THE UNIVERSITY OF CHICAGO

ENGINEERING ANTIBODY GLYCO-ADJUVANT CONJUGATES TO TREAT ESTABLISHED COLD TUMORS

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES

AND THE PRITZKER SCHOOL OF MEDICINE

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

COMMITTEE ON IMMUNOLOGY

BY

TIFFANY MARIE MARCHELL

CHICAGO, ILLINOIS

MARCH 2021

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

CHAPTER 3: RESULTS

ACKNOWLEDGEMENTS

This thesis and my work over the past 5 years would not have been possible without the support and involvement of many people. I feel incredibly fortunate to have had the opportunity to grow as both a scientist and engineer within this academic community.

I am extremely grateful for my mentor Jeff Hubbell. I voted with my feet and came to the University of Chicago for the intellectual environment, but also largely for the opportunity to work in his lab. His unwavering faith in me combined with the (at times, terrifying) freedom to pursue whatever projects, ideas, or careers I found interesting was the best training environment I could have asked for. I'd choose it all again. Thank you for the unlimited opportunities, support, and infectious optimism that always kept me inspired.

Without a doubt, this PhD would figuratively and literally not have been possible without Scott Wilson. Beyond just an intellectual contribution to the adjuvant used in this work, his mentorship had an immeasurably positive influence on my graduate training. Despite always being the busiest person in lab, I am eternally appreciative of all the time he took to teach me what he knew, or to offer guidance and discussion on experiments, and anything else I had on my mind. I feel incredibly lucky to have had the opportunity to work with and learn from you.

Many, many thanks to all of my fellow Hubbell and Swartz lab members who made coming to work every day a pleasure. So many of you were always willing to help in any way that you could and you all were a huge source of support as both friends and colleagues. Special thanks to Aaron Alpar, Taylor Gray, Michal Raczy, and my undergrads Liam Rybicki-Kler and Anja Schempf for all of their work to help push this thesis project forward.

Thank you to the COI community and to my thesis committee members Melody Swartz, Justin Kline, and Bana Jabri. Your training, insightful comments, and collaborations were critical to my development as an immunologist and to the work I've accomplished here.

To all the wonderful friends I've met throughout this experience, thank you for being a continual source of joy and support through all the ups and downs of grad school. All the time we shared will be what I remember most fondly.

Last but certainly not least, thank you to my family for your encouragement and love. Mom, thank you for always supporting me and being my role model of what it means to be a life-long learner. Dad, thank you for always being my reminder to appreciate life daily, to "stop and smell the roses", or to be careful not to "burn the candle from both ends". Casey, having you by my side has made the wins sweeter and the challenges tolerable. Thank you for always believing in me and encouraging me to take the risks that mattered the most.

ABSTRACT

While the advent of checkpoint blockade antibodies has provided a powerful means to reinvigorate pre-existing T cell responses in the treatment of cancer, therapies capable of priming or expanding endogenous antitumor immunity are notably lacking within the current clinical cancer immunotherapy "toolbox". Neoantigen peptide vaccines, dendritic cell-based vaccines, small molecule adjuvants, oncolytic viruses, and GM-CSF secreting tumor cells have demonstrated striking efficacy in murine tumor models yet, thus far, have failed to broadly impact the clinical treatment of advanced cancer. Difficulties faced translating success from the lab to the clinic have highlighted the need for further development of strategies to ignite effective antitumor immunity in formats which are cheaper, more tumor agnostic, and readily available in off-the-shelf formulations. With this aim in mind, we engineered tAb-pManTLR7, comprised of tumor cell-binding antibodies (tAbs) covalently linked to our pManTLR7 agonist, to localize and sustain delivery of a powerful adjuvant to the tumor microenvironment while endowing existing anti-tumor antibodies with a heightened vaccinal effect. Upon intratumoral injection, pManTLR7-armed antibodies bind and opsonize tumor cells, increase the residence time of pManTLR7 in the tumor microenvironment, and functionally link the delivery of endogenous tumor antigens with strong TLR7 activation. Increased tumor-specific cellular responses and intratumoral T cell accumulation upon tAb-pManTLR treatment mirror the salient outcomes of a traditional subunit or peptide vaccine but here, the tumor itself provides an endogenous source of antigen. In aggressive 'cold' models of melanoma and triple-negative breast cancer, tAb-pManTLR treatment slowed or eradicated established tumors in a $CD8^+$ T cell-dependent manner and generated antitumor memory which protected mice against subsequent abscopal tumor challenge. Our engineered approach exemplifies a modular, off-the-shelf immunotherapy

capable of activating systemic antitumor immunity with the sufficient magnitude and functionality required for therapeutic efficacy. This work provides a strong proof-of-principle for the further development of tAb-pManTLR as a therapeutic platform to treat numerous malignancies.

CHAPTER 1

INTRODUCTION

1.1 Cancer Immunotherapy: Historical Background

Immunotherapies for cancer take advantage of the ability of the immune system to recognize and direct cytotoxic responses against tumors. In 1893--far ahead of modern day understanding of the interplay between the immune system and cancer--William Coley first experimented with treating patients with heat-inactivated mixtures of *S. pyogenes* and *S. marcecsens*, dubbed 'Coley's toxins'[1]. Coley's treatment showed variable clinical success, however, his studies remained unappreciated for many decades after his death. In the 1950's and 1970's, a deeper understanding of the immune system's recognition of cancer emerged due to seminal work by Burnet and Thomas [2,3]. As new findings defined the close interplay between tumor biology and the immune system, it became increasingly appreciated that the function of the immune system could potentially be harnessed to fight against cancer. In the 1990's, the advent of CAR-T cells, checkpoint inhibiting antibodies, and dendritic cell vaccines all paved the way for translating our understanding of cancer-immune interactions to the clinical treatment of cancer. Today, cancer immunotherapy is a rapidly growing field for both scientific and therapeutic research that holds promise for significant future improvements to be made in the clinical treatment of malignancies.

1.2 The innate immune system coordinates adaptive immunity

The immune system has evolved to recognize and respond to physical damage as well as invasion from foreign pathogens such as bacteria, viruses, or parasites through the use of molecular patterns. These pathogen-associated molecular patterns (PAMPs) or damage-

associated molecular patterns (DAMPs) serve as 'danger' signals to the immune system [4,5]. Innate immune cells recognize molecular patterns associated with 'danger' via pattern recognition receptors (PRRs) and triggering of these receptors activates the downstream cellular programs to appropriately respond. The PAMPs and DAMPs capable of activating immune cells, as well as the PRR that recognize them, are diverse. These PRRs are expressed in various subsets innate cells in key cellular locations of cell surface, endosome, and the cytosol. Different families of PRRs include toll-like receptors (TLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), AIM-2 like receptors, and nucleotide-binding and leucine-rich-repeat receptors (NLRs) [6 7].

Antigen presenting cells (APCs) are critical for the coordination of antigen specific responses of T cells and B cells. In order to appropriately activate adaptive cellular responses, APCs must first be activated via innate PRRs, which rapidly upregulates a cellular program to enhances their antigen presentation capacity and the necessary co-stimulatory signals to prime naïve T cells [8].

Here, expression of surface MHC class I and class II molecules are increased, presenting peptides generated from antigen processing within discrete cellular locations of the dendritic cell. MHC-I molecules load cytosolic derived peptides which have been imported into the endoplasmic reticulum by the transporter associated with antigen processing (TAP). Peptide antigens loaded onto MHC-I are typically derived from proteins within the cell, but also can potentially be loaded with peptides derived from phagocytosis in a process termed cross-presentation [9]. MHC-II loads peptide antigen from proteins acquired extracellularly, from within the endosome and lysosomes. Peptide-MHC class I complexes are used to prime cytotoxic $CD8^+$ T cells, whereas peptide-MHC class II complexes prime $CD4^+$ T cells. In the lymph node, an

activated dendritic cell will present roughly three times the number antigen peptide-MHC complexes on its surface as compared to a naïve dendritic cell [10]. Antigen-specific recognition of these peptide-MHC complexes by a T cell receptors (TCR), CD3, along with CD4 or CD8 co-receptor is a critical and required step for a T cell's activation. However, to become fully activated, a T cell must also receive additional co-stimulation. Activated DCs also upregulate critical co-stimulatory ligands B7.1 and B7.2 which interact with CD28 and CTLA-4, respectively, present on the surface of T cells [11]. Other activation induced ligands 4-1BBL and OX40L expressed by DCs serve to provide additional signals to T cells which enhance their activation state [12,13]. If antigen-specific T cells recognize their cognate antigen presented on an immature APC which lacks the required co-stimulatory signals for activation, that cell will become functionally inert, undergo abortive proliferation and die, or be induced to become an immune-suppressive regulatory T cell [14–16]. This process is a mechanism of peripheral tolerance and is an important process by which the immune system prevents aberrant attack and destruction of the host tissues in homeostatic conditions.

The crosstalk between innate and adaptive immune cells is essential to initiate the adaptive response to specific antigens and to program the antigen-specific adaptive cells with the appropriate effector functionality. Once a naïve T cell recognizes its cognate antigen and receives appropriate co-stimulation from interacting with a fully activated APC, it clonally expands, giving rise to expanded populations of antigen-specific cells with effector functionality. In the context of cancer vaccination, direct activation of APCs is required for functional priming of cytotoxic $CD8^+$ T cell responses against tumors [17]. These findings highlight the need for efficient co-delivery of tumor antigen with innate activating stimuli for effective immunotherapies to enhance these T cell responses.

In this way, APCs act as gatekeepers in unleashing antigen-specific adaptive immunity or inducing immunological tolerance. These cells use context clues (i.e. DAMPs, PAMPS) during their surveillance of host tissues to make critical decisions to either escalate and coordinate adaptive immune responses to fight a perceived threat or to prevent responses and damage to healthy tissue.

1.3 Tumor antigens can be recognized by T cells

T cells can recognize a multitude of antigenic targets on tumor cells within the context of class I and class II major histocompatibility complexes (MHC). However, due to mechanisms of central tolerance only a restricted subset of proteins within the universe of all tumor proteins will recognizable by ones' own T cells. As a mechanism to prevent aberrant immune responses against healthy tissues, T cells capable of strongly binding or 'recognizing' antigens which are naturally present within the body are purged from ones' repertoire either during thymic development or by mechanisms of peripheral tolerance, occurring constantly in secondary lymphoid tissues and parenchyma. Because malignancies develop from somatic mutations allowing aberrant division and survival in otherwise 'normal' cells, the processes of central and peripheral immunologic tolerance limit the antigenic landscape of tumors which may be recognized by the immune system. Still, tumors do contain antigenic features by which the immune system can respond to. These antigens range in tumor specificity from over expressed 'self' tissue antigens to bona fide tumor-specific novel antigens, termed 'neo-antigens' [18]. Immunogenicity of these antigens and the magnitude of response or potency of T cells specific for them can be influenced by this degree of tumor-specificity, the general level of expression of a given antigen within the tumor itself, and the degree to which that antigen is subjected to mechanisms of tolerance.

Tumor-associated antigens (TAAs) to which T cells respond can be overexpressed self-proteins, differentiation antigens, or cancer/testis antigens. Overexpressed cancer cell proteins are antigens which can also be found on normal tissues that have been expressed at a heightened state on tumor cells (i.e. HER2, hTERT, mesothelin, and MUC-1)[19]. Differentiation antigens such as gp100 or prostate-specific antigen, are overexpressed antigens associated with cell differentiation to specific tissues. Although these antigens can be found in tumors of multiple patients, the low tumor specificity and presence in other non-malignant tissues suggests that T cell clones capable of recognizing these antigens with high affinity should be largely purged from the naïve lymphocyte repertoire. On the other hand, cancer testis antigens such as NY-ESO-1 or MAGE proteins, are aberrantly expressed by tumors and typically would only be found in germline cells. Given that germline cells are 'immuno-privileged' or hidden from systemic immune recognition and attack, these tend to be more tumor-specific and therefore slightly more immunogenic tumor expressed antigens.

Oncogenic viruses can occasionally introduce novel tumor-specific antigens that can be readily recognized by the immune system. Tumors caused by oncogenic viral infection such as HBV or HPV can express viral antigens associated with the infecting virus. These novel proteins are foreign to the immune system and can generate antigen-specific T cell responses.

Lastly, tumors can contain neoantigens, which are mutationally created tumor-specific antigens. Neoantigens are occasionally found in specific mutational hotspots within the tumor genome, and these can be found in a subset individuals with a given cancer [20,21]. These shared neoantigens are promising targets for cancer vaccines or chimeric antigen receptor T cell (CAR-T) as they are highly tumor-specific and thus, have a higher likelihood of being immunogenic than TAAs. However, a majority of tumor neoantigens are specific to a patients' tumor, termed

private neoantigens. The number of tumor neoantigens can vary between different types of cancer, and given their individual nature, are difficult to identify and are often predicted computationally. Given that these neoantigens are highly tumor-specific and can be recognized by high affinity T cells, accumulation of numerous neoantigens does predispose a tumor to having a greater overall immunogenicity. As such, studies of patient responses to checkpoint inhibitor antibodies like anti-CTLA4 or anti-PD1 have found efficacy often correlates with tumor mutational burden [22] [23].

1.4 The cancer-immunity cycle

There is evidence which suggests endogenous immune response to tumors can occur as tumors grow and develop. In 1891, Paul Ehrlich first reported the infiltration of mast cells in human solid tumors. Today, it is widely appreciated that subsets of patients across multiple types of cancers have T cells present within their tumors, suggesting that their immune system has already spontaneously detected and initiated a response against tumor antigens. The mechanistic understanding of how tumor responses are generated and destroy tumor cells, either spontaneously or following therapeutic intervention, has been described as the "cancer-immunity cycle" [24]. At each step there are immunologic cues which are required in order to progress to the following step, ultimately leading to tumor cell killing.

As the first step in this sequential process, tumor cells die and release their antigens for uptake by the immune system. Depending on the context of the tumor cell death, the cellular antigens which can be picked up by an APC will be stimulatory or inhibitory. If there are no DAMPs or PAMPs released in the death of this cell (i.e. apoptosis), the clearance of cellular debris will be immunologically silent. In this scenario, the presentation of these tumor antigens to immune cells result in immunologic ignorance or tolerance of any antigen-specific T cells. At

this point, the cycle has already terminated. However, if the tumor cell dies in an immunologically stimulatory fashion, the corresponding DAMPs released during death will activate the APCs which engulf the debris. These activated cells can then migrate to the draining lymph node and present processed tumor antigen to prime naïve T cells in the lymph node.

In the next steps of the cancer-immunity cycle, the tumor antigen is presented on an activated APC with the appropriate co-stimulatory signals. If a naïve $CD4^+$ or $CD8^+$ T cell then recognizes a tumor antigen in the context of MHC presentation, it will clonally expand and differentiate to a pool of tumor-specific effector T cells. At this point, another potential break in the cancer-immune cycle is introduced: the circulating effector T cells must traffic from circulation after leaving the lymph node and into the tumor. To effectively do this, chemokines such as CXCL9, CXCL10, CCL5 must be released by cells within the tumor environment, creating a diffusion gradient for the activated T cell to follow [25]. The local vessel epithelium must also have upregulated the appropriate integrins LFA1/ICAM1 and selectins, addressing the T cell back to the tumor and promoting its extravasation from circulation and into the tumor tissue. Finally, as the last step to the cancer-immunity cycle, the effector T cells must overcome a host of immunosuppressive stimuli within the tumor to recognize their target antigens and exert their effector functions to kill cancer cells.

Considering the many steps required for effective T cell responses against tumors and the many opportunities for negative feedback mechanisms, it is not surprising that tumors fail to be spontaneously rejected, and only small subsets of patients show signs of endogenous T cell responses to their tumors without any intervention. The overarching goal of immunotherapy is to intervene on this cycle, helping it to initiate or continue through some of the specific checkpoints at which tumors can evade immune responses.

1.5 Mechanisms of tumor-immune suppression

Tumors have multiple redundant mechanisms by which they can to circumvent and evade immunologic destruction. These features directly block or disrupt progression through the cancer-immunity cycle at various steps, by appropriating and amplifying established immunologic pathways that promote self-tolerance and wound healing. Ultimately, tumors cripple the ability of spontaneous immunity to fully eradicate tumors through various complementary mechanisms of immune suppression.

Increased expression of inhibitory ligand PD-L1 on the surface of tumor cells can interact directly with PD-1 on activated effector T cells causing T cell anergy or exhaustion. Interestingly, it's expression can increase in response to inflammatory cytokines and has been shown to be a negative prognostic factor in a number of cancers [26,27]. In humans, tumors have been shown to shed NKG2D stress ligands which limits their ability to be killed by NK and $CD8^+$ T cells [28]. Tumor cells and their stromal cells can also secrete immunosuppressive cytokines such as IL-10 or TGF-β. IL-10 has been shown to prevent effective DC priming of $CD8^+$ responses *in vitro* and was suggested to also play a role in blocking DC recruitment [29,30]. TGF-β can directly act on T cells to limit their effector functionality and has been shown generally to be involved in promoting the differentiation of suppressive regulatory T cells (Tregs). Its expression has also been implicated in tumor progression to a more invasive, metastatic phenotype [31].

Tumors can also recruit a host of immunosuppressive cell types into the tumor environment. Myeloid derived suppressor cells have been shown to mediate a variety of immunosuppressive functions and inhibit T cell function through the expression of arginase and

production of soluble factors such as inducible NOS (iNOS), TGF-β, and IL-10 [32]. Tumor associated macrophages (TAMs) are also an abundant innate immune population within the tumor environments and also play important pro-tumorigenic roles. These tumor-supportive functions are diverse: TAMs have been demonstrated to dampen cytotoxic T cell responses [33], recruit additional immunosuppressive immune cell subsets to the tumor [34], and promote the metastatic potential of tumor cells [35]. Lastly, regulatory T cells are actively recruited to the tumor environment and have been demonstrated to limit T cell responses within the tumor by expression of immune checkpoint (CTLA-4) and inhibitor receptors (i.e. LAG-3, TIM-3), IL-2 consumption, and secretion of immunosuppressive cytokines.

The hypoxic environment within the tumor also reprograms myeloid cells to an immunosuppressive phenotype and inhibits immune killing. This environment enforces the TAM and MDSC phenotypes, as well as decreases aspects of DC maturation (i.e. expression of CD80, CD86, MHC II) in response to low levels of PAMPs like lipopolysaccharide[36]. Hypoxia can also recruit immunosuppressive Treg infiltration into the tumor as well as metabolically disrupt the ability of NK cells or $CD8^+$ T cells to produce cytotoxic Granzyme B[37].

Lastly, tumors can also downregulate surface MHC class I expression which functions as a way to evade T cell recognition and killing. In humans, 40-90% of tumors have been shown to decrease MHC I on the surface and this loss of MHC I has been identified as a negative prognostic factor[38–40]. Although this loss of MHC I sensitizes tumor cells to NK recognition, NK cell cytotoxicity is also limited in the tumor via a number of complementary immunosuppressive mechanisms such as production of soluble immunosuppressive factors such as prostaglandins or TGF-β[41].

Each of these immunosuppressive pathways represent a potential target for intervention with immunotherapy. Further studies in both humans and mice are needed to determine if antagonizing some of these pathways might yield more benefit than others, and in which settings this hierarchy exists. Additionally, their complementary nature highlights the need for rational combinatorial use of immunotherapies and strategies for clinicians to navigate these combinations to provide effective treatment for a given patient.

1.6 Tumor-binding antibodies and their use as therapeutics

Monoclonal antibodies specific to tumor-associated antigens or to over-expressed molecules that support tumor growth are standard clinical treatment options for several types of cancer. These antibodies exert their therapeutic efficacy through various mechanisms of action: (1) exert antibody-dependent cytotoxicity (ADCC) upon binding target cells, (2) block specific growth promoting signals.

Antibody drugs such as Herceptin (anti-HER2) for treatment of HER2+ colorectal and breast cancer, or Rituxan (anti-CD20) for the treatment of B cell malignancies recognize and bind highly expressed tumor antigens and initiate immune-mediated cell killing. Herceptin additionally works by blocking human epidermal growth factor receptor 2 (HER2) on tumor cells, which inhibits intracellular signaling pathways downstream of the receptor that direct cell proliferation and survival [42]. Using a similar mechanism, anti-EGFR antibodies prevent epidermal growth factor receptor signaling to treat EGFR-overexpressing cancers like metastatic colorectal cancers, head and neck squamous cell carcinoma, or squamous non-small cell lung cancer.

Another interesting therapeutic target for tumor-targeting antibodies is CD47. CD47 plays a prominent role as a 'don't-eat-me' signal within the tumor environment which inhibits tumor cell phagocytosis by macrophages. CD47 serves as a marker of healthy 'self' cells and expression on the cell surface has an inhibitory interaction with its co-receptor SIRPα on macrophages which signals to that prevent cell engulfment [43]. CD47 is expressed on many normal tissues and loss of CD47 expression in aged or damaged cells has been described as a mechanism which allows for clearance [44,45]. However, tumors have co-opted overexpression of CD47 as a way to evade this phagocytic surveillance system. Numerous malignancies in both mice and humans overexpress CD47 and clinically, this overexpression serves as an adverse prognostic factor in several types of cancer [46–48]. Antibodies to CD47 which disrupt or block CD47-SIRPα interaction are currently in clinical studies, exploring their safety and efficacy in numerous malignancies as a monotherapy or in combination with other drugs like chemotherapy.

Although they are less relevant for introduction to the thesis work herein, other monoclonal antibodies which are currently used to treat cancer but do not act principally by binding to ligands on the surface of tumor cells include: 1) antibodies which modulate immunoregulatory 'checkpoints' leading to the exhaustion of tumor-specific T cells within the tumor i.e. anti-PD1, anti-CTLA4 or anti-PDL1 or 2) anti-angiogenic antibodies i.e. (anti-VEGF-A) which prevent tumor angiogenic functions.

1.7 Clinical landscape: Immunotherapies to increase antitumor immunity

The immunotherapy landscape is vast and expanding at an accelerating rate. In the past three years alone, the global immune-oncology pipeline has increased by 233% to 4,720 agents in development today in 2020 [49]. In particular, some of the largest areas of pre-clinical

development today are therapies which induce or unleash endogenous tumor immunity such as oncolytic viruses, cancer vaccines, and others which act on the tumor immune environment. These drugs aim to treat classes of tumors with insufficient pre-existing T cell immunity, or with advanced cancer which fail to respond to T-cell-targeted immunomodulators such as anti-CTLA4, anti-PD1, or anti-PD-L1. Following the clinical approval of ipilimumab (a checkpoint inhibitor targeting CTLA4) in 2011, the lack of response seen in some patient populations and types of cancers fueled a deeper scientific understanding of the complex tumor-immune interactions and underscored the need for therapies which act in complementary axes of the tumor-immune cycle [50].

Therapeutic cancer vaccines come in many formats: subunit, peptide, or DC-based. A hallmark feature of all is that they aim to generate antigen-specific immunity to defined tumor-associated antigens, or tumor neo-antigens. In these formats, *a priori* knowledge of immunogenic antigens and formulation with those antigens is required. Advances to cancer vaccine development have primarily come in terms of antigen selection, or improvements to vaccine technology. Thus far, TAA-based vaccines have been largely unsuccessful. Due to the low immunogenicity of these centrally tolerized antigens, most struggle to activate T cell responses of sufficient magnitude for efficacy. One hallmark example of this was the failure of PROSTVAC-VF in a phase III study, in which vaccinations to boost PSA antigen-specific T cells was failed to provide clear therapeutic benefit to patients with metastatic prostate cancer [51]. Neoantigen based vaccines containing patient-specific neoantigen peptides and adjuvant mixes, have shown promise in early clinical trials in melanoma. However, predicting neoantigens and their immunogenicity from sequencing of an individual's tumor is a time and labor intensive process [52–55].

