AUGMENTING TUMOR-SPECIFIC IMMUNE RESPONSES WITH VACCINES THAT EXPLOIT LYMPHOPENIA OR TUMOR AUTOPHAGY

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ABBREVIATIONS USED

AFP: α-Fetoprotein

ADP: antigen-driven proliferation

APC: antigen presenting cell

CEA: carcinoembryonic antigen

CDK4: cyclin dependent kinase 4

CHX: cyclohexamide

CM: complete medium

CML: chronic myelogenous leukemia

CT: cancer/testis

CTL: cytotoxic T lymphocyte

CTLA4: cytotoxic T-lymphocyte antigen 4

DC: dendritic cell

DRiPs: defective ribosomal products

EBV: Epstein-Barr virus

eGFP: enhanced green fluorescent protein

HCC: hepatocellular cancer

HDP: homeostasis-driven proliferation

HER2/neu: human epidermal growth factor receptor 2

HIV: human immunodeficiency virus

HPV: human papillomavirus

HRP: horseradish peroxidase

HSP: heat-shock protein

hTERT: human telomerase reverse transcriptase

ICS: intracellular cytokine staining

IDO: indoleamine 2,3-dioxygenase

IFN: interferon

iNOS: inducible nitric oxide synthase

i.p.: intraperitoneal

i.v.: intravenous

LAG3: lymphocyte-activation gene 3

MAGE: melanoma antigen

MCA: 3-methylcholanthrene

MDSC: myeloid derived suppressor cell

MHC: major histocompatability complex

MHD: MAGE homology domain

mOVA: stable OVA

OFA: oncofetal antigens

OVA: chicken ovalbumin

PBMC: peripheral blood mononuclear cell

PDL1: programmed death ligand-1

p:MHC : peptide-MHC complex

pRB: retinoblastoma

RAG: recombinase activating gene

RAM1: recognized antigen from MCA-induced tumor 1

RIPA: radioimmunoprecipitation assay

RLM: reconstituted lymphopenic mice

rOVA: short-lived OVA

SIV: simian immunodeficiency virus

SLiP: short-lived protein

SV40: Simian Vacuolating Virus 40

TAA: tumor-associated antigens

TAP: transporter associated with antigen processing

TCR: T cell receptor

TGF: transforming growth factor

TLR: toll-like receptor

Treg: regulatory T cell

TRP: tyrosinase related protein

TSMA: tumor-specific mutated antigen

TVDLN: Tumor vaccine-draining lymph nodes

VEGF: vascular endothelial growth factor

VLP: virus-like particles

WT: wild type

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ABSTRACT

Tumor immunotherapy continues to improve by incorporating the advances made in the field of immunology, which provides the basis for many clinical trials. However, the relative lack of success in the clinic warrants further investigation of strategies, especially cancer vaccines, as vaccines often are the cornerstone of many immunotherapies. This body of work represents two unique vaccine approaches with the common goal of augmenting tumor-specific immune responses. The first approach focuses on this goal by exploiting the lymphopenic environment. A vaccine capable of utilizing the entire lymphopenic compartment was achieved by reconstituting irradiated hosts with peptide-pulsed splenocytes. This immunotherapeutic strategy with the addition of a TLR4 ligand primed a potent T cell response as measured on day 8 and could maintain a population of functional antigen-specific T cells for greater than 30 days. A second vaccine strategy made use of the process of autophagy to provide a unique source of antigen capable of generating a cross-reactive immune response. This novel autophagosome vaccine, unlike the intact cells from which it was derived, produced an immune response to not only the vaccinating tumor but also to other independently derived tumors. Short-lived proteins that accumulated in the autophagosomes were shown to be responsible for stimulating this cross-reactive immune response. Further, the ubiquitin/LC3 binding protein p62 is necessary for incorporation of these short-lived proteins and may play a role in this unique immune response. Collectively, these two different studies not only contribute to a better understanding of tumor antigens and the requirements of a successful vaccine but also provide clinically applicable immunotherapeutic strategies.

CHAPTER ONE: INTRODUCTION

Cancer Immunosurveillance

Throughout their lifespan, somatic cells accumulate genetic mutations and epigenetic modifications, which can cause disregulation of cell growth and survival, most often inducing apoptosis/cell death but in rare instances leading to cellular transformation. Genetic instability coupled with selective pressure applied to the transformed cell either intrinsically (34) or by the microenvironment and/or host immune system can drive further mutations causing a benign tumor to become cancerous. Tumorigenesis, while affecting many individuals, occurs at a surprising low frequency if one considers that there are trillions of cells each with hundreds of genetic targets capable promoting transformation, suggesting an active process in restricting this event.

