also shown to improve survival in melanoma.[186]

Through this study, we hope to combine the beneficial effects of checkpoint blockade with the ant-tumor response generated by our “TMV+ adjuvant” vaccine to further enhance and amplify tumor abrogation in vivo.

Initiating an anti-tumor immune response in an immune checkpoint inhibitor resistant murine Lewis Lung Carcinoma

Even though blockade with immune checkpoint inhibitors (ICIs) has shown efficacy in improving survival, prognosis and tumor reduction in a variety of preclinical and clinical cancer studies, a major limitation of the therapy is that it is only responsive in a small fraction of the population.[17, 19, 169, 173, 174, 178, 180, 184-189] Many factors are postulated to contribute to immune checkpoint blockade's limited effectiveness in the patient population. Lack of immune regulatory markers such as PD-L1 expression; lack of TILS, or tumor infiltrating lymphocytes in the tumor microenvironment; immunosuppression induced by other mechanisms such as myeloid derived suppressor cells (MDSC) infiltration and indoleamine-2,3-dioxygenase (IDO) induction; as well as the negative effect of standard of care drugs on checkpoint blockade have all been implicated in the development of ICI resistance.[181, 185, 188]

Additionally, it is also suggested that response to ICI treatment can only be generated when an underlying anti-tumor immune response is present [180]. Allison et al showed that only when anti-tumor immunity was generated via a GMCSF adjuvanted cellular vaccine in a poorly immunogenic melanoma model, could CTLA-4 blockade "release the brakes" and enhance tumor abrogation in vivo. [190, 191]Each therapy when used as a single agent failed to show the same anti-tumor effects that synergized upon combination treatment. [190, 191] Furthermore, checkpoint blockade with aPD-1 has been reported to be more successful in tumors that are particularly known to generate a wide range of immunogenic neoantigens through frameshift mutations due to the lack of mismatch repair mechanisms, rendering them susceptible to immune surveillance.[192] Thus, ICI therapy may be effective only if a pre-existing immune anti-tumor immune

response is present. Therefore, by generating anti-tumor immunity, ICI resistance may be overcome.

In this study, we test this using the ICI resistant [193] murine Lewis Lung Carcinoma Clone LL/2 or LLC1 cell line.[194] By generating a de novo anti-LL/2 immune response using a tumor membrane vesicle based “TMV+ Adjuvant + ICI” vaccine, we hope to induce a response to checkpoint blockade and induce protection against tumor challenge and/or abrogation of existing tumors in vivo.

Lung cancer is the leading cause of cancer related deaths in both sexes, with almost 15,0000 patients succumbing to the disease, and over 200,000 new cases diagnosed every year. Small cell lung cancer makes up about 20% of the cases and are fast growing and highly metastatic, while the more common non small cell lung carcinomas make up approximately 80% of the cases and encompass a wide range of manifestations and growth characteristics.[195, 196] The standard of care chemotherapy drugs so far have been non-specific anti-cancer alkylating agent such as 5-FU, cisplatin and carboplatin, which given way to targeted therapies such as tyrosine kinase inhibitors that antagonize EGFR RTK mediated carcinogenesis. [197, 198] Even though the standard of care therapies for lung cancer that include surgery, radiation, chemotherapy and palliative care, have become more refined over time, 5-year survival is abysmal and remains poor, resistance to chemotherapy is common, and surgery and radiation based interventions are insufficient to control the cancer; indicating a clear need for the development of better therapies to manage lung cancer.[197-199] A paradigm shift in the treatment of lung cancer arose only in past few years, with the FDA approval of aPD-1 antibody for NSCLC, which has shown to increase overall survival in patient groups that respond to treatment. [200, 201]Thus, the need for novel strategies to combat lung cancer is never ending, and the previous success shown by using checkpoint blockade

in lung cancer might suggest the use of novel immunotherapies to combat this disease.[200, 201]

We hope to test our the efficacy of our immunotherapeutic cancer vaccine approach in a lung cancer model using the LL/2 or LLC1 murine lung cancer cell line in , syngeneic , immunocompetent C57BL/6 mice. It is one of the many clones harvested from metastatic lung nodules of Lewis Lung Carcinoma (LLC), a spontaneously developed murine lung carcinoma discovered by Dr. Margaret Lewis in the 1951. [194] There is no bonafide or well-characterized tumor associated antigen for Lewis Lung Carcinoma, and many pre-clinical studies have tried to develop tumor antigen based vaccines to induce tumor protection in vivo with limited success.

Potential TAAs for Lewis Lung Carcinoma? : A brief review of Lewis Lung Carcinoma based immunotherapy

In the LL/2 / LLC1 model, a few TAA based cancer vaccine strategies have had success in generating an anti-tumor immune response despite lack of a known TAA. A few of these vaccine therapies are briefly discussed below.

Among the oldest tumor antigens that have been implicated as potential TAAs for LLC are MUT 1 and MUT 2, which were recognized to be mutants of the cell junction protein Connexion 37. In 3LL, a metastatic clone of LLC, mice immunized with cell constructs loaded with MHC-MUT1/MUT2 constructs induced tumor protection and reduced metastasis, suggesting the presence of an immunogenic TAA.[202, 203] 10 years later, when MUT 1 and MUT2 were sequenced using PCR, it was suggested that Connexion 37 is not mutated in 3LL, contradicting previous proposals.[204]

Peng et al created an adenovirus encoding Cytokeratin 19, a cytoskeletal protein associated with lung neoplasms, and transduced DCs to express CK19, which were then used to immunize mice. Upon challenge with LL/2, immunized mice had smaller tumors than controls, although complete tumor protection was not achieved. Others have approached a more holistic method to develop a TAA based vaccine for LL/2 by isolating tumor antigen containing exosomes from cell culture supernatants, which were then pulsed onto dendritic cells ex vivo. Mice were then immunized with membrane vesicles prepared from TAA exosome pulsed DCs. Upon tumor challenge, mice showed protection from tumors via a CTL response.[205] Others have also used the adjuvant properties of TLR agonists to enhance immunity – a combined CpG and basic growth factor (an angiogenesis promoting factor) based vaccine successfully reduced tumor growth in vivo, although complete protection was not seen.[206]

Thus, there has been marginal success in developing successful immunotherapeutic strategies for the LL/2 cell line/ Lewis Lung Carcinmoa Model. However, these studies suggest that a suboptimal anti-LL/2 response may be generated, implying that Lewis Lung Carcinoma is indeed targetable by anti-tumor immunity despite the lack of a bonfaide, immunogenic TAA.

We hope to enhance this anti-tumor immunity generating potential in the Lewis lung Carcinoma LL/2 model by intervening at strategic points of the cancer immunity cycle with adjuvants and checkpoint blockade to generate a robust anti-LL/2 specific immune response in vivo. By combining our TMVs with adjuvants to boost host immunity, we also hope to reverse the ICI resistant property of the LL/2 cell line that has been reported by Charles River, and generate a long lasting, and self-renewing humoral and T and B cell mediated adaptive immune response.

Experimental Design

The following studies were undertaken to induce an anti-tumor response in the immune checkpoint inhibitor resistant LL/2 (a clone of Lewis Lung Carcinoma) lung cancer model. The first phase of study began with characterization of the LL/2 cell line both in vitro and in vivo. LL/2 tumors were then grown subcutaneously in syngeneic, immunocompetent C57BL/6 mice and harvested for TMV preparation (Experiments 1 and 2).

The second phase of study involved the production and characterization of Tumor Membrane Vesicles, or TMVs derived from both LL/2 tumor tissue and LL/2 cell pellets grown in vitro (Experiment 3). Once TMVs were prepared and characterized, we tested whether prophylactic vaccination with LL/2 TMVs protected against subcutaneous LL/2 tumor challenge in vivo, and also determined the dose of TMVs to utilize for future studies (Experiment4).

The next phase of study involved screening various synthetic, commercially available adjuvants and immune checkpoint inhibitor (ICI) antibodies in combination with our TMVs to adjudge their abilities to abrogate LL/2 tumor growth in vivo (Experiments 5 and 6).

Once the most efficacious “TMV+Adjuvant” and “TMV+ICI” combinations were ascertained, we combined vaccination approaches to create a trivalent “TMV + Adjuvant +ICI” vaccine, where mice were vaccinated prophylactically with LL/2 tumor TMVs in combination with adjuvants selected from our previous screen, followed by therapeutic

administration (used interchangeably with therapeutic vaccination) of ICI antibodies after LL/2 tumor challenge (Experiment 7).

We further tested if whether LL/2 cells respond to IFN - γ treatment, in vitro, to try and simulate the potential effect TMV mediated immune activation could have on LL/2 tumors in vivo (Experiment 8).

Lastly, as a pilot study (Pilot Study 1, Pilot Study 2, Pilot Study 3 - data not shown), we incorporated LL/2 TMVs with the GPI anchored form of the Th1 response inducing cytokine IL-12 through Protein Transfer to create a biologically/genetically adjuvanted "LL/2-GPI-IL-12 TMV" vaccine (Pilot Study 1). To ascertain the anti-tumor properties of LL/2-GPI-IL-12 TMVs in vivo, we prophylactically vaccinated mice with LL/2-GPI-IL-12 TMVs and co-administered ICI intraperitoneally upon subcutaneous tumor challenge in a manner similar to the "TMV+ Adjuvant+ ICI" study (Pilot Study 2). Lastly, we also tested the effects of prophylactic vaccination with LL/2 GPI-IL-12 vaccination in an experimental metastasis model where we vaccinated mice intravenously with LL/2-GPI-IL-12 followed by LL/2 challenge through lateral tail vein injection.

Each experiment is listed below:

- **Experiment 1: Characterization of the LL/2 cell line through flow cytometry**
- **Experiment 2: Characterization of LL/2 tumor growth in vivo**
- **Experiment 3: Production and Characterization of LL/2 cell derived and tumor tissue derived TMVs**
- **Experiment 4: Determining the LL/2 TMV Dose for vaccine studies:** *Prophylactic vaccination with varying amounts of LL/2 TMVs followed by tumor challenge*
- **Experiment 5: "TMV+ Adjuvant" vaccine Prophylactic Vaccination Study:** *Prophylactic vaccination with "TMV + Adjuvant" vaccine followed by LL/2 tumor challenge*
- **Experiment 6: "TMV + ICI" vaccine Therapeutic vaccination study:** *LL/2 tumor inoculation followed by therapeutic vaccination with "TMV + ICI" vaccines*
- **Experiment 7: "TMV + Adjuvant + ICI" Study:** *Prophylactic vaccination with "TMV + Adjuvant" vaccine followed by therapeutic vaccination with "ICI" upon tumor challenge*
- **Experiment 8: In Vitro treatment of LL/2 cells with IFN-γ:** *Characterization of MHC I and PD-L1 expression on LL/2 tumors "in vivo"*

Materials and methods

Mice

6-8 week old C57BL/6 mice were purchased from Jax Laboratories, and were maintained with strict adherence to IACUC guidelines.

Cell Lines

LL/2 (also known as LLC/1) cell line was purchased from ATCC (Catalog number CRL-1642). Cells were grown in Dulbecco's Modified Eagle's Medium - high glucose (Sigma-Aldrich Catalog Number D6429) supplemented with 10% FBS (Atlanta Biologicals, Catalog Number S11150H). LL/2 cells were frozen in medium (described above) supplemented with 5% Dimethyl Sulfoxide or DMSO (Fisher, Catalog Number BP231-1). The LL/2-RFP cell line was developed in the Shanmugam Lab at Winship Cancer Institute, Emory University.

Tumor inoculation

Subconfluent LL/2 cells were split the day before and harvested with a 0.25%Trypsin-EDTA solution (Sigma Aldrich, Catalog Number T4049) and resuspended in sterile PBS. Cells were then counted, and viability was determined using Trypan Blue exclusion. The amount of cells needed per mouse was suspended in a total volume of 100uμ/ injection, and cells were maintained on ice until needed.

For subcutaneous injections, C57BL/6 mice were anaesthetized and a 26 G syringe was used to inoculate cells onto the flanks of the mice after shaving the fur and cleaning the skin with an alcohol swab. All tumor cell injections were performed on the flank contralateral (or opposite) to the flank were the vaccine was administered. Mouse weight and tumor size were measured every 3 days using digital calipers, and mice were

sacrificed if one of four conditions were met: if the mouse was sick (according to IACUC guidelines), if the mouse had tumors that reached 2 cm in diameter, the mouse had a necrotic tumor or if the mouse's body weight reduced by 20% of its original body weight (recorded prior to tumor inoculation).

Vaccine Immunization

All of our TMV based vaccines were prepared in PBS, and were injected in 100µl final volumes through subcutaneous or intravenous routes (lateral tail vein) injections. For prophylactic vaccinations, naive mice were injected with TMV, or "TMV+Adjuvant" vaccines on Day 0, and then boosted 2 weeks later. For therapeutic vaccination, TMV and ICI were administered post tumor challenge. TMV was administered on Day 2 and 9 post challenge, while ICI was administered on Days 3,6 and 9 post challenge.

Measuring Tumors

Once mice were inoculated with tumor cells, tumor growth was measured every three days by using vernier calipers to measure length and breadth of the tumors. Tumor area was calculated by multiplying breadth x length.

Measuring Survival in mice

Mouse weights were taken every three days and survival was recorded. Mice were sacrificed if one of four enpoints were met: if the mouse was sick (according to IACUC guidelines), if the mouse had tumors that reached 2 cm in diameter, the mouse had a necrotic tumor or if the mouse's body weight reduced by 20% of its original body weight (recorded prior to tumor inoculation).

Bio Imaging of LL/2 –RFP Tumors and Lungs

Lungs and tumors from mice bearing LL/2 RFP tumors (2 cm in diameter) were resected and imaged to determine the presence of RFP using the IVIS Spectrum imaging platform and an Andor Ikon Camera, Model number I1410N6549 at the Emory Bioimaging Core.

India Ink Staining

India Ink was purchased from Speedball art and media supplies. It was then diluted to a 15% solution in PBS. 1 L of Fekete's solution was prepared by combining 580 ml 95% ethanol, 200 ml H_2O, 80 ml 37% formaldehyde solution and 40 ml glacial acetic acid. [207] After sacrifice, india ink was injected intratracheally into the lungs in situ. Lungs were resected, rinsed in distilled water and developed in Fekete's solution to visualize metastatic nodules. Lungs were then stored in Fekete's solution.

TMV preparation

Briefly, minced LL/2 Tumor tissue or cultured cell pellets were suspended in homogenization buffer (20 mM Tris pH 8.0, 10 mM NaCl, 0.1 mM $MgCl_2$, 0.1mMPMSF) and homogenized using a mechanical homogenizer. The supernatant was collected from the homogenate after centrifugation, and the remaining pellet was passed through the homogenization process once more. The collected supernatant was layered carefully on a 41% sucrose solution prepared with homogenization buffer, and ultracentifuged at 23000 RPM for 1 hour at 4°C using a Beckman SW41 rotor. Once centrifuged, TMVs were aspirated carefully from the interface between the supernatant and sucrose solutions and washed in PBS two times by centrifugation at 13200 RPM at 4°C for 1 hour each in a microcentrifuge (Eppendorf, Catalog Number 5415 D). Micro BCA was performed to quantify protein concentration and FACS analysis was performed to characterize antigen expression profiles of prepared TMVs.

TMV Quantification via BCA

Micro BCA was performed to quantify protein concentration in TMVs and tumor tissue/ cell homogenates using the Thermo Fisher MicroBCA Kit Catalog number 23235.

Flow cytometry analysis of LL/2 cells, LL/2 cell derived TMVs and LL/2 tumor derived TMVs

Cultured LL/2 cells were incubated for 30 minutes with Direct-conjugate antibodies diluted 1:100 in FACS Buffer (PBS, 2-5% serum, 0.09% sodium azide, 5mM EDTA) or with 100uL hybridoma supernatants. Cells were then washed twice (FACS buffer was added to cells which were then pelleted by centrifuging for 5 minutes, at 5000 RPM, 4°C; supernatant was aspirated; cells were resuspended in FACS buffer again for second wash) and fixed in 2% formalin, or stained with secondary antibody of choice diluted 1:100 in FACS buffer. After the secondary staining, cells were washed again and fixed for storage in 4°C until acquisition of samples. All samples were acquired in BD FACS Calibur Cell Analyzer, and results were analyzed using Flow Jo version 10. Compensation was calculated and applied to all samples.

TMVs were stained in an identical manner expect that 5-10µgof TMVs was used and the centrifugation during the washes was performed for 15 minutes at 13200 RPM at 4°C. LL/2 tumor derived TMVs were incubated for 10 minutes with 1µg/ sample of rat anti-mouse CD16/32 Ab (clone 2.4G2) F_C Block before any staining was performed.

The following antibodies (clone names) were used: MHC 1 (M1/42), CD44 (IM7), CD49d (9F10), PD-L1 (10F.9G2), SCA-1 (D7), ICAM-1 (15.2), CD24 (M1/69), CD47 (miap301), MHC II (M5/114.15.2), CTLA-4 (L3D10) and rat anti-mouse CD16/32 Ab (clone 2.4G2).

Electron Microscopy Imaging of LL/2 TMVs

Tumor tissue derived and cell pellet derive TMVs were prepared and imaged by Hong Li at the Robert P. Apakarian Electron Microscopy Core. Briefly, a drop of LL/2 TMVs was placed on a formvar-carbon coated copper grid and was gently blotted to remove excess. Then a drop of sodium phosphotungstate, also known as PTA, was added, the excess blotted away, and the grid was left to dry before imaging with a transmission electron microscope. PTA is a contrast agent that provides a dark background for samples being imaged. The negative staining and imaging was performed by Hong Li at the About the Robert P. Apkarian Integrated Electron Microscopy Core at Emory University.

Reconstitution of adjuvants

All adjuvants (catalog number) - Alhydrogel (vac-alu-250), Quil A/Saponin (vac-quil), Imiquimod (vac-imq), MPLA (vac-mpla), ODN 2395 CpG (vac-2395-1), Poly I:C (vac-pic) and Addavax (vac-adx-10)- were purchased from InVivogen. Adjuvants were reconstituted and stored according to manufacturer's instructions. Alhydrogel came ready to use, and is a cloudy, colloidlal gel. Imiquimod is a white crystalline powder and was reconstituted in sterile endotoxin-free physiological water (NaCl 0.9%). MPLA came as a clear lipidic film and was reconstituted in sterile endotoxin-free physiological water (NaCl 0.9%) and followed by 5 cycles of sonication until dissolved. CpG came as a white lyohilized powder and was reconstituted in sterile endotoxin-free physiological water (NaCl 0.9%). Poly I:C was a white film and was reconstituted in sterile endotoxin-free physiological water (NaCl 0.9%), annealed for 10 minuets at 65 -70C to dissolve. Quil A was a white powder reconstituted in sterile endotoxin-free physiological water (NaCl 0.9%).Finally, AddaVax came ready to use and is a milky, white elmulsion. Once reconstiituted, all adjuvants were aliquoted, labelled and stored according to manufacturer's instruction until later use.

Preparation of ICI antibodies

Antibodies against PD-1 (Clone: RMP1-14 Catalog Number: BE0146) and CTLA-4 (Clone: 9D9 Catalog Number: BE0164) were acquired from BioXCell, and diluted in PBS to make 1mg/mL solutions before injecting 200µl 1(200 ug) per mice through intraperitoneal injection.

Blood Collection from mice

Blood was collected in BD Microtainer Blood Collection Tubes from mice under anesthesia through the facial vein using a 5mm Golden Rod Lancet. Once blood was collected, hemostasis was ensured by applying gentle pressure at the puncture site. IACUC guidelines were followed. Mice were monitored until they were ambulatory.

Serum Preparation

Blood was collected in BD Microtainer Blood Collection Tubes and centrifuged at 5000 RPM for 5 minutes. The separated serum was then aliquoted in 50µl increments in labeled tubes and stored at -20C until needed. Freeze thawing of samples was avoided.

Detection of anti-LL/2 antibodies in serum via flow cytometry

Cultured LL/2 cells were incubated for 30 minutes with serum diluted 1:10 in FACS Buffer, allowing anti-LL/2 antibodies in the serum (if any) to bind to LL/2 cells. Excess serum was washed out and cells were stained with a FITC conjugated goat anti-mouse IgG (Jackson Immunoresearch catclog number 115-095-146) or a FITC conjugated goat anti-mouse IgG + IgM antibody (BD Catalog number 555988) for 30 minutes, washed twice and then fixed in 2% Formalin until acquisition.

Detection of IFN-γ and IL-4 in serum via ELISA

Serum was diluted 1:10 and ELISA was performed with BD OptEIA™ set for mouse IL-4 Catalog Number. 555232, and the BD OptEIA™ set for mouse IFN-γ Catalog Number. 555138.