Oncolytic viruses are another form of cancer immunotherapy which has demonstrated the ability to enhance antitumor immunity to tumors. These therapies are genetically modified viruses which infect, replicate, and lyse tumor cells. Through this mechanism, oncolytic viruses can provide effective tumor control through the direct killing of tumor cells as well as through the generation of antitumor immunity resulting from this immunogenic cell death. In this way locally treated tumors can generate systemic tumor-specific responses that can act on distant metastatic lesions. These viruses can also be engineered to express additional therapeutic modalities such as cytokines, chemokines, or antibodies as part of their viral genome, or to gain improved tumor infectivity. Today, one oncolytic viral therapy T-VEC (Imlygic) is FDA approved for use in metastatic melanoma. Although T-VEC demonstrates significant benefit, it's clinical use has largely been hampered by the fact it is not yet approved for use in combination with standard-of-care (SOC) checkpoint inhibitor antibodies in this setting. Several other 'second generation' oncolytic viral platforms are well within the clinical studies to assess their safety and efficacy in treating melanoma or other advanced, unresectable malignancies as a second line therapy. Largely, tumor-selective infection has been achieved by local, intratumoral injection of oncolytic viral therapies. Further developments in tumor-targeting or tumor-specific replication strategies could potentially allow for systemic treatment and ease of application to treat a greater range of tumor types [56].

There are several other classes of drugs in the immune-oncology space which can act by igniting or unleashing adaptive immunity to tumors. These agents can include cytokines, chemokines, TLR agonists, immune stimulating antibodies or other small molecule adjuvants. Each of these classes of immunomodulators may have distinct mechanisms of action, however

the ultimate effect is to create a more pro-inflammatory tumor environment and promote the immunologic destruction of tumors.

Cytokine engineering for the treatment of cancer is a growing area of focus. Several inflammatory cytokines have already been clinically approved to treat cancer. Recombinant interferon alpha (IFNα) or engineered forms of IFNα have been approved for use in subsets of patients with various malignancies such as leukemia, sarcoma, or melanoma. In development, engineered forms of other pro-inflammatory cytokines such as IL-15, IL-12, seek to improve their tumor-specific actions while limiting systemic toxicity.

Adjuvants and specific PRR agonists are also studied for their utility in activating the local innate cells within the tumor and enhancing cellular immune responses. These therapeutics activate local innate cells directly to produce desired inflammatory cytokines, increase antigen presentation, and ultimately, T cell priming. Creating an inflammatory milieu within the tumor environment via adjuvants may also help with effector responses, by providing the necessary stimuli to counterbalance the natural immunosuppressive intratumoral environment [57]. Here, immune adjuvants can come in a variety of formats: lipids (i.e. MPL, oil), protein (i.e. flagellin, versican), nucleic acids (dsRNA, ssRNA, poly I:C, STING ligand), small molecules (i.e. TLR agonists) or minerals (i.e. alum).

Given the massive scope of cancer immunotherapies and approaches to immunotherapy in a wide variety of cancers, these highlights represent a few of the largest areas of clinical development that fall within the overarching goal of enhancing immune recognition of tumors for therapeutic benefit. Many or all of these therapies which seek to rev up of the immune system to fight cancer could work synergistically with those that seek to remove immunosuppressive mechanisms which limit proper effector functionality within the tumor, such as checkpoint

inhibitor antibodies, modalities to prevent immune evasion, and those that might enhance or prolong effector functionality.

1.8 Biomaterial design for exploiting adaptive and innate immunity to tumors

Our antibody-adjuvant conjugate (tAb-pManTLR) utilizes tumor-binding antibodies (tAb), conjugated with our polymeric adjuvant, pManTLR7. Our previously published p(Man-TLR7) polymer is a small (<20kDa) random linear copolymer of monomeric units of mannose and TLR7-agonist. Our antibody-adjuvant conjugate is designed to bind to tumor cells upon injection, localizing the adjuvant to the tumor environment. Tumor antigen complexed with antibody-p(Man-TLR7) conjugates can be then efficiently internalized via mannose-binding C type lectins and, from within the endosome, activate APCs via TLR7 (Figure 1). All components of the designed conjugate work complementary to each other to link innate immune activation with efficient priming of adaptive immune responses.

A. Tumor-binding antibodies

In our construct, p(ManTLR7) adjuvant can be chemically conjugated to various antibodies that can bind ligands expressed and enriched on the surface of malignant cells. The ligands that can be used as tumor-cell targets are diverse: over expressed tissue antigens (i.e. TRP1, mesothelin), tumor enriched immune-evasion proteins (CD47), integrins, cluster of differentiation antigens (i.e. CD19 or CD20), growth factor receptors (i.e. HER2, EGFR), over-expressed immune inhibitory ligands (i.e. PD-L1), or even glycoproteins (i.e. MUC-1). Our conjugation strategy attaches at free amines, and as such, is readily adapted to accommodate virtually any antibody. The modularity of design which allows any antibody moiety to be used can be used to tailor the therapeutic to be as broadly applicable or tumor specific as needed.

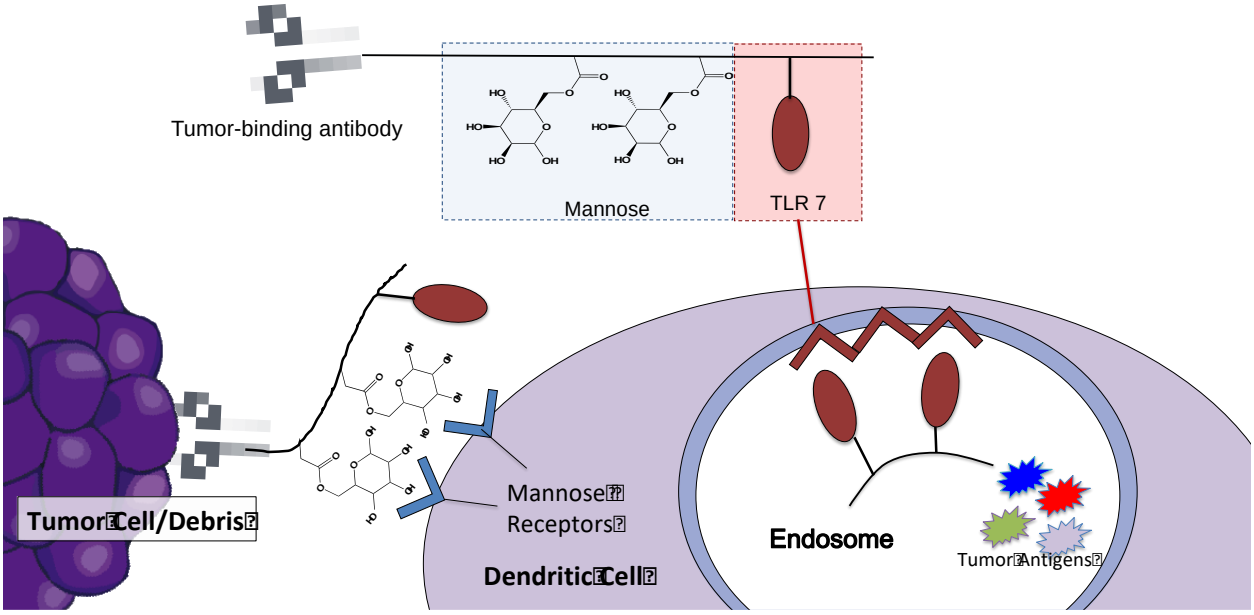

Figure 1 tAb-pManTLR schematic

Upon intratumoral injection, the antibody component of tAb-pManTLR binds its cognate ligand expressed on the surface of tumor cells. The mannose monomers contained within the pManTLR7 polymer can be recognized by endocytic mannose receptors (MR) on intratumoral dendritic cells as well as other APC subsets, to promote internalization of tumor antigen:tAb-pManTLR immune complexes and direct delivery to the endosome. Within the endosome, pManTLR7 polymers release from the attached antibody as the chemical linkage undergoes a pH-sensitive disulfide reduction within the endosomal environment. The TLR7 agonists can then bind their endosomal TLR7 receptor, thus activating the APC. Tumor antigens present within the endosome will then be processed and presented by the activated cell to prime naïve T cell responses.

Principally, this antibody component serves as a cell surface anchor, slowing the adjuvant drainage from the tumor as it binds its ligands within the environment. Here, the antibody functionally increases the intratumoral concentration of the conjugated adjuvant and its availability to activate intratumoral APCs. This slowed antigen drainage also closely mimics the sustained inflammation of a natural infection rather than a bolus dose immunization, leading to enhanced functional T and B cell priming[58]. As seen in murine studies with LCMV infection and chronic hepatitis infection in humans, large doses of viral antigen can lead to T cell exhaustion and hampered immunity whereas low doses preferentially induce longer-lived T cell responses [59].

In addition to the basic utility of augmenting tissue localization and biodistribution of our adjuvant, each monoclonal antibody may contribute its own additional functional profile to overall antitumor efficacy. Depending on the choice of antibody, it could additionally serve to block immune-inhibitory ligands and prevent intratumoral T cell exhaustion, initiate ADCC of tumor cells, increase adjuvant uptake through Fc interactions, or increase tumor cell engulfment by blocking anti-phagocytic signals on tumor cells. Any of these defined antibody functions could provide additive benefit to our primary goal of enhancing innate immune activation within the TME.

There are a few lines of evidence that suggest the tumor-binding antibody may play an additional role in enhancing downstream T cell priming to tumor antigens. A previous study from the Engleman lab demonstrated activated tumor-associated DCs endocytosed and presented tumor antigen when loaded with antibody-bound tumor cells than tumor cell lysate [60]. *In vivo* studies showed that administration of tumor-binding antibody along with DC activating stimuli vs. the activating stimuli alone significantly enhanced tumor eradicating immune responses in a

variety of syngeneic murine models. Interestingly, under homeostatic conditions in which tumor-associated DCs were not activated, treatment with tumor-binding antibodies did not lead to significant uptake of tumor antigen and failed to provide strong therapeutic benefit. Although the tumor-binding antibodies and APC activating stimuli were separately administered in these studies, it is likely that linked antibody-adjuvant will act in a similar manner to enhance DC tumor phagocytosis, antigen processing, and downstream T cell priming against tumor antigen.

B. TLR 7/8 Agonists

The small molecule TLR agonist used in our pManTLR polymer was also previously developed in our lab. It demonstrates the capacity to stimulate TLR7 and TLR8, with slightly biased activity toward TLR7[61]. The choice to use a TLR7 agonist in the preclinical testing of our antibody-adjuvant platform is largely based on TLR7/8 biology, expression, and its translational potential for clinical use.

TLR7 and TLR8 are endosomal receptors, expressed in various APC subsets in both human and mice, which recognize single-stranded RNA molecules. Stimulation of these receptors in humans and mice signaling via a MyD88-dependent pathway to activate APCs. In humans, TLR7-mediated APC activation results in production of multiple pro-inflammatory cytokines including TNFα, IL-6, IL-1β, IL-12, and IFNα. Interesting, TLR7 activation has been demonstrated to increase antigen cross-presentation and $CD8^+$ T cell priming in multiple DC subsets through the induction of type I interferon and IL-12 cytokine secretion [62].

Small molecules with stimulatory capacity for both TLR7 and TLR8 are ideal candidates in clinical vaccine development given their broad receptor expression in antigen presenting cell subsets. In humans, TLR 8 is broadly expressed in all major conventional DC subsets, monocytes, neutrophils and monocytes, with TLR7 expression in plasmacytoid DCs (pDC) and

B cells [63]. In mice, monocytes, DC subsets except CD8α$^+$, B cells, plasmacytoid DCs are responsive to TLR7 stimuli [64]. In this way, our TLR7 as an adjuvant in mice should perform most similarly to a TLR8 or TLR7/8 adjuvant in translation to humans.

Rapid diffusion and biodistribution pose a technical challenge to the clinical use of small molecule TLR7/8 agonists. Upon subcutaneous injection of resiquimod, the drug was detectable in the serum within minutes, and showed a half-life of around 6h [65]. This quick diffusion away from the site of vaccination and suggests a loss of co-delivery with relevant proximal vaccinal antigens, which is an important feature in successful vaccination. Furthermore, this promiscuous biodistribution and off-target activation increases the propensity of these TLR agonists to induce systemic toxicity [66]. Thus far, the only FDA approved imidazoquinolinone-based small molecules are formulated as topical creams or gels. These therapeutics show clinical benefit in treating superficial malignancies as well as other dermatologic conditions as a result of TLR7/8-mediated activation [67]. The second generation of TLR7/8-agonists now in development, formulated within liposomes or nanoparticles, are under exploration for improved pharmacokinetics, safety, and overall efficacy for use in oncology and/or infectious disease settings.

Our polymeric formulation, pManTLR7, overcomes both of these technical limitations: copolymerization with mannose along a physiologically inert HPMA backbone increases the solubility of our TLR7/8 agonist and brings the overall adjuvant size to 20kDa. Conjugation to protein moieties such as a vaccinal antigen or targeting agent conveniently tailors our TLR7/8 agonist for close physical and temporal delivery codelivery with relevant antigens.

C. Mannose

Mannose is used in our pManTLR7 adjuvant as a means to encourage phagocytosis, co-localization of our TLR7 agonist with its endosomal receptor, and the cross-presentation of co-delivered antigen. Specifically, the mannose monomers within p(Man-TLR7) targets cargo antigen for uptake by APCs via C-type lectin scavenging receptors capable of recognizing the mannose.

Most specifically, mannose receptor (MR; CD206) is a member of a family of C-type lectin receptors that recognize carbohydrates [68] . MR is expressed predominantly on myeloid cells, including macrophages and professional APCs, as well as some additional non-classical APCs (Kerrigan and Brown, 2009). The extracellular portion of MR consists of the 8 carbohydrate recognition domains (CRD) followed by a single fibronectin type II repeat domain and an N-terminal cysteine-rich domain [70]. The CRDs 4-8 of the MR recognize mannose-rich residues of a variety of pathogens including viruses, bacteria, fungi and helminths, and recognition of allergens as well as auto-antigens by MR has also been reported [69,71]. The intracellular tyrosine-based fragment of MR does not contain any downstream signaling motifs, however it promotes the delivery of mannosylated ligands to early endosomes [72]. Hence, ligation of the MR by mannosylated molecules induces its internalization and delivery to this compartment enhanced antigen cross-presentation on MHC class-I [72,73].

Given the capacity of mannose to direct antigen for cross presentation, targeting of antigen to DCs using MR-specific antibodies or surface mannosylation has be explored in vaccine design to enhance $CD8^+$ T cell responses [74–76]. Previous work from our lab demonstrated the inclusion of mannose in our polymeric adjuvant enhanced uptake of protein-pManTLR7

conjugates in various APC subsets and resulted in increased activation status of the recipient cells in the draining lymph node [61].

1.9 Summary

Recognition of malignancies by the immune system is widely accepted as critical to the elimination of cancer. As a result, development of novel therapies to enhance antitumor immunity are a major clinical focus for the treatment of cancer. Cancer vaccines are one form of immunotherapy by which tumor proteins, or antigens, are used to activate cellular and humoral immune responses against cancer. As a therapy, cancer vaccines seek to activate the necessary adaptive responses to drive tumor rejection.

Several major barriers have thus far precluded the success of cancer vaccines in clinical translation. Predicting tumor-specific immunogenic epitopes to use in vaccine formulation is a labor-intensive and costly process [53,54]. Furthermore, vaccines which seek to activate T cells to broadly expressed, yet weakly tolerized 'self' antigens have shown some success, but only within in a limited subset of cancers. Ultimately, many therapeutic cancer vaccines fail to activate sufficient magnitude and functionality of cytotoxic $CD8^+$ T cell responses required for therapeutic efficacy [77–79]. For this reason, we reimagine the classical cancer 'vaccine format and have developed a novel antibody-TLR7 agonist conjugate (tAb-pManTLR) that adjuvants tumor cells *in situ* to induce durable, curative tumor-specific T cell responses in a modular design, adaptable to treat a variety of solid tumors. Our engineered material exploits multiple existing immunologic pathways for pathogen defense, and physically links innate and adaptive immunity to drive tumor-specific responses.

Our recently developed low molecular weight (<20kDa) polymeric glyco-adjuvant, termed pManTLR7 is comprised of multiple residues of TLR7 agonist and mannose [61]. We demonstrated that chemical conjugation of protein antigens to pManTLR7 via a self-immolative linkage provides an effective subunit vaccine for use in infectious disease. Specifically, mannose-targeting of antigen and TLR7 agonist to antigen presenting cells (APCs) enhanced cross-presentation of pManTLR7 linked antigens and generated robust antigen-specific T cell responses. Given the critical importance of cellular responses to the control and destruction of cancer [80], we focused our attention on translating our pManTLR7 adjuvant to improve antitumor immunity in tumor settings that lack these endogenous responses. Overall, this work provides a novel means by which to stimulate potent cellular immunity against endogenous tumor antigens and provide therapeutic benefit in poorly immunogenic tumor models in which the current standard-of-care checkpoint blockade antibodies are ineffective.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mice

C57BL/6 female mice and BALB/c female mice aged between 8-12 weeks were obtained from Charles River. *Tyr:Cre-ER^{+}/LSL-Braf^{V600E}/Pten$^{fl/fl}$* mice, ages 8-16 weeks were provided by T. Gajewski (University of Chicago) and the line was bred and maintained within our facility. All studies with animals were carried out in accordance to procedures approved by the Institutional Animal Care and Use Committee at the University of Chicago and housed in a specific pathogen-free environment at the University of Chicago.

2.2 Reagents

CpG-B 1826 was purchased from InvivoGen. Mouse anti-rat/mouseTRP1 (Clone TA99), mouse anti-mouse CD47 (Clone MIAP410), and mouse IgG2a Isotype control (Clone C1.182) antibodies were purchased from BioXCell. Rat anti-mouse PD-1 (Clone 29F.1A12, Bio X Cell) and hamster anti-mouse CTLA4 (Clone 9H10, Bio X Cell) were used for checkpoint blockade antibody studies. Before administration to mice, endotoxin levels of all formulations were tested via HEK-Blue hTLR4 cells from InvivoGen. NHS Ester Sulfo-Cy7 dye (Lumican), AlexaFluor 647 (Invivogen), or DyLight 800 NHS Ester (ThermoFisher) was used to label antibodies and antibodies used in the creation of antibody-pManTLR for flow cytometry, immunofluorescence staining, and in vivo imaging analysis according to manufacturer's recommendations. A detailed explanation of the synthesis of the pManTLR polymer, linker, and intermediates is provided in the supplementary methods of our previous publication [81].

2.3 Cell Culture

B16F10 melanoma and EMT6 breast cancer cell lines were obtained from ATCC and B16.F10 cells were engineered in house to express the model antigen OVA (B16.OVA). Cells were cultured in "complete media": DMEM supplemented with 10% FBS, and 1% penicillin and streptomycin. C1498 cells were cultured in DMEM + 10% FBS + 1% P/S + 0.05mM Beta-mercaptoethanol with density maintained between 10^5-10^6 cells/mL. Prior to inoculation of tumors cells were removed from culture, washed twice with cold PBS, counted, and resuspended at the appropriate concentration for tumor inoculation.

2.4 Tumor inoculation and treatment

$5x10^5$ B16F10 cells resuspended in 50uL of PBS were inoculated intradermally on the left side of the back, or left flank, of each C57BL/6 mouse. $5x10^5$ EMT6 cells resuspended in 50μL of PBS were inoculated *s.c.* into the mammary fat pad of BALB/c mice. For the melanoma GEMM, tumors were induced on the back of 8-16 week old *Tyr:Cre-ER*$^+$*/LSL-Braf*^{V600E}*/Pten*$^{fl/fl}$ mice. Fur was shaved prior to application of 50 μg 4-OH-tamoxifen (Sigma-Aldrich) at 10 mg/mL topically, as previously described (Spranger et al., 2015). For inoculation with systemic AML, $1x10^6$ C1498 cell resuspended in 100uL of PBS were delivered intravenously via tail vein injection to C57BL/6 mice.

Tumors were measured every other day starting at day five after inoculation with digital caliper. Tumors were measured with digital calipers and the tumor depth, the widest tumor measurement from side to side, and longest length perpendicular to that width were recorded. Volumes were calculated as volume V(mm^3) = length x width x height. Mice were sacrificed when tumor volume had reached over 1000 mm^3. Treatments were performed on days described in figures

and in figure legends. tAb-pManTLR7 vaccination or control treatment was administered in described doses via intratumoral injection in a total volume of 35 μL. Checkpoint inhibitor antibodies (CPI) were administered as a mix of 100 μg of anti-PD1 and 100 μg of anti-CTLA4 intraperitoneally. Prior to initial treatment, mice were randomized into treatment groups with each treatment group split up between cages to reduce cage effects.

2.5 Antibody Depletion Experiments

$CD8^+$ T cells were depleted by administration of 400μg anti-CD8α (clone 2.43, BioXCell) depleting antibody i.p. twice per week. Macrophages were depleted by administration of 300μg of anti-CSF1R (clone AFS98, BioXcell) depletion antibody every other day. Control group (tAb-pManTLR treatment only) received 300μg of IgG2a isotype control antibody every other day. Depletion antibodies were administered through the entire vaccination treatment window, starting one day prior to vaccination. Cellular depletions were confirmed via flow cytometry analysis of tumor, spleen, or LN populations.

2.6 Immunofluorescence of tumor tissue.

Harvested tumors were fixed with 4% paraformaldehyde (PFA) and flash frozen embedded in OCT medium and stored at -20 degrees Celsius until sectioning. Serial sections of the tumor (10μm thick) were cut starting from the side until middle of tumor was reached. Slide mounted sections were then blocked with 10% casein solution, then with 20% rat serum prior to incubation with primary antibodies: biotinylated anti-collagen IV Ab (Jackson ImmunoResearch), rat anti-mouse CD47 (Bio X Cell), and Sulfo-Cy7 (Lumican) labelled mouse anti-mouse TRP1 Ab for 2 hours at room temperature, followed by staining with Alexa Fluor 750-conjugated streptavidin (BioLegend), and goat anti-rat-647 (Invitrogen) (1:400 final

concentration for all) for 1 hour. Slides were mounted with ProLong gold antifade medium with DAPI (Invitrogen) before imaging on Olympus confocal microscope. Images were taken with 20x oil lens, composite images and scale bar overlays were made using ImageJ.

2.7 Serum cytokine concentration analysis.

B16F10 melanoma tumors were inoculated using $3x10^5$ cells and vaccinated every 4 days starting on day 5 post inoculation with 30 µg of TLR7 as anti-TRP1-pManTLR7 and molar equivalent dose of controls CpG, anti-TRP1 and free pManTLR7 polymer, or saline. 24 hours after the 2nd vaccination, 200 µL of blood was collected in heparin-coated tubes and serum was separated by centrifugation and stored at -20 degrees Celsius until cytokine ELISAs were performed. Sera was assessed for IL-6 and IL-12p70 using ELISA, Ready-Set-Go Kit (eBioscience), following the manufacturer's instructions.

2.8 Flow cytometric analysis of tumor-binding antibodies and conjugates

Flow cytometry analysis was done using a BD FACS LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). For staining, tumor cells were washed twice with PBS, then stained in PBS + 0.2% FBS containing AlexaFluor647-labeled anti-TRP1, anti-CD47, mouse IgG2a isotype control antibodies at 30 µg/mL or with 5 µg/mL antibody as pManTLR7 conjugate for 20 min on ice. Stained cells were washed twice and resuspended in PBS + 2% FBS for analysis.

2.9 Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry

First, saturated solution of the matrix, α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich), was prepared in 50:50 acetonitrile:1% TFA in water as a solvent. The analyte in PBS (5 µl, 0.1 mg/ml) and the matrix solution (25 µl) were then mixed and 1 µl of that mixture was deposited

on the MTP 384 ground steel target plate. The drop was allowed to dry in the nitrogen gas flow which resulted in the formation of uniform sample/matrix co-precipitate. All samples were analyzed using high mass linear positive mode method with 2500 laser shots at the laser intensity of 75%.