The role of immunity in limiting the frequency of malignant cells was initially a hypothesis of Paul Ehrlich who in 1909 predicted that the immune system could protect the host from a neoplastic disease (43). There was unknowingly support for this hypothesis based on observations made by William Coley in 1891 who demonstrated that some cancer patients with advanced disease underwent regression or prolonged survival when injected with heat-inactivated bacteria (30, 31). These "Coley Toxins" are now understood to indeed augment tumor immunity by the activation and maturation of tumor loaded dendritic cells via the pathogen associated molecular patterns originating from the bacterial endotoxins (11, 67).

However it wasn't until nearly 50 years after Ehrlich's hypothesis was posited that strong evidence of tumor-mediated immunity was demonstrated (50, 137).

These observations were later shown to be dependent upon lymphoid cells (123) and helped lay the groundwork for Burnet and Thomas' cancer immunosurveillance hypothesis which predicted that lymphocytes were responsible for eliminating continuously arising, nascent transformed cells (21, 174). The hypothesis was initially discredited by studies that demonstrated that nude athymic mice had the same frequency of chemically-induced (3-methylcholanthrene (MCA)) sarcoma formation and non-viral spontaneous tumorigenesis as immunocompetent littermates (146, 168). The controversy was later determined to be unfounded as nude CBA/H mice were shown to have an impaired yet functional immune system (74, 106) and an increased sensitivity to the MCA carcinogen (65). The cancer immunosurveillance hypothesis gained strong support with experimental models that could incorporate monoclonal antibodies or transgenic mice to block specific immune components and thus demonstrate their necessity in restraining tumorigenesis. Blocking or genetic deletion of IFN-γ or perforin, two molecules uniquely associated with activated immune cells, made mice more susceptible to transplanted fibrosarcomas and to chemically-induced or spontaneous tumors (79, 161, 165). Furthermore, the use of mice deficient in the recombinase activating gene (RAG)-2 which lack αβ T cells, NKT cells, and B cells (155) provided an accurate model to conclusively test the cancer immunosurveillance hypothesis. Similar to the IFN-γ and perforin studies, $RAG^{-/-}$ mice developed MCA-induced sarcomas quicker and with a higher frequency than wild type controls (150, 159), providing

indisputable evidence of a lymphocyte-dependent immunosurveillance. Indirect evidence supports the role of immunosurveillance in humans. Examples include cancer patients generating an adaptive and innate immune response to their tumors (53), an increased incidence of nonviral cancers in immunosuppressed transplant recipients compared to immunocompetent control populations (120), and the presence of tumor infiltrating lymphocytes (TIL) as a positive indicator of patient survival (29, 54, 120).

The cancer immunosurveillance hypothesis, now well defined and accepted, has evolved into a broader model termed cancer immunoediting (160) which suggests that a relationship exists between the immune system and a tumor that has escaped the initial immunosurveillance phase (elimination). Depending on many factors, this equilibrium can be tipped in either direction, resulting in the elimination or outgrowth of the tumor. The implication of this model is that the immunogenicity of tumors will be altered by the immune system as it eliminates the most immunogenic clones (42). Although the role of tumor immunogenicity as it relates to cancer immunosurveillance and immunoediting, has recently been challenged (182, 183), reports continue to support immunosurveillance and cancer immunoediting (86) and thus the possibility of redirecting or augmenting an immune response to impact tumor growth.

Defining the Rejection Antigens of MCA-induced Sarcomas

Fundamental to both cancer immunosurveillance and immunoediting are the tumor antigens that drive the immune response. An understanding of the work that established the

existence of tumor antigens is therefore necessary to fully appreciate the immunoediting paradigm. Although Ehrlich was the first to predict the involvement of the immune system with cancer, the initial experiments could not adequately prove his prediction. In fact, early transplantation experiments using outbred animals discredited the idea of tumor antigens, as the "marketplace" rats and mice immunized with a tumor could reject normal tissue from the tumor donor and reciprocally, immunization with normal tissue from a donor could protect the host against a tumor challenge (101, 187, 188). This confusion necessitated the development of inbred mouse strains, which in addition to providing the means to understand the major histocompatability complex (MHC) (59, 162), allowed researchers to properly demonstrate tumor-specific rejection of transplanted tumors. However, for more than 20 years after the establishment of the inbred C3H strain of mice, (167), studies were still performed with tumors of genetically unknown origin, thus precluding any meaningful conclusions due to the potential alloresponse. The pivotal studies of Gross eliminated this issue by generating MCA-induced sarcomas in the same C3H mice used for the study (61). These experiments demonstrated for the first time a controlled tumor immunization capable of protecting mice from further tumor challenges.