DTH Response in vivo

Delayed Type Hypersensitivity, or DTH response was assessed 3 weeks after the first dose of TMV, and one week after the booster dose. Footpad thicknesses of all the mice were measured in triplicate using Vernier Calipers before injecting 50µgof LL/2TMV suspended in 25µl of PBS, into one of the footpads of immunized mice. On the other footpad, 25µl of PBS was injected as control. 24 hours later mice were checked for swelling and footpad measurements were made like before.

IFN- γ Tx of LL/2 Cells

125,000 LL/2 cells were plated in a 12 well plate, and 1 mL of a 500U/mL rmIFN- γ (eBioscience catalog number BMS326) solution was added. Cells were incubated at 37C for 24 hours, and flow cytometry analysis was performed the following day.

Graphs and Statistical Analysis

All graphs have been made either using GraphPad or Microsoft Excel. All survival curves have been nudged on both the x and y axes to make individual survival curves of all the groups more clear. Statistical Analyses was performed using GraphPad software. One-Way and Two-Way ANOVA with Bonferroni or Tukey post-tests were performed to determine statistical significance. The Mantel-Cox test was performed on Kaplan Meir survival curves to assess statistical significance.

Results

Results from Experiment 1: Characterization of the LL/2 Cell Line through flow cytometry

LL/2 cells do not express MHC I, CD24, CD49d, ICAM-1 or SCA-1 but expresses CD44, CD47 and PD-L1 in vitro

LL/2 cells in vitro grow in a mixed adherent and suspended cell population; show foci formation and double their population in about 21 hours.

The antigenic profile of LL/2 cell line was characterized using flow cytometry to ascertain if they express various proteins of biological significance – essential antigen presentation proteins such as MHC Class I; potential stem cell markers such as CD24/HSA, CD44 and SCA-1; markers of metastatic potential such as CD49d and ICAM-1, and immune regulation proteins such as CD47 and PD-L1.

The results from flow cytometry (Figure 6) indicate that LL/2 cells do not express MHC I, CD24, CD49d or SCA-1 but express varying levels of CD44, ICAM-1, CD47 and PD-L1 in vitro. The biological significance of these antigens is discussed in the following paragraphs.

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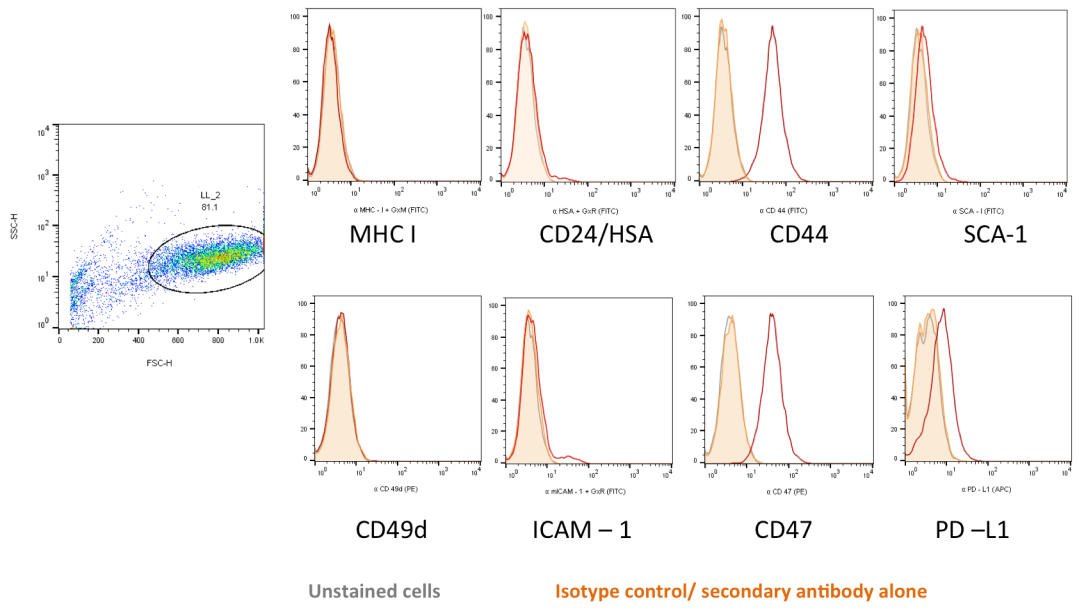

Figure 6: Antigenic profile of LL/2 cells. LL/2 cells do not express MHC I, CD24, CD49d, ICAM-1 or SCA-1 but expresses CD44, CD47 and PD-L1 in vitro.

Major Histocompatibility Class I (MHC I) proteins are expressed on all nucleated cells and present endogenous antigen to surveying CD8 T cells. CD8 T cells recognize and mount cytotoxic activity against a cell that displays aberrant self-antigen, pathogenic antigen or tumor associated antigens on MHC class I molecules. Thus, MHC Class I is important for recognition of neoplastic cells by T cells [208-210].

CD44 is a hyaluronic acid receptor that may be a potential stem cell marker [210-212]. It is widely expressed in a variety of cancers, and it is suggested that CD44 allows tumor cells adopt a pro-oncogenic phenotype by modulating a variety of proliferation and gene expression pathways, in addition to migration, motility and invasive ability through association with components of the extracellular matrix such as fibronectin and laminin[210-212].

A low expression of CD24 (Heat Stable Antigen) is a marker for stem cells in breast cancer [209, 213], and is associated with a “partial stem cell phenotype” in lung cancer [214]. CD24 expression is also known to promote metastasis and extravasation by being a ligand for P-selectin, an adhesion receptor found on blood cells, that helps tumor cells enter into the blood stream and metastasize [209, 210].

SCA-1 or Stem Cell Antigen - 1 is a progenitor hematopoietic cell and stem cell marker that is well studied in breast cancer. It is associated with the sphere formation of stem cells in breast cancer models [215]. A subset of SCA-1 positive cells are also implicated in prostate cancer to produce and secrete HIF-1a, a master transcription factor that turns on TWIST and MET to promote the development of angiogenesis, stemness, invasive ability and dedifferentiation [216]. HIF-1a is also associated with enhanced EGFR signaling in Non Small Cell Lung Cancer [217].

CD49d is an integrin molecule expressed on tumor cells and has been implicated in homing of cancer cells to metastatic sites in chronic lymphocytic leukemia. It is associated with enhanced metastasis and poor prognosis in CLL, and also in several other myeloproliferative blood neoplasms [218, 219].

ICAM-1 is an adhesion molecule whose upregulation on tumor cells induces chemokine and cytokine signaling that homes in granulocytes to the tumor/endothelial barrier. Upon their entry, granulocytes such as neutrophils and macrophages degranulate, Breaking down the extracellular matrix and loosening the tight endothelial junctions, allowing the tumors cells to extravastate into the blood stream and migrate to distant sites [220].

CD47 or the “don’t-eat-me” signal is a mechanism by which tumor cells evade detection by tumor infiltrating macrophages.[221] It is highly expressed in many cancers including ovarian and prostate cancer and ligates to receptors on tumor infiltrating macrophages inhibiting the phagocytosis of the tumor cells evading immune detection [221-223].

PD-L1, Programmed Death Ligand- 1, is a checkpoint inhibitor protein that is expressed by tumor cells as a mechanism to evade immune surveillance [224]. PD-L1 ligates with its receptor, PD-1, on activated T cells and induces T cell anergy, culling its effector function and proliferative abilities [224].

Results from Experiment 2: Characterization of LL/2 tumor growth in vivo

A subcutaneous challenge of one million LL/2 cells in vivo produces palpable tumors in 3 to 5 days, and a 1 cm diameter tumor in 15-20 days.

LL/2 cells were grown subcutaneously in 30 mice to produce tumor tissue for the preparation of tumor tissue derived TMVs. One million LL/2 cells harvested from subconfluent flasks, and with a determined viability of at least 95% via trypan blue exclusion, were inoculated subcutaneously on both flanks of naive C57BL/6 mice. Mouse weight and tumor size were measured every 3 days using digital calipers, and mice were sacrificed if one of four conditions were met: if the mouse was sick (according to IACUC guidelines), if the mouse had tumors that reached 2 cm in diameter, the mouse had a necrotic tumor or if the mouse’s body weight reduced by 20% of its original body weight (recorded prior to tumor inoculation). For this particular study, all mice were sacrificed when either of the flank tumors reached 1 cm in diameter (and not 2 cm in diameter since tumor burden was doubled upon inoculation of LL/2 cells on both flanks).

LL/2 tumors exhibit a very aggressive growth pattern – we observed a few tumors that were necrotic before even reaching the size of 1 cm.

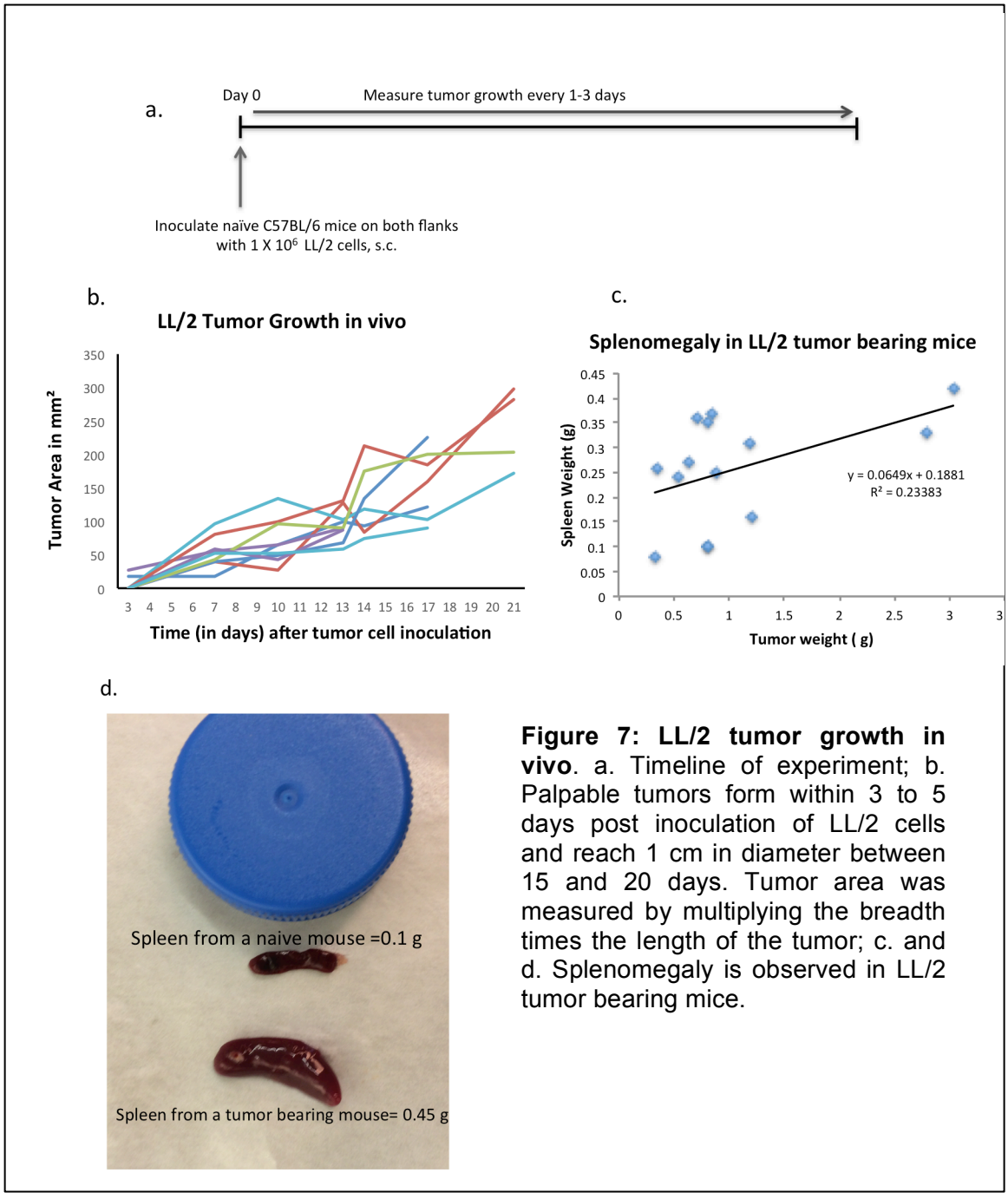

Figure 7: LL/2 tumor growth in vivo. a. Timeline of experiment; b. Palpable tumors form within 3 to 5 days post inoculation of LL/2 cells and reach 1 cm in diameter between 15 and 20 days. Tumor area was measured by multiplying the breadth times the length of the tumor; c. and d. Splenomegaly is observed in LL/2 tumor bearing mice.

Groups of mice (n=5), were inoculated on both flanks with LL/2 cells and observed daily (Fig. 7a). All tumors became palpable about 3-5 days post challenge, and all mice grew

tumors progressively (Fig 7b.). One million cells produced a 1 cm diameter tumor between 15 and 20 days, and weighed about 1 g upon harvest. A total of 48 g of tumor tissue was collected from tumor bearing mice, and skin and necrotic tissue if present was removed before freezing at -80°C until TMV preparation.

LL/2 tumors induce splenomegaly

Splenomegaly, or enlargement of the spleen, was observed in all tumor bearing mice. (1 cm diameter wide) Tumor bearing mice had spleens that were approximately 3 to 5 times larger in size and weight compared to naive (tumor-free, unvaccinated) mice. (Fig 7c. and 7d.)

Although we did not investigate into what specific splenic populations induce splenomegaly in LL/2 tumor bearing mice, in our other tumor models such as the 4TO7 [56] and 4T1 (unpublished) murine triple negative breast cancers, we have implicated an infiltration of MDSCs to account for enlargement of the spleen upon tumor formation. Myeloid derived suppressor cells, MDSCs, are progenitor granulocytic cells that secrete ROS, NOS and arginase that impede TCR function and induce immunosuppression[63]. Once enriched in the spleen, this population of MDSCs may infiltrate tumor tissue as well.

Furthermore, other studies [225] have shown that a splenectomy performed after Lewis Lung Carcinoma tumors have established prevents further growth, suggesting a pro-tumorigenic role of splenomegaly. Furthermore, they discovered enriched MDSC populations in the spleen, and showed Lewis Lung Carcinoma tumors could be controlled in a manner similar to the splenectomies by using an anti-MDSC (anti-Gr1+) antibody [225]. Thus, splenomegaly could be a mechanism through which LL/2 tumors propagate [225].

Further tests would be needed to determine the exact cause of splenomegaly, but in this study, we hypothesize that it may be indicative of an immunosuppressive mechanism that enables tumor growth via enrichment of MSDC populations.

LL/2 tumors may not metastasize to the lung when tumors are less than or equal to 2 cm in diameter

The presence of lung metastases in mice bearing LL/2 subcutaneous tumors was studied using four techniques: visual inspection, measuring lung weight, india ink staining and by ex vivo imaging using an LL/2-RFP cell line (Figure 8).

Upon tumor harvest, we studied if subcutaneous LL/2 tumors had the ability to metastasize to the lungs, or any other distant organs. We resected and visually inspected lungs, liver, brain, heart and kidneys of tumor bearing mice, and saw no metastatic nodules (in mice bearing tumors less than or equal to 2 cm in diameter).

Resected lungs from tumor bearing mice were weighed upon tumor harvest, and there was no significant difference between the lung weights of tumor bearing mice and lung weights of naive, unvaccinated, tumor-free mice, suggesting that 1 cm wide LL/2 tumors may not metastasize to the lung. (Fig 8a.)

The lack of metastatic nodules in the lungs of LL/2 tumor bearing mice was further validated by performing intratracheal injections (Figure 8c. and 8d.) into the lungs with a

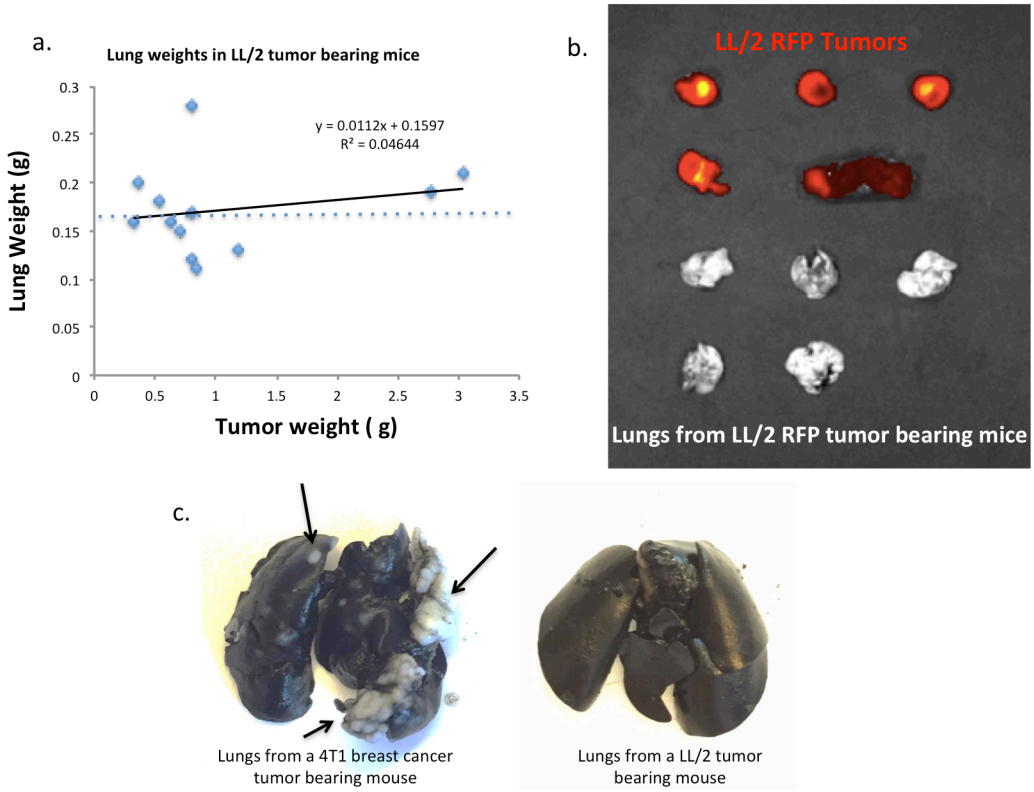

Figure 8: Subcutaneous LL/2 tumors up to 2 cm in diameter may not metastasize to the lungs: a. Lung weights of tumor bearing mice are similar to lung weight of a naive mouse (dotted blue line); b.lungs harvested from 2 cm large LL/2 RFP tumors do not exhibit any RFP signal (in red), suggesting the lack of LL/2-RFP micrometastases; c. India ink staining of lungs from LL/2 bearing mice (right) did not show the presence of white metastatic nodules the can be clearly seen in lungs harvested from 4T1 tumor bearing mice (white metastatic nodules in white can be seen clearly on the black, healthy lung tissue. Metastatic nodules are indicated with the black arrows.)

15% india ink solution. Because of their non-conformity to the highly organized architecture of lung tissue, metastatic nodules, if present can be visualized in the lung through india ink staining. Once India ink, a black colored dye, is diluted to a 15% solution with distilled water and successfully injected through the trachea, it selectively stains the healthy lung tissue black while leaving the metastatic nodules unstained and pearly white. Lungs from 1 cm large and 2 cm large LL/2 tumor bearing mice did not

have any white metastatic nodules, suggesting that subcutaneous LL/2 tumors may not metastasize. (Figure 8c. and 8d.)

To adjudge the presence of micrometastasis in the lung upon subcutaneous injection of LL/2 cells, we utilized an RFP-LL/2 cell line that was developed at the Shanmugam lab at Winship Cancer Institute. We inoculated 500,000 LL/2 – RFP cells on the flanks of naive mice. LL/2 RFP tumors grew progressively (data not shown), and mice were sacrificed when tumors reached 2 cm in diameter. Tumors and lungs from tumor bearing mice were harvested, and imaged using the IVIS bioimaging platform at the Emory Bioimaging Core in collaboration with members of the Shanmugam laboratory. LL/2 RFP tumors showed high expression of RFP as seen in Figure 8b., but we could not see any RFP signal in the lungs of the same mice, suggesting that subcutaneous LL/2 tumors may not metastasize to the lungs. However, this does not rule out the possibility of micrometastases in the lungs of tumor bearing mice – our readout (the bioimaging) was simply not sensitive enough to detect micrometastasis in the lungs. A clonogenic assay, in which tumor bearing mice's lungs would be digested with collagenase and then cultured in vitro with an appropriate selection agent that kills all fibroblasts (healthy lung tissue) but not the metastatic clones, would be necessary to confirm this.