2.10 Tissue Processing

Spleens, lymph nodes, and tumors were collected and kept on ice, in IMDM until processing. Tumors were digested in 1mL DMEM supplemented with 2% FBS, collagenase D (2 mg/mL; Gibco), Dnase I (40 µg/mL; Roche) for 45 min at 37℃ mixing. Lymph nodes were mechanically disrupted and digested at 37℃ for 45 min in collagenase D. Digested tumors, digested lymph nodes, or spleens were processed into single-cell suspensions via mechanical disruption and passage through a sterile 70 µm screen. Red blood cells in tumor cells and splenocytes were lysed by resuspending in ACK lysing buffer (Quality Biological) and incubating for 5 min at room temperature. Lysis reaction was quenched using 15 mL DMEM + 10% FBS. The single cell suspensions for tumor, lymph nodes, or splenocytes were then washed once with PBS or DMEM and resuspended in DMEM. These single cell suspensions were then used in restimulation experiments or directly stained for flow cytometry analysis.

2.11 *Ex vivo* T cell restimulation

Single-cell suspensions from spleen or lymph nodes were prepared as described above. $1x10^6$ cells from spleen or lymph node were restimulated in vitro with the addition of 2.0 µg/mL of peptide epitopes as described: $gp100_{25-33}$ (EGSRNQDWL), Trp2 (SVYDFFVWL), OVA MHC class I (SIINFEKL) and MHC class II (ISQAVHAAHAEINEAGR) (Genscript).

For ELISA analysis, following a 72hr restimulation cells are spun down and supernatant collected for the measurement of secreted cytokines. Cytokine ELISAs were performed using the Ready-Set-Go Kit (eBioscience), according to manufacturer's protocol. All cell restimulations were done in duplicate for each biological replicate with an unstimulated (no peptide added) control well to determine background levels of non-specific activation.

For flow cytometric analysis, cells were restimulated with peptide for 6 hrs. total. Cytokines were retained intracellularly via addition of GolgiPlug and GolgiFix (BD) for final 4hrs of restimulation. Following 6hr restimulation, cells were immediately washed and stained for flow cytometry analysis.

2.12 Production and characterization of antibody-pManTLR7 conjugates

Antibody (at >5mg/mL) was mixed with 20 to 30 molar equivalence of 2 kDa self-immolative PEG linker in phosphate buffer solution (pH 7.7) and reacted for one hour, mixing at RT. The reaction solution was then purified twice via Zeba spin desalting columns with 7 kDa cutoff to remove unreacted linker (Thermo Fisher). Successful linker conjugation was confirmed using gel electrophoresis and comparison to a size standard of the unmodified antibody. Antibody-linker construct in PBS (pH 7.4) was then reacted with 7 molar excess of p(Man-TLR7) polymer in an endotoxin-free Eppendorf tube for 2 hours, mixing, at RT. Excess p(Man-TLR7) polymer was removed using FPLC size-exclusion chromatography Superdex 200 column (GE). Fractions containing species with MW higher than 150 kDa (as assessed by gel electrophoresis) were then pooled and concentrated in 100 kDa Amicon centrifuge unit to create a final product of an appropriate concentration for injection in *in vivo* studies.

To determine the concentration of TLR7 agonist (TLRa) content in the pManTLR7 polymer and in the final polymer-antibody conjugates, absorbance at 327nm was measured. Known quantities of mTLR7 in saline was measured (n=3 independent samples) at 327nm in several concentrations ranging from 8 mg/mL to 1 mg/mL to calculate a standard curve as previously published in Wilson et al. 2019. The determined standard curve [TLR7a (mg/mL) = 1.9663* A_{327} + 0.0517] was then used to calculate TLR7 agonist concentration in the prepared pManTLR7 conjugate.

To determine the antibody content in our antibody-pManTLR7 conjugates, SDS-PAGE was performed on 4-20% gradient gels (Bio-Rad) using antibody of a standard curve of 4 concentrations of antibody (2, 1.5, 1, and 0.5 mg/mL) and two dilutions of antibody-pManTLR7 were fully reduced with 10mM dithiothreitol. Reducing conditions liberated conjugated pManTLR7 allowing for the band intensity of the reduced antibody to be analyzed. Band density of reduced antibody and antibody standard curve was then measured using ImageJ and antibody concentration of sample was calculated using standard curve generated.

2.13 Flow cytometric analysis

Prior to antibody staining, cells were washed with PBS and stained for 15 min on ice with an eFluor 455UV (eBioscience) fixable viability dye. The cells were washed twice with PBS then stained in PBS + 2% fetal bovine serum (FBS) containing the antibody cocktail (BD Biosciences and Biolegend) for 20 min on ice. Stained cells were washed twice with PBS + 2% FBS, and the cells were then fixed for 15 min in PBS + 2% paraformaldehyde. Cells were washed twice and resuspended in PBS + 2% FBS. If required, intracellular staining of FoxP3 was carried out using the eBioscience Foxp3 Transcription Factor Staining Buffer Set, per the manufacturer's

instructions. Flow cytometry measurements were performed using an LSR Fortessa flow cytometer (BD Biosciences), and data were analyzed using FlowJo software (Tree Star).

2.14 Tumor retention studies

C57BL/6 mice B16F10-tumor bearing mice or BALB/C EMT6-tumor bearing mice (100 mm^3) were injected intratumorally with fluorescently labeled antibody-pManTLR7 conjugates of $\alpha CD47_{647}$-pManTLR7, $\alpha TRP1_{647}$-pManTLR7, or $\alpha IgG2a$ isotype $control_{647}$-pManTLR7. To block antigen-specific binding sites non-fluorescent αCD47 or αTRP1 antibody was pre-injected 2 h prior to injection of fluorescently labeled corresponding antibody-pManTLR7 conjugate. Starting at 4 hours post injection, mice were imaged via IVIS Spectrum in vivo imaging system (Perkin Elmer) at regular intervals until the fluorescent signal was undetectable over background. Images were processed and Radiant Efficiency was quantified for a region of interest (ROI) selection of the tumor area using Living Imaging 4.5.5 software (Perkin Elmer). Radiance Efficiency is measured in units of "photons/second/cm^2/steradian" normalized to the incident excitation power. All imaging parameters were kept consistent between all images taken and animal autofluorescence was subtracted from the tumor ROI. Background radiant efficiency from an identical sized ROI placed over the sample stage was also subtracted from radiant efficiency of the tumor to normalize readings across images. To account for any differences in the degree of fluorescent labeling across the three tAb-pManTLR constructs used, comparisons between groups were only made using % of initial radiant efficiency. Loss of fluorescence in tumors was tracked by calculating the % of initial (4hr) fluorescent signal radiant efficiency at various timepoints mice were imaged (12h, 24h, 36h, 48h, 60h, 72h, 84h, and 96h post injection). Intratumoral half-life was calculated using phase decay curve fitting of %Radiant Efficiency loss over time.

2.15 Statistical Analysis

Statistical analysis and graphs were generated using Prism software (V7; GraphPad Software). For single comparisons, a two-tailed t test was used. Data were also analyzed using one-way ANOVA with Bonferroni *post hoc* test. Differences in survival curves were analyzed using log-rank (Mantel Cox) test. Group size (*n*) used to calculate significance is indicated in figure legend. Significance is reported with respect to vehicle control group, unless stated otherwise in figure legend. For showing statistical significance ***P≤0.001; **P≤0.01; *P≤0.05, unless otherwise stated.

CHAPTER 3

RESULTS

3.1 Preparation and characterization of tAb-pManTLR7 conjugates

In the design of tAb-pManTLR7, the antibody component must be specific to tumor antigens to localize the complex within the tumor. We hypothesized that following intratumoral administration, the tumor-binding antibody (tAb) of our conjugate would bind to surface molecules expressed by tumors, thereby increasing persistence of the adjuvant within the tumor. Given the ease of conjugation of pManTLR7 to various antibodies, our *in situ* tAb-pManTLR therapeutic platform is tunable in specificity from highly tumor-specific to widely applicable, depending on the choice of antibody used. As a proof of concept, and to demonstrate the modularity of this approach, we created and characterized two tAb-pManTLR7 constructs with different tumor-binding antibodies—one in which targets a broadly expressed yet tumor-enriched surface antigen, CD47, and another which targets a melanocyte-specific antigen, TRP1.

The use of anti-CD47 in pre-clinical testing affords the ability to test the same construct across a number of murine tumor models. In humans, elevated surface CD47 expression has been observed for various malignancies, including lymphoma, bladder cancer, breast cancer and colon cancer[46–48,82]. In addition to typical antibody opsonization, antibody blockade of CD47 improves clearance of cells by macrophages and dendritic cells, resulting from the loss of inhibitory CD47 interactions with the prophagocytic SIRPα receptor on APCs[83,84]. Consistent with previous reports of elevated CD47 expression in various malignancies across both human and mouse, flow cytometric analysis or immunofluorescence staining confirmed the ability of anti-CD47 (clone MIAP 410) to bind to tumor cells from every tumor model tested: B16F10 melanoma, EMT6 and

PyMT breast cancer, and genetically engineered melanoma model $Braf^{V600E}/Pten^{fl/fl}$ (Figure 2B-E).

For testing in models of melanoma, we chose a well characterized monoclonal recognizing tyrosinase-related protein 1, αTRP1 (clone TA99), which has been demonstrated in numerous settings to bind this TAA[85–87]. I confirmed the ability of TA99 to bind melanocytes from two murine tumor models of melanoma: B16F10 and the genetically-engineered murine model *Tyr:Cre-ER^{+}/LSL-Braf^{V600E}/Pten$^{fl/fl}$* (Figure 2A, E).

Our pManTLR polymers were chemically linked to these tumor-binding antibodies as previously published[81], using a 2 kDa bifunctional linker (Fig. 3A). Here, we reproducibly created tAb-pManTLR7 conjugates and observed consistent shifts in protein mobility corresponding to increasing overall kDa following the reaction of antibody to linker and pManTLR7 to antibody-linker. After pManTLR7 was reacted with antibody-linker, excess unconjugated pManTLR7 polymer was separated from full tAb-pManTLR7 conjugate using size exclusion chromatography (SEC) (Fig. 4A). All fractions post SEC containing species with a molecular weight higher than antibody alone (150kDa) were then pooled and concentrated to a final product (Fig. 4B, C).

Unlike typical antibody-drug conjugates, our tAb-pManTLR7 constructs contain a high TLR7 agonist payload per antibody molecule. Using MALDI-TOF-MS, we determined approximately 10-12 polymers are attached per antibody (Fig. 3B). This quantitation of BCN-decorated linker conjugated to each antibody corresponds with final number of polymers per antibody as the cycloaddition reaction of the bicyclononyne moiety on the linker reacts with the terminal azide of pManTLR7 at a >95% yield[61]. The antibody concentration in our prepared batches of tAb-pManTLR7 conjugate was determined using gel electrophoresis and densitometry analysis.

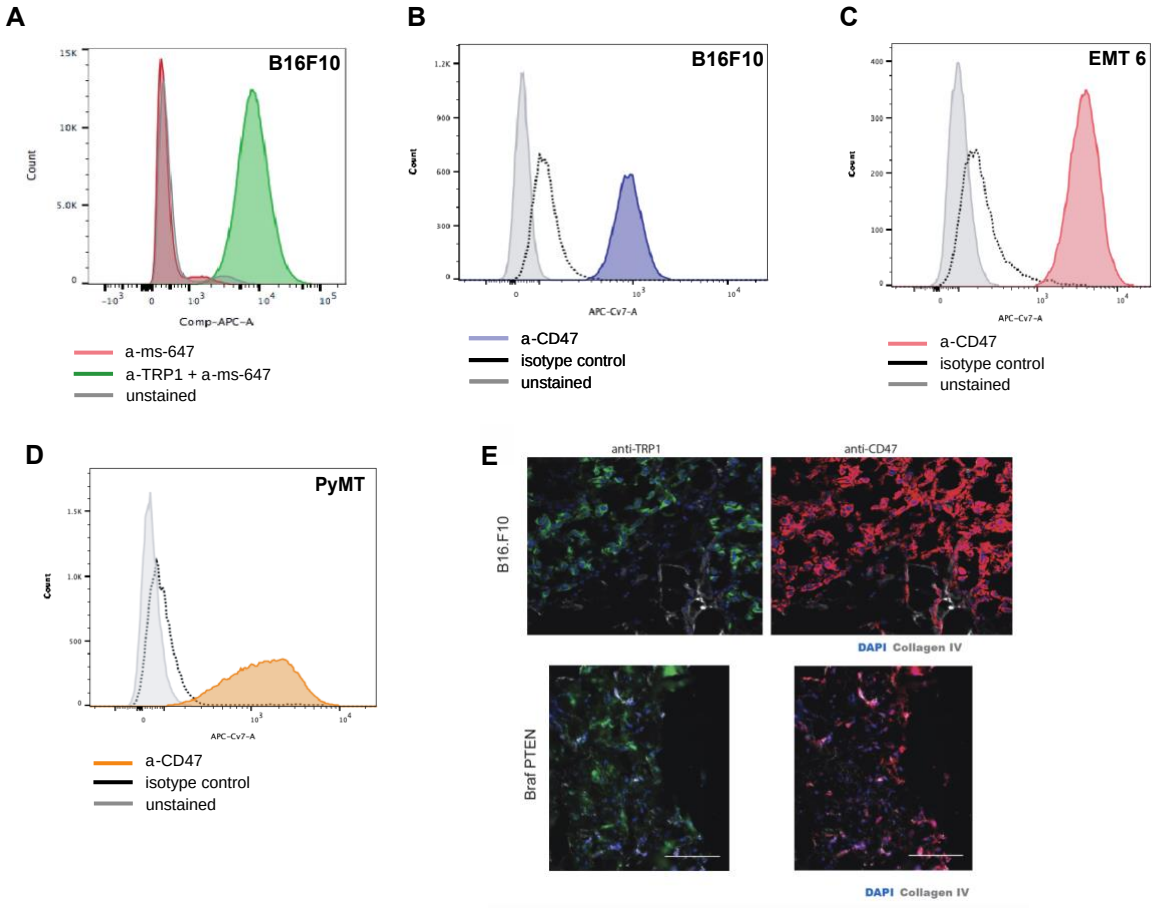

Figure 2 Selection of monoclonal antibodies for tAb-pManTLR testing in murine tumor models.

A) B16F10 melanoma cells were incubated with 30µg/mL αTRP1 (TA99) for 30 minutes at 4 degrees Celsius, washed, and stained with AlexaFluor647 labeled anti-mouse IgG followed by flow cytometry analysis. **B)** B16F10, **C)** EMT6, and **D)** PyMT tumor cells were stained with Cy7-labeled αCD47, mouse IgG2a isotype control antibodies at 30 µg/mL for 30 minutes at 4 degrees Celsius, washed, followed by flow cytometry. **E)** Frozen tissue sections of B16F10 tumor or *Braf*^{V600E}/*Pten*$^{fl/fl}$ tumor stained with αTRP1-647, αCD47-Cy7, and biotinylated anti-collagen IV antibodies. Primary staining of collagen was detected via streptavidin$_{594}$. Scale bar shows 70µm.

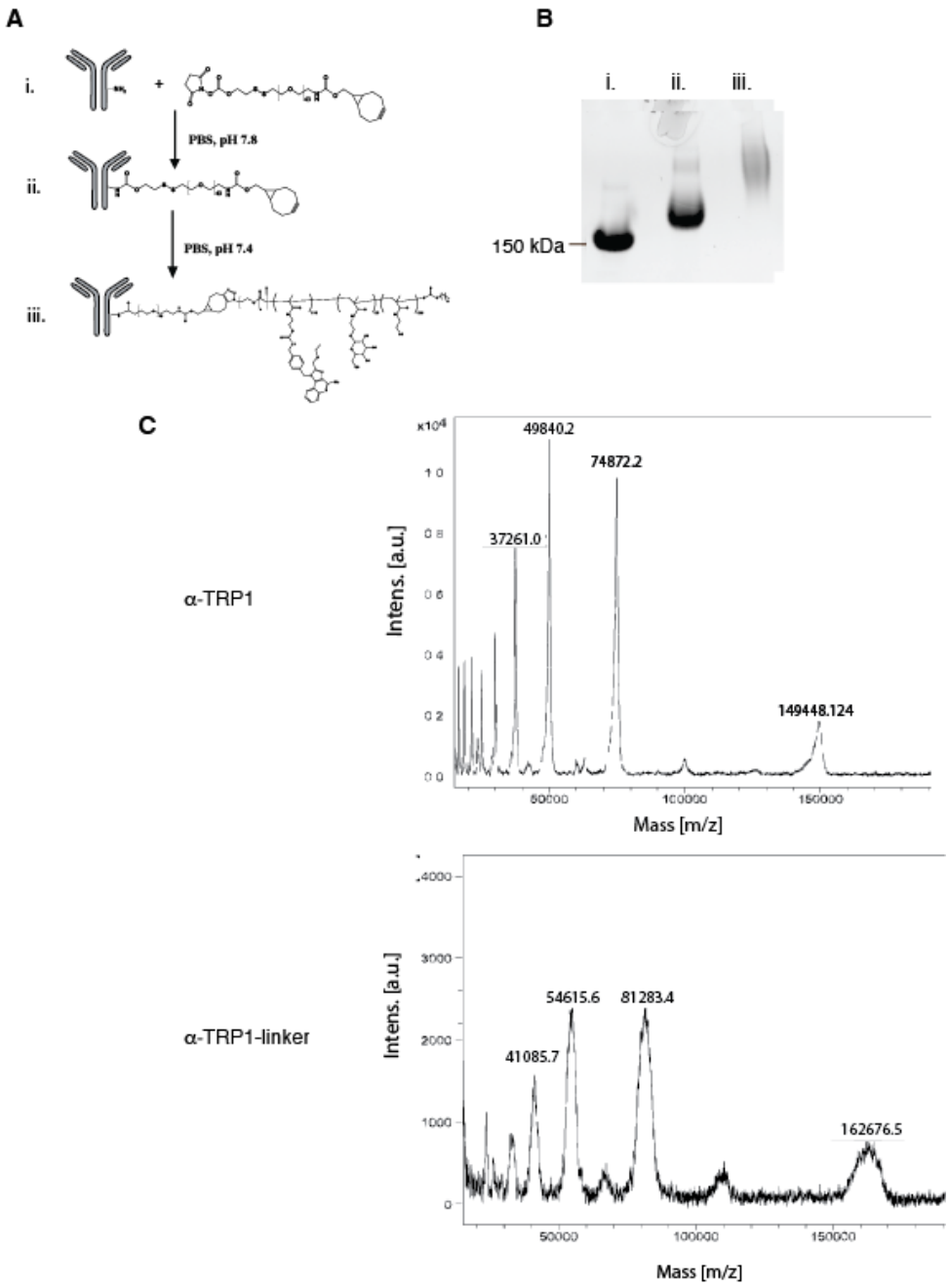

Figure 3 Antibody conjugation and characterization.

A) Representative schematic of stepwise conjugation of antibody-pManTLR7 polymer, composed of mannose and TLR7 monomers. **B)** Gel electrophoresis analysis of i. free antibody, ii. αCD47-Linker, iii. αCD47-pManTLR7. The conjugation was repeated with αTRP1 with similar results. **C)** MALDI-TOF-MS analysis of TRP1 antibody alone (left) and after conjugation to 2kDa Dithiol pyridyl-PEG-BCN linker (right). Change in molecular weight before and after linker addition was used to quantify the number of linker molecules per antibody and subsequent estimation of the number of pManTLR7 polymers per antibody of final product.

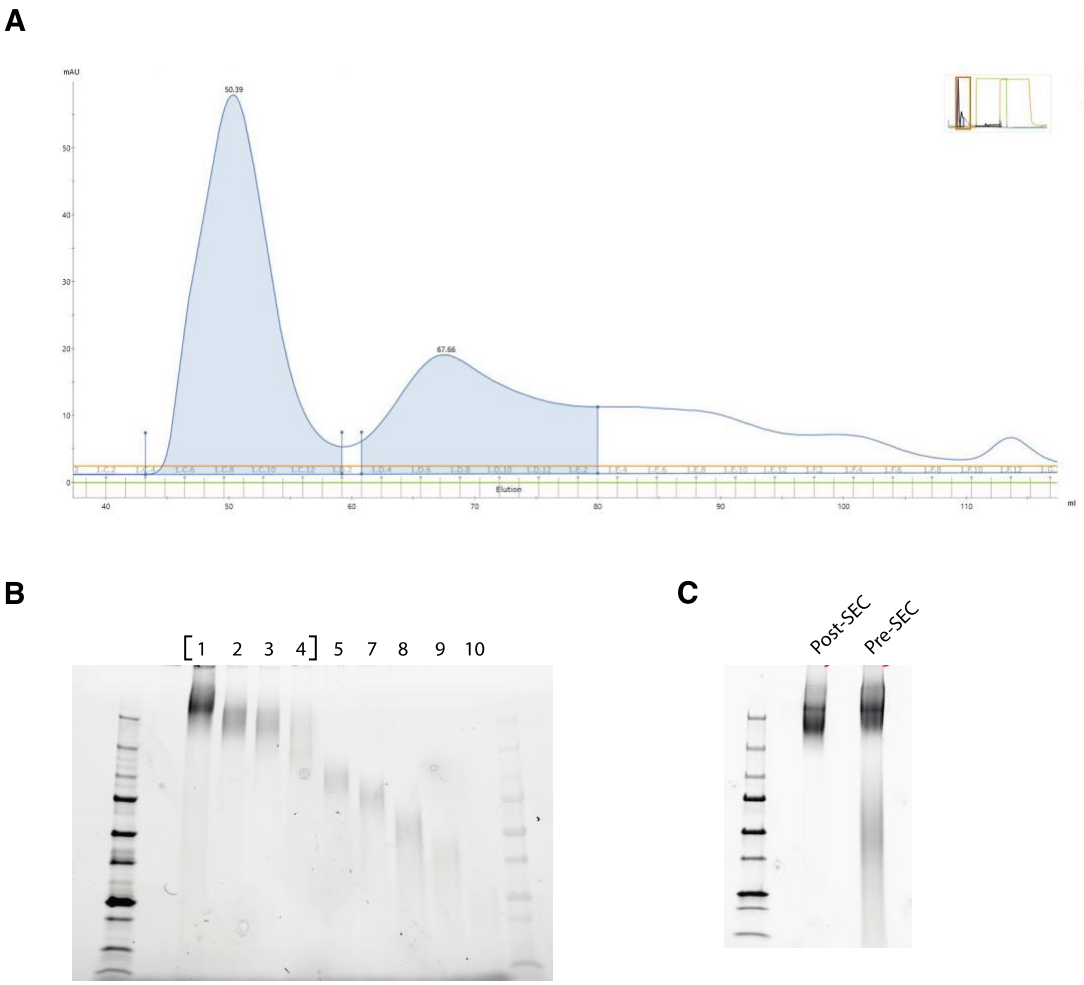

Figure 4 Purification of tAb-pManTLR7 conjugates

A) Representative SEC trace of αCD47-pManTLR7 and excess free pManTLR7 polymer at UV280nm. **B)** Representative gel electrophoresis analysis of protein fractions eluted from SEC. brackets delineate fractions corresponding to the first large peak of UV_{280} chromatogram which contained αCD47-pManTLR7 conjugate **C)** Gel electrophoresis of tAb-pManTLR conjugate before and after SEC to remove free pManTLR7 polymer. These analyses were repeated for each preparation of tAb-pManTLR7 conjugate with similar results.

Four dilutions of parental tAb at known concentrations as well as a sample of tAb-pManTLR were fully reduced and run on 4-20% gradient gels (Fig. 5A). The band density of reduced antibody fragment was then determined using image analysis for all samples. The known antibody concentrations of the parental tAb bands were then used to create a standard curve by which to calculate the antibody content of the tAb-pManTLR7 conjugate samples (Fig. 5B). TLR7 agonist content of the final tAb-pManTLR7 product was also determined in a similar manner by measuring UV-VIS absorbance of tAb-pManTLR7 at 327nm and calculating the final concentration using a standard curve of UV_{327} absorbance of known TLR7 monomer quantities.