Subsequent studies extended these findings by vaccinating mice via ligation of a progressively growing tumor, resulting in a greater frequency of immunized mice compared to the previous study which relied on rejection of a small viable tumor dose to achieve immunization (50). Furthermore, the timing involved to achieve immunity in these experiments suggested the involvement of the adaptive immune response, as 6 or more days

from the time of ligation of the primary tumor to the implantation of the challenge dose was necessary to protect the mice. The definitive experiments of Prehn and Main demonstrated that the immunity achieved with tumor immunization was due to antigens associated only with the tumor (figure 1) (137). Vaccination with tumor and not non-malignant syngeneic donor tissue led to the rejection of a subsequent viable tumor inoculation but not the rejection of a donor skin graft. The antigens responsible for this protection were therefore described as tumor rejection antigens. These experiments helped dispel any skepticism by excluding the

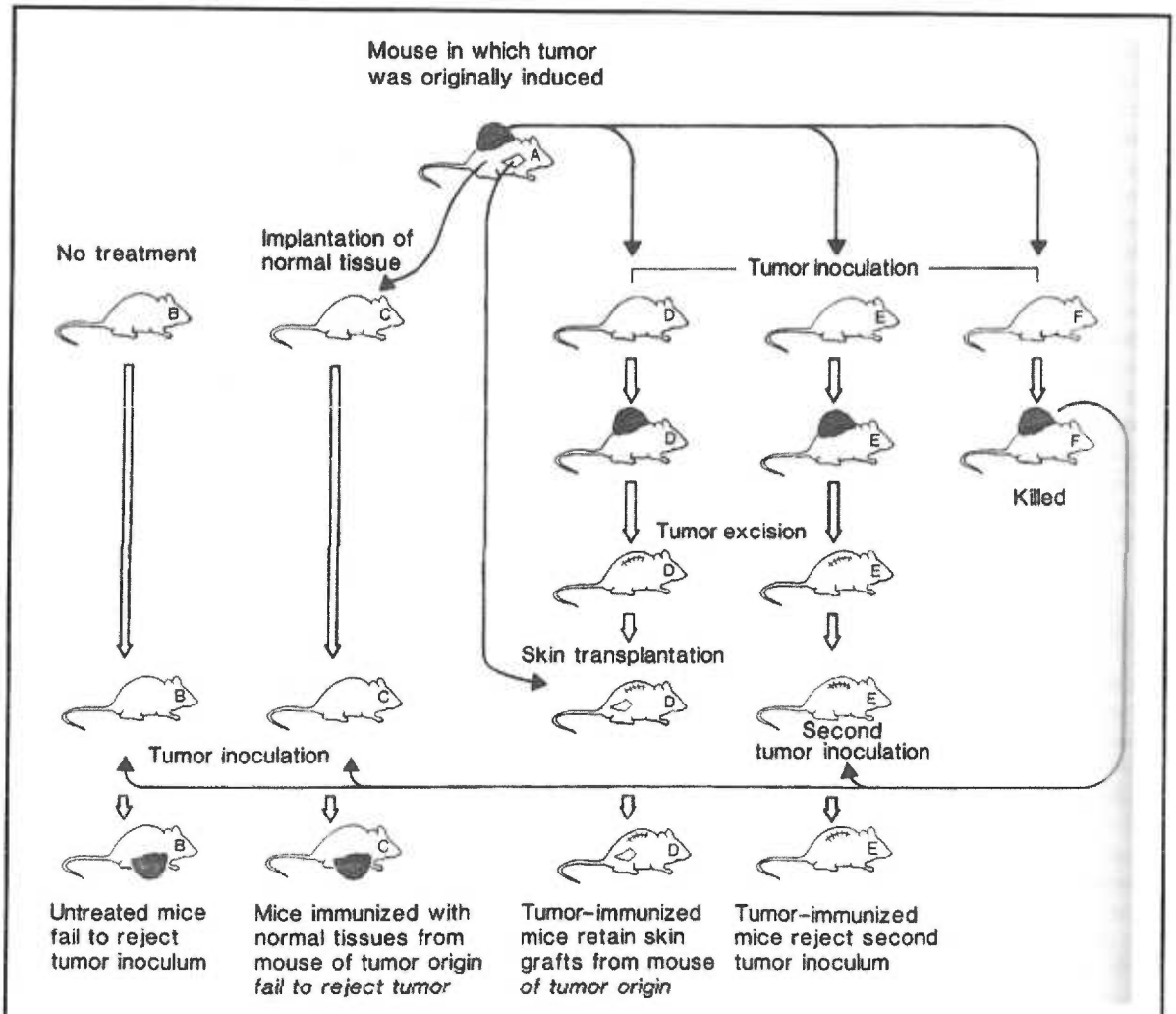


Figure 1: Overview of Prehn and Main's notable experiment.

possibility that the protection gained by immunization was due to genetic heterogeneity of the inbred mice. Further, this study demonstrated that the rejection antigens associated with each independent sarcoma were generally unable to provide protection against a subsequent challenge with a different sarcoma. This observation was validated by other studies that demonstrated a high degree of antigenic individuality using a large panel of MCA-induced sarcomas (13, 181).

Using MCA-induced sarcomas, other researchers were also able to demonstrate immunity in isologous hosts by either vaccination with irradiated cells (140) or in primary autochthonous hosts by amputation of the limb hosting the progressively growing tumor (85). Importantly, these studies as well as those of Old *et al.* (125) demonstrated that adoptive transfer of splenocytes or lymph node (LN) cells but not serum from immunized mice were able to eliminate or blunt the growth of a subsequent tumor challenge. This tumor protection was lost if the LN cells were freeze/thawed before transfer. Additionally, control mice made lymphopenic with irradiation prior to a sarcoma inoculation