For all our vaccination studies, in which mice were sacrificed when tumors reached 2 cm in diameter, visual inspection and/or Indian Ink staining was performed at the time of sacrifice, and no metastatic nodules were observed. Mice bearing subcutaneous LL/2 tumors up to 2 cm in diameter (the pre-determined IACUC endpoint) may not matestasize to the lungs. However, this does not rule out the development of metastasis in mice that survive with tumors larger than 2 cm, or the presence of micrometastases in

the lungs. Thus, LL/2 cells are highly tumorigenic in vivo, but may not metastasize to the lungs or distant organs (in a subcutaneous tumor model).

To better understand the dynamics of in vivo growth of LL/2 cells, and to determine an appropriate tumor challenge dose for our prophylactic and therapeutic vaccination studies, we inoculated varying numbers of LL/2 cells in mice (n=3/group) and observed their growth every 3 days. (Fig. 9) We hoped to find a dose where tumors would grow less aggressively than when inoculated with 1000,000 cells. This would allow time for anti-tumor immunity to generate before tumors become too large, and thus, allow the vaccine effect to be more apparent in vivo. From Figure 9, we observed that a tumor cell inoculation between 400,000 and 600,000 cells may be appropriate for tumor challenge. We also observed that mice inoculated with the lowest cell number of 50,000 cells did not develop any tumors, suggesting the existence of a threshold for the implantation of LL/2 tumors in vivo. For all our tumor challenge studies, consistent with literature, we decided to use 500,000 LL/2 cells for tumor cell challenge.

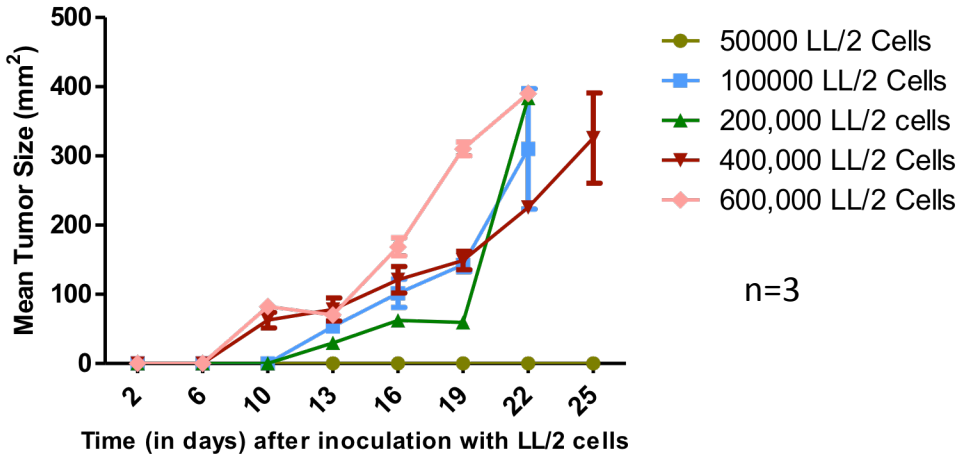

Figure 9: Tumor challenge dose was ascertained by inoculating varying amounts of LL/2 cells in vivo.

Results from Experiment 3: Preparation and characterization of LL/2 cell pellet derived and LL/2 tumor tissue derived TMVs

TMVs were prepared, as described in materials and methods, through the process of tumor or cell pellet homogenization followed by high-speed centrifugation through a 41% sucrose gradient. This process results in the accumulation of TMVs at an interface, which is then carefully aspirated to harvest, washed in PBS and protein concentration quantified with Micro BCA (Figure 10).

Approximately 120 mg of tumor tissue derived TMV was generated from 45 grams (about 15 batches of TMVs, 3g tumor tissue/batch) of tumor tissue for all the experiments described in this study. The percent yield averaged at 12.9% ((protein concentration in TMVs/protein concentration in tissue homogenate) x100).

9 mg of LL/2 Cell derived TMVs was also prepared from 5.6 g LL/2 cultured cell pellet. For initial vaccination studies, cell derived TMVs were used since LL/2 tumors were still growing in mice, and were not ready to harvest for preparation of tumor derived TMVs.

LL/2 tumor tissue derived TMVs express CD24 and CD44. LL/2 cell pellet derived TMVs do not seem to express any of the antigens present on LL/2 cells.

LL/2 cell pellet derived TMVs and LL/2 tumor tissue derived TMVs were characterized using flow cytometry with the same panel used for LL/2 cells in addition to a few other markers – CTLA-4 and MHC II (Figure 11). CTLA-4, as described in the introduction, is the immune checkpoint protein that is found on activated T cells and regulatory T cells. MHC II is the other Major Histocompatibility Complex protein that presents peptides

derived from extracellular antigen to T cells. MHC II is usually expressed by mature DCs as well as a range of tumor cells (as a byproduct of aberrant cell signaling). Since TMVs

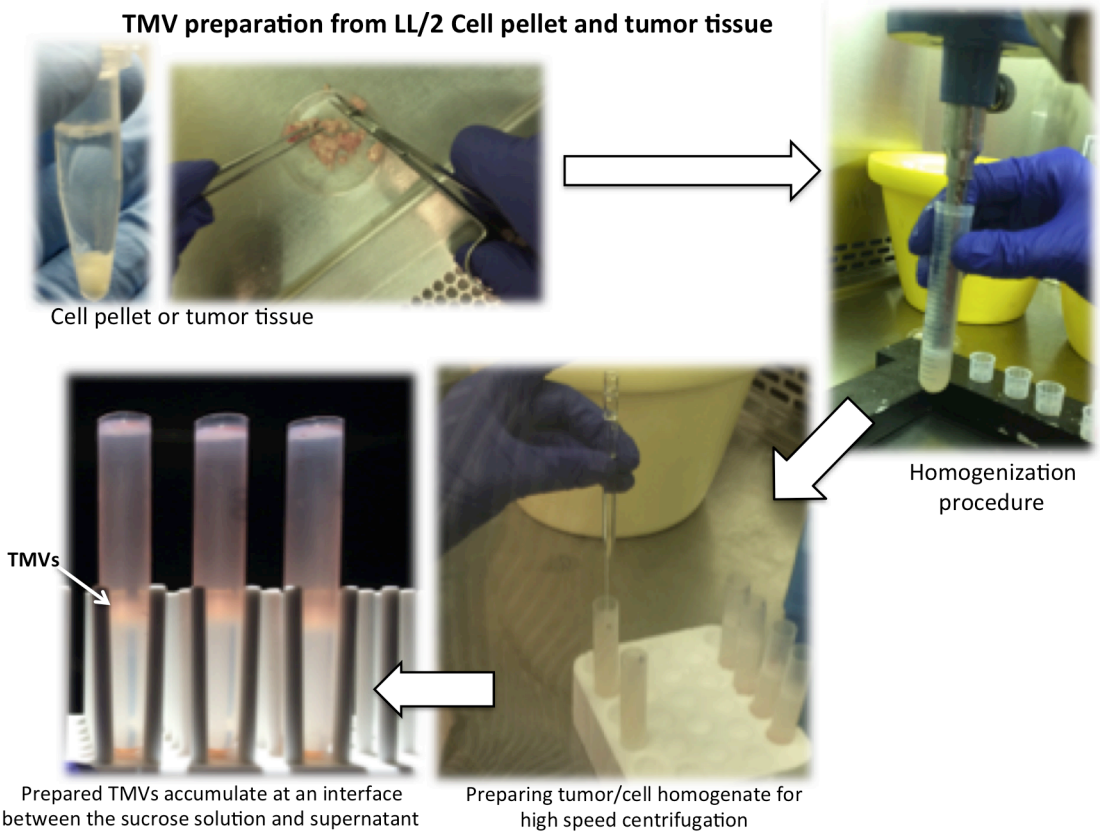

Figure 10: Preparation of LL/2 cell derived TMVs from cell pellet or tumor derived TMVs from fresh or frozen tumor tissue

may encompass the antigenic profile of the tumor tissue, which include tumor infiltrating leukocytes and other stromal tissue, both CTLA-4 and MHC II staining on tumor derived TMVs may be used to gain insight into the type of cells present in the tumor microenvironment.

Tumor derived TMVs express CD24 and CD44. Cell derived TMVs do not seem to express any of the antigens present on LL/2 cells (Figure 11). The stem cell marker CD24 is selectively expressed on tumor TMVs, but not on LL/2 cells or cell derived TMVs,

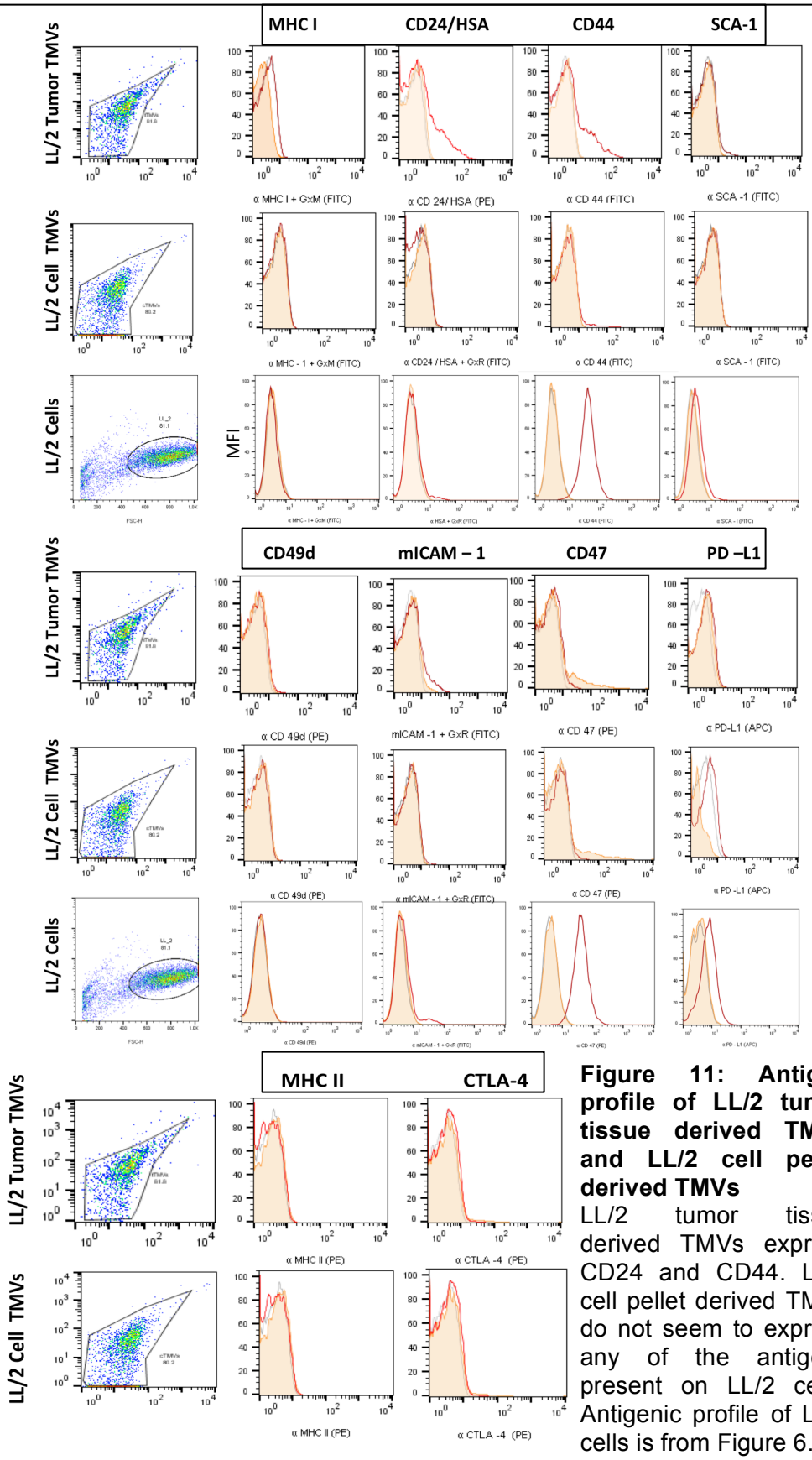

Figure 11: Antigen profile of LL/2 tumor tissue derived TMVs and LL/2 cell pellet derived TMVs

LL/2 tumor tissue derived TMVs express CD24 and CD44. LL/2 cell pellet derived TMVs do not seem to express any of the antigens present on LL/2 cells. Antigenic profile of LL/2 cells is from Figure 6.

suggesting that LL/2 tumors may express CD24 in vivo. The stem cell marker CD44 is expressed by tumor derived TMVs, just like the cell line, but it is not present on the surface of cell derived TMVs. Similarly, CD47 and PD-L1 expression also seems to be "lost". The explanation for these antigenic mismatches may be resolved if one attributes this as an artifactual development of TMV preparation. We hypothesize that these antigens are not lost, and are still present on the TMVs but on the

inner leaflet of the vesicles, or are not concentrated enough on the surface of miniscule TMVs to fluoresce at a detectable threshold for a positive signal. Tumor and cell derived TMVs did not express any of the other antigens tested. Tumor TMVs also stained negative for MHC II and CTLA-4.

TMVs are heterogeneous in shape and size

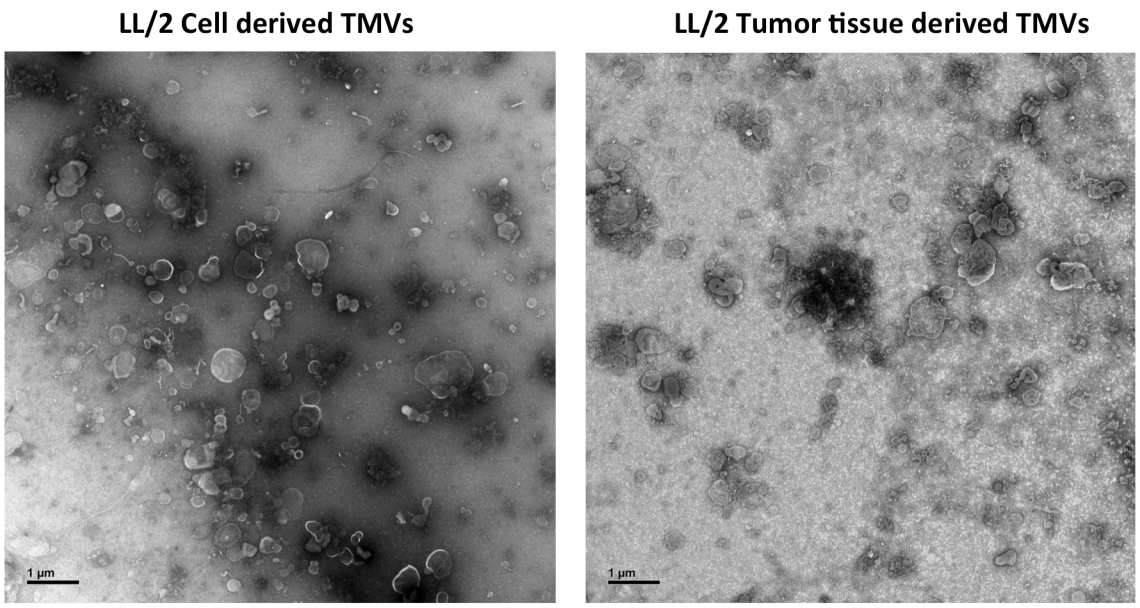

Figure 12: Negative staining of TMVs was performed and images were acquired using transmission electron microsccopy. Images reveal the vesicle structure of TMVs and heterogeneity in shape and size.

Lastly, TMVs were imaged using transmission electron microscopy (Figure 12) with negative staining performed on samples at the Robert P. Apakarian Electron Microscopy Core. As apparent from the images, TMVs are varied in shape and size, and are approximately less than 1 um in diameter.

Results from Experiment 4: Determining the LL/2 TMV Dose for vaccine studies

Whilst we were growing tumors in vivo for preparation of tumor tissue derived TMVs, we prepared TMVs from LL/2 cell pellets as described in materials and methods. These cell derived TMVs were used in our first vaccination study to see if prophylactic vaccination with LL/2 cell TMVs could protect from LL/2 tumor challenge. We immunized mice with varying amounts of cell TMVs, to titrate the tumor protective effects, if present. This strategy would help us decide how much TMV we needed to use for our future studies.

In this prophylactic vaccination study, groups of mice (n=3) were immunized subcutaneously with 25ug, 50ug, 100ug, or 200 µg of LL/2 cell derived TMVs in 100uL volume of PBS, or with 100µl PBS alone as scaffold controls. The week the first immunization is delivered is denoted as Week 0 in all our experiments. A booster dose of the same amount of LL/2 cell derived TMVs was given 2 weeks after the first dose, and is denoted as Week 2. Four weeks after the first dose, mice were inoculated with one million LL/2 cells (Figure 9 was still in progress during this time) suspended in 100µl PBS, on the flank contralateral to the site of immunization. Tumor growth and survival was observed as described earlier.

Through prophylactic vaccination with various doses of TMVs, we hoped to select the LL/2 TMV dose at which tumor protection was suboptimal – where immunization produced incomplete tumor protection. Combining adjuvants and ICI with this dose of TMVs would allow us to visualize and study the enhancement of the anti-tumor response in a more pronounced, clear manner.

Additionally, to determine if TMV vaccination led to the production of anti-LL/2 antibodies in vivo, blood was collected from the submandibular vein or the facial vein of the immunized mice at various time points before tumor challenge. Blood was collected right before the first dose was given, and then at weeks 1, 2 and 4. The time the first dose of the vaccine is given is denoted as Week 0. If a booster vaccination coincided with a blood collection week, mice were bled first, and then vaccinated. The results from this study are summarized in Figure 13.

Prophylactic vaccination with LL/2 cell derived TMVs does not protect from subcutaneous LL/2 tumor challenge or enhance survival

Prophylactic vaccination with LL/2 cell derived TMVs did not protect immunized mice from subcutaneous tumor challenge, and did not reduce mean tumor size significantly in vivo.

None of the doses ranging from 25 μg/dose to 200 μg/dose completely prevented tumor growth, although we did see a (non-significant) reduction in tumor size of mice immunized with 200 μg TMV compared to all other groups. Two-way ANOVA with Bonferroni post test was performed, and showed no significant differences in tumor size between the different groups of mice at any time point.

The Mantel-Cox test on Kaplan Meir survival curves could not be performed due to an insufficient number of mice, but the 100µg/dose group had the highest survival, while the 50µg/dose group had the lowest with a difference of about 20 days.

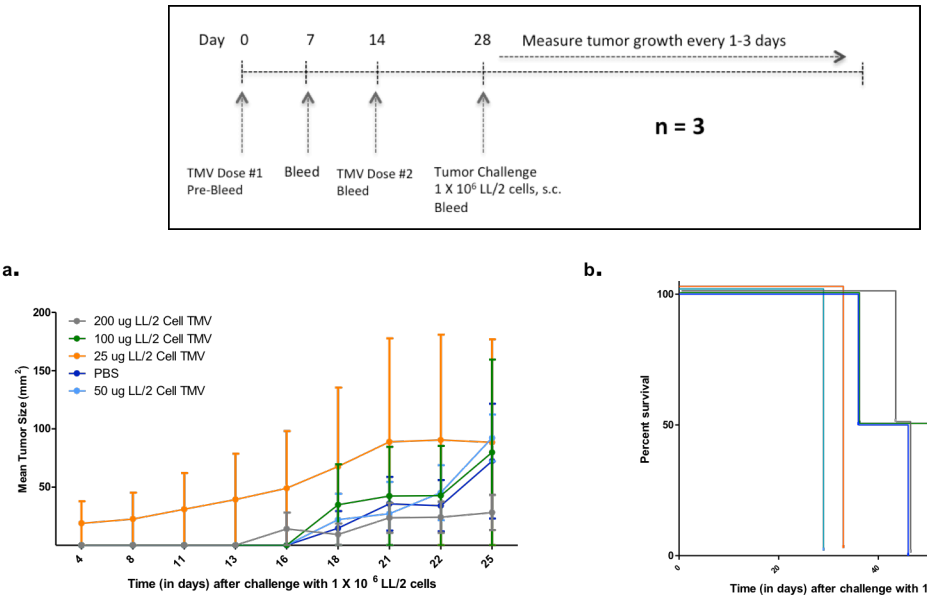

Figure 13: Prophylactic Vaccination with LL/2 TMVs does not protect against tumor challenge. Groups of mice (n=3) were immunized s.c. with various doses of LL/2 cell derived TMV, boosted, and then challenged with 1 X10^6 LL/2 cells s.c. on the contralateral flank. The timeline of this experiment is represented above. a. Tumor growth over time.; b. Survival post tumor challenge.