3.2 tAb-pManTLR conjugates bind tumors and are retained within the TME

Importantly, conjugation of pManTLR7 to αCD47 or αTRP1 antibodies did not disrupt their antigen-specific binding as our fluorescently labeled tAb-pManTLR7 conjugates bound tumor cells with the same intensity as the unmodified parental antibodies (Fig. 6A, B). After confirming our tAb-pManTLR7 binding *in vitro*, we then looked to assess the ability of our tAb-pManTLR7 to persist within the tumor environment by binding cells *in situ* upon intratumoral injection. Once injected, we hypothesized that the antibody in tAb-pManTLR7 would interact with cognate ligands expressed throughout the tumor environment, slowing drainage through from the tumor. Tumor retention studies tracked the persistence of fluorescently labeled tAb-pManTLR upon injection using an *in vivo* imaging system (IVIS) to quantitatively measure the fluorescent signal in the tumor of each mouse over time following injection. Calculated loss of this signal over time showed both antibody constructs are retained within the tumor as much as 3-fold longer than isotype control antibody-pTLR7 constructs (Fig. 6 C-E).

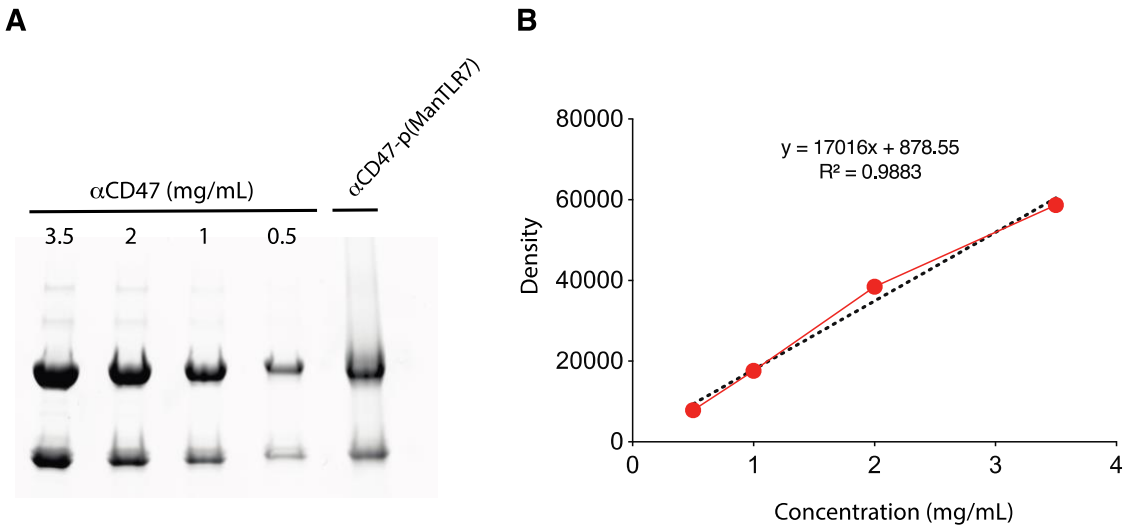

Figure 5 Quantification of antibody concentration in tAb-pManTLR

A) Gel electrophoresis analysis of fully reduced samples of αCD47 antibody at various concentrations and αCD47-pManTLR7. **B)** Standard ladder correlating band intensity from gel analysis to known antibody concentrations with the calculated linear regression curve as shown.

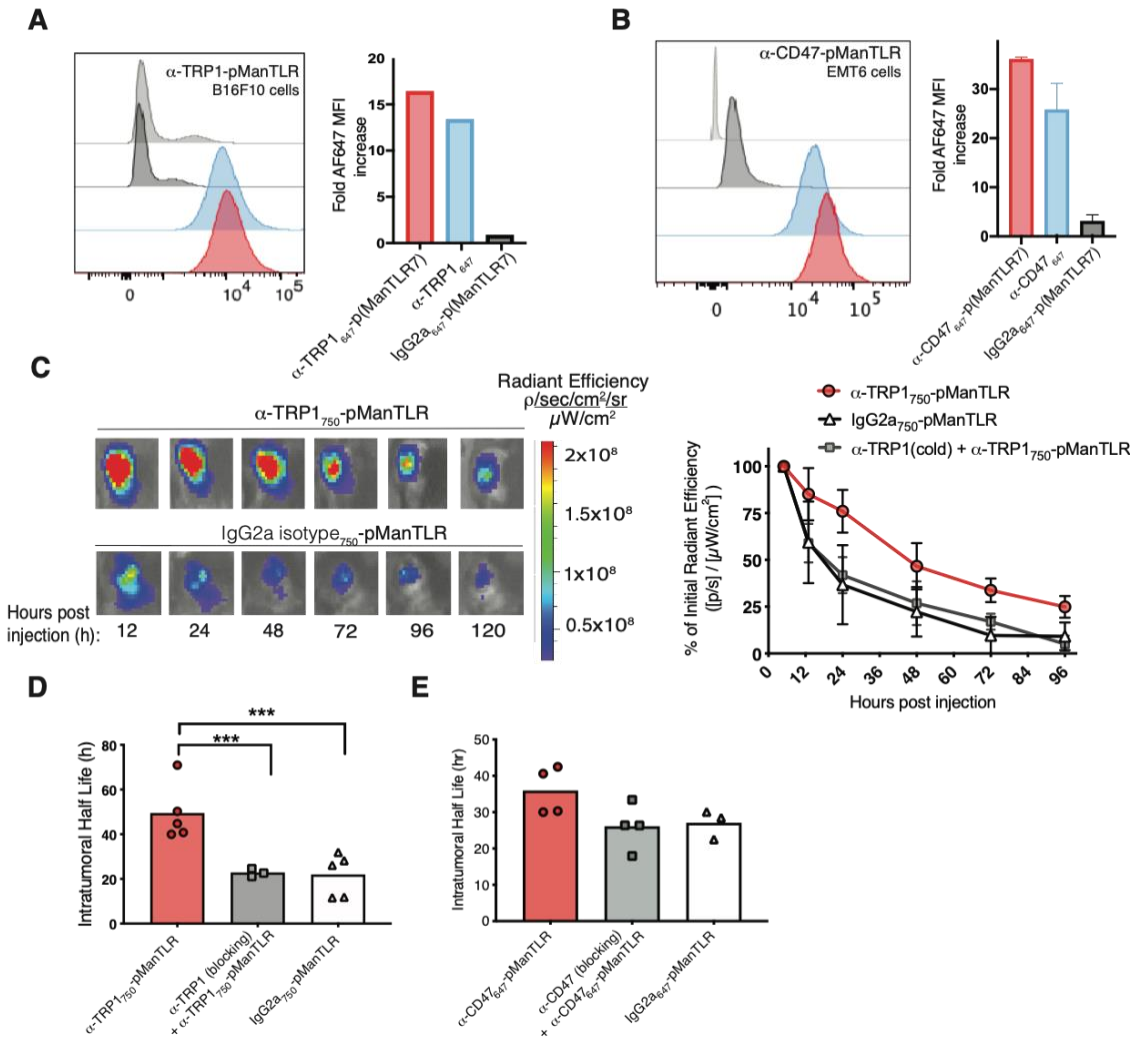

Figure 6 tAb-pManTLR7 conjugates bind to the surface of tumor cells and prolong tumor retention in an antigen-specific manner.

A) B16F10 tumor cell staining with equimolar amounts of fluorescently labeled αTRP1$_{647}$-pManTLR7, αTRP1$_{647}$, or isotype control antibody **B)** EMT6 tumor cell staining with equimolar amounts of fluorescently labeled αCD47$_{647}$-pManTLR7, αCD47$_{647}$, or isotype control$_{647}$-pManTLR7 conjugate. **C)** Tumor bearing mice imaged 4 h post injection with fluorescently labelled conjugates of αCD47$_{647}$-pManTLR7, αTRP1$_{647}$-pManTLR7, αIgG2a isotype control$_{647}$-pManTLR7 and imaged via IVIS at regular intervals to measure loss of tumor fluorescent signal over time. To block antigen-specific binding sites non-fluorescent αCD47 or αTRP1 antibody was pre-injected 2 h prior to injection of fluorescently labeled corresponding antibody-pManTLR7 conjugate. Loss of tumor fluorescence was calculated as % of initial (4 h) fluorescent signal. Intratumoral half-life was calculated using phase decay curve fitting of %Radiant Efficiency loss over time in **D)** B16F10 and **E)** EMT6 tumor bearing mice. Data were compiled from 2 independent experiments for **D**. Statistical analyses were performed using one-way ANOVA with significance shown.

To further demonstrate tAb-pmanTLR7 tumor retention was mediated by antigen-specific binding of the antibody, we prophylactically blocked TRP1 and CD47 ligands within the TME by injecting a surplus of unlabeled αTRP1 or αCD47 antibody into B16F10 or EMT6 tumors, respectively. tAb_{750}-pManTLR7 injection into these antigen-blocked tumors completely abrogated the retention of our conjugate and mimicked the intratumoral kinetics as the nonspecific $isotype_{750}$-pManTLR controls (Fig 6 C-E).

3.3 tAb-pManTLR7 is endocytosed by and activates local APCs

We next wanted to assess the cellular populations which endocytosed tAb-pManTLR after treatment and if the prolonged intratumoral kinetics of our conjugate would improve local APC activation within the tumor and tumor-draining lymph node (tdLN). To determine which populations of antigen presenting cells internalize tAb-pManTLR7, we vaccinated EMT6 tumor-bearing mice with fluorescently labeled $\alpha CD47_{647}$-pManTLR7 and, 24 h later, assessed APC subsets for uptake. Likely due to mannose recognition by broadly expressed endocytic C-type lectin receptors, we found $\alpha CD47_{647}$-pManTLR7 was present in all tumor APC subsets assessed, with a slight enrichment for uptake within the monocyte linage subsets (Fig. 7A). In the tdLN, a majority of $CD11c^+$ DCs were tAb-$pManTLR7^+$. Notably, greater than 50% of all cross-presenting $CD103^+$ DCs and LN-resident $CD8a^+$ DCs had endocytosed our fluorescently labeled conjugate (Fig. 7A). Enhanced delivery of our TLR7 agonist to, and activation of, these cross-presenting DC subsets is critical to the priming of $CD8^+$ T cells. Multiple mechanistic studies of tumor immunology have defined $CD103^+$ DCs as crucial to tumor antigen trafficking and priming of T cell immunity[88].

Based on these data, we then wanted to assess any changes in the activation status and frequencies of various APC populations in the tumor and its draining lymph node as a result of tAb-pManTLR or an unconjugated component formulation treatment. Consistent with the observed increase in endocytosis, we observed a trend of increased $CD103^+$ DCs in the tdLN of tAb-pManTLR7 treated mice (Fig. 7E) as well as significantly enhanced activation of this population in tdLN at the 24 h timepoint (Fig 7F), as compared to antibody and unconjugated pManTLR or saline treated controls. Treatment also significantly increased frequencies of inflammatory monocytes and macrophages within the draining lymph node (Fig. 7B-D). Lastly, at this timepoint, tumor-retained αCD47-pManTLR treatment resulted in improved intratumoral activation of macrophages and $CD11c^+CD11b^+$ DCs over treatment with αCD47 and unconjugated pManTLR7 controls. Together, our data demonstrated uptake of our conjugate by various APC subsets confirmed enhanced activation of these subsets upon treatment with our tAb-pManTLR7 conjugate.

3.4 tAb-pManTLR7 treatment eradicates established tumors in EMT6 model of triple negative breast cancer

We first evaluated the efficacy of our tAb-pManTLR7 vaccination in the subcutaneous syngeneic triple negative breast cancer model, EMT6. This model represents immunologically 'excluded' tumors in which T cells are localized to the stromal boundaries with very few numbers infiltrating deeper within the tumor bed. Similar to human tumors with this same immune-excluded phenotype, this model does not respond strongly to treatment with checkpoint inhibition. Tumor cells ($5x10^6$) were injected into the mammary fat pad and allowed to grow for 6d at which point tumors were ~$70mm^3$. EMT6 tumor-bearing mice were treated intratumorally

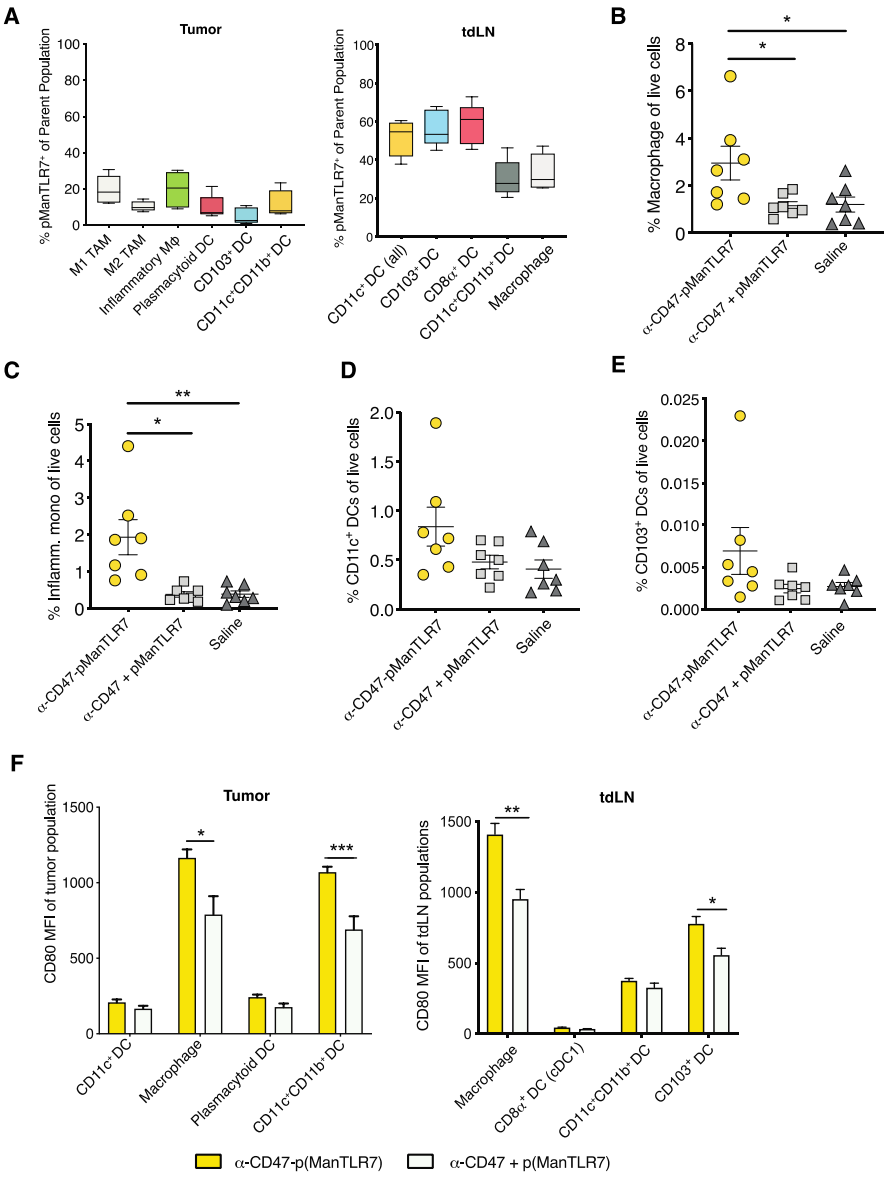

Figure 7 tAb-pManTLR7 activates local APCs and is endocytosed by multiple APC subsets **A)** Percentage of αCD47$_{647}$-pManTLR+ cells of DC populations and macrophages in the tumor draining lymph node 24 hours after intratumoral injection. APC subsets defined as CD11c+ all: $CD11c^+CD11b^-$, $CD103^+$ DC: $CD11c^+CD11b^-CD103^+$, $CD8^+$DC: $CD11c^+CD11b^-CD8\alpha^+$, $CD11c^+CD11b^+$ DCs, Macrophage: $CD11b^+F480^+$. (n=5, mean ± SD) **B)** Percentage of macrophages and inflammatory monocytes ($CD11b^+Ly6C^{hi}$) in tumor draining lymph node 24 hours after vaccination with αCD47-pManTLR7, αCD47 + pManTLR7, or saline. (n=7, mean ± SD) **C)** Activation of cell populations in tumor (left) and tumor draining lymph node. Cell populations defined by same markers as in **A)**, Plasmacytoid DC: $CD11c^+B220^+$. For **B, C** statistical differences were determined via two-tailed t-test. Experiments were repeated twice with similar results.

four times, with αCD47-pManTLR7 or control treatments of substituent components until their tumors were no longer palpable (Fig. 8A). αCD47-pManTLR7 treatment alone induced significant tumor reduction or complete regression, resulting in 50% survival of treated animals (Fig. 8B, C). Complete tumor eradication in established 'cold' tumor models—as seen with our treatment— are rarely observed with vaccination or adjuvant treatment alone with many requiring combination with 2 or 3 additional modalities to achieve tumor regression[89–91]. Combination treatment of αCD47-pManTLR7 with checkpoint inhibitor antibodies (CPI) resulted in similarly impressive tumor control to αCD47-pManTLR7 monotherapy, with 75% survival seen in treated mice. To further demonstrate that conjugating pManTLR7 to tAbs improves therapeutic efficacy, mice were treated with equivalent amount of antibody and unconjugated pManTLR7 as in the αCD47-pManTLR7 conjugate. Tumor growth and mouse survival showed the unconjugated mixed components of tAb-pManTLR was ineffective at controlling or eradicating tumors to the same extent as the full conjugate. Importantly, these data suggest the requirement of tumor retention of pManTLR7 and improved APC activation to provide functional tumor control.

Clinically, late stage malignancies are prone to relapse, even after initial tumor regression. Thus, the ability to generate systemic memory is a crucial attribute of an effective therapeutic within this disease setting. To assess the ability of tAb-pManTLR7 to generate anti-tumor memory, surviving mice treated with αCD47-pManTLR7 were re-challenged with a second EMT6 tumor on the abscopal mammary fat pad 30 days after initial tumors were no longer palpable (Fig 8A). Strikingly, 75% and 80% of mice from αCD47-pManTLR7 and αCD47-pManTLR7 + CPI treated animals, respectively, rejected secondary abscopal tumor outgrowth whereas all naïve animals developed tumors (Fig. 8D). Together these data suggest, local treatment with

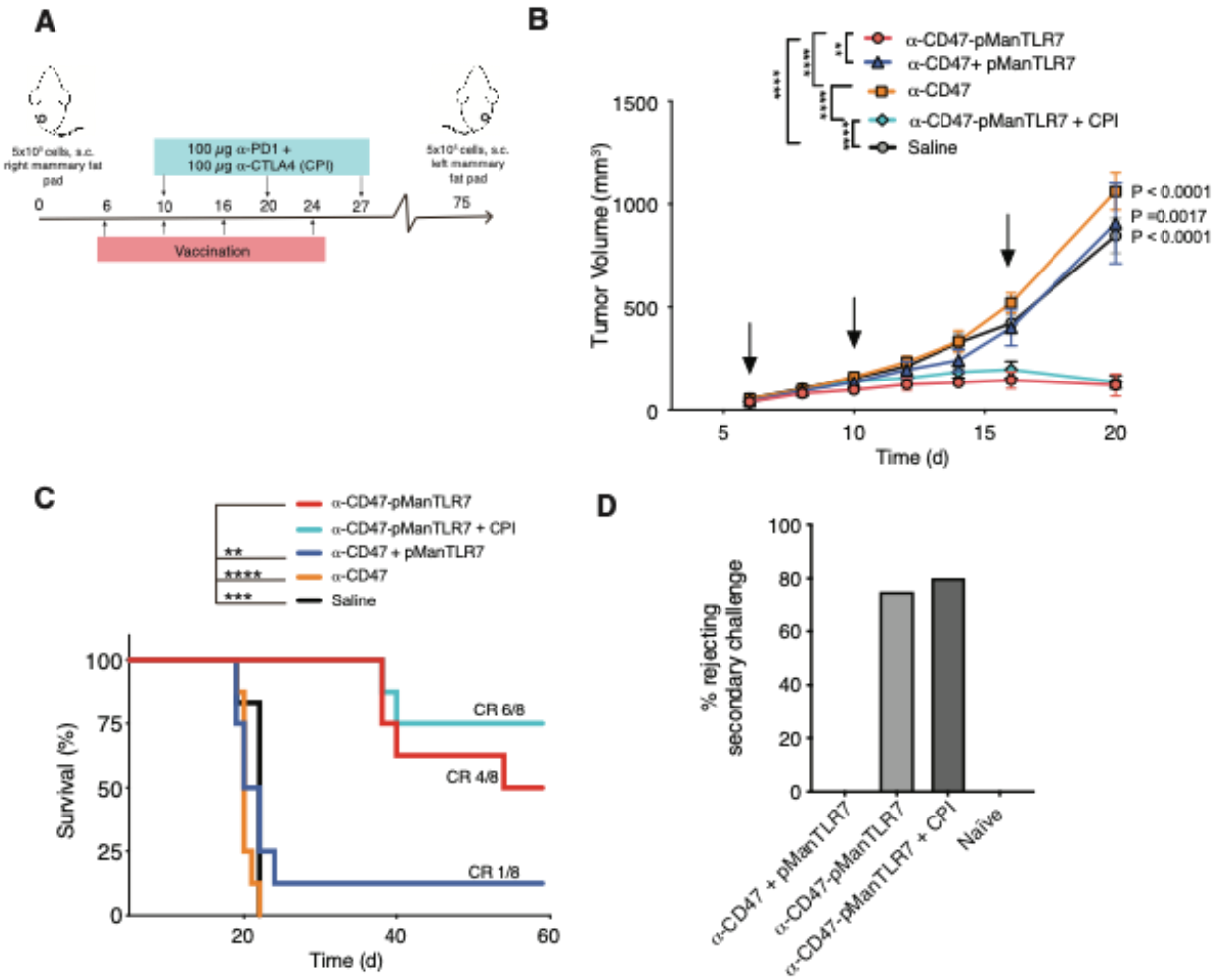

Figure 8 tAb-pManTLR7 treatment eradicates established tumors in EMT6 model of triple negative breast cancer

A) Treatment schema. BALB/c mice were implanted with EMT6 mammary carcinoma cells ($5x10^5$) in right mammary fat pad. On d6 tumors (~60-75mm^3) were injected with 20μg TLR7 as αCD47-pManTLR7, equimolar unconjugated mix of αCD47 + pManTLR7, equimolar αCD47 as contained in αCD47-pManTLR7 conjugate, or vehicle control (saline). Combination therapy with CPI was administered via *i.p.* injection of 100μg αPD1+ 100μg αCTLA4. **B)** Tumor growth curves (volume±SEM) and **C)** survival plots of the treated mice (n=8 mice per group). Experiment was repeated twice with similar results. **D)** Surviving mice from initial treatment were implanted with a second injection of EMT6 cells ($5x10^5$) into left mammary fat pad. Mice were assessed for tumor growth after 14 days, % tumor free mice shown. Statistical significance as shown with respect to mice treated with αCD47-pManTLR7. Statistical differences were determined by one-way analysis of variance (ANOVA) using Bonferroni's post hoc test in **B)**, or by log-rank (Mantel-Cox) test in **C** (n= 8 mice per group).

tAb-pManTLR7 generates bona fide systemic immune memory capable of recognizing and controlling distant tumor growth.

3.5 Efficacy of tAb-pManTLR7 treatment is dependent on $CD8^+$ T cells

To better understand how tAb-pManTLR treatment provides antitumor efficacy, we performed antibody depletions of effector cell subsets that have been defined in the immunologic rejection of tumors. For cancer vaccines, the initiation of $CD8^+$ T cell responses are critically important to tumor control and rejection[92,93]. Macrophages are also capable of engulfing antibody opsonized tumor cells and given the strong activation of macrophages we observed upon tAb-pManTLR7 treatment, changes in their polarization may lead to loss of immunosuppressive TAM functions.