had an increase rate of tumor growth. Collectively these studies established that the protection elicited by the rejection antigens is dependent upon cellular immunity. The role of immunity and specifically cytotoxic T lymphocytes (CTL) was further demonstrated as both tumor-infiltrating lymphocytes (TIL) cultured from a MCA-induced sarcoma (12) and lymphocytes isolated from tumor vaccine-draining lymph nodes (TVDLN) (193) displayed *ex-vivo* CTL activity and could mediate regression of experimentally-induced lung metastasis upon adoptive transfer. Similar to the previous studies, these tumor-primed lymphocytes while cross-

reactive with heterologous clones of a parental sarcoma (110), were specific for only the sarcoma from which they were primed.

A greater understanding of the antigenic properties of the MCA-induced sarcomas became possible with the exploitation of tumor-specific CTL clones. Using a cloned CTL line to screen a cDNA library isolated from the immunizing sarcoma, a mutated mitogen-activated protein kinase (MAPK) was revealed as the rejection antigen (75). Additionally, CTL clones also were used to generate tumor antigen-loss variants in heterozygous MCA sarcomas. These antigen-loss variants could elude CTL detection by genetic loss of hemizygously expressed tumor-specific epitopes which could be detected by high density genetic analysis (4, 39). This technique revealed that a chromosomal region rich in tumor-modifier genes can encode the MCA-induced tumor rejection antigens (4). This observation coupled with the mutated MAPK data suggests that the rejection antigens of at least some MCA-induced sarcomas play a role in the transformation process or maintenance of the malignant phenotype.

Development and implantation of immunological and molecular techniques have allowed our understanding of MCA-induced tumor antigens to reach the genetic level with more breakthroughs likely to follow. However, it was the early experiments and resulting insights gained that were particularly important as they provided a proof of concept that tumors are antigenic and hold the potential to generate immunity. In this regard, the tumor rejection antigens associated with MCA-induced sarcomas have influenced tumor immunology by helping to define tumor antigens associated with all types cancers.