A major limitation of this study was that there was a delay in tumor growth in the PBS immunized (scaffold control) group. The PBS group was last to develop tumors, at around day 15 after inoculation (instead of between days 3 and 5). This suggests that tumor cell inoculation was inconsistent or incomplete or induced tolerance. Thus, we were not able to assess with certainty the effect TMV vaccination had on tumor growth. The differences seen in tumor sizes could have been caused due to anti-tumor activity via TMV immunization or because of differences in tumor growth due to technical errors during tumor inoculations. Furthermore, 3 mice in total (one each from the 200 µg/dose, 50 µg/dose and 25 µg/dose groups) did not develop any tumors at all. Upon rechallenge

with LL/2 cells, these mice grew tumors progressively (data not shown) suggesting that tumor inoculation was not successful in these groups.

Nevertheless, we tried to study tumor abrogation via TMV immunization in mice that developed tumors around the same time, by comparing groups other than the 25μg/dose group. Compared to the PBS and other TMV groups, mice vaccinated with 200 μg/dose had the smallest tumors, suggesting the presence of an anti-tumor response mediated via TMVs.

However, for further studies, we decided to choose the highest immunization dose we tested, 200μg/dose, as the amount of TMV to produce our “TMV+ Adjuvant”, “TMV+ICI” and “TMV+ Adjuvant +ICI” vaccines.

Vaccination with LL/2 cell TMVs does not produce an anti-LL/2 antibody response

Serum collected from immunized mice at Weeks 0 (pre-immunization), 1, 2 and 4 were screened for the presence of antibodies through flow cytometry. Briefly, cultured LL/2 cells were incubated for 30 mins with serum diluted 1:10 in FACS Buffer, allowing anti-LL/2 antibodies in the serum (if any) to bind to LL/2 cells. Excess serum was washed out and cells were stained with a FITC conjugated goat anti-mouse IgG antibody to visualize the serum antibodies bound on LL/2 cells.

Results from flow cytometry (Figure 14) showed that an antibody response was not generated upon immunization with none of the doses of LL/2 cell derived TMVs, compared to the PBS controls. There was a lot of intra group heterogeneity with a trend of the 25μg/dose having the highest antibody, but also the largest tumors. Inversely, the

group with the smallest tumors had one of the lowest antibody levels consistent throughout all four weeks. Statistical analysis using a Two-Way ANOVA showed no

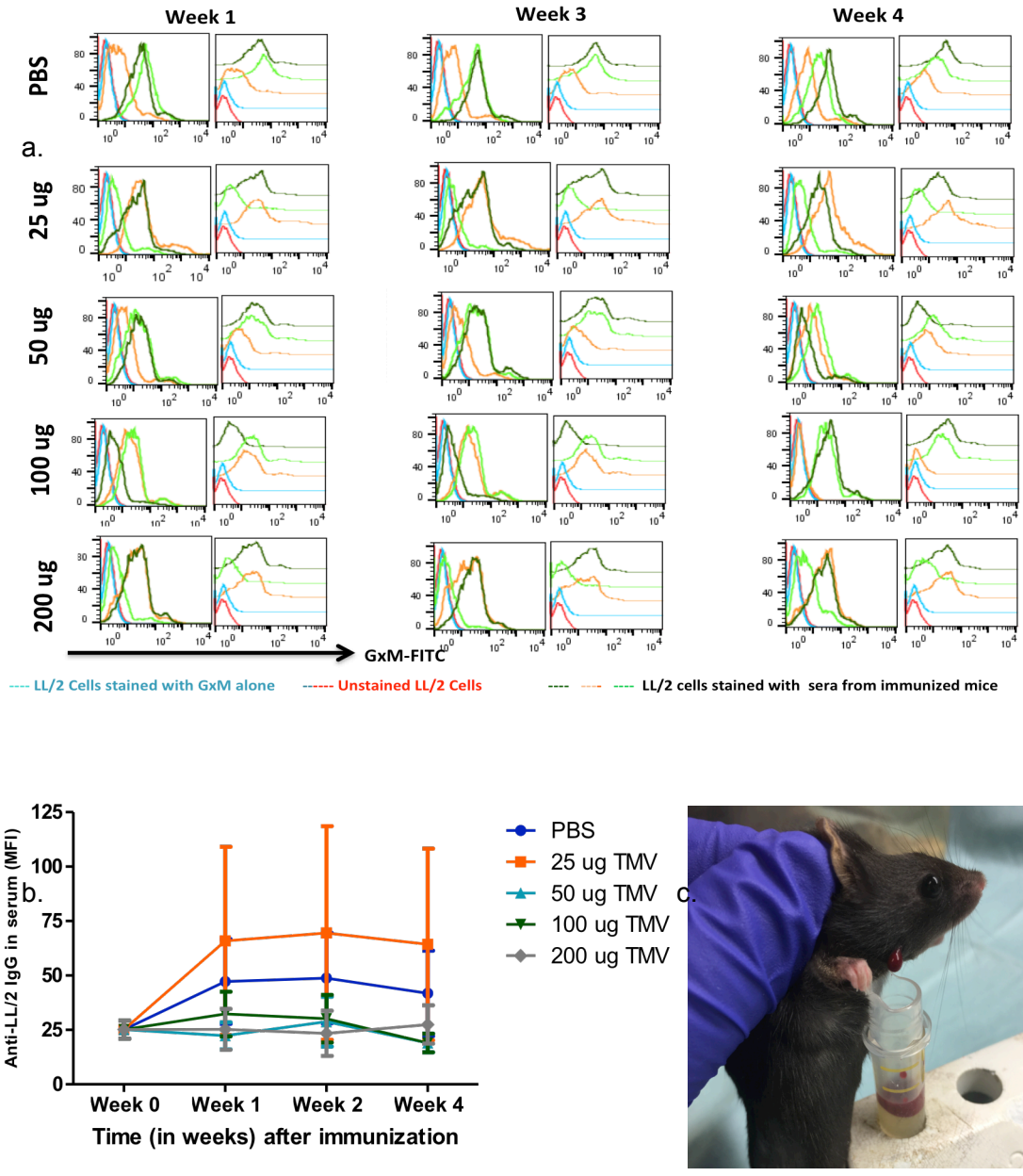

Figure 14: Immunization with LL/2 TMVs did not induce antibodies against LL/2 . a. Individual anti-LL2 antibody levels in mice.; b. Compilation of data from a.; c. Blood collection from the facial vein. Antibody levels were determined via flow cytometry and one-way ANOVA was performed to assess statistical significance.

significant difference in antibody levels between all treatment groups, at all time points. For this screen, a FITC conjugated goat anti-Mouse IgG secondary antibody was used, which was specific to only the IgG isotype anti-LL/2 antibodies. For our next screens, we used a secondary antibody specific to both mouse IgG and mouse IgM isotypes to enrich our screen.

Our next phase of study involved performing in vivo screens to test the efficacies of various “TMV+Adjuvant” and “TMV+ ICI” vaccines in protecting against tumor challenge, and inducing regression of established tumors, respectively.

Results from Experiment 5: “TMV + Adjuvant” Prophylactic Vaccination Study

Seven adjuvants – 4 TLR agonists MPL, Imiquimod, CpG, Poly I:C; depot effect inducing alum/Alhydrogel; immune cell trafficking agent AddaVax/MF59 and CTL inducing Quil A/Saponin – were each combined with LL/2 tumor derived TMVs to produce seven distinct “TMV+ Adjuvant” constructs.

Each vaccine dose consisted of 200 µg of tumor tissue derived LL/2 TMVs combined with the selected adjuvants. Adjuvants were reconstituted following manufacturer’s instructions and the dosing guidelines for each was followed. Literature was also surveyed to ensure that manufacturer’s dosing guidelines were reflective of convention. Each of the adjuvants’ dosing instruction was provided as a working range per mouse. To prevent risk of anaphylaxis, we chose the amount of adjuvant per dose as the mid value of the prescribed working range. Thus, cumulatively, over two doses of “TMV+Adjuvant” vaccine, each mouse received a total of 400 µg of LL/2 tumor tissue

derived TMVs and the maximum amount of adjuvant in the working range recommended by the manufacturer.

“TMV+Adjuvant” vaccines were freshly prepared the day of vaccination. Like our previous prophylactic vaccination study, groups of mice (n=5) were challenged with “TMV+Adjuvant” vaccines at Week 0, boosted 2 weeks later, and then challenged subcutaneously with 500,000 LL/2 cells on the contralateral flank four weeks after the first dose of vaccine.

Blood was collected from the facial vein of immunized mice every week until tumor challenge. Serum analysis was performed to quantify levels of anti-LL/2 antibodies via flow cytometry, and the levels of the Th1 cytokine IFN-γ, and the Th2 cytokine IL-4 in serum were determined via ELISA. After tumor challenge, tumor size and survival were measured and recorded.

The table below describes the adjuvants, and the amount that was used per dose of “TMV+Adjuvant” Vaccine. Each of these adjuvants was combined with 200 μg LL/2 TMV:

Table 2: The properites and dosage of adjuvants selected to make "LL/2 TMV + Adjuvant" Vaccines

Adjuvant Name	Mechanism of Action	Expected Immune Response	Amount/Dose	FDA Approved?
AddaVax	Immune cell homing to site of vaccination through chemokine induction	Th1 and Th2 [2, 3]	25 µl	Yes
Alhydrogel	Depot Effect	Th2[8]	30 µl	Yes
MPL	TLR4 Agonist	Th1 and Th2 [9]	10 µg	Yes
Imiquimod	TLR 7/8 agonist	Th1[9]	50 µg	Yes
CpG	TLR 9 agonist	Th1[9]	35 µg	No
Poly I:C	TLR 3 agonist	Th1[9]	50 µg	No
Quil A/ Saponin	?	Th1/ CTL [10]	7.5 µg	No

Prophylactic Vaccination with "TMV+Adjuvant" vaccines do not significantly reduce LL/2 tumor growth in vivo nor enhance survival

Immunization with none of the "TMV + Adjuvant" vaccines provided complete tumor protection upon LL/2 tumor challenge. Furthermore, none of the "TMV+Adjuvant" vaccines reduced tumor size significantly when compared to the control groups. (Figure 15a.)

Two-way ANOVA with a Bonferroni post test was performed at Day 20 and 25 after tumor challenge, and none of "TMV+Adjuvant" vaccinated groups had a significant difference in tumor size when compared to the PBS and TMV immunized groups. Tumor implantation rate was 100% - all mice, including PBS controls, developed palpable tumors within 5 days after tumor challenge. Once established, tumors grew

progressively although TMV+ CpG, TMV + MPL and TMV + AddaVax immunized mice showed a modest reduction in tumor size when compared to controls.

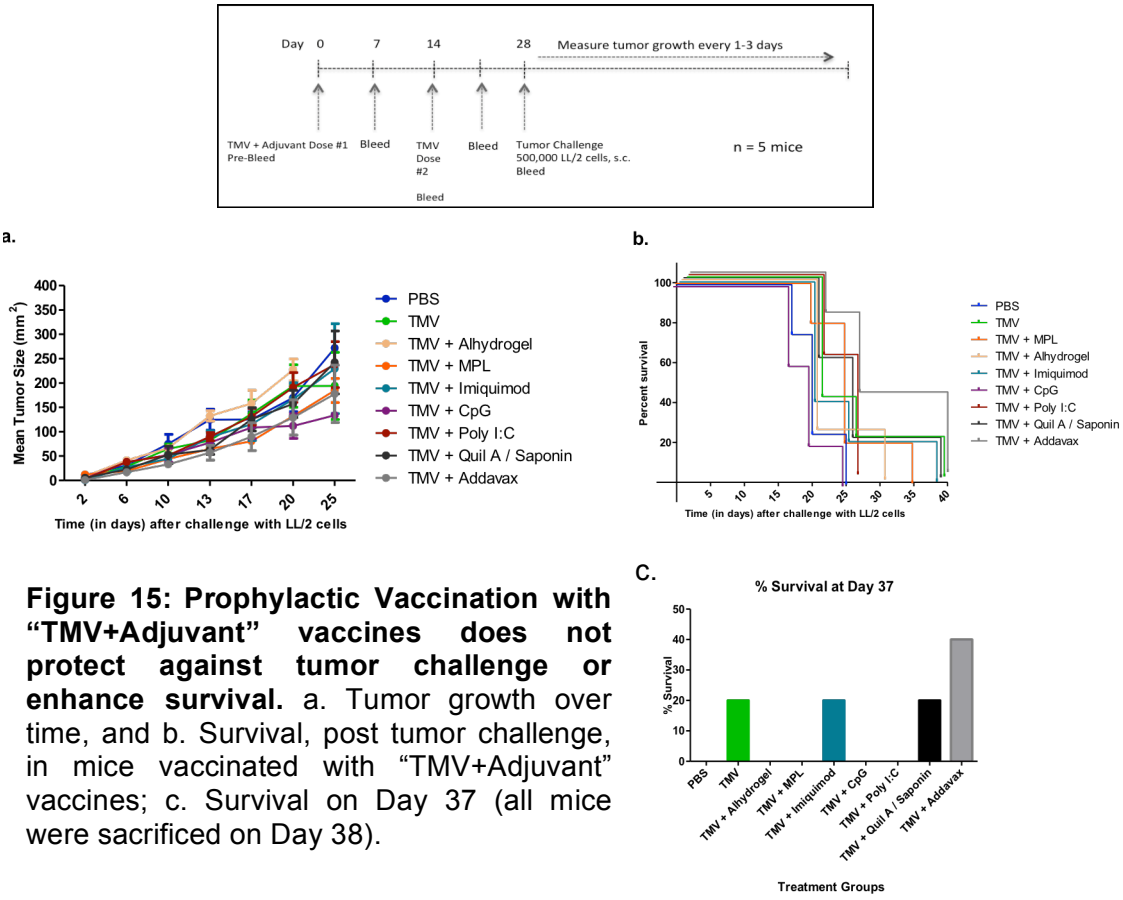

Figure 15: Prophylactic Vaccination with "TMV+Adjuvant" vaccines does not protect against tumor challenge or enhance survival. a. Tumor growth over time, and b. Survival, post tumor challenge, in mice vaccinated with "TMV+Adjuvant" vaccines; c. Survival on Day 37 (all mice were sacrificed on Day 38).

Likewise, survival was not enhanced significantly by combining TMV with adjuvants. The Mantel Cox test was used to compare Kaplan Meir survival curves of all the groups, and no significant difference in survival was observed between groups. (Fig15b.) However, the TMV+AddaVax group had the highest number of tumor bearing mice that lived until Day 37, one day before all surviving mice were sacrificed since their tumor sizes had reached the IACUC prescribed endpoint. (Fig 15c.)

Although the reduction in tumor size was not statistically significant TMV co-administered with MPL, AddaVax and CpG produced tumors with the smaller mean sizes compared to controls (PBS and TMV groups). For combining with checkpoint blockade in our next in vivo studies, we chose MPL and AddaVax as vaccine adjuvants. CpG was not chosen because of the relative reduction in survival that it produced.

The following trends in tumor growth and survival were observed for each group; all differences were non-significant (Figures 16 a. - 16p.). Compared to PBS controls, immunization with TMV decreased tumor size and enhanced survival. Compared to TMV immunization alone, co-administration of Alhydrogel increased tumor sizes and decreased survival. Co-administration of MPL decreased tumor sizes and survival; Imiquimod increased tumor sizes and had no effect on survival; CpG induced the smallest tumors among all the groups, but also induced the lowest survival rates. Poly I:C co administration with TMV increased tumor size and decreased survival compared to TMVs alone; Quil A/Saponin increased tumor size, but did not affect survival; and lastly, AddaVax, in combination with TMVs, produced smaller tumors, and enhanced survival more than any other group in this prophylactic vaccination study. (Figures 16 a. - 16p.)

Prophylactic vaccination with "TMV+Adjuvant" vaccines does not induce anti-LL/2 antibody responses

Serum diluted 1:10 was screened for anti-LL/2 antibodies as described above, with one change. For this study, a FITC conjugated Goat antibody against Mouse *IgG and IgM* was used. Antibodies with the IgM isotype are induced in the earlier in the immune response when compared to the IgG antibodies. Thus, we hoped to use this strategy to enrich our antibody screen.

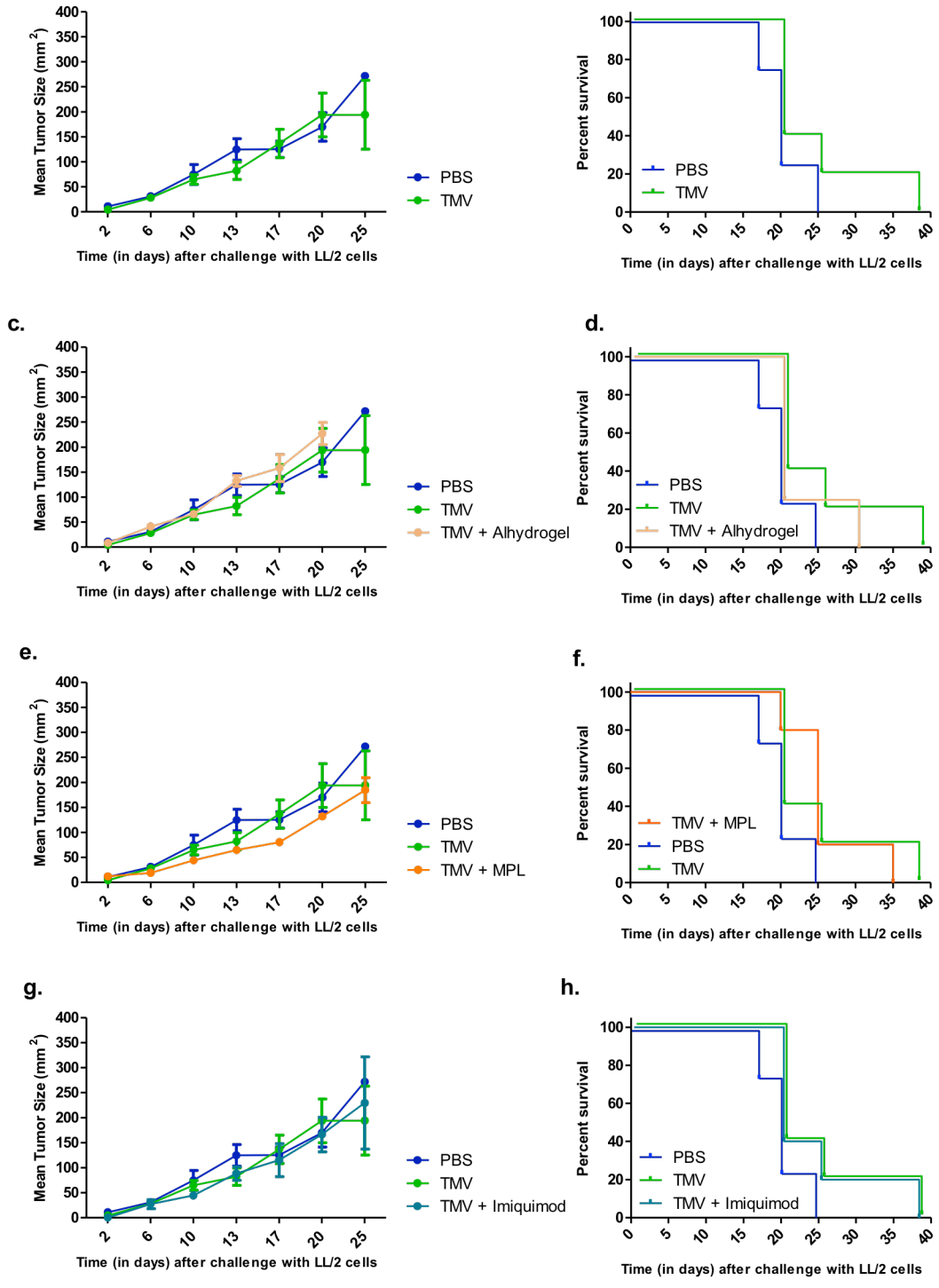

Figure 16: Prophylactic Vaccination with "TMV+Adjuvant" vaccines does not protect against tumor challenge or enhance survival: Vaccination with LL/2 Tumor tissue derived TMVs alone (a., b.) or in combination with Alhydrogel (c., d.), MPL (e., f.) and Imiquimod (g., h.) does not protect against LL/2 tumor challenge.