To interrogate the mechanism behind tAb-pManTLR7 treatment efficacy, we next examined the requirement for these two effector subsets in the ability of tAb-pManTLR7 to clear established tumors. To test the requirement of macrophages and $CD8^+$ T cells we depleted these populations through the administration of CD8α or CSF1R antibodies (Fig. 9A). Depletion antibodies were administered to EMT6 tumor-bearing mice throughout the course of treatment, starting one day prior to the first injection of tAb-pManTLR. Depletion of $CD8^+$ T cells abolished treatment-mediated tumor control and survival, indicating the requirement of $CD8^+$ T cells in the antitumor efficacy of tAb-pManTLR7 treatment (Fig. 9B, C). Macrophage depletion via CSF1R antibody did not significantly alter the efficacy of tAb-pManTLR7 treatment. Here, tumor growth in macrophage-depleted mice tracked closely with isotype control-treated mice and 33% of mice ultimately still cleared their tumor, suggesting macrophages do not play a critical role in mediating tAb-pManTLR7 treatment efficacy.

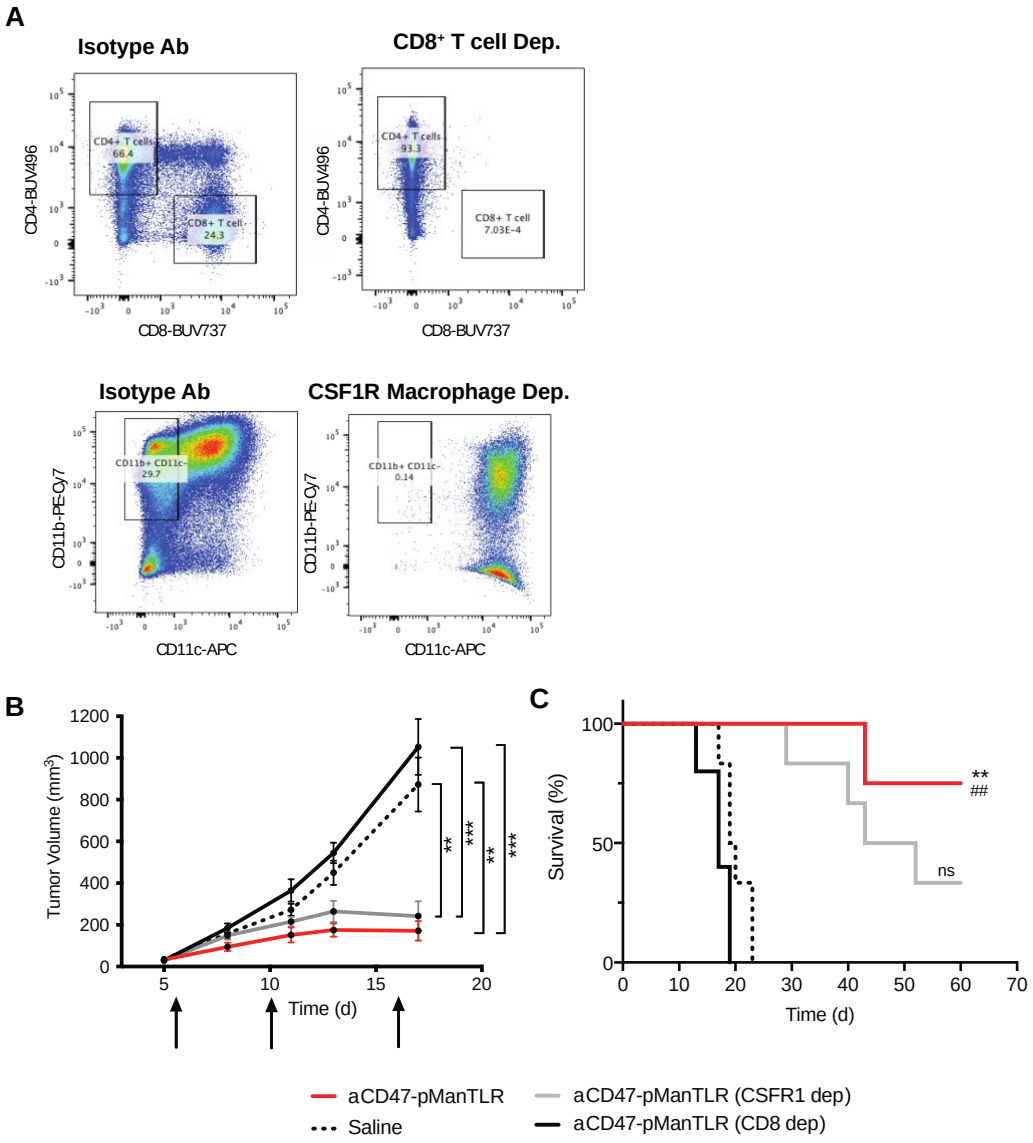

Figure 9 Efficacy of tAb-pManTLR7 treatment is dependent on CD8⁺ T cell responses

A) Confirmation of CD8⁺ T cell (top) and macrophage (bottom) depletion 24h post treatment of mice with α-CD8α or α-CSF1R antibodies with comparison to isotype control treatment. Flow cytometric analysis of CD8⁺ T cells in spleen and CSF1R macrophage depletion in tumors shows comparison to isotype control treated animals. Mice were implanted with EMT6 tumors and treated three times with αCD47-pManTLR7 every 4 days starting at day 6. Tumor growth **B)** and survival over time **C)** for mice treated with depleting antibodies administered *i.p.* starting 1 d before initiation of αCD47-pManTLR7 therapy, to deplete CD8⁺ T cells (CD8), or macrophages (CSF1R) (n=7 mice per group for all groups). Arrows indicate treatment timepoints. Statistical significance by Welch's *t*-test in **C** or by log-rank (Mantel-Cox) test vs. αCD47-pManTLR7 treatment in **C.**

3.6 tAb-pManTLR treatment reduces tumor growth and increases intratumoral T cell accumulation in established B16F10 melanoma

To show the generalizability of our approach and the ability to utilize different tumor-binding antibodies, we next tested our tAb-pManTLR treatment in another 'cold' tumor model, B16F10 melanoma. We tested the efficacy of tAb-pManTLR conjugates using either αCD47 or αTRP1 as both antibodies are capable of binding B16F10 cells and in doing so, can functionally retain the construct intratumorally (Fig. 10B, C). Comparable efficacy was seen with either αCD47-pManTLR or αTRP1-pManTLR treatment in B16F10-tumor bearing mice. Both tAb-pManTLR constructs showed the ability to significantly slow tumor growth relative to intratumoral saline treatment.

In this model, we also benchmarked αTRP1-pManTLR against two other clinically tested therapies for advanced melanoma: intratumoral treatment with equimolar CpG (a TLR9 agonist) and α-PD1 and α-CTLA4 combination checkpoint blockade antibodies (CPI). Here, αTRP1-pManTLR monotherapy showed improved tumor control over both of these clinical treatments (Fig. 10C). Again, in this second tumor model, we failed to see improved tumor control upon combination of αTRP1-pManTLR treatment with αPD1 and αCTLA4 over αTRP1-pManTLR treatment alone. Also similar to what was observed in the EMT6 breast cancer model, treatment with tAb-pManTLR conjugates as αCD47-pManTLR or αTRP1-pManTLR provided improved antitumor efficacy over unconjugated substituent antibody and pManTLR7 components and further highlights the necessity of the linkage between the two (Fig. 10B, C).

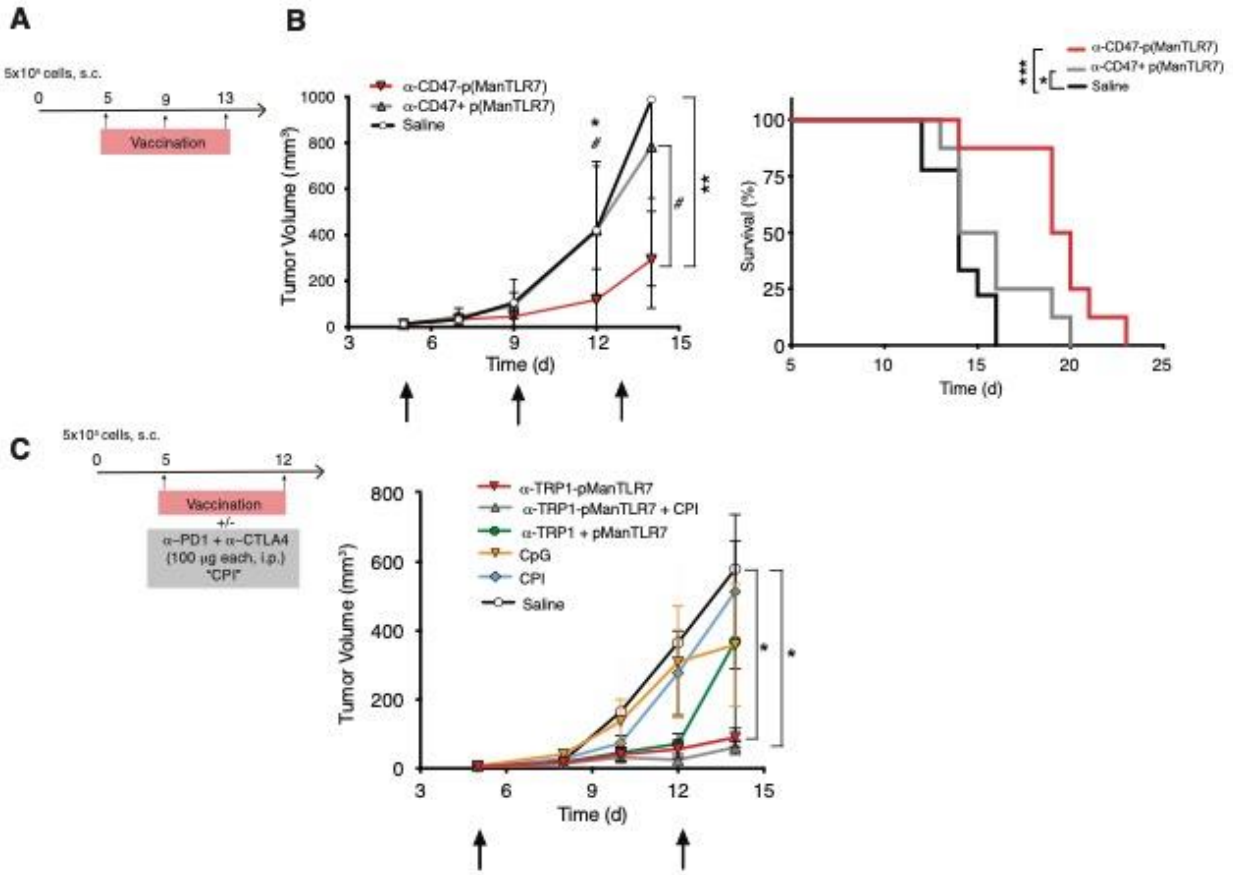

Figure 10 tAb-pManTLR treatment reduces tumor growth in established B16F10 melanoma

A) Treatment schema for B16F10 tumor bearing mice treated with αCD47-pManTLR7, equimolar unconjugated mix of αCD47 + pManTLR7, or vehicle control (saline). **B)** Tumor growth curves of the treated mice with arrows indicating treatment times (average tumor volume ±SD) and survival over time. (n=8 mice for all groups; day 14 significance shown by Welch's *t*-test comparison with αCD47 + pManTLR7) or by log-rank (Mantel-Cox) test vs. αCD47 + pManTLR7. Italics indicate significance vs. saline treatment. **C)** Mice with B16F10 tumors were treated with αTRP1-pManTLR7 or control group as indicated. Intratumoral CpG treatment was dosed as equimolar to TLR7 content in αTRP1-pManTLR7 conjugate. Experiments were repeated at least twice with similar results. Shown are mean tumor volume curves±SD (n=7 for all groups).

3.7 tAb-pManTLR7 treatment remodels T cell responses within the tumor

Given the requirement of $CD8^+$ T cells for therapeutic efficacy of tAb-pManTLR, we next wanted to assess if treatment could increase the numbers of tumor-infiltrating T cells or alter their phenotype. We treated B16-OVA tumor bearing mice with αCD47-pManTLR and four days following the last treatment, assessed the intratumoral T cells using flow cytometry. tAb-pManTLR7 treated mice showed significantly increased numbers of both $CD8^+$ and $CD4^+$ T cells in tumors, with no increase in $Foxp3^+$ $CD4^+$ Tregs (Fig. 11A, B). The intratumoral balance of $CD8^+$ T cells to Tregs was significantly increased upon treatment suggesting a reduced ability of these suppressor cells to limit CTL functionality (Fig. 11C). Of note, these increases in intratumoral T cells were only observed in response to αCD47-pManTLR but not αCD47 and unconjugated pManTLR7 mix. We also assessed nuclear staining of TOX in intratumoral T cells, a transcription factor which been shown to drive a transcriptional program consistent with T cell exhaustion[94,95]. Importantly, high TOX expression is correlated with failure to produce effector cytokines and increased expression of inhibitory receptors on T cells, both of which are hallmark characteristics of dysfunctional intratumoral T cells. At this timepoint, αCD47-pManTLR treatment did not increase TOX expression in $CD4^+$ or $CD8^+$ T cells as compared to unconjugated and saline controls, suggesting these intratumoral T cells are functional (Fig. 11D).

To determine if tAb-pManTLR treatment could similarly impact intratumoral T cells in a wildtype B16F10 model--without the expression of a foreign immunogenic antigen--tumor-bearing mice were treated twice and at day 14 tumors were assessed via flow cytometry (Fig. 12A). Here we compared the effects of αTRP1-pManTLR, αPD1 + αCTLA4 (CPI), and combination treatment of αTRP1-pManTLR + CPI on infiltrating T cell numbers and phenotype. αTRP1-pManTLR treated mice showed increased numbers of intratumoral $CD4^+$ and $CD8^+$ T

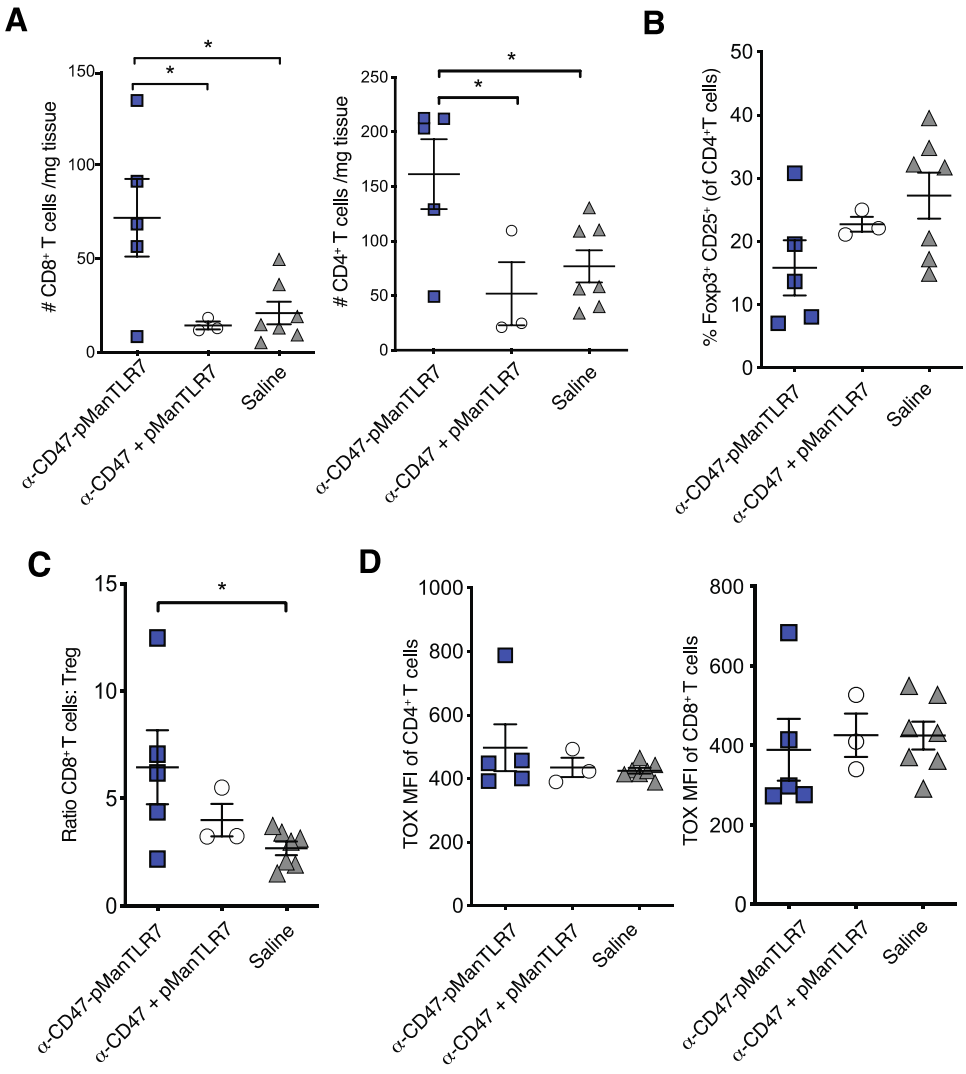

Figure 11 αCD47-pManTLR treatment increases intratumoral T cells in B16-OVA model

C57Bl/6 mice were implanted subcutaneously with $5x10^5$ B16-OVA tumor cells on left shoulder and treated twice (days 5 and 9) with αCD47-pManTLR7, equimolar unconjugated mix of aCD47 + pManTLR7, or vehicle control (saline). Four days after the 2nd treatment (d13) animals were sacrificed and tumors and tumor-draining LN harvested. Tumors were homogenized and a single cell suspension was stained and quantified via flow cytometry. Shown are individual mice ± SEM for **A)** numbers of $CD8^+$ and $CD4^+$ T cells per mg of tumor tissue**, B)** frequency of $FoxP3^+CD25^+$ Tregs of $CD4^+$ T cells, **C)** the ratio of $CD8^+$T cells to $CD4^+FoxP3^+CD25^+$ Tregs, and **D)** TOX MFI of $CD4^+$ and $CD8^+$ T cell populations. Significance between groups shown by Welch's *t*-test; n=5 mice for αCD47-pManTLR7 and saline treated groups, and n=3 mice per group for equimolar αCD47 and unconjugated pManTLR7 treatment)

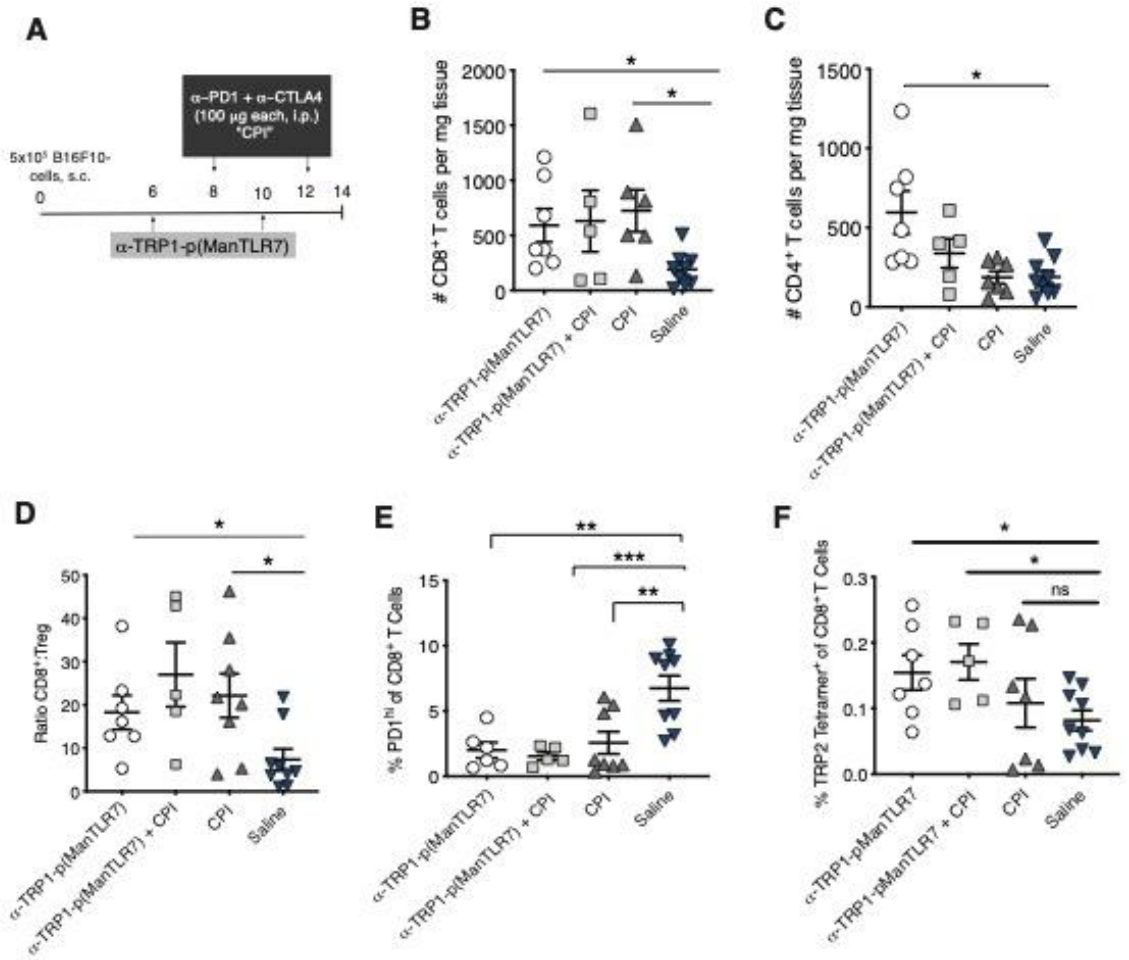

Figure 12 αTRP1-pManTLR treatment in B16F10 melanoma improves intratumoral T cell accumulation

A) Mice inoculated with B16F10 tumors were treated with 25µg TLR7 as αTRP1-pManTLR or saline on days 6 and 10 post tumor inoculation. CPI was administered i.p. on d8 and 12 post tumor inoculation as the only treatment or in addition to αTRP1-pManTLR. Tumors were excised and analyzed on day 14 via flow cytometry. Shown are mean ± SEM for numbers of **B)** $CD8^+$T cells and **C)** $CD4^+$T cells per mg of tumor tissue, **D)** the ratio of $CD8^+$T cells to $CD4^+FoxP3^+CD25^+$ Tregs, **E)** frequency of $PD1^{hi}$ of $CD8^+$T cells, and **F)** frequency of TRP2-tetrameter$^+$ of $CD8^+$ T cells. This experiment was repeated twice with similar results. Significance between groups shown by Welch's *t*-test; n=5 mice for αTRP1-pManTLR7 + CPI treatment, n=9 for saline treatment, and n=7 mice per group for all other treatments).

cells as well as improved $CD8^+$ T cells:Treg ratios (Fig. 12B-D). Combined treatment of αTRP1-pManTLR with αPD1 and αCTLA4 did not appear to affect the frequency of exhausted $PD1^{hi}$ $CD8^+$ T cells, as αTRP1-pManTLR treatment alone seemed to reduce the frequency of these cells relative to saline (Fig. 12E). We also assessed the frequency of intratumoral $CD8^+$ T cells recognizing an endogenous B16F10 melanocyte antigen, Trp2. Staining with K^b/TRP2 tetramer revealed only αTRP1-pManTLR treatment, with or without additional CPI, was able to increase these tumor-specific CTLs (Fig. 12F). Together, these data highlight the ability of tAb-pManTLR conjugates formulated with either αTRP1 or αCD47 to improve the magnitude of intratumoral T cell responses in model antigen-expressing and wildtype tumor models.