Tumor Antigens

Tumor antigens fall into three broad classes defined by the genes unique to, or differentially expressed by, the transformed cell: viral genes, mutated genes, and tumor-associated genes (differentially expressed "normal" genes). Polyomaviruses such as Simian Vacuolating Virus 40 (SV40) are well-studied DNA viruses, capable of transforming infected cells due in large part to the expression of the large T antigen which disregulates the retinoblastoma (pRB) and p53 tumor suppressor proteins (6). Mice immunized with SV40 or polyoma viruses are protected from a challenge with an SV40 or polyoma-induced tumor, suggesting that viral antigens provide the targets associated with tumor protection (19). These tumor antigens are unlike the chemically-induced rejection antigens described above, as they are not unique to individual tumors. Infection with various DNA viruses have been associated with human cancers, including hepatitis B virus with liver cancer, Epstein-Barr virus (EBV) with immunoblastic lymphomas, and human herpes virus 8 with Kaposi's sarcoma (148). Persistent infection with oncogenic human papillomavirus (HPV) is a cause of virtually all cervical cancers (178) and prophylactic HPV vaccines comprised of virus-like particles (VLP) derived from several HPV genotypes have proven highly effective in reducing the incidence of intraepithelial neoplasia in young women (142).

Although tumorigenesis only needs a few altered genes to occur (63), individual tumors accumulate an average of 90 mutant genes (158). These mutations, deletions or translocations can yield a mutated protein with a novel peptide sequence, creating a tumor-specific mutated antigen (TSMA) (148). Some TSMA can contribute to the process of

transformation as seen with the first reported human TSMA, a mutated cyclin dependent kinas 4 (CDK4) that inhibited binding of the p16 tumor suppressor (189). Targeting antigens involved in tumorigenesis or maintaining the malignant state are immunologically important (see "challenges of a tumor vaccine" sub-chapter below). TSMAs are not immunogenic by default, although dominant immune responses have been observed with human TSMAs (96, 180). TSMAs tend to be unique to each tumor but there is evidence of T cell responses against conserved antigenic determinants resulting from common genetic alterations occurring at distinct chromosomal locations such as the bcr-abl translocation involved with chronic myelogenous leukemia (CML) (194), the recognized antigen from MCA-induced tumor 1 (Ram1) region of chromosome 4 (4), and the constitutively active mutant BRAF found in 60% of human melanomas (7). Mutations to p53 are common in many cancers and tend to cluster in conserved regions but the specific location and mutation are quite heterogeneous (119, 148). While an "off the shelf" vaccine approach for common TSMA is clinically appealing, the limited occurrence of conserved TSMA antigens restricts the use of most TSMA to an autologous vaccine unique to the individual.

This common vaccine approach is more applicable to the third category of tumor antigens since normal cellular genes that have become disregulated during the process of tumorigenesis encode these proteins. These antigens are called tumor-associated antigens (TAA) because they are not unique to the tumor (148). TAA fall into 4 groups based on their expression profile. The first TAA group, oncofetal antigens (OFA), contains proteins normally expressed in embryonic or fetal cells which are absent in nonmalignant adult cells.

The first defined oncofetal protein was α-Fetoprotein (AFP) and was associated with elevated serum levels in patients with cancer of the liver and testis (115). Carcinoembryonic antigen (CEA) is also elevated in the serum of patients with various carcinomas and like AFP, is a useful diagnostic marker of disease occurrence/progression. Although some groups report that AFP can be used with specific tumor therapies (115), others have demonstrated a lack of AFP-specific T cell function and differentiation to effector or memory phenotype in AFP peptide vaccinated Hepatocellular cancer (HCC) patients (22).

The second TAA group, cancer/testis (CT) antigens, are expressed in a non-lineage-specific manner as genes normally expressed only in the human germ line encode them. The first identified TAA was melanoma antigen (MAGEA1) (175) and has been detected in many melanomas, some breast carcinomas, and other tumor types, but not in any normal tissues except testis (119, 157). Interactions with different proteins via the MAGE homology domain (MHD) allows MAGE proteins to influence a range of signaling pathways and its expression is thought to contribute to the malignant phenotype of cancer cells (157). Because of their limited expression in normal tissue but wide range of expression in many tumors, CT antigens such as MAGE and the highly immunogenic NY-ESO-1 are ideal candidates for vaccines and have been used in clinical trials (157).