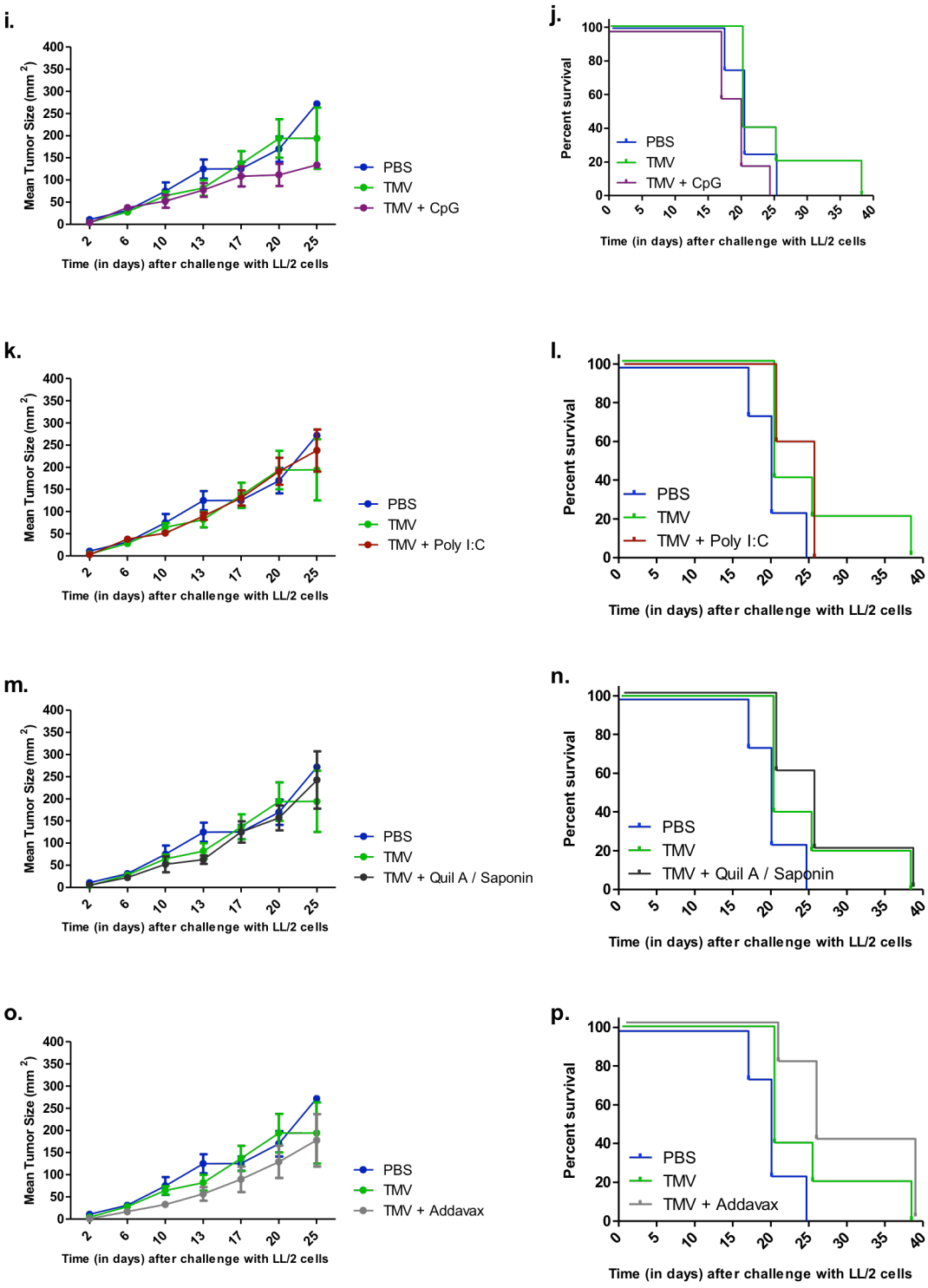

Figure 16 (continued): Prophylactic Vaccination with "TMV+Adjuvant" vaccines does not protect against tumor challenge or enhance survival: Vaccination with LL/2 Tumor tissue derived TMVs in combination with CpG (i., j.), Poly I:C (k., l.), Quil A/ Saponin (m., n.), or AddaVax (o., p.) does not protect against LL/2 tumor challenge.

When compared to the IgG + IgM levels present in PBS vaccinated mice, none of the "TMV+ Adjuvant" vaccines generated an increase in antibody levels at both time points evaluated(Fig.17). Two-way ANOVA was performed at both time points, and no statistical difference was observed. Antibody levels did not increase significantly after vaccination with 'TMV+ Adjuvant" from Week 2 to Week 4 in all groups.

Even in groups where we combined our TMVs with adjuvants like alhydrogel, that are known to exclusively enhance antibody responses in vivo, we did not observe a significant increase in antibody levels. Surprisingly, the TMV+ Alhydrogel group had the lowest antibody levels and even decreased from Week2 to Week 4. Addavax, which is also known to induce a Th2 response, did have one of the higher antibody levels but only negligibly higher than the PBS controls (Figure 17).

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ELISA to measure IFN-γ and IL-4 levels in serum

Serum collected from Week 3 three weeks after the first dose of vaccine and one week after the booster dose) and stored at -20 C was diluted 1:10 and ELISA was performed to determine IFN-γ and IL-4 levels. For both tests, serum cytokine levels were below detection limit in all groups. ELISA was repeated twice to confirm results (data not shown). This observation further suggests, that only a modest or no anti-tumor immune response may have been generated.

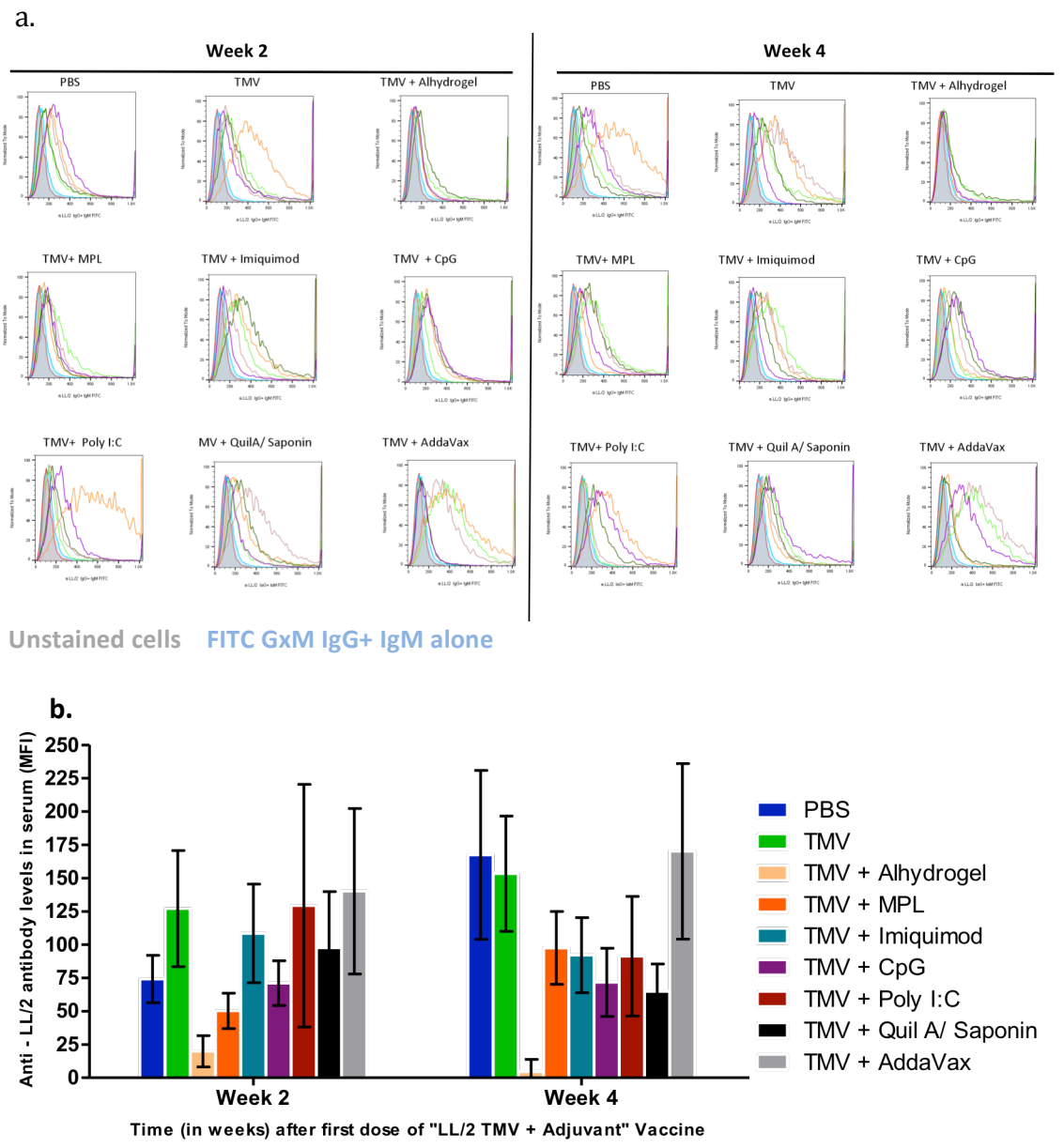


Figure 17: Prophylactic vaccination with "TMV + Adjuvant" vaccines did not induce an anti-LL/2 antibody response.

a. Anti-LL/2 antibodies in each individual group during Week 2 and Week 4 determined through flow cytometry; b. Compilation of data from a.

Immunization with LL/2 tumor TMVs and "TMV + Adjuvant" vaccines does not reduce splenomegaly in tumor bearing mice

Spleen weights were measured upon sacrifice/ death. Tumor induced splenomegaly was not abrogated through any of our vaccination approaches (Figure 18) Statistical significance was tested using one-way ANOVA, and all p values were greater than 0.05 (non-significant).

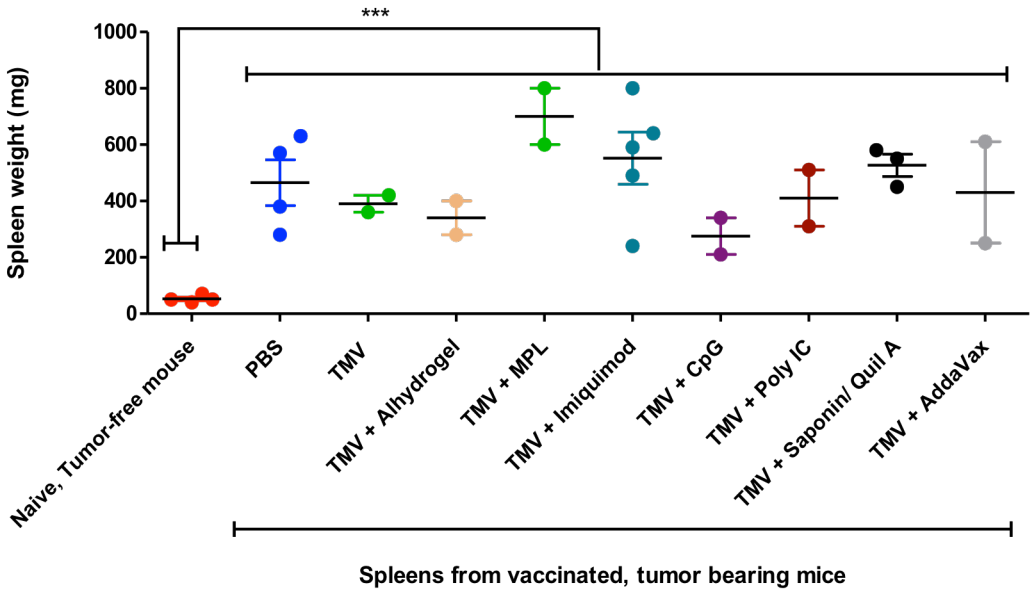


Figure 18: Vaccination with both LL/2 tumor tissue derived TMVs and "TMV+ Adjuvant Vaccines" does not prevent LL/2 tumor induced splenomegaly.

In conclusion, prophylactic vaccination with neither LL/2 tumor derived TMVs alone nor in combination with any of the seven "TMV+Adjuvant" vaccines that we tested, afforded protection against LL/2 tumor challenge. Furthermore, vaccination did not significantly reduce mean tumor size, did not induce an anti-LL/2 antibody response nor induce polarization of the immune response by induction of Th1 and Th2 cytokines IFN-γ and IL-4, respectively. Thus, adjuvants did not enhance the anti-tumor immunity mediated by LL/2 tumor derived TMVs. For combination with ICI therapy, MPL and AddaVax were

chosen because their co-administration with LL/2 TMVs reduced mean tumor size without reducing survival.

Results from Experiment 6: "TMV + ICI" vaccine Therapeutic vaccination study

To characterize and validate the nature and extent of immune checkpoint inhibitor resistance in the LL/2 model, we conducted a comprehensive therapeutic vaccination screen of aPD-1, aCTLA-4 and combination therapy of aPD-1 and aCTLA-4. The LL/2 cell line is marketed as an ICI resistant model by Charles River [193], and we first tested whether ICI therapy was indeed ineffective in abrogating LL/2 tumor growth in vivo.

We also combined the ICIs administraton with LL/2 tumor tissue derived TMV vaccines to initiate regression of established tumors in a therapeutic setting. Our aim was to choose the ICI or ICI combination that abrogated tumor growth when co-administered with LL/2 TMVs. By finding the optimal ICI that complements LL/2 TMV immunotherapy, we hoped to combine the " TMV+ICI" vaccine effects with the "TMV+ Adjuvant" vaccine and induce an enhanced anti-tumor immunity via a "TMV+ Adjuvant + ICI" vaccine.

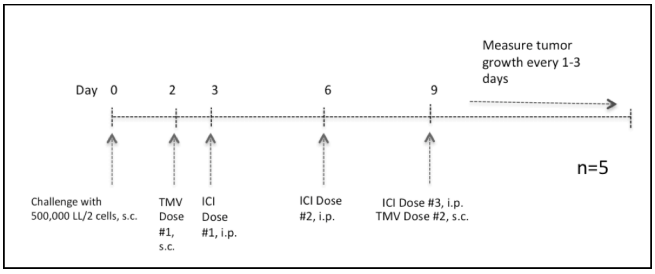
Both aPD-1 and aCTLA-4 antibodies were procured from BioXCell, and a 200 µg/dose of each ICI was used in combination with 200 µg of LL/2 tumor derived TMVs. The dose of ICI conformed to what has been used conventionally in pre-clinical studies to achieve tumor abrogation. To defy immunosuppression on the PD-1/PD-L1 and PD-L2 axis, we strategically chose to use the aPD-1 antibody to ensure elimination of rescue PD-1 signaling mediated by PD-L2 engagement, which is another ligand for PD-1 in addition to PD-L1.

We inoculated naive mice with 500,000 live LL/2 cells subcutaneously. 2 days after tumor inoculation, we delivered our first dose of 200 μg LL/2 TMVs subcutaneously. This was followed by intraperitoneal injections of ICI therapy (either 200 μg aPD-1, 200 μg aCTLA-4, or the combination) on days 3, 6 and 9 after tumor inoculation. On day 9, a LL/2 TMV booster dose was also delivered subcutaneously. Tumor size and survival was measured every 3 days from the day of tumor cell inoculation, and mice were sacrificed if any of the endpoints described earlier were reached.

Therapeutic vaccination with a combination of aPD-1 and aCTLA-4 significantly reduces mean tumor size in vivo, but does not inhibit tumor growth in combination with LL/2 TMVs

LL/2 tumor inoculation rate for this study was 100 % - all tumors were palpable within the first five days, and grew progressively despite our therapeutic vaccination strategies. Our goal was to find a checkpoint blockade therapy that in combination with LL/2 TMVs induced regression of established tumors.

Tumor growth over time and survival is represented in Figures 19 and 20. A two-way ANOVA was performed, and the only treatment regimen that significantly reduced mean tumor size compared to controls was combination therapy with aPD-1 and aCTLA-4 alone. Interestingly, in combination with TMVs, combination ICI therapy produced tumors larger than the controls groups, were really necrotic compared to any other group, and decreased survival. Mantel-Cox test was performed to compare Kaplan-Meir survival curves, but no significant difference in survival was found to exist between any of the groups.



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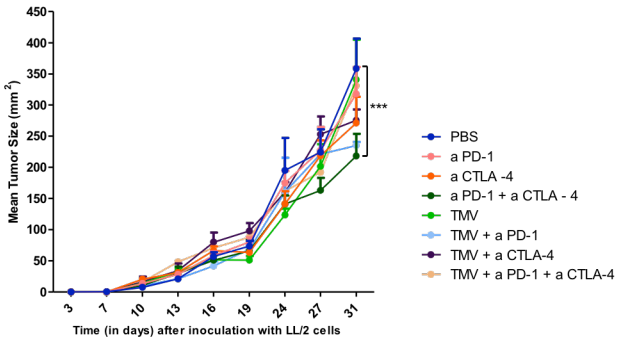

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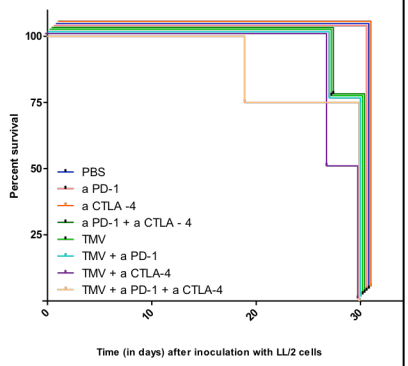
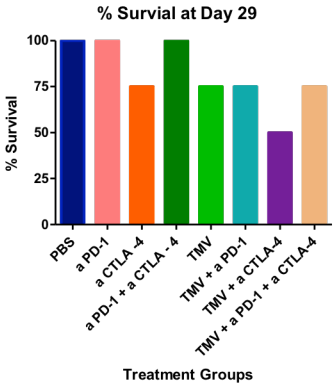


Figure 19: Therapeutic Vaccination with ICI and "TMV + ICI" Vaccines: Therapeutic vaccination with a combination of aPD-1 and aCTLA-4 reduces mean tumor size significantly; none of the other vaccination regimens reduced tumor growth. **a.** Tumor growth post LL/2 cell inoculation; b. Survival; c. Survival on Day 29; All mice were sacrificed on Day 30.

c.



Therapeutic Vaccination with aPD-1 or aCTLA-4 alone; or with any of the "TMV + ICI" vaccines, does not induce regression of tumors in vivo, or enhance survival

The following trends were also observed. Therapeutic vaccination with TMVs did not reduce tumor burden, and decreased survival compared to PBS controls. (Fig 20a.) Therapeutic vaccination with ICIs alone (without TMV) decreased tumor sizes compared to TMV and PBS groups.(Fig.205b.) aPD-1, aCTLA-4 and the combination treatment with aPD- 1 and CTLA-4 decreased tumor size, the combination therapy producing the smallest tumors among the groups, as mentioned before. When combined with TMVs, aPD-1 produced smaller tumors. (Fig 20 c.) aCTLA in combination with LL/2 TMVs produced tumors similar to the controls, but decreased survival. (Fig 20d.) And lastly, the combination ICI therapy in combination with LL/2 TMVs seemed to reverse the tumor inhibitory effects seen with ICI combination therapy alone. (Fig 20e.) The TMV+ aPD-1 +aCTLA-4 group was also the group with the lowest overall survival.

When comparing all the "TMV + ICI" vaccines, aPD-1 was the only ICI that reduced tumor growth, and also had higher survival than the other two "TMV+ ICI" vaccines. (Fig 20f.) Thus, we chose aPD-1 as the ICI to combine with "TMV + Adjuvant" vaccines to produce "TMV + ICI + Adjuvant" vaccines for our next in vivo study. Furthermore, two aPD-1 antibodies Keytruda and Opdivo, have been FDA approved for the treatment of Non Small Cell Lung cancer, 2014 and 2015, respectively, making aPD-1 an appropriate choice for translation to the clinic.[184] There are currently no aCTLA-4 antibodies that are approved for lung cancer treatment.