3.8 Immunizing effect of tAb-pManTLR expands functional T cell responses to tumor antigens in the draining lymph node

We next wanted to assess the ability of tAb-pManTLR7 to expand tumor-specific T cell responses and interrogate the functionality of those tumor-specific T cells. The magnitude and functionality of therapy-induced cellular response is highly predictive of the overall efficacy in tumor control as well as in protective vaccine responses [96,97]. To address this, we treated mice bearing B16F10 tumors engineered to express OVA as a model antigen (B16-OVA) so we could evaluate cytokine production via flow cytometry following T cell restimulation with OVA peptides, forty-eight hours after the last treatment. Post restimulation with K^b OVA peptide, the αCD47pManTLR7 treated cohort showed significantly increased frequencies of $CD8^+$ T cells producing IFNγ, TNFα, and perforin over saline treated controls (Fig. 13A-C). Importantly, our therapy also significantly increased in multifunctional, $IFN\gamma^+TNF\alpha^+$ $CD8^+$ T cells over both unconjugated mix and saline treated control cohorts (Fig. 13D). Similar results were observed in

$CD4^+$ T cells post restimulation with MHC class II OVA peptide, with the frequency of $IFN\gamma^+TNF\alpha^+$ $CD4^+$ T cells showing greatest increase in response to αCD47pManTLR7 treatment (Fig. 13E-G).

We repeated this experiment in wildtype B16F10-tumor bearing mice to verify if there were also detectable cellular responses to endogenous melanoma antigens gp100 and trp2. Consistent with the increased frequencies of intratumoral K^b TRP2-$tetramer^+$ $CD8^+$ T cells at this timepoint (Fig. 12F), we also observed significantly more TNFα secreting $CD8^+$ T cells in the lymph node following restimulation with Trp2 peptide. Restimulation with tumor antigen gp100 peptide showed relatively weak responses, with only a slight trend of increased $TNF\alpha^+$ $CD8^+$ observed in the αTRP1pManTLR7 treated groups (Fig. 14A, B). The expansion of multifunctional antigen-specific $CD8^+$ T cells in the tumor-draining lymph nodes of tAb-pManTLR7 treated mice further supported the sum of our data and suggests treatment enhances the priming of potent tumor-specific effector responses.

3.9 Tumor retention of tAb-pManTLR7 prevents systemic dissemination of adjuvant and treatment toxicity

Given the rapid dissemination and poor pharmacokinetics of unformulated small molecule adjuvants, we next wanted to assess if our intratumorally retained tAb-pManTLR therapy could reduce the systemic toxicity resulting from this. We treated B16F10 tumor bearing mice with αTRP1-pManTLR7 or an equimolar dose of unformulated CpG, a TLR 9 agonist, then measured the serum concentration of IL-6 and IL-12p70 at 24h post treatment. CpG induced detectable levels of these proinflammatory cytokines in the serum while serum IL-6 and IL-12 remained undetectable in the αTRP1pManTLR7 treated cohort (Fig. 15A, B).

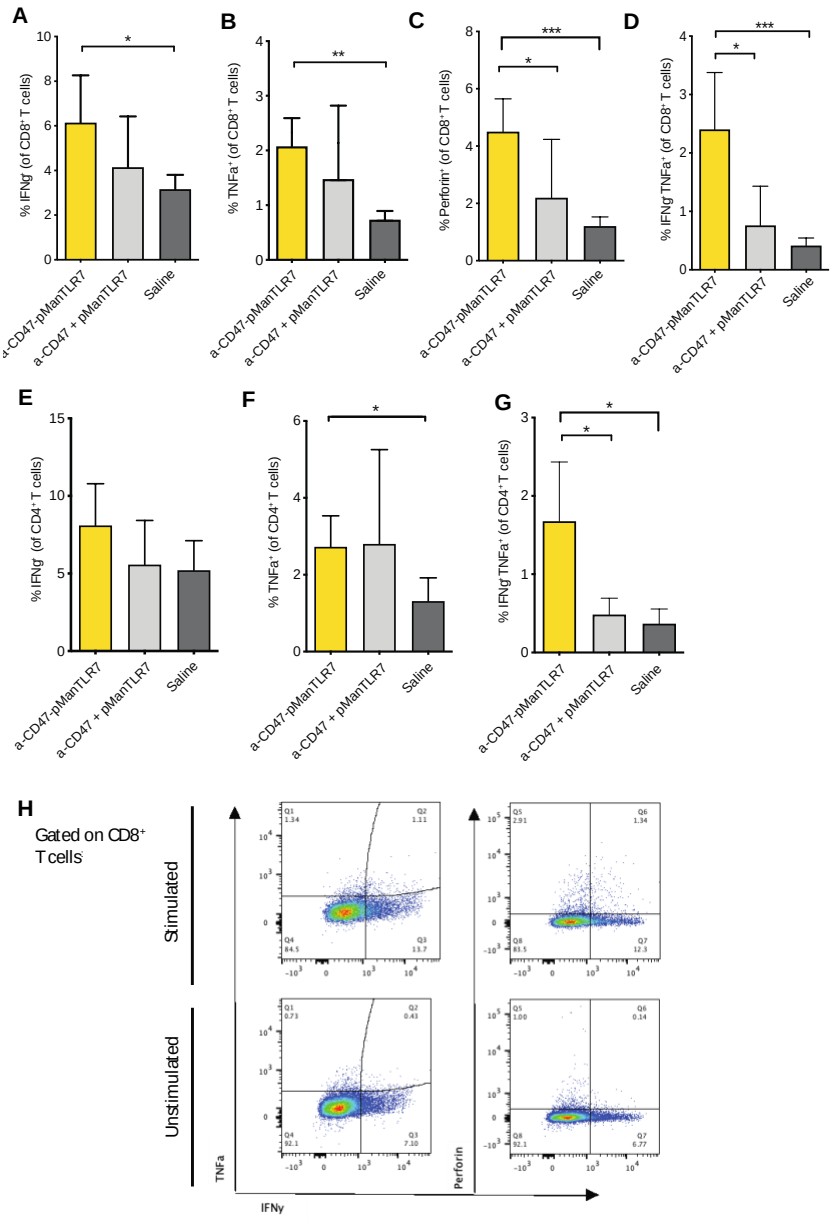

Figure 13 OVA-specific T cell responses following treatment of B16-OVA with αCD47-pManTLR7

Tumor-draining LN cells were restimulated with OVA MHC class I and class II peptide epitopes (SIINFEKL, ISQAVHAAHAEINEAGR) for 6 h total, in the presence of GolgiStop for final 4 h. Shown are quantifications of intracellular cytokine staining following restimulation for **A)** %IFNγ$^+$ of CD8$^+$ T cells, **B)** %TNFα$^+$ of CD8$^+$ T cells, **C)** %Perforin$^+$ of CD8$^+$ T cells **D)** %IFNγ$^+$TNFα$^+$ of CD8$^+$ T cells, **E)** %IFNγ$^+$ of CD4$^+$ T cells, **F)** %TNFα$^+$ of CD4$^+$ T cells and **G)** %IFNγ$^+$TNFα$^+$ of CD4$^+$ T cells. **H)** Gating strategy for cytokine$^+$ restimulated lymphocytes. Statistical significance shown by one-way ANOVA.

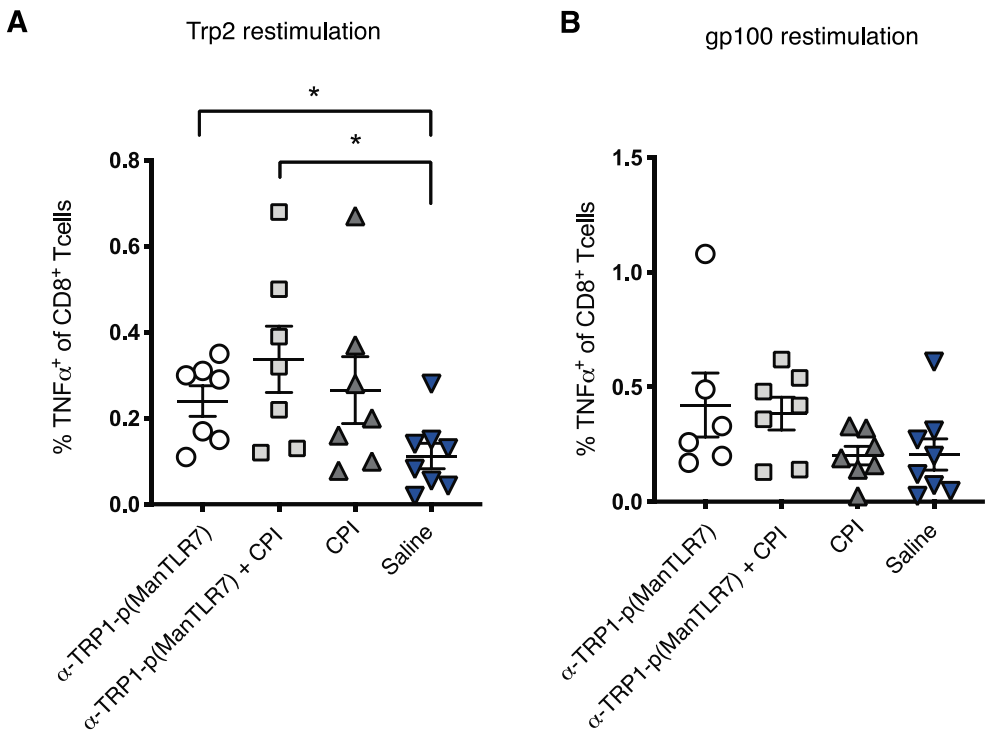

Figure 14 Antigen-specific responses to endogenous B16F10 tumor antigens following treatment with αTRP1-pManTLR7

Mice inoculated with B16F10 tumors were treated with 25μg TLR7 as αTRP1-pManTLR or saline on days 6 and 10 post inoculation. CPI was administered i.p. on d8 and 12 post tumor inoculation. Tumor-draining lymph nodes were harvested and single cell suspensions were restimulated with MHC class I peptides from Trp2 or gp100 at 2μg/mL for 6h, in the presence of GolgiStop for the final 4h. Cells were stained for intracellular cytokine production and quantified the **A)** % TNFα$^+$ of CD8$^+$ T cells upon restimulation with Trp2 peptide and **B)** % TNFα$^+$ of CD8$^+$ T cells upon restimulation with gp100 peptide. N=7 for all groups with statistical significance shown by one-way ANOVA.

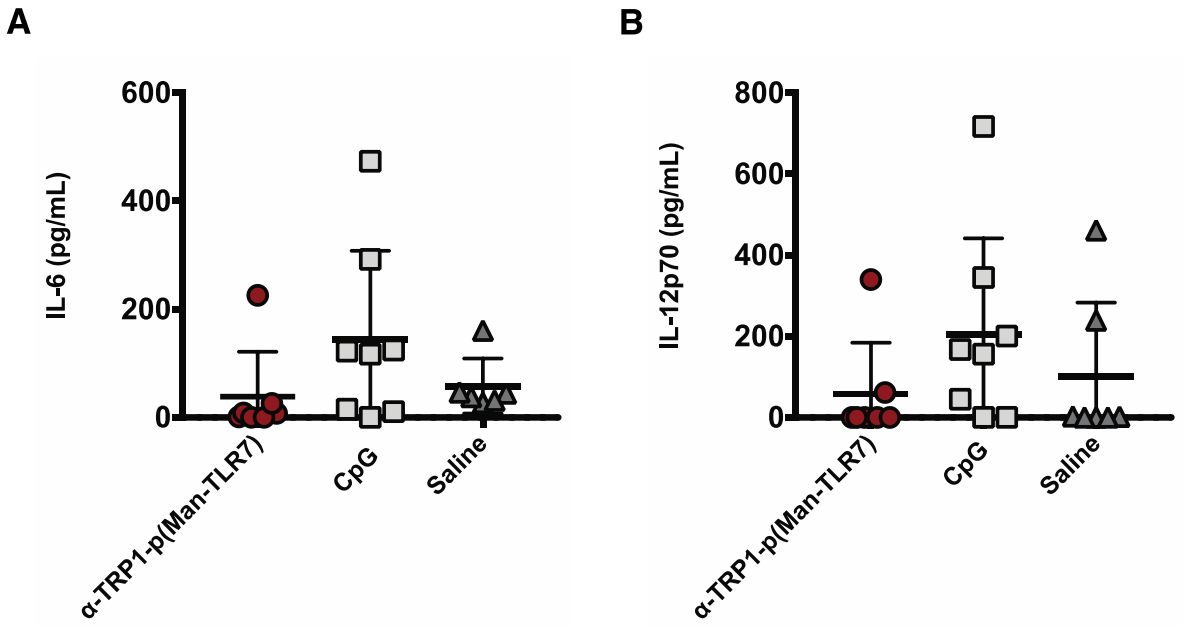

Figure 15 Inflammatory serum cytokines following tAb-pManTLR7 treatment

Mice bearing B16F10 tumors were treated with 25μg of TLR7 as αTRP1-pManTLR, equimolar dose of unformulated CpG, or saline on days 5 and 9 post tumor inoculation. 24h after the second treatment on d9, mice were bled, and ELISA was performed on mouse sera to quantify systemic **A)** IL-6 and **B)** IL-12p70 to compare the unformulated CpG adjuvant and αTRP1-pManTLR treatment. N=8 mice per treatment group.

To further assess if tAb-pManTLR induced inflammation-related systemic toxicity, we tracked the weight of EMT6 tumor-bearing mice over the course of treatment with αCD47-pManTLR7, component formulations, αCD47-pManTLR7+ CPI, or saline (Fig. 16A-E). Overall bodyweight of treated mice remained unchanged relative to saline-treated controls over the course of repeated doses (Fig. 16F).

3.10 Intravenous delivery of tAb-pManTLR for the treatment of systemic C1498 model of acute myeloid leukemia

Although intratumoral administration can be used to treat a number of injectable, superficial solid tumor malignancies, the ability to deliver tAb-pManTLR7 systemically through intravenous (i.v.) injection would significantly benefit the platform in its ease of use in the clinic and application to treat additional tumor types. In systemic administration, the antibody could target the therapy to the tumor. For disseminated malignancies, the ability to administer tAb-pManTLR7 i.v. could bind circulating tumor cells or additionally target the therapy to treat distant metastases concurrently with primary tumors.

As a pilot experiment of using tAb-pManTLR in *i.v.* delivery, we first explored the safety and efficacy of αCD47-pManTLR in the treatment of mice with a systemic model of acute myeloid leukemia (AML), C1498 (Fig. 17A). This model is poorly immunogenic and has been shown to be refractory to treatment with checkpoint inhibitor antibodies, mimicking the tumor phenotype in human AML patients. We chose αCD47 as our tumor-targeting antibody for this model as αCD47 is currently in clinical development for the treatment of AML[98] and has been shown to be overexpressed on malignant cells in this cancer as well as other hematologic neoplasms[47,99].

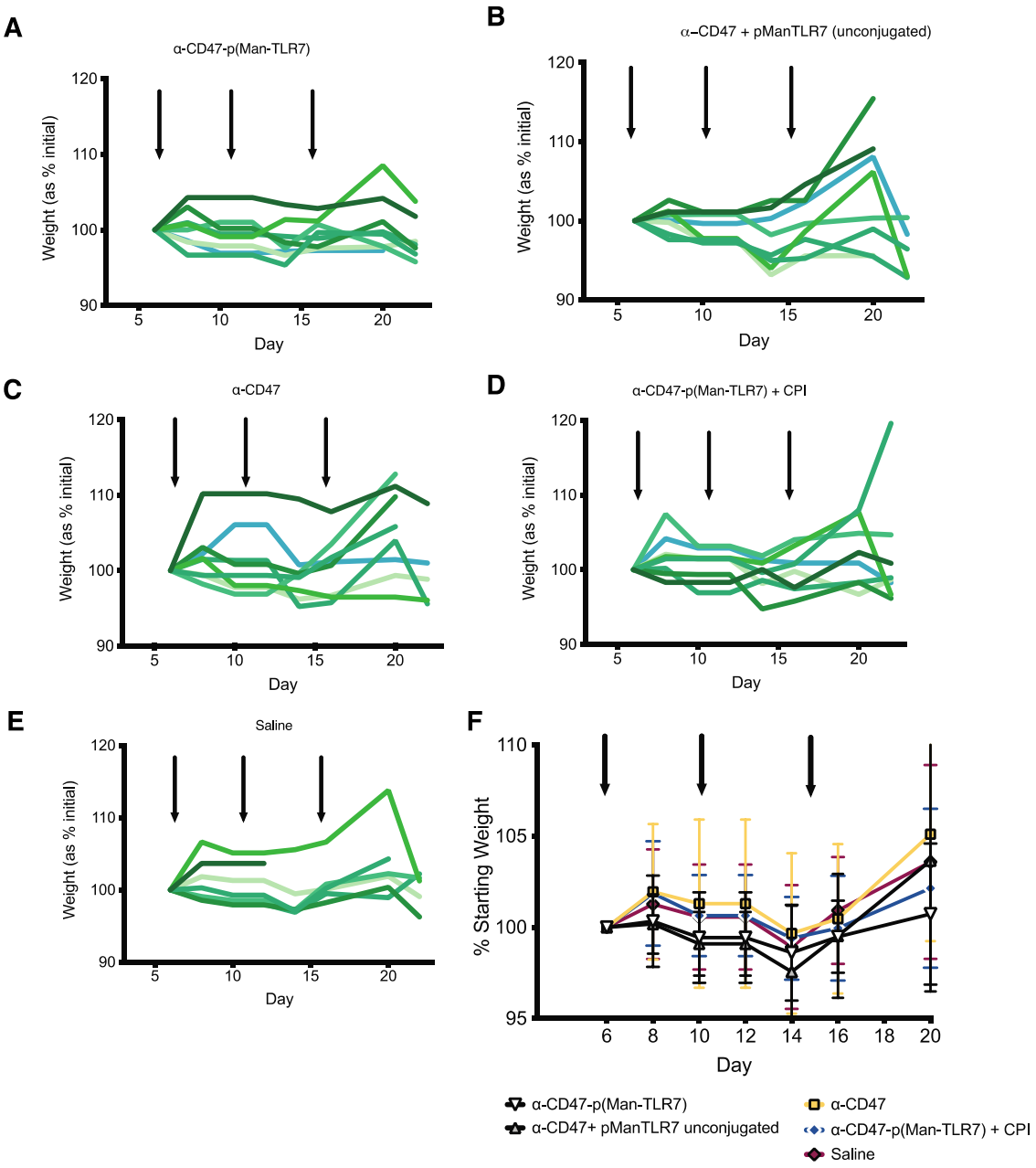

Figure 16 Effect of tAb-pManTLR treatment on bodyweight after repeated dosing

Body weight of EMT6 tumor-bearing BALB/c mice were monitored over time as % of initial weight (before initiating treatment d6), following repeated intratumoral administration of **A)**20μg of TLR7 as αCD47-pManTLR, **B)** equimolar αCD47 and unconjugated pManTLR7, **C)** equimolar αCD47 alone (29μg), **D)** αCD47-pManTLR + CPI, or **E)** saline. **F)** Averaged body weight per treatment cohort with SD shown, with arrows indicating the days of treatment. N=8 mice per group, with n=7 in the saline treated cohort.

For this study, we chose an intermediate dose for αCD47-pManTLR, containing 15μg of TLR7 per dose to minimize the potential for systemic toxicity given the ability of our conjugate to activate multiple subsets of APCs in the blood. We also hoped this lower dose could magnify potential differences in efficacy between αCD47-pManTLR and our untargeted αCD47 and pManTLR mix control treatment. We hypothesized that nonspecific APC activation would result in some baseline immune activation and marginal antitumor effect and that efficacy would be enhanced over the baseline effect if there was active tumor-targeting and co-delivery of tumor cells with adjuvant. Here, similarly to how our therapeutic functions in intratumoral administration, APCs which engulfment of CD47-blocked tumor cells would receive strong activating signals from our TLR agonist, leading to presentation and priming of cellular responses to tumor antigens. After three treatments with αCD47-pManTLR7, equimolar αCD47 and unconjugated pManTLR7, or saline, we tracked mouse survival over time (Fig. 17B). Interestingly, the cohort treated with an unconjugated mix of αCD47 and pManTLR7 fared the worst, with a median survival of 23 days. No significant differences in survival were observed between saline and αCD47-pManTLR7 treated animals, although one mouse did survive from the αCD47-pManTLR7 cohort.

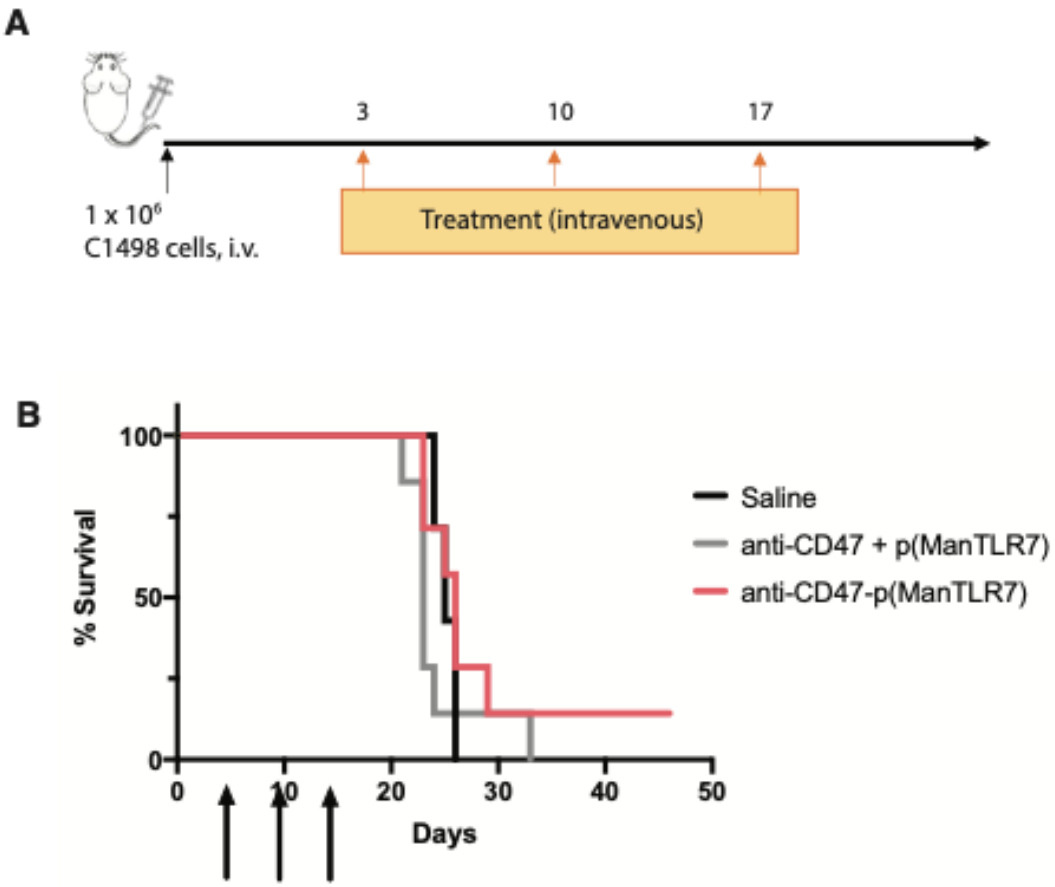

Figure 17 Treatment of systemic C1498 AML with intravenous αCD47-pManTLR7

A) C57Bl6J mice were inoculated with 10^6 C1498 AML tumor cells and treated three times, every 7 days starting at d3 post tumor inoculation with: 15μg of TLR7 as αCD47-pManTLR, equivalent dose of mixed αCD47 and unconjugated pManTLR7, or saline. **B)** Mouse survival over time. N=7 mice per treatment group.