The third group of TAA are proteins expressed in various nonmalignant cell types but are specifically overexpressed in cancer cells. Human telomerase reverse transcriptase (hTERT) is an example of a gene expressed in regenerative tissues but is overexpressed in many cancers (62). Genes involved in the differentiation of a specific cell type that are

(over)expressed in tumors of the same lineage makeup the fourth group of TAA called differentiation antigens. These include the melanocyte restricted antigens gp-100/pmel17, Melan-A/MART-1, tyrosinase, and tyrosinase related protein (TRP)-1 and -2. Human epidermal growth factor receptor 2 (HER2/neu) is another well studied differentiation antigen that is normally involved in the signal transduction pathways leading to cell growth and differentiation but is overexpressed in breast cancer, ovarian cancer, adenocarcinomas, and other cancers. Unlike the melanocyte-restricted antigens, overexpression of this known proto-oncogene in $Her2^{+}$ breast cancer has been associated with a more aggressive phenotype and with decreased survival (37).

Great strides have been made in the discovery, classification and role of tumor antigens. Although a better understanding of tumor antigens can help define mechanisms involved in tumor immunity, the prevention of tumorigenesis or regression of established tumors will likely take more than a tumor antigen, regardless of which group it belongs to. An appropriate antigen can direct the immune response towards a tumor but the suppressive network associated with most established cancers prevents a strong and durable response (49, 57). The challenge of designing a successful vaccine, is not only the choice of antigen but determining how to best support the ensuing immune response while reducing the tumor associated regulatory elements.

Challenges of a Tumor Vaccine

A simple yet powerful way to shift the balance of cancer immunoediting towards immunity is with a vaccine. The concept of a vaccine is simple; a biological preparation that improves immunity to a particular disease. However, choosing an appropriate "preparation" capable of driving the strongest immune responses requires not only an appropriate tumor antigen but also an understanding of the biology involved with the cancer and its relationship with the immune system. While there are obstacles associated with vaccines for nearly all chronic diseases, the additional hurdles associated with developing an effective cancer vaccine has challenged researchers for more than a hundred years (30, 49).

One of the most fundamental immunological complications associated with cancer is the immune system's ability to discriminate between self and non-self. Unlike pathogens that express foreign antigens, most tumors predominately express self-antigens, which provides an advantage for the tumor since the immune response must contend with mechanisms of tolerance designed to limit self or autoreactive responses (84). Both central and peripheral tolerance are efficient at limiting self responses but each mechanism is not absolute and can be exploited to generate a tumor-specific immune response. Central tolerance eliminates overtly self-reactive T cells in the thymus by negative selection (53, 163). However, the diverse repertoire of T cells required to react to the nearly infinite range of foreign antigen is capable of T-cell receptor (TCR) degeneracy or cross-reactivity, allowing T cells to recognize a range of epitopes unrelated to and with affinities that differ from the cognate antigen (32, 35, 81). Mostly due to TCR degeneracy, self-reactive or

tumor-specific clones do exist in the periphery but often have TCRs with a low affinity for tumor antigen (78, 91) that limits the strength of the immune response (5).

Tumor-specific T cells can be primed to generate an effective anti-tumor response but without understanding and modulating tumor-related peripheral tolerance, the efficacy of a tumor vaccine can be greatly diminished. The tumor and/or its microenvironment can initiate and reinforce various mechanisms of peripheral tolerance but equally important to tumor immunity is the context in which antigens are presented to the immune system. Unlike microbial or viral infections, which provide inflammation as well as strong immune activating signals (77, 164), tumorigenesis is immunologically "silent" and lacks the molecular stimuli necessary to activate professional antigen presenting cells (APC), a critical factor in creating tolerance (112, 164). Additionally, tumors can produce IL-6, IL-10, and vascular endothelial growth factor (VEGF) that can further suppress DC function (52, 88). DC/tumor fusion (156), *ex vivo* pulsing/maturation of DCs (46, 113), antibody-directed delivery of antigen in vivo (18, 164), and many other promising strategies have been described yet promoting immunostimulatory APCs remains a critical obstacle of tumor immunity and in vaccine development in particular (14, 129).