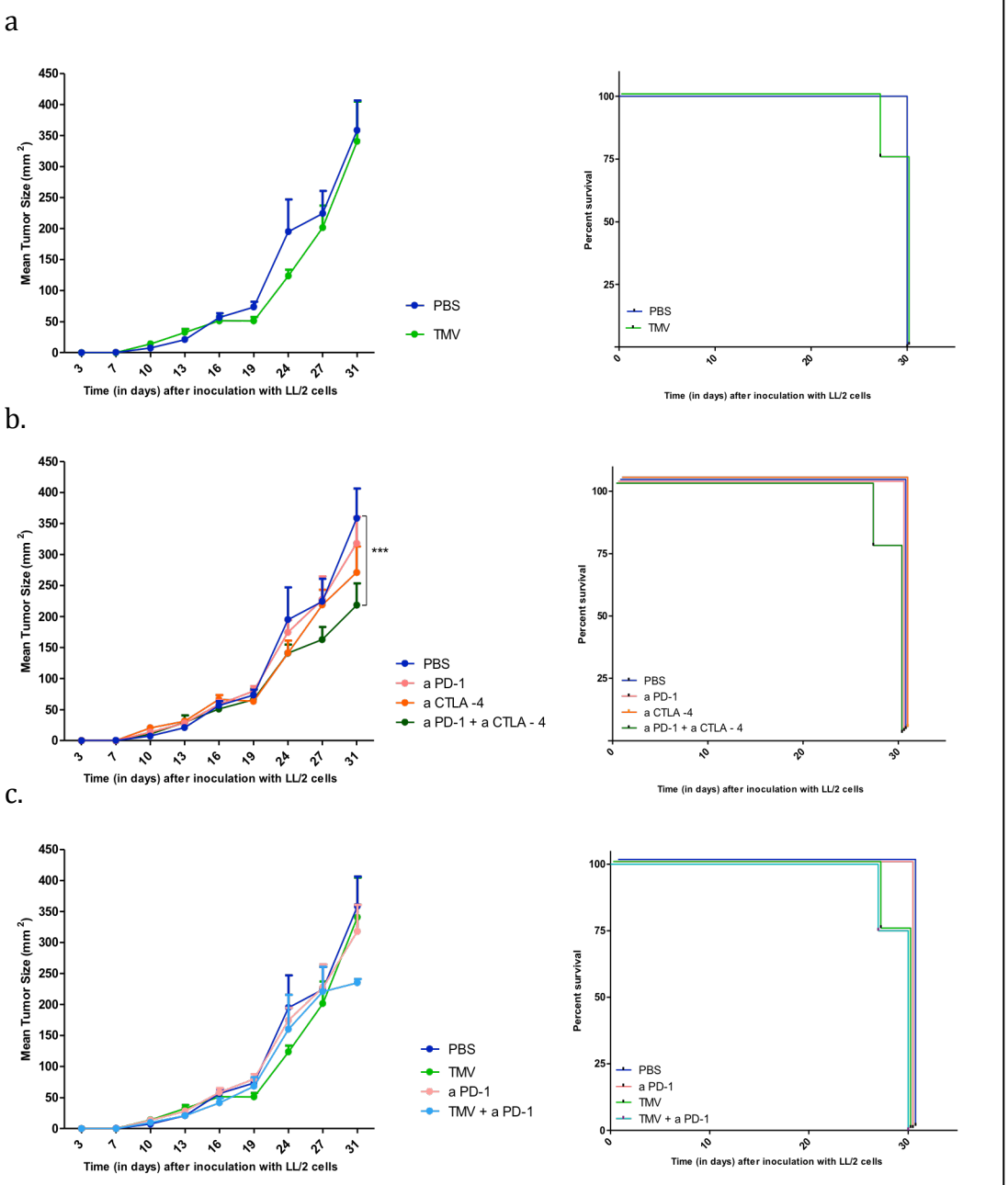


Figure 20: Therapeutic Vaccination with ICI and "TMV + ICI" Vaccines: a. Therapeutic vaccination with LL/2 TMV alone does not induce regression of established tumors; b. Therapeutic vaccination with the combination of aCTLA-4 and aPD-1, but not each of the ICIs on their own, significantly reduces tumor size in vivo; c. Therapeutic vaccination of aPD-1 in combination with TMVs reduces mean tumor size, but not significantly.

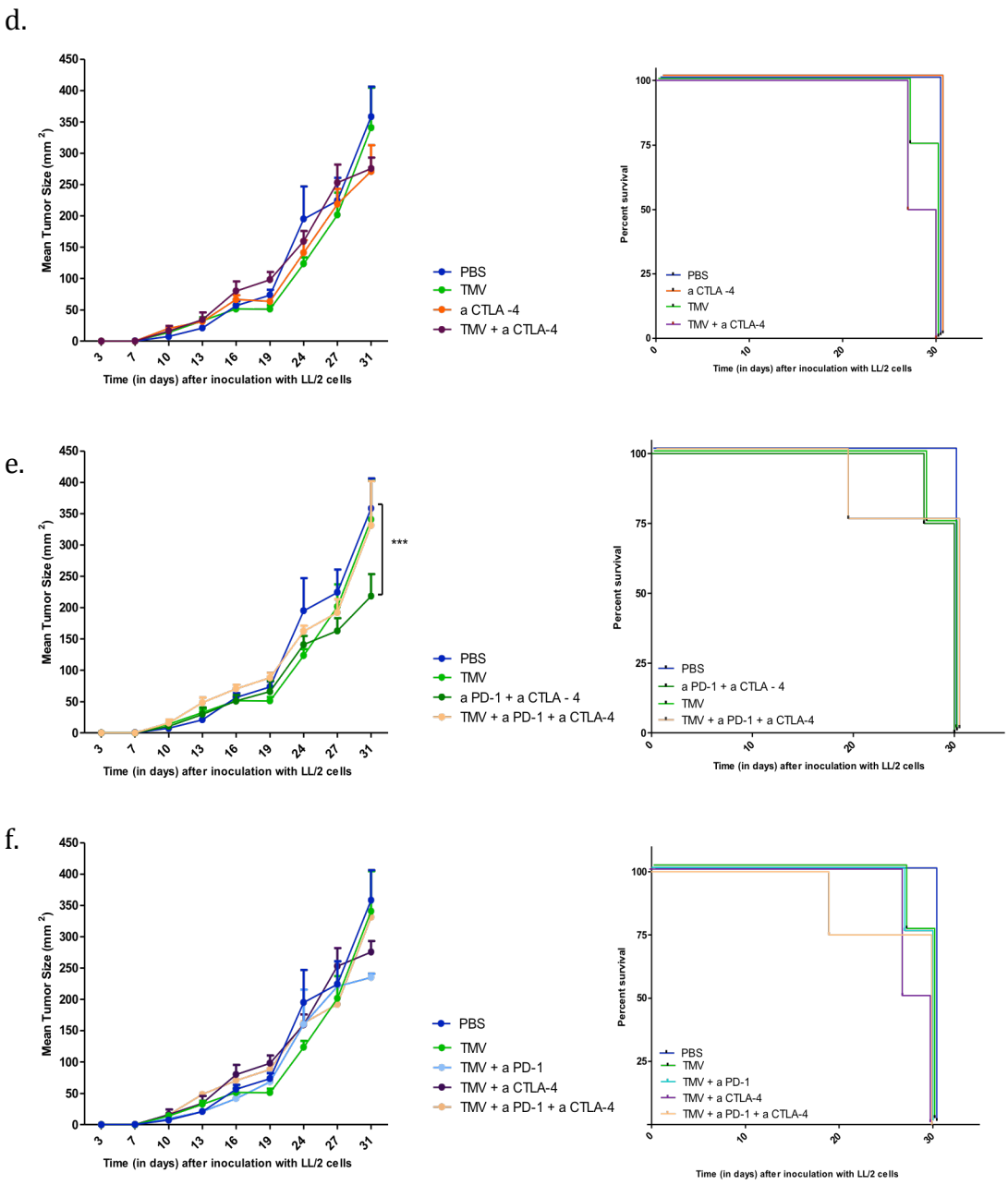


Figure 20 (continued): Therapeutic Vaccination with ICI and "TMV + ICI" Vaccines: d. Therapeutic vaccination of aCTLA-4 in combination with TMVs does not reduce mean tumor size; e. Therapeutic vaccination of aCTLA-4 and aPD-1 in combination with TMVs does not reduce mean tumor size; f. aPD-1 is the only ICI that induces a reduction, albeit modest, of tumor growth in vivo.

Therapeutic vaccination with ICIs and "TMV + ICI" vaccines did not reduce splenomegaly in tumor bearing mice

Spleen weights were measured upon sacrifice/ death. Tumor induced splenomegaly was not abrogated through any of our vaccination approaches (Figure 21). Statistical significance was tested using one-way ANOVA, and all p values were greater than 0.05 (non-significant).

In conclusion, The only ICI that reduced tumor growth in combination with L/2 TMVs was aPD-1, and was chosen to combine with "TMV + Adjuvant" vaccines to produce "TMV + ICI + Adjuvant" vaccines for our next in vivo study.

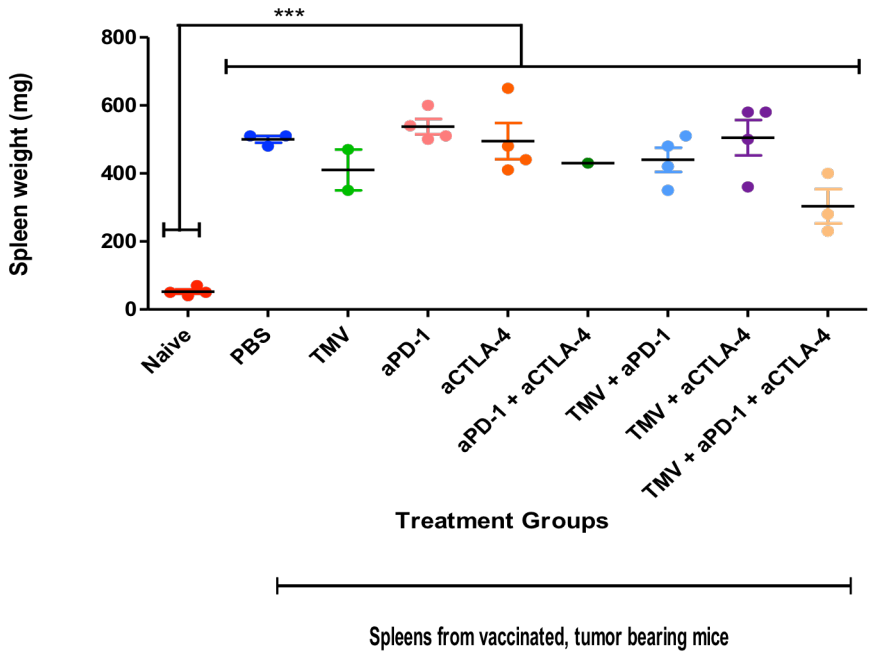


Figure 21: Therapeutic vaccination with ICIs and "TMV + ICI" vaccines does not reduce splenomegaly in LL/2 tumor bearing mice

Results from Experiment 7: "TMV + Adjuvant + ICI" Study

In this final in vivo study, we combined the most trending "TMV+ Adjuvant" vaccines with the most effective "TMV + ICI" vaccines from our previous two screens to produce "TMV+ Adjuvant + ICI" vaccines. With this vaccination strategy, we hoped to induce regression of LL/2 tumors in vivo by effectively revving up anti-cancer immune response (the Cancer – Immunity Cycle). We wanted to "step on the gas" of the suboptimal anti-tumor immunity generated via LL/2 TMVs, with the help of adjuvants, and "release the brakes" using checkpoint blockade. More importantly, we hoped to make ICI resistant LL/2 tumors respond to ICI by generating a de novo immune response via our "TMV +Adjuvant" vaccines.

We combined prophylactic and therapeutic vaccination models for this study. Mice were first vaccinated prophylactically with "TMV+ Adjuvant" vaccines ("TMV + MPL" and "TMV+ AddaVax") and boosted after two weeks. Two weeks after the booster dose, mice were challenged subcutaneously on the contralateral flank with 500,000 live LL/2 cells. On days 3,6 and 9 after tumor challenge, therapeutic administration in the form of 200 µg of aPD-1 antibody was delivered through intraperitoneal injections.

Tumor size and survival was measured every 3 days from the day of tumor cell inoculation, and mice were sacrificed if any of the endpoints described earlier were reached. Blood was collected during the immunization period until tumor challenge, and tested for antibodies via flow cytometry, and the cytokines IL-4 (Th2 cytokine) and IFN-γ (Th1 cytokine) via ELISA.

We doubled the dose of adjuvants for this study hoping to see a more potent immune response. We also included control groups in the form of groups vaccinated with adjuvants alone. Both aPD-1 and LL/2 TMV amounts remained the same at 200μg/dose.

Table 3: A summary of adjuvants selected to make "LL/2 TMV + Adjuvant + ICI" Vaccines

Adjuvant Name	Mechanism of Action	Expected Immune Response	Amount/Dose	FDA Approved?
AddaVax	Immune cell homing to site of vaccination through chemokine induction	Th1 and Th2 [2, 3]	50 ul	Yes
MPL	TLR4 Agonist	Th1 and Th2 [9]	20 ug	Yes

Thus, through combining vaccination approaches, we created two "TMV +Adjuvant + ICI" vaccines: "TMV+ MPL+ aPD-1" and "TMV+ AddaVax+ aPD-1" vaccines.

"TMV + Adjuvant + ICI" vaccines do not protect against tumor challenge nor induce regression of tumors in vivo, nor enhance survival

LL/2 tumor inoculation rate for this study was 100 % - all tumors were palpable within the first five days, and grew progressively despite our vaccination strategies. Tumor growth over time and survival is represented in Figures 22 and 23. A two-way ANOVA was performed, and no significant differences in tumor size were observed at any time point, despite the lack of combining TMVs with adjuvants and checkpoint blockade, and also doubling the adjuvant dosage. Mantel-Cox test was performed to compare Kaplan-Meir survival curves, and no significant difference in survival was found between any of the groups.

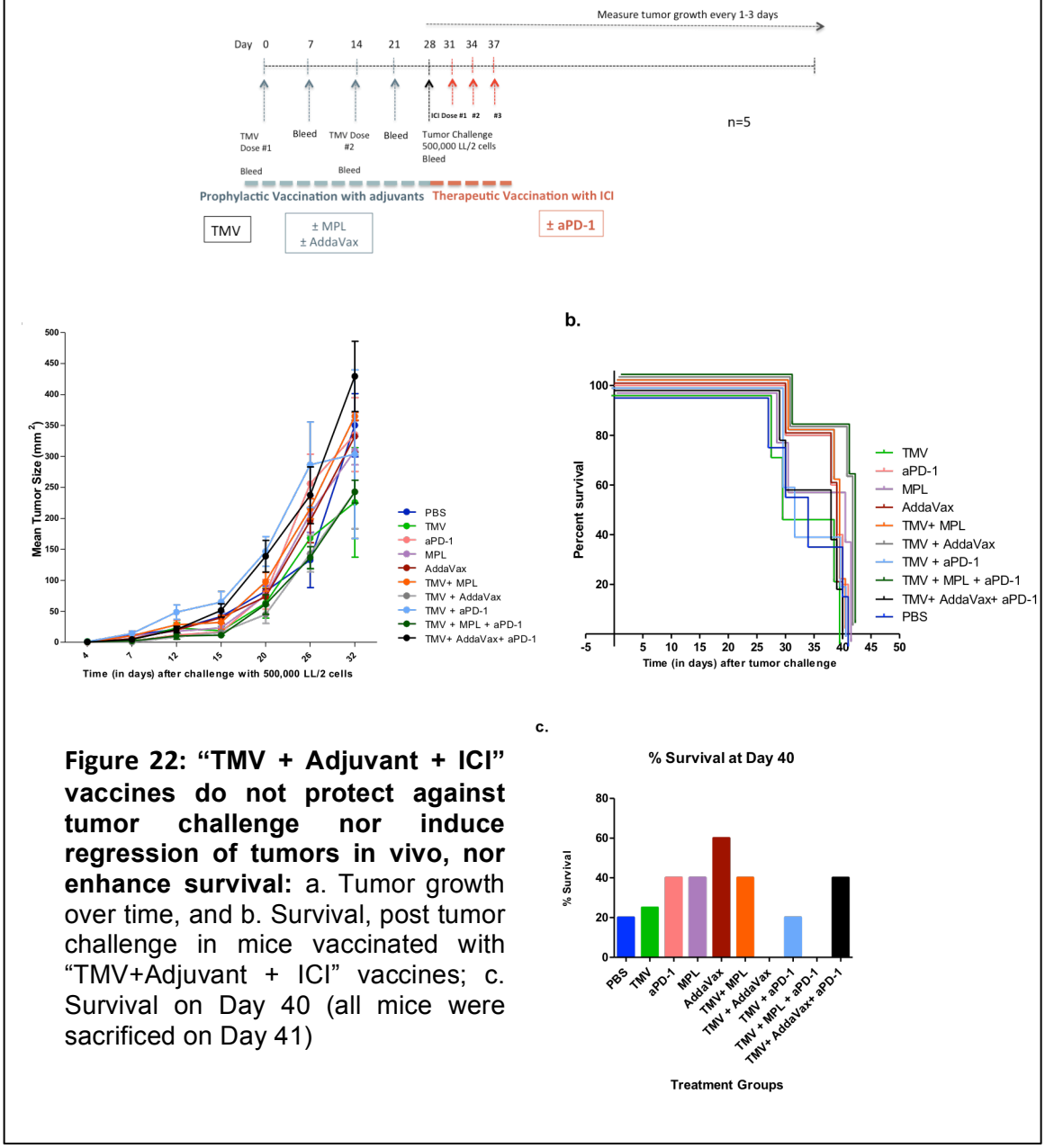


Figure 22: "TMV + Adjuvant + ICI" vaccines do not protect against tumor challenge nor induce regression of tumors in vivo, nor enhance survival: a. Tumor growth over time, and b. Survival, post tumor challenge in mice vaccinated with "TMV+Adjuvant + ICI" vaccines; c. Survival on Day 40 (all mice were sacrificed on Day 41)

Surprisingly, the TMV group had the smallest tumors, although their survival was similar to the PBS control group. Even more surprisingly, "TMV+Addavax+aPD-1" vaccinated mice had the largest tumors of the lot, although most of them survived longer. Between the two "TMV+Adjuvant+ ICI" vaccines that we tested, only the MPL based vaccine

showed a reduction in tumor size, albeit modest, compared to TMV, aPD-1, TMV+MPL or MPL alone. Overall survival advantage was not observed. (Fig. 22)

The following trends were observed: (Figures 23a. to 23i.) Compared to PBS vaccinated mice, TMV vaccinated mice had smaller tumors (smallest among all the groups), but comparable survival (Fig. 23 a.). aPD-1 combined with TMVs produced larger tumors than TMV alone, but smaller tumors than PBS controls and aPD-1 alone. (Fig. 23 b.) Vaccination with MPL, AddaVax, or aPD-1 alone did not reduce tumor size compared to TMV (Fig. 23 c.)

MPL in combination with LL/2 TMVs larger than even the PBS control mice, but did increase survival (Fig. 23 d.). When combined with aPD-1, MPL+ TMV vaccine (i.e., the TMV + MPL +aPD-1 vaccine) did reduce tumor size, but not more than TMV alone. Survival was increased compared to controls. (Fig. 23 e.)

Likewise, AddaVax in combination with LL/2 TMVs did not reduce tumor growth compared to TMV alone, but did increase survival (Fig. 23 f.). The “TMV + AddaVax +aPD-1” tumors grew even larger than the PBS mice. Survival was also decreased compared to controls. (Fig. 23 g.)

Thus, combining LL/2 TMVs with AddaVax, MPL and aPD-1 did not reduce tumor growth or increase survival. LL/2 tumors remained resistant to checkpoint blockade even after an immunostimulatory “TMV+ Adjuvant” vaccine was co-delivered.