CHAPTER 4

DISCUSSION

4.1 Summary

In this work I successfully developed and tested a novel antibody-adjuvant conjugate as a cancer immunotherapy, using a mannosylated TLR7 agonist polymer as the adjuvant payload. Combining tumor binding antibodies (tAb) with a strong TLR7-agonizing adjuvant (pManTLR7), tAb-pManTLR7 conjugates are reproducibly generated, and our chemical conjugation strategy easily accommodates to use various tumor-targeting antibodies. Upon intratumoral administration, tAb-pManTLR7 strategically localizes and sustains the delivery of our adjuvant to the tumor microenvironment and is endocytosed by multiple APC subsets within the local tumor microenvironment (TME) and tumor-draining lymph node (tdLN). In turn, this potent innate immune activation provides the necessary signals for functional T cell activation against co-delivered tumor antigens, which then provides tumor-specific cytoxicity (Figure 18). Treatment of B16-OVA, or wildtype B16F10-tumor bearing mice with tAb-pManTLR7, increased tumor infiltrating $CD4^+$ and $CD8^+$ T cells and beneficially equilibrated the intratumoral ratio of $CD8^+$ T cells:$FoxP3^+$ Tregs. We also observed increased frequencies of polyfunctional, tumor-specific T cells in the tumor draining lymph node. Most importantly, tAb-pManTLR7 treatment resulted in superior antitumor efficacy compared to formulations of substituent components in subcutaneous (s.c.) B16F10 melanoma and EMT6 mammary carcinoma models. As a monotherapy, αCD47-pManTLR7 induced complete remission (CR) in 50% of EMT6-tumor bearing animals and generated systemic anti-tumor memory capable of protecting them from a subsequent challenge with an abscopal tumor. Complete tumor eradication in established ‘cold’ tumor models—as seen with our treatment—are rarely observed with vaccination or

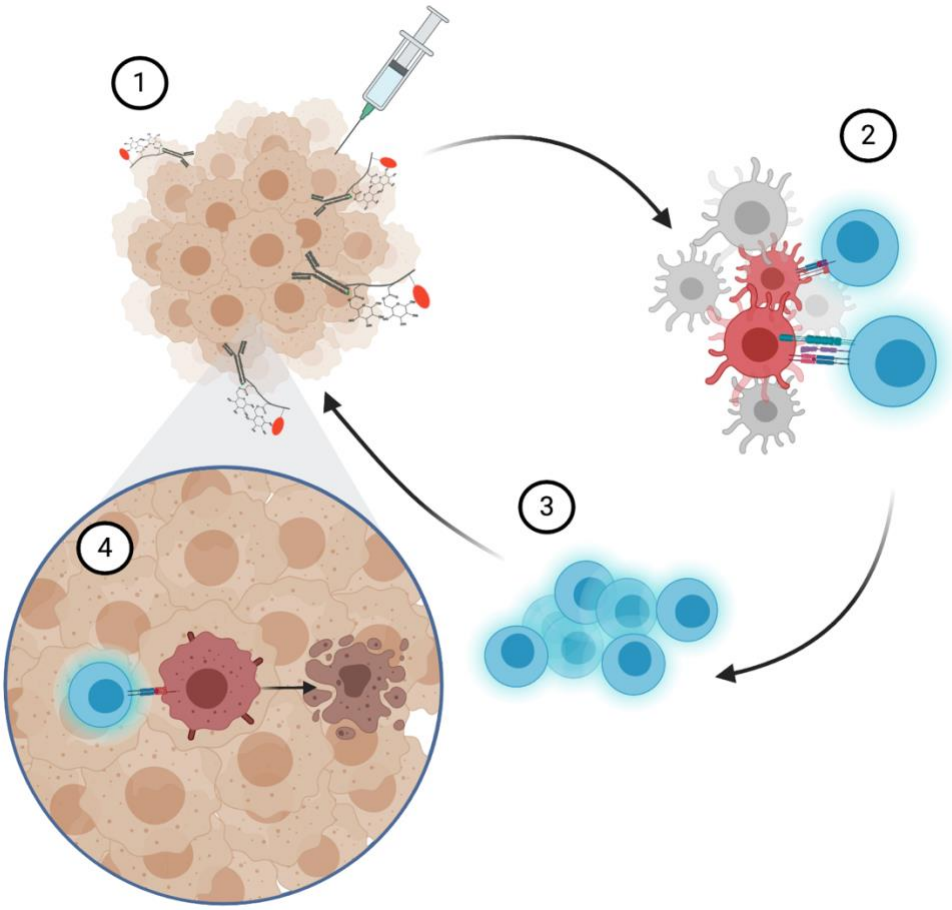

Figure 18 Proposed mechanism of tAb-pManTLR treatment

1) Intratumorally delivered tAb-pManTLR bind tumor cells and activate dendritic cells and other APCs. 2) Tumor antigens are presented to prime and activate naïve T cells. 3)T cells return to the tumor to 4) kill tumor cells presenting their cognate ligand.

adjuvant treatment alone, with many requiring combination with two or three additional modalities to achieve tumor regression[89–91]. With tAb-pManTLR7, efficacy was only modestly improved when administered in combination with αPD1 and αCTLA4 antibodies.

In sum, our engineered antibody-adjuvant conjugate offers a modular, off the shelf formulation by which to improve tumor-specific T cells and cure established, aggressive 'cold' tumors. When tumors are treated locally, tAb-pManTLR7 activates robust cellular immunity, provides durable anti-tumor memory, and provides proof-of-concept for a highly translational strategy to enhance antitumor immunity in numerous malignancies.

4.2 tAb-pManTLR Antibody-Adjuvant Design

tAb-pManTLR7 builds upon many of the same elements of material design used in antibody-drug conjugates (ADCs) which are clinically used to deliver cytotoxic payloads to tumor cells. tAb-pManTLR7 extends the potential for antibody conjugate therapies to modulate immunity selectively within the tumor. In our design, tAb-pManTLR7 overcomes a few technical challenges which traditional ADCs commonly face. The polymeric TLR7 agonist and its formulation with mannose solves two key challenges which ADC development typically face: i)excessive hydrophobicity and ii) increased potential for higher drug-antibody ratios[100].

Increased ADC hydrophobicity, occurring as a result of chemical conjugation of small molecule drugs, can cause issues with cell internalization of the ADC as well as with aggregation after . Our adjuvant polymers' formulation with HPMA and mannose improves the solubility of hydrophobic imidazoquinoline-based agonists and, in our hands, chemical conjugation of our pManTLR7 polymers to antibodies have been performed dozens of times without significant aggregation.

Additionally, our tAb-pManTLR design effectively increases the antibody-adjuvant ratio in its formulation. Because several monomers of TLR7 agonist are co-polymerized within pManTLR7, the amount of TLR7 per conjugation site on the antibody is expanded. For comparison, two companies developing antibody-adjuvant conjugates using TLR7/8 or TLR8 agonists have attached single agonists per conjugation site whereas tAb-pManTLR7 has an estimated 6 to 7 TLR7 agonist monomers per polymer, with roughly 10 polymers per antibody (Fig. 3B).

4.3 tAb-pManTLR Conjugation

For the three tumor-binding antibodies (αCD47, αTRP1, and αCD19) we have used to create tAb-pManTLR7 conjugates, the final average molecular weight was similar and showed the reproducibility of this conjugation strategy for antibodies. The extent of pManTLR7 grafting per antibody was easily controlled by modifying the molar ratio of the BCN-decorated linker in the first step of the conjugation reaction. Interestingly, although this method of conjugation is not site-specific and polymers can be attached to any free lysine resides on the antibody, the addition of pManTLR7 never appeared to significantly disrupt the antigen-specific binding of the antibody component following reactions at these molar ratios. It is certainly possible that for some antibodies with high lysine content in the antigen-binding variable regions or at higher densities of pManTLR7 grafting, that this could occur. Additionally, this conjugation strategy may need to be reassessed if smaller protein components are used as tumor-targeting agents such as fragment antibodies (fAbs) or single-chain variable regions (scFv). These smaller antibody-derivative proteins would contain reduced numbers of accessible lysine residues for conjugation and as such, might bias the pManTLR7 addition to sites which disrupts their capacity for antigen-specific binding. For clinical translation and more precise control over the

antibody:TLR7/8 agonist dose ratio, the antibody and chemical linker may need to be re-engineered to include site-specific conjugation for pManTLR7 attachment.

4.4 Antibody-pManTLR linkage is a critical component of tAb-pManTLR efficacy

At each stage of our proposed mechanism of action for tAb-pManTLR7, we compared the effect of an equivalent dose of tAb and unconjugated pManTLR7, referred to as our 'unconjugated control', to that of the full conjugate. Comparisons made to this unconjugated control treatment were used to determine the effect of the antibody-adjuvant linkage, and the intratumoral retention of our pManTLR7 adjuvant resulting from this linkage.

Analysis of the intratumoral retention kinetics of tAb-pManTLR showed the greatest differences in antigen-specific tumor retention of our conjugate at the 24h timepoint. To determine the effect of this intratumoral retention on APCs in the local environment, I chose this same timepoint to compare the activation state of various APCs in the tumor and draining lymph node between tAb-pManTLR7 and unconjugated control treated mice. Strikingly, we observed improved activation in all APC subsets analyzed in tAb-pManTLR treated tumors over the unconjugated control treatment, as measured by CD80 expression, with the most pronounced differences found in the macrophage, $CD11b^+$ DC, and $CD103^+$ DC subsets. Further analysis of T cell activation after two doses of tAb-pManTLR7 vs. unconjugated mix also demonstrated that antibody-adjuvant linkage was important for maximizing tumor-specific responses. tAb-pManTLR7 treatment showed significantly more tumor infiltrating $CD4^+$ and $CD8^+$ T cells, tumor-specific $pentamer^+$ $CD8^+$ T cells, and % $cytokine^+$ T cells in the dLN upon antigen restimulation than unconjugated control treated animals. Given this, in conjunction with data demonstrating that tAb-pManTLR7 efficacy is dependent on $CD8^+$ T cell responses, it was unsurprising that we observed tAb-pManTLR7 outperformed the unconjugated control treatment

in the ability to eradicate tumors or slow their growth in every tumor model and tAb-pManTLR7 formulation tested. Together, these data provide strong evidence that our antibody-adjuvant conjugate mediates its antitumor efficacy through this physical linkage which enforces the intratumoral retention and co-localization of our agonist within the TME.

4.5 Impact of tAb-pManTLR treatment on cells within the tumor microenvironment

Our data show that intratumorally administered tAb-pManTLR is engulfed by many subsets of antigen presenting cells in the tumor and draining lymph node, and that this uptake results in strong cellular activation of both myeloid and monocytic cells. This observation was made 24 hrs. following treatment with tAb-pManTLR, but it is still unknown if tumor retention prolongs the duration cellular activation in either location. Given that fluorescent tAb-pManTLR7 was still detectable within the tumor for three to four days post injection (Fig. 4C), it is possible that cells in the tumor and lymph node continue to show an activated phenotype for several days longer than mice treated with a bolus dose of antibody and unconjugated pManTLR7 which would drain quickly to the lymph node.

Given the magnitude of literature surrounding tumor-associated macrophage (TAM) immunosuppressive functions and the data demonstrating the ability of our tAb-pManTLR7 treatment to activate macrophages, further interrogation in the ability of treatment to reprogram TAM phenotypes may yield interesting insights. Indeed, work from others have reported the profound ability of TLR7/8 agonists to activate TAMs, re-polarizing them to a pro-inflammatory state with antitumor efficacy[101,102]. In concert, our data suggests that intratumoral T cells following treatment are fully functional and do not appear to be exhausted or suppressed, which may be interpreted as a reduction in the immunosuppressive networks present in the tumor environment, including TAMs. RNA-seq or other transcriptional analysis of the intratumoral

macrophage populations pre- and post-tAb-pManTLR treatment may show a shift the intratumoral macrophage polarization from pro-tumorigenic towards an inflammatory tumoricidal program. Alternatively, we can assess the ability of pManTLR7 to directly impact macrophage polarization *in vitro*. Here, M2 polarized macrophages can be differentiated from BMDM by culturing in the prescence of IL-4. Following the incubation with varying amounts of pManTLR7 the transcriptional profiles of the macrophages can be assessed to determine the relative changes in key M1 vs. M2 genes. Analysis of supernatant cytokines following this *in vitro* stimulation can also supplement transcriptional data: M1 macrophages secrete both IFNγ and IL-12p70 whereas M2 macrophages produce IL-6 upon activation.

More global changes to the immunosuppressive networks within the TME in response to tAb-pManTLR7 treatment can be also be explored through RNAseq analysis of all immune populations within the tumor, multiplex cytokine analysis from digested tumors, or quantitative mRNA analysis of gene transcripts related to specific immunosuppressive pathways.

With an eye to clinical translation of this approach, it would be important to understand the contribution of type I IFN, particularly IFNα, to the efficacy of this platform. In mice, our pManTLR7 adjuvant indeed has the potential to activate plasmacytoid dendritic cells (pDC) via TLR7. When activated this DC subset is well understood to be able to secrete huge amounts of type I IFN, which is an important cytokine in the context of cancer immunotherapy and the activation of innate and adaptive immunity for T cell priming against tumors. For this reason, adjuvants that activate type I IFN responses (i.e. STING agonists, TLR9 agonists) have been explored clinically, formulated as a cancer vaccines or administered intratumorally. We can test the contribution of IFN responses to the efficacy of our treatment by testing efficacy of tAb-pManTLR7 in IFNAR knockout mice, in which cells cannot respond to type I IFN cytokines. If

this pathway is indeed critical to our enhanced T cell priming and activity it would then be prudent to ensure that our TLR7/8 adjuvant has the capacity to activate pDC. In humans, pDCs express TLR7 and TLR9, so it is important that our adjuvant maintain the ability to stimulate both TLR7 and TLR8 to mimic the activation profile seen in mice. Activation of human plasmacytoid DCs, isolated from peripheral blood, can be mixed with our adjuvant for clinical translation *in vitro* to confirm activitiy. The monomeric TLR 7/8 agonist contained within the pManTLR7/8 polymer can additionally be tested for hTLR7 activity using a cell-based reporter assay.

4.6 Endocytic pathways of tAb-pManTLR7

Two features of tAb-pManTLR7 can theoretically promote its uptake by antigen presenting cells: the Fc region of the antibody or the mannose contained in the adjuvant polymer. It is possible that either the antibody Fc, the mannose residues within the polymer, or the combination of two, are required for endocytosis and ultimately, efficacy of our treatment.

In our previous work in which antigen-pManTLR7 conjugates were tested as a subunit vaccine for infectious disease applications, mannose was shown to be required for efficient pManTLR7 uptake and DC activation *in vitro* and *in vivo*[61]. The contribution of mannose to overall vaccine efficacy was demonstrated by comparing cellular and humoral responses following immunization with OVA-p(ManTLR7) vs. OVA-p(HPMA-TLR7). Although OVA-p(HPMA-TLR7) did generate small OVA-specific IgG, $CD4^+$ and $CD8^+$ responses, OVA-p(Man-TLR7) treated animals showed significantly heightened humoral responses as well as higher-quality OVA-specific T-cell responses compared with this non-targeted formulation. To assess if mannose is similarly important to the generation of cellular responses in my formulation, benchmarking tAb-pManTLR7 treatment against tAb-p(HPMA-TLR7) in overall therapeutic

efficacy would provide insight into the requirement of mannose in this formulation. Further analysis to observe if any deficit seen between the mannosylated or non-mannosylated formulations occurs at the stage of APC activation or at $CD8^+$ T cell priming, by assessing these responses at the appropriate timepoints following treatment.

Work done by Engleman and colleagues also suggests that antibody recognition via Fc receptors on dendritic cells could be important to the efficacy of tAb-pManTLR treatment. Here, they showed treatment of B16F10 tumors with tumor-binding allogenic IgG along with DC-activating stimuli (αCD40 + TNFα) mediated tumor regression in a FcγR-dependent manner[60]. In tAb-pManTLR, it is unknown to what extent pManTLR7 conjugation impacts antibody Fc accessibility and its recognition by Fc receptors on various cell types. Functionality of tAb Fc following polymer conjugation can be easily explored in an *in vitro* assay, assessing DC uptake of fluorescently labeled tumor cells coated with parental tAbs or tAb-p(HMPA-TLR7) conjugates of similar 20 kDa size. Here, tAb-p(HPMA-TLR7) would show equal to or improved tumor cell endocytosis to the parental antibodies if the Fc receptor is functionally accessible. To determine if these Fc receptor interactions are required for the antitumor efficacy of tAb-pManTLR7, tumor control following treatment in Fcγ-receptor deficient mice could then be assessed.

Given that antibody-mediated endocytosis of tumor antigen may be at play, further experiments to interrogate how the antibody might influence the overall magnitude of tumor antigen presentation or the APC subsets which prime T cell responses would be interesting. Here, mice bearing B16-OVA tumors can be treated with tAb-pManTLR7 and 24hrs later, different APC subsets from the tdLN can be sorted and incubated with OTI or OTII T cells, which recognize ovalbumin in the context of MHC class I or MHC class II, respectively. The

extent of T cell proliferation--as determined by CFSE or CellTrace Violet dilution—following incubation with these various subsets could be compared across populations, as well as to APC subsets from B16-OVA tumor-bearing mice treated with antibody and unconjugated pManTLR7.

4.7 tAb-pManTLR improves tumor-reactive T cell responses

The ability of our platform to enhance functional tumor-specific T cell responses increases the translational potential to treat patient populations and aggressive cancers that lack these critical endogenous responses. By exploring the ability of our therapy to induce these responses in two 'cold' murine tumor models (EMT6 and B16F10) both of which are poorly immunogenic and lack pre-existing intratumoral T cells, supports that tAb-pManTLR7 could provide therapeutic benefit in this subset of patients. In these two distinct tumor models, using two different tumor-targeting we showed tAb-pManTLR treatment was capable of enhancing tumor-specific T cell responses, and that efficacy was dependent on $CD8^+$ T cells, suggesting this is indeed a salient outcome of our therapy. Our data shows profound increases in the numbers of intratumoral T cells and the frequency of tumor-specific T cells in the tumor and draining lymph node upon treatment with tAb-pManTLR7. Restimulating these cells with tumor antigen, we demonstrated our treatment improved the frequency of tumor-reactive T cells that were functional and capable of secreting multiple effector cytokines upon activation.

In further studies it would be interesting to explore to what extent the treatment-enhanced intratumoral T cell responses were due to increased T cell priming, or improved T cell survival or proliferation within the tumor. To formally test this, tumor-bearing mice can be given FTY720 following treatment with tAb-pManTLR7, to prevent the egress of recently primed T cells back into circulation and to the tumor. If tAb-pManTLR7 treatment still results in tumor control, it is likely acting through T cells already present within the tumor, either by promoting their survival

or clonally expanding their numbers. If FTY720 treatment abolishes tAb-pManTLR7 efficacy, these data confirm that our treatment acts by priming new antitumor T cell responses. Given that in these tumor models we see few intratumoral T cells prior to treatment with tAb-pManTLR7 and demonstrate increased T cell responses in the tumor-draining lymph node following treatment, we do expect FTY720 treatment would hamper the efficacy of tAb-pManTLR7 to some extent, if not completely.

Our antibody depletion experiments demonstrated a clear requirement of $CD8^+$ T cells for tAb-pManTLR efficacy, however, it does not rule out the possibility that other immune cells may also contribute to tumor regression. It is possible that NK cells can respond directly to tAb-pManTLR7 opsonized tumor cells via CD16 engagement of tAb Fc and provide ADCC functionality. Alternatively, the increased APC activation in the TME following treatment might provide NK cells with the additional cytokine signals required for licensing cytotoxic responses through ligation of their multiple activating receptors[103]. In a similar manner, gamma delta T cells may also be involved in antitumor responses upon tAb-pManTLR7 treatment, through ligation of activating receptors similar to NK cells in response to stress-induced ligands on tumor cells[104] or priming conventional αβ T cells[105]. Here, NK cell requirement can be tested using an NK1.1 depletion antibody in the context of tAb-pManTLR7 treatment. Contribution of gamma delta T cells can also be explored by testing treatment efficacy in tumor-bearing TCRδ chain knockout mice.

4.8 Combining tAb-pManTLR7 treatment with checkpoint inhibitor antibodies

The literature describing the combinatorial benefit of novel immunotherapies (i.e. adjuvants or vaccines) with checkpoint inhibitor therapeutics is vast. In the past few years from

our lab alone, we had several publications describing the use of engineered inflammatory cytokines and chemokines to improve tumor responsiveness to checkpoint inhibition[106–108]. A great deal of evidence from both a therapeutic development and from a biological standpoint support the combinatorial use of checkpoint inhibitors with other immunotherapies which enhance complementary steps of the cancer-immunity cycle. Given the strong immunosuppressive microenvironments present within tumors, the delivery of immunostimulatory molecules into tumors can beneficially enhance the immunogenicity of the tumor and thereby improve responsiveness to checkpoint inhibition. Surprisingly, our studies exploring the combined efficacy of tAb-pManTLR7 with checkpoint inhibitor antibodies in two poorly immunogenic murine tumor models did not demonstrate a significant benefit or synergy over tAb-pManTLR7 treatment alone. These findings can be interpreted in two ways: 1) tAb-pManTLR7 treatment provides sufficient activation to overcome immunosuppressive mechanisms in tumors which drive T cell exhaustion and dysfunction, or 2) the murine tumor models used in these studies do not recapitulate the tumor biology or time course over which would capture synergy between the tAb-pManTLR7 and checkpoint inhibition.

The syngeneic tumor models of EMT6 breast cancer and B16F10 melanoma used in our efficacy studies show aggressively quick growth after inoculation, and as such, do not accurately replicate the biology of our treatment in slower growing human malignancies. To compare the effects of tAb-pManTLR7 to control treatments, our analyses of were performed 3-4 days following our last treatment, prior to the tumors of control treated mice reaching sac criteria. It is likely that at these early timepoints, our treatment-induced T cell responses had not yet experienced the chronic stimulation or immunosuppressive influence of the TME for long enough to become exhausted, for which the use of checkpoint blockade antibodies would be

beneficial. At the same time, data demonstrating the ability of tAb-pManTLR7 alone to completely eradicate EMT6 also highlights the potency of our therapeutic. For tumor regression there must have been sufficient disruption of tumor immunosuppression along with a large enough magnitude of adaptive immunity generated for the T cells to never become functionally exhausted. Exploring our therapeutic efficacy in a genetically-engineering murine model (GEMM) of cancer which more closely mimics the features of human disease may allow us to determine if T cells generated from tAb-pManTLR therapy eventually do become exhausted and if, at later timepoints, can benefit from combination with checkpoint blockade antibodies.

4.9 Design modifications to optimize systemic administration

With systemic administration, optimizing biodistribution of tAb-pManTLR to maximize tumor localization while minimizing off-target effects will be paramount to success. As the conjugate is only tumor-specific in its targeting antibody, it can still theoretically activate any antigen presenting cell upon endocytosis. The inclusion of mannose as a promiscuous APC-targeting agent may increase tAb-pManTLR7's opportunity to activate non-target cells outside of the tumor environment. Mannose recognition by mannose receptor (MR) or other C-type lectins (DC-SIGN, L-SIGN, mSIGNR1, Langerin, BDCA-2, DCIR, Dectin-2, MCL, LSECtin and MINCLE[109]) prior to tumor engagement may trigger internalization by scavenging APCs in the spleen or liver as it circulates in the bloodstream. Indeed, many of these receptors are found on APCs in the liver and in lymphoid tissues and are thought to be important in liver immunosurveillance by facilitating antigen uptake and presentation.

Due to this, for a next generation tAb-p(TLR7) conjugate for systemic administration, new formulations will likely be required. Off-target liver or spleen uptake of tAb-pManTLR can

be overcome by formulating tAb conjugates with an alternative polymeric adjuvant formulation, p(HPMA-TLR7). In this formulation, active TLR7 agonist monomers are co-polymerized with a biologically inert monomer (N-(2-hydroxypropyl) methacrylamide, HPMA) without the added D-mannose monomers. Given the size of the adjuvant polymer, receptor-mediated endocytosis will still be required for internalization and TLR7-mediated cell activation from the endosome. Here, instead of relying on polymeric mannose residues, the Fc region of the tumor-targeting antibody may be able to provide this functionality. tAb Fc may bind and crosslink Fcγ receptors on DCs or other APCs to promote the engulfment of tAb-p(TLR7):antigen immune complexes. In the creation of tAb-p(HPMA-TLR7), polymer conjugation to tAbs may need to be optimized or engineered to be more site-specific, to ensure this region remains in accessible, native form with the capacity engage these endocytic receptors.

Lastly, to determine if antitumor responses and efficacy can be achieved if tumor cells are appropriately targeted in models of systemic malignancy (i.e. AML), tAb-pManTLR7 can be pre-incubated with a small number of tumor cells prior to injection. In this setting, pre-coating of tumor cells with tAb-p(TLR7) ensures the adjuvant will be co-delivered with tumor antigen wherever it is endocytosed, functionally removing the technical barrier of tumor-targeting. Performing an *in vivo* experiment to determine antitumor efficacy with tumor cell pre-incubation should theoretically give a maximum for the therapeutic window which could be achieved if tumor-targeting of i.v. delivered tAb-adjuvant conjugates is optimized.

4.10 Safety and tolerability of tAb-pManTLR treatment

Biodistribution studies, serum cytokine analysis, and overall body weight maintenance, suggest that intratumoral Ab-pManTLR7 treatment has minimal risk of toxicity due to systemic, inflammatory activation. These data, as well as data from our previous study in lab have shown

that pManTLR7 is rapidly endocytosed due to mannose-targeting of APCs and fluorescent-labelled antigen-pManTLR conjugates largely accumulates within the draining lymph nodes[81]. To further confirm the lack of tAb-pManTLR7-mediated toxicities, further detailed assessment of serum chemistry for biomarkers of tissue damage or gross histopathology can be performed.

Although systemic dissemination from the tumor does not appear to be a concern for toxicity, antibody responses against the tAb-pManTLR construct could pose a challenge to repeat dosing in clinical translation. We were able to administer our treatment up to 4 times intratumorally, however, mice appeared hunched and slowed following the 3rd treatment. Although treatment-related reactions were not dose-limiting in these experiments, analysis of serum antibodies did show detectable levels of anti-drug antibodies (ADA) to either the targeting antibody itself, or to the pManTLR polymer and were likely the cause of these transient reactions. Given the use of a protein targeting moiety and potential for linked protein-chemical delivery, ADAs which developed against the antibody or pManTLR polymer of our conjugate could have resulted from T-cell dependent germinal center reactions. For systemic applications, these ADA responses might be long-lived and result in reduced tumor-targeting or efficacy over time as the conjugate is rapidly cleared from circulation upon injection. In future work, the development and use of chemical moieties for achieving intratumoral retention of pManTLR7 would be largely beneficial for clinical translation of this approach. As a fully chemical therapeutic, lacking any proteinaceous components, theoretical ADA responses which could develop against the chemical intratumorally anchored pManTLR7 therapy would do so in a T cell-independent manner, and thus would theoretically be transient and of low affinity.

REFERENCES

1. Decker, W. K. *et al.* Cancer immunotherapy: Historical perspective of a clinical revolution and emerging preclinical animal models. *Frontiers in Immunology* (2017) doi:10.3389/fimmu.2017.00829.

2. Burnet, F. M. Immunological Surveillance in Neoplasia. *Immunol. Rev.* (1971) doi:10.1111/j.1600-065X.1971.tb00461.x.

3. Lewis, T. Cellular and Humoral Aspects of the Hypersensitive States: A Symposium at the New York Academy of Medicine. *J. Am. Med. Assoc.* **529**, (1959).

4. Matzinger, P. The Danger Model: A Renewed Sense of Self. *Science (80-.).* **296**, 301–305 (2002).

5. Gallucci, S., Lolkema, M. & Matzinger, P. Natural adjuvants: Endogenous activators of dendritic cells. *Nat. Med.* **5**, 1249–1255 (1999).

6. Rathinam, V. A. K., Vanaja, S. K. & Fitzgerald, K. A. Regulation of inflammasome signaling. *Nature Immunology* (2012) doi:10.1038/ni.2237.

7. Iwasaki, A. & Medzhitov, R. Control of adaptive immunity by the innate immune system. *Nature Immunology* (2015) doi:10.1038/ni.3123.

8. Reis E Sousa, C. Dendritic cells in a mature age. *Nature Reviews Immunology* (2006) doi:10.1038/nri1845.

9. Vyas, J. M., Van Der Veen, A. G. & Ploegh, H. L. The known unknowns of antigen processing and presentation. *Nature Reviews Immunology* (2008) doi:10.1038/nri2368.

10. Cella, M., Engering, A., Pinet, V., Pieters, J. & Lanzavecchia, A. Inflammatory stimuli induce accumulation of MHC class II complexes on dendritic cells. *Nature* (1997) doi:10.1038/42030.

11. Guermonprez, P., Valladeau, J., Zitvogel, L., Théry, C. & Amigorena, S. Antigen presentation and T cell stimulation by dendritic cells. *Annual Review of Immunology* (2002) doi:10.1146/annurev.immunol.20.100301.064828.

12. Shuford, W. W. *et al.* 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. *J. Exp. Med.* (1997) doi:10.1084/jem.186.1.47.

13. Lane, P. Role of OX40 signals in coordinating CD4 T cell selection, migration, and cytokine differentiation in T helper (Th)1 and Th2 cells. *Journal of Experimental Medicine* (2000) doi:10.1084/jem.191.2.201.

14. Lee, J.-W. *et al.* Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. *Nat. Immunol.* **8**, 181–190 (2007).

15. Bonifaz, L. *et al.* Efficient Targeting of Protein Antigen to the Dendritic Cell Receptor

DEC-205 in the Steady State Leads to Antigen Presentation on Major Histocompatibility Complex Class I Products and Peripheral CD8 + T Cell Tolerance. *J. Exp. Med.* **196**, 1627–1638 (2002).

16. Mueller, D. L. Mechanisms maintaining peripheral tolerance. *Nature Immunology* (2010) doi:10.1038/ni.1817.

17. Kratky, W., Reis E Sousa, C., Oxenius, A. & Spörri, R. Direct activation of antigen-presenting cells is required for CD8 + T-cell priming and tumor vaccination. *Proc. Natl. Acad. Sci. U. S. A.* (2011) doi:10.1073/pnas.1108945108.

18. Ilyas, S. & Yang, J. C. Landscape of Tumor Antigens in T Cell Immunotherapy. *J. Immunol.* (2015) doi:10.4049/jimmunol.1501657.

19. Hollingsworth, R. E. & Jansen, K. Turning the corner on therapeutic cancer vaccines. *npj Vaccines* (2019) doi:10.1038/s41541-019-0103-y.

20. Chheda, Z. S. *et al.* Novel and shared neoantigen derived from histone 3 variant H3.3K27M mutation for glioma T cell therapy. *J. Exp. Med.* (2018) doi:10.1084/jem.20171046.

21. Wang, Q. J. *et al.* Identification of T-cell receptors targeting KRAS-mutated human tumors. *Cancer Immunol. Res.* (2016) doi:10.1158/2326-6066.CIR-15-0188.

22. Snyder, A. *et al.* Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N. Engl. J. Med.* (2014) doi:10.1056/NEJMoa1406498.

23. Rizvi, N. A. *et al.* Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science (80-.).* (2015) doi:10.1126/science.aaa1348.

24. Chen, D. S. & Mellman, I. Oncology Meets Immunology: The Cancer-Immunity Cycle. *Immunity* **39**, 1–10 (2013).

25. Franciszkiewicz, K., Boissonnas, A., Boutet, M., Combadière, C. & Mami-Chouaib, F. Role of chemokines and chemokine receptors in shaping the effector phase of the antitumor immune response. *Cancer Research* (2012) doi:10.1158/0008-5472.CAN-12-2027.

26. Abiko, K. *et al.* IFN-γ from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer. *Br. J. Cancer* (2015) doi:10.1038/bjc.2015.101.

27. Hamanishi, J. *et al.* Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. *Proc. Natl. Acad. Sci. U. S. A.* (2007) doi:10.1073/pnas.0611533104.

28. Kaiser, B. K. *et al.* Disulphide-isomerase-enabled shedding of tumour-associated NKG2D ligands. *Nature* (2007) doi:10.1038/nature05768.

29. Qin, Z., Noffz, G., Mohaupt, M. & Blankenstein, T. Interleukin-10 prevents dendritic cell accumulation and vaccination with granulocyte-macrophage colony-stimulating factor gene-modified tumor cells. *J. Immunol.* (1997).

30. Steinbrink, K. *et al.* Interleukin-10-treated human dendritic cells induce a melanoma-

antigen- specific anergy in CD8+ T cells resulting in a failure to lyse tumor cells. *Blood* (1999) doi:10.1182/blood.v93.5.1634.

31. Yang, L. TGFβ and cancer metastasis: An inflammation link. *Cancer and Metastasis Reviews* (2010) doi:10.1007/s10555-010-9226-3.

32. Marvel, D. & Gabrilovich, D. I. Myeloid-derived suppressor cells in the tumor microenvironment: Expect the unexpected. *Journal of Clinical Investigation* (2015) doi:10.1172/JCI80005.

33. Kuang, D. M. *et al.* Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J. Exp. Med.* (2009) doi:10.1084/jem.20082173.

34. Curiel, T. J. *et al.* Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* (2004) doi:10.1038/nm1093.

35. DeNardo, D. G. *et al.* CD4+ T Cells Regulate Pulmonary Metastasis of Mammary Carcinomas by Enhancing Protumor Properties of Macrophages. *Cancer Cell* (2009) doi:10.1016/j.ccr.2009.06.018.

36. Noman, M. Z. *et al.* Hypoxia: A key player in antitumor immune response. A review in the theme: Cellular responses to hypoxia. *Am. J. Physiol. - Cell Physiol.* (2015) doi:10.1152/ajpcell.00207.2015.

37. Noman, M. Z. *et al.* Blocking hypoxia-induced autophagy in tumors restores cytotoxic T-cell activity and promotes regression. *Cancer Res.* (2011) doi:10.1158/0008-5472.CAN-11-1094.

38. Hicklin, D. J., Marincola, F. M. & Ferrone, S. HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. *Molecular Medicine Today* (1999) doi:10.1016/S1357-4310(99)01451-3.

39. Chowell, D. *et al.* Patient HLA class I genotype influences cancer response to checkpoint blockade immunotherapy. *Science (80-.).* (2018) doi:10.1126/science.aao4572.

40. Mizukami, Y. *et al.* Downregulation of HLA Class I molecules in the tumour is associated with a poor prognosis in patients with oesophageal squamous cell carcinoma. *Br. J. Cancer* (2008) doi:10.1038/sj.bjc.6604715.

41. Lee, J.-C., Lee, K.-M., Kim, D.-W. & Heo, D. S. Elevated TGF-β1 Secretion and Down-Modulation of NKG2D Underlies Impaired NK Cytotoxicity in Cancer Patients. *J. Immunol.* (2004) doi:10.4049/jimmunol.172.12.7335.

42. Hudis, C. A. Trastuzumab - Mechanism of action and use in clinical practice. *New England Journal of Medicine* (2007) doi:10.1056/NEJMra043186.

43. Chao, M. P., Weissman, I. L. & Majeti, R. The CD47-SIRPα pathway in cancer immune evasion and potential therapeutic implications. *Current Opinion in Immunology* (2012) doi:10.1016/j.coi.2012.01.010.

44. Oldenborg, P. A. *et al.* Role of CD47 as a marker of self on red blood cells. *Science (80-.*

). (2000) doi:10.1126/science.288.5473.2051.

45. Reinhold, M. I. *et al.* In vivo expression of alternatively spliced forms of integrin-associated protein (CD47). *J. Cell Sci.* (1995).

46. Zhao, H.-J. *et al.* Prognostic significance of CD47 in human malignancies: a systematic review and meta-analysis. *Transl. Cancer Res.* **7**, 609–621 (2018).

47. Majeti, R. *et al.* CD47 Is an Adverse Prognostic Factor and Therapeutic Antibody Target on Human Acute Myeloid Leukemia Stem Cells. *Cell* (2009) doi:10.1016/j.cell.2009.05.045.

48. Li, Y. *et al.* Overexpression of CD47 predicts poor prognosis and promotes cancer cell invasion in high-grade serous ovarian carcinoma. *Am. J. Transl. Res.* (2017).

49. Upadhaya, S., Hubbard-Lucey, V. M. & Yu, J. X. Immuno-oncology drug development forges on despite COVID-19. *Nat. Rev. Drug Discov.* (2020) doi:10.1038/d41573-020-00166-1.

50. Ledford, H. Melanoma drug wins US approval. *Nature* (2011) doi:10.1038/471561a.

51. Gulley, J. L. *et al.* Immunologic and prognostic factors associated with overall survival employing a poxviral-based PSA vaccine in metastatic castrate-resistant prostate cancer. *Cancer Immunol. Immunother.* (2010) doi:10.1007/s00262-009-0782-8.

52. The problem with neoantigen prediction. *Nat. Biotechnol.* **35**, 97 (2017).

53. Sahin, U. *et al.* Personalized RNA mutanome vaccines mobilize poly-specific therapeutic immunity against cancer. *Nature* **547**, 222–226 (2017).

54. PA, O. *et al.* An immunogenic personal neoantigen vaccine for patients with melanoma. (2017).

55. Zhang, X., Sharma, P. K., Peter Goedegebuure, S. & Gillanders, W. E. Personalized cancer vaccines: Targeting the cancer mutanome. *Vaccine* **35**, 1094–1100 (2017).

56. Wong, H. H., Lemoine, N. R. & Wang, Y. Oncolytic viruses for cancer therapy: Overcoming the obstacles. *Viruses* (2010) doi:10.3390/v2010078.

57. Mesa, C. & Fernández, L. E. Challenges facing adjuvants for cancer immunotherapy. *Immunology and Cell Biology* (2004) doi:10.1111/j.0818-9641.2004.01279.x.

58. Bachmann, M. F. & Jennings, G. T. Vaccine delivery: a matter of size, geometry, kinetics and molecular patterns. *Nat. Rev. Immunol.* **10**, 787–796 (2010).

59. Zinkernagel, R. M. On differences between immunity and immunological memory. *Current Opinion in Immunology* (2002) doi:10.1016/S0952-7915(02)00367-9.

60. Carmi, Y. *et al.* Allogeneic IgG combined with dendritic cell stimuli induce antitumour T-cell immunity. *Nature* (2015) doi:10.1038/nature14424.

61. Wilson, D. S. *et al.* Antigens reversibly conjugated to a polymeric glyco-adjuvant induce protective humoral and cellular immunity. *Nat. Mater.* (2019) doi:10.1038/s41563-018-0256-5.

62. Oh, J. Z., Kurche, J. S., Burchill, M. A. & Kedl, R. M. TLR7 enables cross-presentation by multiple dendritic cell subsets through a type I IFN-dependent pathway. *Blood* (2011) doi:10.1182/blood-2011-04-348839.

63. Vasilakos, J. P. & Tomai, M. A. The use of Toll-like receptor 7/8 agonists as vaccine adjuvants. *Expert Review of Vaccines* (2013) doi:10.1586/14760584.2013.811208.

64. Edwards, A. D. *et al.* Toll-like receptor expression in murine DC subsets: Lack of TLR7 expresion of CD8α+ DC correlates with unresponsiveness to imidazoquinolines. *Eur. J. Immunol.* (2003) doi:10.1002/eji.200323797.

65. Soria, I. *et al.* Effect of food on the pharmacokinetics and bioavailability of oral imiquimod relative to a subcutaneous dose. *Int. J. Clin. Pharmacol. Ther.* (2000) doi:10.5414/CPP38476.

66. Savage, P. *et al.* A phase I clinical trial of imiquimod, an oral interferon inducer, administered daily. *Br. J. Cancer* (1996) doi:10.1038/bjc.1996.569.

67. Gaspari, A. A., Tyring, S. K. & Rosen, T. Beyond a decade of 5% imiquimod topical therapy. *Journal of Drugs in Dermatology* (2008).

68. Sancho, D. & Reis e Sousa, C. Signaling by myeloid C-Type lectin receptors in immunity and homeostasis. *Annual Review of Immunology* (2012) doi:10.1146/annurev-immunol-031210-101352.

69. Kerrigan, A. M. & Brown, G. D. C-type lectins and phagocytosis. *Immunobiology* **214**, 562–575 (2009).

70. Martinez-Pomares, L. The mannose receptor. *J. Leukoc. Biol.* (2012) doi:10.1189/jlb.0512231.

71. Hoving, J. C., Wilson, G. J. & Brown, G. D. Signalling C-type lectin receptors, microbial recognition and immunity. *Cellular Microbiology* (2014) doi:10.1111/cmi.12249.

72. Burgdorf, S., Kautz, A., Bohnert, V., Knolle, P. A. & Kurts, C. Distinct Pathways of Antigen Uptake and Intracellular Routing in CD4 and CD8 T Cell Activation. *Science (80-.).* **316**, 612–616 (2007).

73. Joffre, O. P., Segura, E., Savina, A. & Amigorena, S. Cross-presentation by dendritic cells. *Nat. Rev. Immunol.* **12**, 557–569 (2012).

74. Keler, T., Ramakrishna, V. & Fanger, M. W. Mannose receptor-targeted vaccines. *Expert Opinion on Biological Therapy* (2004) doi:10.1517/14712598.4.12.1953.

75. Ramakrishna, V. *et al.* Mannose Receptor Targeting of Tumor Antigen pmel17 to Human Dendritic Cells Directs Anti-Melanoma T Cell Responses via Multiple HLA Molecules. *J. Immunol.* (2004) doi:10.4049/jimmunol.172.5.2845.

76. Lam, J. S., Mansour, M. K., Specht, C. A. & Levitz, S. M. A Model Vaccine Exploiting Fungal Mannosylation to Increase Antigen Immunogenicity. *J. Immunol.* (2005) doi:10.4049/jimmunol.175.11.7496.

77. Appay, V., Douek, D. C. & Price, D. A. CD8+T cell efficacy in vaccination and disease.

Nat. Med. **14**, 623–628 (2008).

78. Koup, R. A. & Douek, D. C. Vaccine design for CD8 T lymphocyte responses. *Cold Spring Harb. Perspect. Med.* **1**, (2011).

79. Banchereau, J. & Palucka, K. Immunotherapy: Cancer vaccines on the move. *Nat. Rev. Clin. Oncol.* (2017) doi:10.1038/nrclinonc.2017.149.

80. Dunn, G. P., Old, L. J. & Schreiber, R. D. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* **21**, 137–148 (2004).

81. Wilson, D. S. *et al.* Antigens reversibly conjugated to a polymeric glyco-adjuvant induce protective humoral and cellular immunity. *Nat. Mater.* **18**, 175–185 (2019).

82. Tong, B. & Wang, M. CD47 is a novel potent immunotherapy target in human malignancies: Current studies and future promises. *Future Oncology* (2018) doi:10.2217/fon-2018-0035.

83. Contreras-Trujillo, H. *et al.* Anti-CD47 antibody-mediated phagocytosis of cancer by macrophages primes an effective antitumor T-cell response. *Proc. Natl. Acad. Sci.* (2013) doi:10.1073/pnas.1305569110.

84. Liu, X. *et al.* CD47 blockade triggers T cell-mediated destruction of immunogenic tumors. *Nat. Med.* (2015) doi:10.1038/nm.3931.

85. Thomson, T. M., Mattes, M. J., Roux, L., Old, L. J. & Lloyd, K. O. Pigmentation-associated glycoprotein of human melanomas and melanocytes: Definition with a mouse monoclonal antibody. *J. Invest. Dermatol.* **85**, 169–174 (1985).

86. Takechi, Y., Hara, I., Naftzger, C., Xu, Y. & Houghton, A. N. A melanosomal membrane protein is a cell surface target for melanoma therapy. *Clin. Cancer Res.* **2**, 1837–1842 (1996).

87. Bevaart, L. *et al.* The high-affinity IgG receptor, FcγRI, plays a central role in antibody therapy of experimental melanoma. *Cancer Res.* **66**, 1261–1264 (2006).

88. Roberts, E. W. *et al.* Critical Role for CD103 + /CD141 + Dendritic Cells Bearing CCR7 for Tumor Antigen Trafficking and Priming of T Cell Immunity in Melanoma. *Cancer Cell* **30**, 324–336 (2016).

89. Sagiv-Barfi, I. *et al.* Eradication of spontaneous malignancy by local immunotherapy. *Sci. Transl. Med.* **10**, eaan4488 (2018).

90. Saenger, Y. M. *et al.* Improved Tumor Immunity Using Anti-Tyrosinase Related Protein-1 Monoclonal Antibody Combined with DNA Vaccines in Murine Melanoma. *Cancer Res* **68**, 9884–9891 (2008).

91. Moynihan, K. D. *et al.* Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. *Nat. Med.* **22**, 1402–1410 (2016).

92. Sato, E. *et al.* Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer.

Proc. Natl. Acad. Sci. U. S. A. **102**, 18538–43 (2005).

93. Yee, C. *et al.* Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 16168–73 (2002).

94. Scott, A. C. *et al.* TOX is a critical regulator of tumour-specific T cell differentiation. *Nature* (2019) doi:10.1038/s41586-019-1324-y.

95. Khan, O. *et al.* TOX transcriptionally and epigenetically programs CD8+ T cell exhaustion. *Nature* (2019) doi:10.1038/s41586-019-1325-x.

96. van Duikeren, S. *et al.* Vaccine-induced effector-memory CD8+ T cell responses predict therapeutic efficacy against tumors. *J. Immunol.* **189**, 3397–403 (2012).

97. Ossendorp, F. *et al.* against Tumors Cell Responses Predict Therapeutic Efficacy T + Vaccine-Induced Effector-Memory CD8. *J Immunol Ref.* **189**, 3397–3403 (2018).

98. Sallman, D. A. *et al.* The First-in-Class Anti-CD47 Antibody Magrolimab (5F9) in Combination with Azacitidine Is Effective in MDS and AML Patients: Ongoing Phase 1b Results. *Blood* (2019) doi:10.1182/blood-2019-126271.

99. Eladl, E. *et al.* Role of CD47 in Hematological Malignancies. *J. Hematol. Oncol.* **13**, 1–14 (2020).

100. Perez, H. L. *et al.* Antibody-drug conjugates: Current status and future directions. *Drug Discovery Today* (2014) doi:10.1016/j.drudis.2013.11.004.

101. Rodell, C. B. *et al.* TLR7/8-agonist-loaded nanoparticles promote the polarization of tumour-associated macrophages to enhance cancer immunotherapy. *Nat. Biomed. Eng.* **2**, 578–588 (2018).

102. Chi, H. *et al.* Anti-tumor activity of toll-like receptor 7 agonists. *Frontiers in Pharmacology* (2017) doi:10.3389/fphar.2017.00304.

103. Shimasaki, N., Jain, A. & Campana, D. NK cells for cancer immunotherapy. *Nature Reviews Drug Discovery* (2020) doi:10.1038/s41573-019-0052-1.

104. Raverdeau, M., Cunningham, S. P., Harmon, C. & Lynch, L. γδ T cells in cancer: a small population of lymphocytes with big implications. *Clinical and Translational Immunology* (2019) doi:10.1002/cti2.1080.

105. Collins, R. A. *et al.* Gammadelta T cells present antigen to CD4+ alphabeta T cells. *J. Leukoc. Biol.* (1998).

106. Ishihara, J. *et al.* Matrix-binding checkpoint immunotherapies enhance antitumor efficacy and reduce adverse events. *Sci. Transl. Med.* **9**, eaan0401 (2017).

107. Mansurov, A. *et al.* Collagen-binding IL-12 enhances tumour inflammation and drives the complete remission of established immunologically cold mouse tumours. *Nat. Biomed. Eng.* (2020) doi:10.1038/s41551-020-0549-2.

108. Ishihara, J. *et al.* Targeted antibody and cytokine cancer immunotherapies through collagen affinity. *Sci. Transl. Med.* (2019) doi:10.1126/scitranslmed.aau3259.

109. Lee, R. T. *et al.* Survey of immune-related, mannose/fucose-binding C-type lectin receptors reveals widely divergent sugar-binding specificities. *Glycobiology* (2011) doi:10.1093/glycob/cwq193.