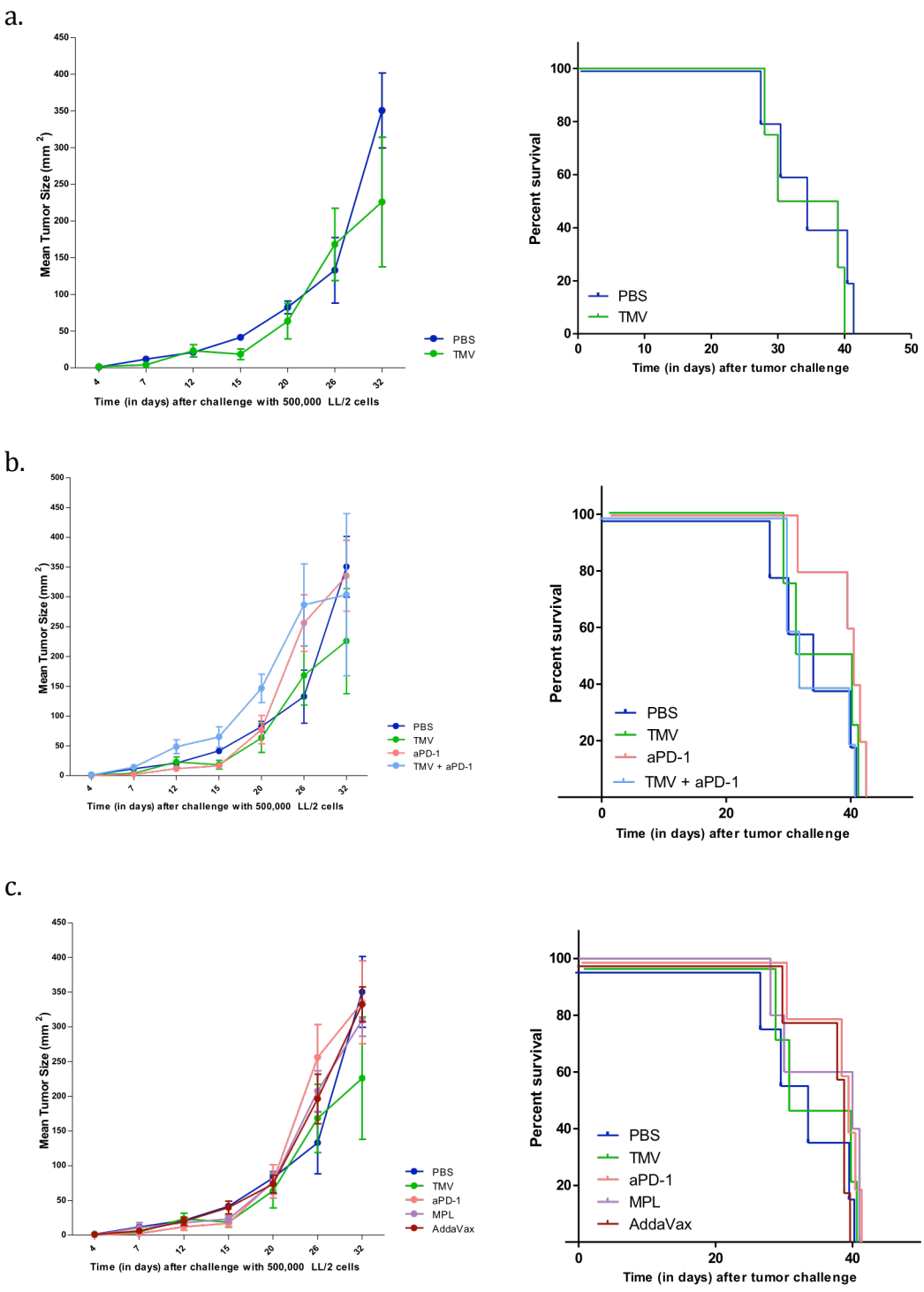
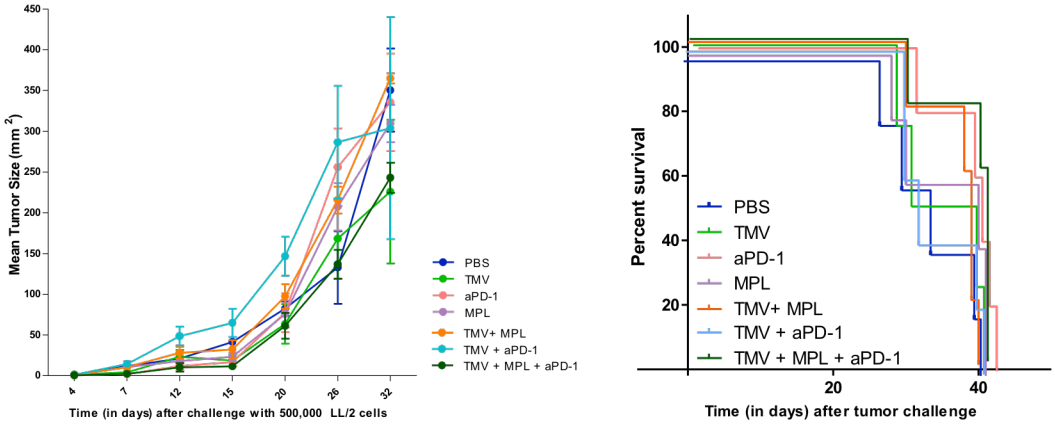


Figure 23: Vaccination with "TMV+ Adjuvant+ ICI" vaccines does not protect from tumor challenge or regress established tumors: a. Vaccination with LL/2 TMVs alone; b. aPD-1 alone, "TMV + aPD-1" vaccine, or c. or with aPD-1, MPL and AddaVax alone do not reduce tumor growth or enhance survival.

d.



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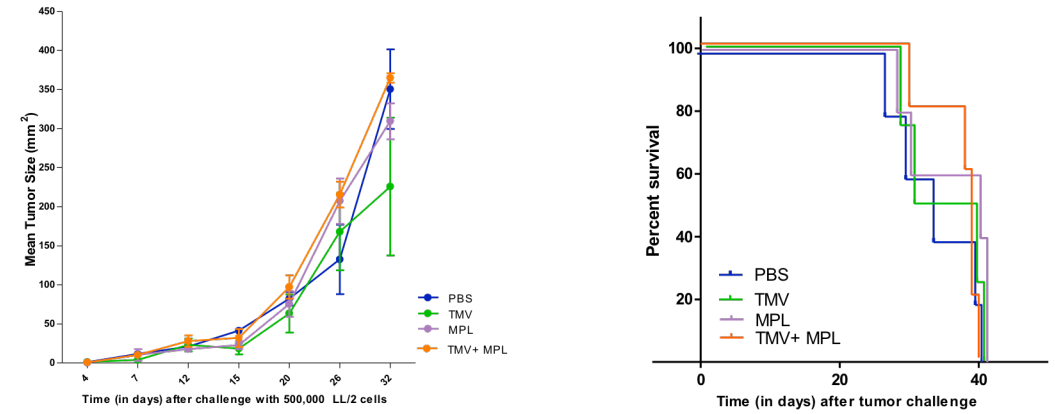

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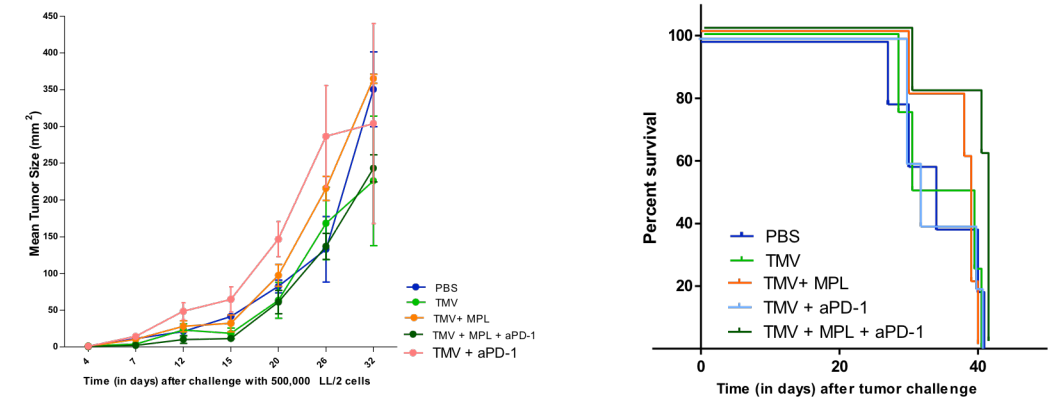

Figure 23 (cont.): Vaccination with "TMV+ MPL+ aPD-1" vaccines does not protect from tumor challenge or regress established tumors: a. "TMV + MPL + aPD-1" tumors are not significantly different from control groups; b. " TMV+MPL" vaccine does not abrogate tumor growth; c. "TMV+ MPL+ aPD-1" vaccine does not abrogate tumor compared to the " TMV+MPL" and "TMV+ aPD-1" vaccine.

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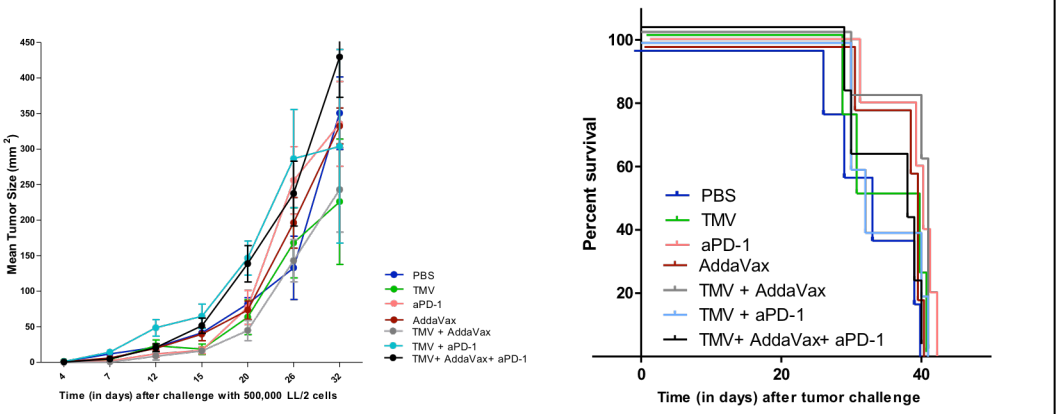

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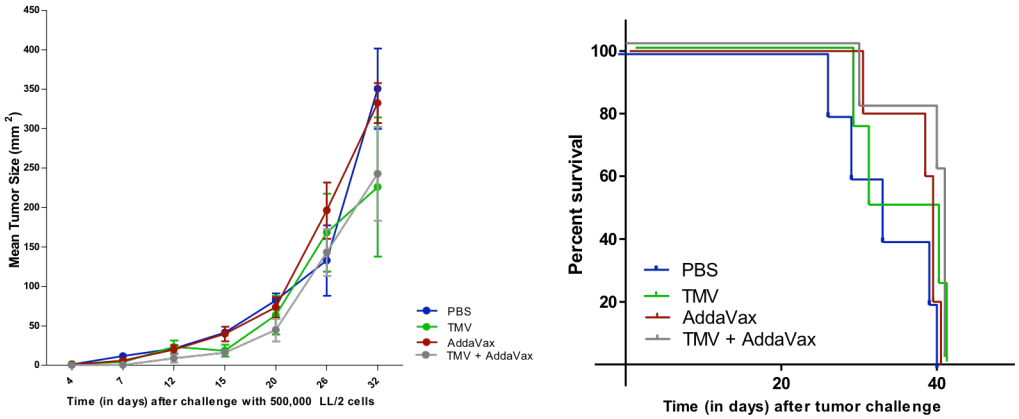

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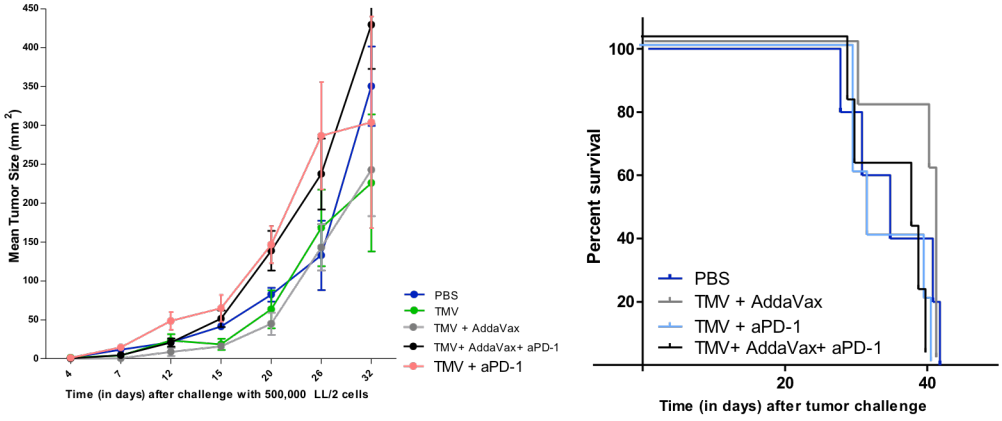

Figure 23 (cont.): Vaccination with "TMV+ AddaVax + aPD-1" vaccines does not protect from tumor challenge or regress established tumors: g. "TMV + AddaVax + aPD-1" tumors are not significantly different from control groups; h. " TMV+AddaVax" vaccine does not abrogate tumor growth; i. "TMV+ AddaVax+ aPD-1" vaccine does not abrogate tumor compared to the " TMV+AddaVax" and "TMV+ aPD-1" vaccine.

Vaccination with "TMV+ Adjuvant (double the dose)" does not induce an anti-LL/2 antibody response.

Even after doubling the dose of adjuvants, we could not see the production of an antibody response. (Figure 24) We expected an antibody response because both MPL and AddaVax can promote a Th2 response (humoral immunity promoting) in addition to a Th1 response (cellular immunity promoting). Two-Way ANOVA was performed and no significant differenced between the PBS and vaccine immunized mice was found. Mice immunized with TMV + Addavax were the only mice that had higher antibody levels than the PBS controls, and also seemed to increase from Week 2 to Week 4, although there was no significant difference between groups.

No DTH response was detected in vaccinated mice

To test, in vivo, if a T Cell - mediated immune response has been generated through immunization with TMV ± adjuvant constructs, we decided to elicit Delayed-Type Hypersensitivity responses in our vaccinated mice 3 weeks after immunization, and one week before tumor challenge. Delayed Type Hypersensitivity, or DTH, is a Type IV hypersensitivity response, an immunopathological reaction classified by Gell and Coombs in 1963 [226]. It is a measure of the T cell immune response generated upon vaccination, and is characterized by the production of local inflammation and swelling at the site of antigen contact or exposure, in a subject that has been sensitized to the antigen through prior vaccination or exposure[226]. Similar to a TB Spot Test, DTH challenge with a small dose of antigen should induce swelling mediated by the infiltration of immune cells at the site of injection

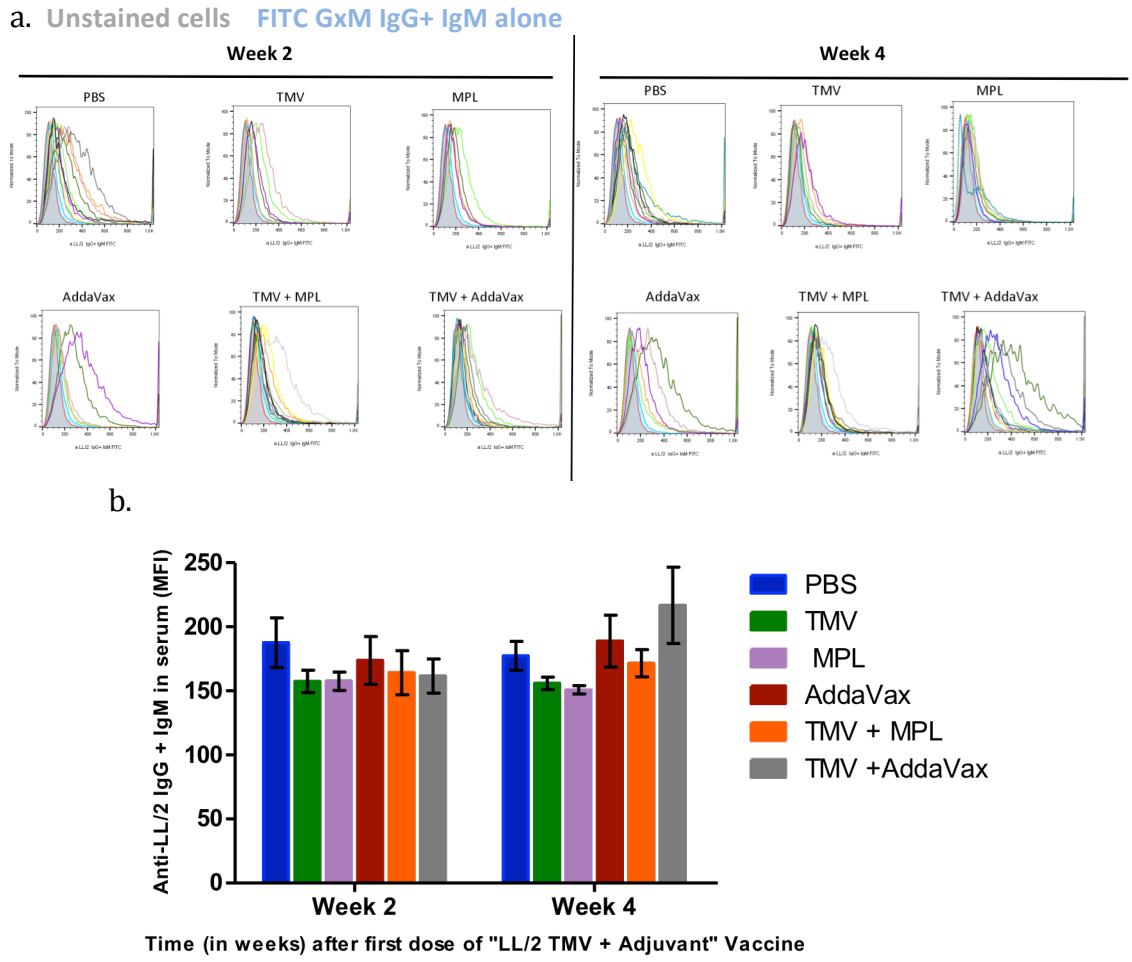

Figure 24: Vaccination with "TMV+ MPL (2 0 µg)" and "TMV+ Addavax (50uL) does not induce an antibody response: a. Anti-LL/2 antibodies in each individual group during Week 2 and Week 4 determined through flow cytometry; b. Compilation of data from a.

.

DTH responses are seldom used in cancer vaccinology as a measure of immunity, and based on the vaccine system, can vary a lot between test subjects. After vaccination with a HER-2 peptide vaccine, patients developed DTH responses and were shown to have higher amounts of CD4 and CD8 T cells in the peripheral blood [227]. On the other hand, in Wilms Tumor antigen-1 based vaccine for prostate, lung and colon cancer, DTH

response was a poor predictor of response, and only developed in 3 out of the 7 patients tested [228].

We inoculated 50 µg of TMV in 25µl of PBS into one of the footpads of each immunized mouse. On the other footpad, we injected PBS to control for injection related swelling and inflammation (Figure 25). Footpad thickness was assessed before and after DTH challenge with the help of vernier calipers and we found no swelling or redness to be present in any of the TMV and TMV+Adjuvant immunized groups, compared to the naive mouse that we also challenged similarly. Thus, no DTH response could be elicited after vaccination with "TMV +Adjuvant" vaccines (that were combined with ICI only upon tumor challenge).

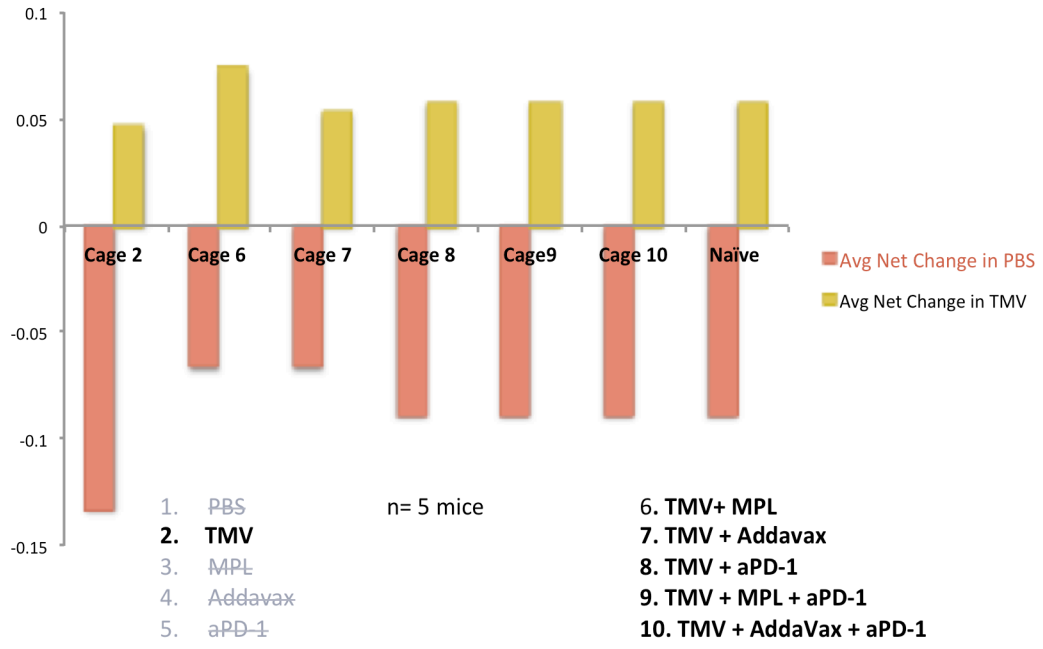

Figure 25: DTH response is not generated in vaccinated mice; no swelling or redness was observed in mice footpads either, in comparison to a naive mouse controls.

IL-4 levels are significantly decreased in mice immunized with "TMV+ Adjuvant" vaccines.

Serum was diluted 1:5, and ELISA was performed to measure levels of IFN-γ and IL-4 in the sera of immunized mice. One-Way ANOVA was performed to ascertain statistical significance. (Figure 26)

A favorable anti-tumor immune response is mediated by the Th1 response. The Th1 effector cytokine, IFN-γ, helps activate macrophages and dendritic cells, and promotes CTL differentiation [42]. Increase in IL-4 levels may be indicative of a Th2 response, which leads to the generation of an antibody mediated response, which only accounts for modest anti-tumor immunity[42]. Thus, reduced levels of IL-4 and increased levels of IFN-γ may be associated favorable anti-tumor immune activation.

As mentioned before, immunization with TMV+MPL and TMV+AddaVax produced tumors of comparable sizes, and were not significantly different from each other or from the PBS groups.

In both our groups, PBS mice had the highest amount of both cytokines. IL-4 and IFN-γ levels seemed to decrease upon TMV immunization, although this decrease was not significant.

In comparison to the TMV group, immunization with TMV + MPL vaccines seemed to induce a non-significant increase in IFN-γ levels, accompanied by a *significant* decrease in IL-4 levels, suggesting a possible skew towards the formation of a Th1 immune response, although more cytokines need to be assayed before any conclusion can be

made. TMV+MPL vaccinated mice also had the highest IFN-γ to IL-4 ratio, and the lowest IL-4 to IFN-γ ratio among the rest of the treatment groups

In comparison to the TMV group, immunization with TMV+ AddaVax vaccines seemed to induce a non significant decrease in IFN-γ levels, and a *significant* decrease in IL-4 levels, although there was no significant difference between IL-4 levels in TMV + MPL and TMV+ AddaVax sera. TMV+ Addavax vaccination also induced IFN-γ to IL-4 ratio higher than the rest of the treatment groups, although no increase in IFN-γ was observed.

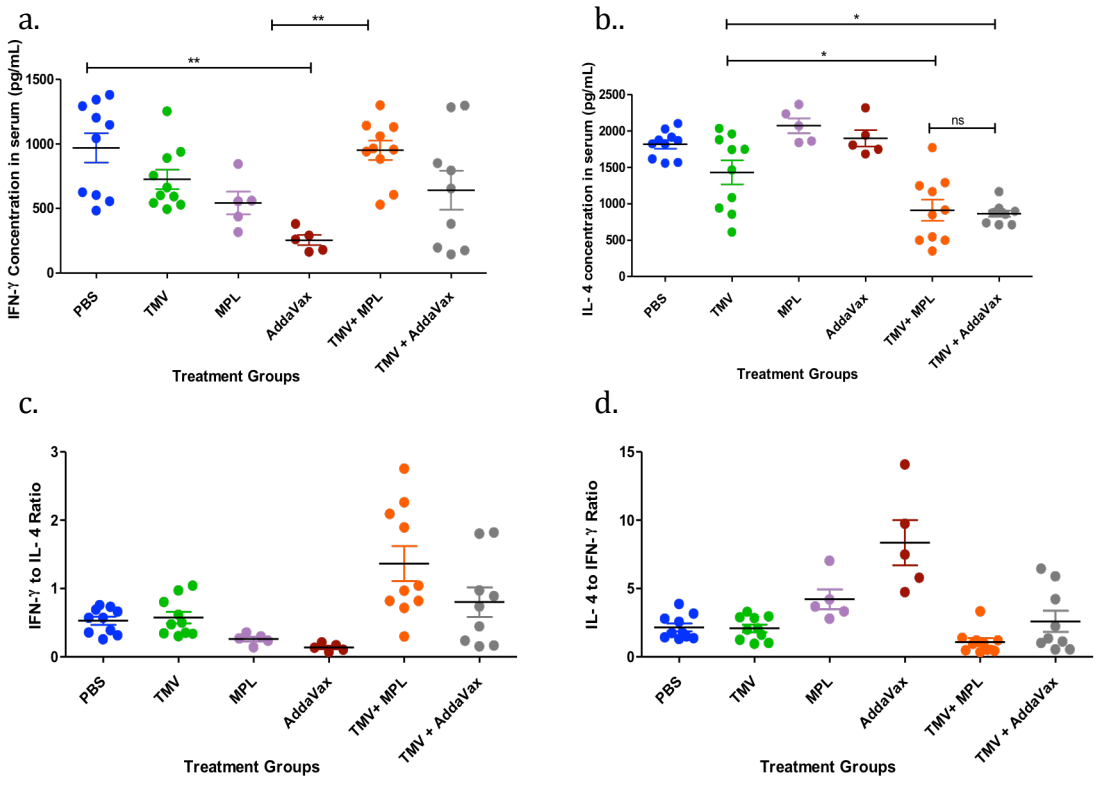

Figure 26: Cytokine levels in sera of mice immunized with "TMV + Adjuvant" vaccines
a. IFN-γ concentration (pg/mL); b. IL-4 concentration (pg/mL); c IFN-γ to IL-4 ratio; d. IL-4 to IFN-γ ratio in sera of vaccinated mice. Sera was diluted 1:5 and ELISA was performed to determine cytokine levels. One way ANOVA was performed to asses statistical significance.

Both TMV+MPL and TMV+AddaVax immunization decreased IL-4 levels in a similar manner compared to TMVs and PBS, and had similar levels of IL-4 compared to each other. IFN-γ levels increased (non-significantly) in TMV+MPL immunized groups but did not rise in a similar manner with TMV+AddaVax vaccination, which was reflective in the IFN-γ to IL-4 ratios itself. Although more cytokines need to surveyed before making any conclusion, this trend of TMV+AddaVax immunization not inducing an increase in IFN-γ levels in a manner similar to TMV+MPL immunized mice, may be associated with TMV+AddaVax+aPD-1 treated groups forming much larger tumors than TMV+MPL+aPD-1 immunized mice.

"TMV + Adjuvant + ICI" vaccines do not reduce splenomegaly in tumor bearing mice

Spleen weights were measured upon sacrifice/ death. Tumor induced splenomegaly was not abrogated through any of our "TMV + Adjuvant + ICI" vaccination approaches (Figure 27). Statistical significance was tested using one-way ANOVA, and all p values were greater than 0.05 (non-significant).

In conclusion, we were not able to enhance the potential anti-tumor properties of the "TMV+Adjuvant" vaccine through co-administration with aPD-1. More importantly, we were not able to overcome the ICI resistant property of LL/2 tumors even though we tried to generate an anti-tumor immune response with "TMV+Adjuvant" vaccines.

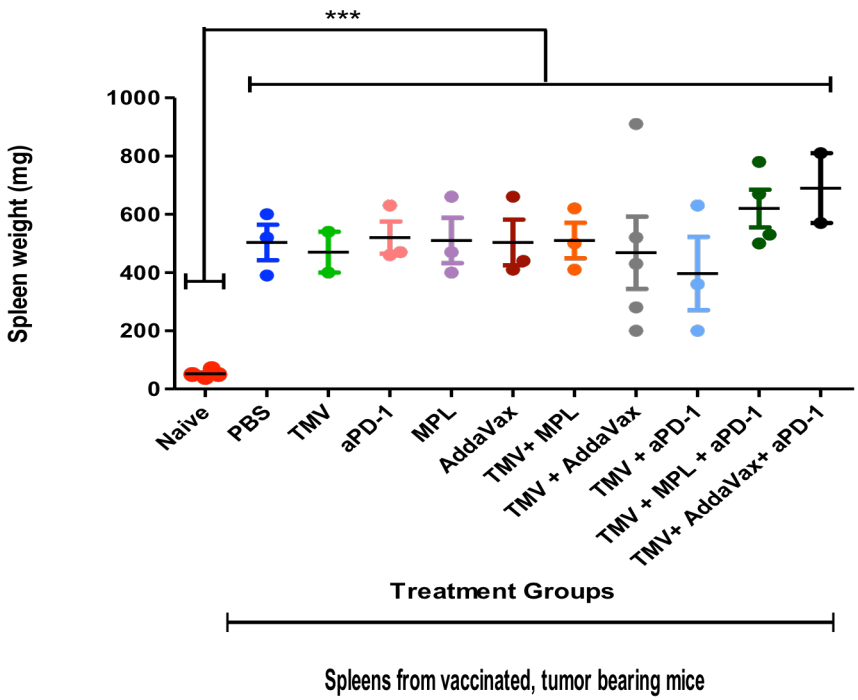

Figure 27: "TMV + Adjuvant + ICI" vaccines do not reduce splenomegaly in tumor bearing mice

Results from Experiment 8: In vitro treatment of LL/2 cells with 500U/ml of IFN-γ

Through this study, we tried to gain insight into the in vivo properties of LL/2 tumors by emulating how LL/2 tumor cells would respond to the presence of IFN-γ in the tumor microenvironment, which would presumably be produced by activated T cells or activated NK cells, and activated Th1 CD 4 Helper T cells [229]. During our characterization of LL/2 cells we noticed that LL/2 cells did not express MHC I, which is essential for TCR ligation of effector T cells on tumor cells, and subsequent CTL mediated lysis. Through this study, we wanted to determine if MHC I was inducible in vitro through IFN-γ treatment. This crude assay may be used to assess the extent of LL/2 tumors' susceptibility to T cell mediated tumor cell clearance. As mentioned in the introduction, IFN-γ is known to upregulate MHC I and PD-L1 on tumor cells through STAT signaling [229]. IFN-γ also upregulates ICAM-1 in vitro [230].

MHC I is not upregulated with IFN-γ treatment of LL/2 cells

We treated LL/2 cells with 500 U/mL of recombinant mouse IFN-γ and did flow cytomteric analysis of the markers PD-L1, MHC I, ICAM-1, CD47 and CD44, 24 hours after stimulation (Figure 28). Staining for CD44 and CD47 expression was included as controls.

Figure 28 suggests that LL/2 cells do not upregulate MHC I nor PD-L1 expression by IFN-γ in vitro treatment. However, the change in ICAM-1 expression levels suggest that LL/2 cells have the ability to intercept and respond to IFN-γ in the tumor microenvironment. This treatment was done at varying concentration of IFN-γ (250 U/mL to 1500 U/ml) to confirm results. Thus, LL/2 cells have the ability to response to IFN-γ cytokine signaling in vivo, but may not upregulate MHC I as a result, suggesting the existence of a possible immune evasion mechanism adopted by LL/2 tumors in vivo.

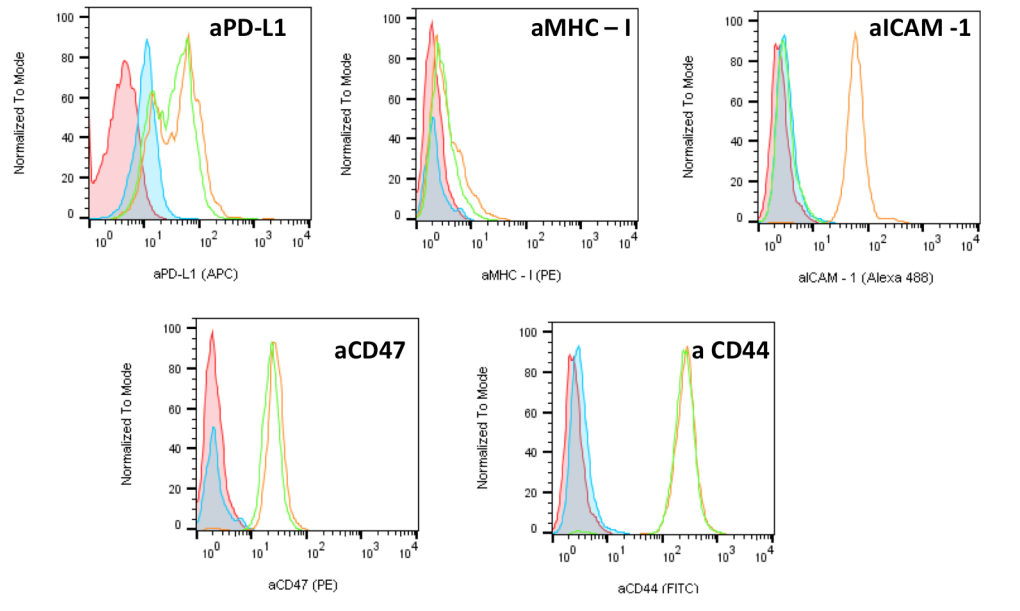

Figure 28: MHC I expression does not increase upon treatment with IFN- γ

Discussion

Through this study, we attempted to enhance the anti-tumor activity of LL/2 Tumor Membrane Vesicles by co-administering them with various vaccine adjuvants, or synthetic immunstimulatory compounds. This “TMV+ Adjuvant” vaccine was then combined with immune checkpoint blockade therapy to generate de novo anti-tumor immunity in an ICI resistant murine lung cancer model to induce tumor abrogation/ protection in vivo.

Results from our comprehensive in vivo vaccine studies indicate that we were not able to mount anti-tumor immunity potent enough to exhibit complete protection from LL/2 tumor challenge, reduce tumor size significantly or regress established tumors in vivo. Tumor growth was also not abrogated when TMVs were delivered in combination with vaccine adjuvants, or with immune checkpoint inhibitor antibodies. The lack of success of our vaccine could be attributed to many reasons: the lack of immunogenic TAA in LL/2 TMVs; the lack of MHC I on LL/2 tumors or presence of additional immunosuppressive mechanisms in the immuno-tumor interactosome.

The lack of an immunogenic TAA (tumor associated antigen) may have been the key factor that contributed to the inability of LL/2 tumor derived TMVs to mount anti-tumor activity even when combined with adjuvants that license LL/2-antigen-presenting DC mediated T cell activation, expansion and effector function. Hopwell et.al found that only in LL/2 tumor models that were made immunogenic with OVA transfection to produce the immunogenic LL/2-OVA tumor model system, did a consecutively active NFKB overexpression mediated tumor rejection arise [231]. Even a simple inoculation of LL/2-OVA tumors produced CD8 T cells that that were OVA specific [231]. Their NFKb

overexpression based interventions selectively abrogated tumor growth in LL/2-OVA systems, and not in unmodified LL/2 models [231]. Mice bearing NFKb subunit modified LL/2-OVA tumors when compared to NFKb subunit modified LL/2 tumors had reduced metastatic clones in the lung in a experimental metastasis model, as well as higher anti-tumor CD8 responses [231]. Thus, for anti-tumor immune responses to be generated and amplified, the presence of an immunogenic antigen to target may be required [231]. The lack of the same in the LL/2 tumor model makes it non-immunogenic, and escape immunosurvaillence[232]. Thus, even though our TMVs are potentially superior delivery systems for TAAs, LL/2 TMVs may not mount an immune response due to lack of an immunogenic TAA on LL/2 tumors.

In addition to LL/2 tumors undergoing immunoediting to escape immunosurvaillance as described by Schrieber [233], LL/2 cells in vitro also do not express the most important molecule needed for CTL recognition of LL/2 cell in vivo – MHC I, the Major Histocompatibility Complex I. Even if an anti-LL/2 CTL army is mobilized in vivo, cytotoxic CD8 T cells cannot exert their effector functions unless they ligate their TCRs with tumor antigen presenting MHC I molecules on tumor cells. To test if MHC I could be upregulated in LL/2 cells, we conducted an in vitro stimulation of LL/2 cells with IFN-γ. IFN-γ, as described earlier, is the effector cytokine secreted by activated T cells and Natural Killer cells, and is known to induce MHC I expression in vivo, making tumor cells amenable to lysis by CTLs[229]. Through our in vitro stimulation, we did not see an upregulation of MHC I on the surface of LL/2 cells, even though LL/2 cells respond to IFN-γ (we saw upregulation of ICAM-1 upon treatment with IFN-γ). If our in vitro screen accurately emulates in vivo conditions, then MHC I may not be inducible in vivo either, making tumors invisible and undetectable by T cells. Furthermore, Lechner et al also implicated the role of MHC I expression as a limiting factor to the efficacy of LL/2

immunotherapy in vivo. [234]Even in their study, LL/2 tumors did not respond to their immunotherapy strategy mediated by a tumor-targeted chemokine LEC (LEC/chTNT-3) fusion protein combined with a dendritic cell vaccine and a TLR agonist, which they attributed to modest MHC I expression on LL/2 tumor cells in vivo, as determined by immunohistochemistry. [234]

Thus, T cell mediated tumor clearance is contingent upon MHC class I expression on tumor cells, and since LL/2 tumors may not exhibit MHC I expression, MHC I independent immune effector cells such as Natural Killer Cells might be better strategies to target for inducing LL/2 tumor abrogation in vivo [235-237]. Natural Killer Cells recognize the “lack of self” phenotype – they recognize cells that lack or downregulate MHC I expression and selectively eliminate these populations through cell lysis, making them favorable effector cells to boost fir LL/2 immunotherapy [235-237]. In preclinical studies, NK cell stimulation has been attempted by treatment with cytokines IL-12, IL-15, IL-18 and IL-21, as well as using HDAC inhibitors, but safety has been a matter of concern [235-237]. Several clinical trials have also relied on ex vivo stimulation and autologous transplant of NK cells. In a phase I clinical trial for lung cancer, NK cells were stimulated ex vivo with heat shock proteins and reinfused into lung cancer patients [40, 238]. High amounts of NK cells were observed in vivo, and reinfusion was correlated with no negative effects, but anti-tumor activity was not observed[40, 238]. Thus, NK based immunotherapies might be well suited for lung cancers, or any other cancer model, that downregulates MHC I expression as an immune evasion mechanism. Further research and development of NK cell targeted therapies is much needed, as it is still in its nascent stages compared to other branches of immunotherapy[236].

Additionally, other mechanisms of immune suppression may be subduing the anti-tumor immune response if it were generated. In lung cancer, immunosuppressive myeloid derived suppressor cells, an intrinsic Th2 polarization of the tumor microenvironment, over expression of Prostaglandin E2 as well as induction of Indoleamine 2,3-dioxygenase mediated immunosupression has been implicated as factors contributing to anti-tumorimmunity dysregulation.[239-247]

MDSCs, or myeloid suppressor cells, are immature granulocytic cells that induce imunosupression in the tumor microenvironement by secreting a variety of metabolic and immune response regulating enzymes and cytokines [239, 240, 246]. In lung cancer, high levels of MDSCs have been found in the blood of patients, and are suggested to impair T cell effector function as well as T cell migration to tumor sites[239]. MDSC infiltration at the tumor site can also induce regulatory polarization of T cells by secretion of TGF-b by MDSCs [240, 246]They release reactive radicals such as ROS and NOS that impair T cell function and promote genetic instability [240, 246]T cell anergy can be induced when MDSCs release the enzyme Arginase[240, 246]. And lastly, MSDCs are also known to deplete Tryptophan from the tumor microenvironment, an essential metabolite needed for T cell proliferation and effector function[240, 246].

Additionally, lung cancer cells may be intrinsically promoting a Th2 polarized microenvironment, by producing cytokine IL-4, 5, 10 and 13, as suggested by Huang et.al.[239]. When they performed rtPCR of lung tissue from five human lung cancer tissue samples, they found a cytokine milieus that was highly Th2 favored. Thus, tumor cells may be producing a Th2 cytokine profile that would be inhibiting the formation of a Th1 response, preventing the formation of a cell mediate immune response[241]. Lung cancers are also known to express large amounts of Prostaglandins that have been

implicated in dysregulating and increasing the angiogenesis, proliferation and invasive capacity of tumors [242].

Last but not the least, Indoleamine 2,3-dioxygenase, or IDO, an enzyme that metabolizes tryptophan, an metabolite essential for T cell survival, effector function and expansion, may be may be induced in the lung microenvironment and at the site of APC maturation and T cell activation [243-245]. At the site of antigen uptake and antigen presentation by mature DCs, IDO may prime DCs to convert from a immunogenic phenotype to a tolerogenic phenotype [243-245]. IDO primed DCs may hamper antigen processing and presenting and ultimately T cell activation because they tend to produce immunosuppressive cytokine such as TGF-b and IL-10 instead of pro-inflammatory, DC maturation inducing cytokines IL-2 and IL-12. Thus, the DC maturation ability of our adjuvants may have been abrogated due to the presence of the IDO mediated immunosuppressive cytokine milieus [243-245].

If we were not successful in generating a base line anti-tumor immune response owing to the severe deficiencies in immunogenicity that the LL/2 tumor model exhibits, the adjuvant-mediated, and ICI-mediated enhancement of the anti-tumor immune response may have also not been apparent. To truly study and simulate the individual adjuvant effect, we would have to utilize a model system with a strong immunogenic antigen, such as the D2F2/E2 mouse mammary carcinoma cell line expressing human HER-2, which is currently used in our lab. Furthermore, these sets of experiments need to be repeated with a larger number of mice to gain statistical strength and validate results.

Nevertheless, this study highlights the importance of characterizing and working with immunotherapy resistant models such as the LL/2 model because unlike most

immunogenic, homogenous and highly responsive models of carcinoma that researchers favor for testing new therapies, the non-responsive, poorly immunogenic and aggressive phenotype of the LL/2 tumor reflects the status and nature of many of the tumors that doctors encounter in the clinic. Thus, research and development of immunotherapeutic strategies to invoke anti-tumor immunity in poorly responsive models are of utmost importance to the future and success of cancer immunotherapy and to expand the knowledge of tumor immunology.

Conclusion

In conclusion, generating a potent anti-tumor response in a poorly immunogenic, ICI resistant mouse model - which is representative of a significant population of cancer patients in the clinic- is quite challenging even when conditions are optimized in the laboratory. Through our study, even though we were unsuccessful in generating a TMV mediated anti-tumor response that could be enhanced using vaccine adjuvants and checkpoint blockade therapy, we gained valuable insight into the capability of LL/2 tumors in evading immunosurvaillance and propagating in vivo in an “insidious, mysterious way” to “destroy life”[11].

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