While APCs are clearly important in determining tolerance versus immunity, other subsets of lymphocytes also contribute to tumor-induced peripheral tolerance. Suppression of immune responses by tumor-induced or sensitized $CD4^+$ regulatory T cells (Tregs) (20, 131) can occur via multiple mechanisms, including those that directly impact T cells by direct perforin-mediated cytolysis (23) or with immunosuppressive molecules such as TGF-β (25,

48), IL-10 (103, 111), and IL-35 (33). Tregs can also diminish APC function with cytotoxic T-lymphocyte antigen 4 (CTLA-4) (103, 114, 149) or LAG-3 (100). Modulation of these potent suppressors has become the focus of many studies (134, 198) as well as being incorporated into clinical trials (136). Tumor-associated myleoid derived suppressor cells (MDSCs) can suppress tumor immune responses by production of reactive oxygen species and nitric oxide or local depletion of arginine (107). Similarly, indoleamine 2,3-dioxygenase (IDO)-expressing plasmacytoid DCs can induce peripheral tolerance in tumor setting by direct induction of T cell anergy due to local tryptophan depletion (116) and/or by activating Tregs (151, 152). Encouraging studies have demonstrated a correlation between inhibition of IDO and antitumor responses (69).

Identifying the different cell types and mechanisms involved with peripheral tolerance is requisite for developing a successful vaccine strategy, but the tumor's ability to directly restrict immune responses also needs to be considered. Many of the immunosuppressive cytokines and molecules expressed by the suppressive lymphocytes are also expressed by tumors such as TGF-β (17), IL-10 (90, 117), IDO (141, 192), and inducible nitric oxide synthase (iNOS) (154, 197). Additionally, tumors may also express programmed death ligand-1 (PD-L1)/B7-H1, which engages an inhibitory receptor on activated T cells and limits their function (51, 195, 196). Tumors can also express galectins, a family of lectins capable of inducing apoptosis in T cells and associated with malignant tumor development and metastasis (89, 102, 130). Blocking antibodies and inhibitors for PD-L1 and galectins, respectively have shown promise in augmenting tumor immunity (8, 66).

Tumors can limit a vaccine's efficacy by not only exerting peripheral tolerance but also by directly evading the immune response. Tumors under immunoselective pressure can down-regulate the expression of proteasome components, transporter associated with antigen processing-1 (TAP1)/TAP2, β2-microglobulin, or MHC class I molecules, which reduces the visibility of tumors to $CD8^+$ T cells and thereby limit their CTL function (108). A related mechanism that occurs in tumors is loss of responsiveness to IFN-γ signaling which promotes proliferation and angiogenesis while limiting apoptosis and immunogenicity (79, 150, 160). Tumors can also directly lose expression of antigens needed for recognition by CTLs, creating antigen-loss variants (95, 176, 179). As described above in the cancer immunosurveillance sub-chapter, these immune evasion tactics can drive tumors from equilibrium towards escape. This underscores the necessity of a vaccine to incorporate antigen(s) that are critical to maintaining the oncogenic phenotype if possible and/or priming a response that can recognize more than a single epitope or antigen.

Using a vaccine to establish a strong tumor-specific immune response is an uphill battle that must contend with, but by no means is limited to, the ubiquitous mechanisms of tolerance and tumor immune escape described here. While many of the challenges tied to tumors or tumor-associated suppressive cell types have been elucidated and often resolved by researchers working in a defined model system, it is unlikely that any one strategy will release the immune response from this pervasive network of tolerance. Prophylactic tumor vaccines have the advantage of generating an immune response without many of the tumor-associated peripheral tolerance or escape mechanisms described herein, yet are limited in

their application (104). Looking forward, it is clear that a combinatorial vaccine approach should include the appropriate antigen and target multiple mechanisms of tolerance while also augmenting the immune response. Coupling a vaccine with either an agonist antibody targeting OX-40, a co-stimulatory receptor capable of augmenting T-cell expansion and survival, while limiting T cell anergy (60, 138) and the suppressive activity of regulatory T cells (132, 144), is a straightforward yet effective example of combinatorial immunotherapy approach (139, 147). Alternatively, researchers have demonstrated impressive clinical success with the induction of lymphopenia by radiation and chemotherapy combined with adoptive transfer of autologous tumor-infiltrating lymphocytes (TIL), a strategy using adoptive cellular therapy to provide a strong tumor-specific population while limiting mechanisms of tolerance (40, 143). The design of a complimentary vaccine strategy minimally needs to evaluate how the antigen source and immunomodulatory approach can compliment each other. The biology and stage of the cancer, as well as the condition and prior treatment(s) of the patient should influence this choice. While basic research is essential to understand the efficacy and mechanism of a specific vaccine or immunomodulatory strategy as single modality, the cooperative or synergistic effects of a combinatory approach will likely prove necessary to overcome the complex suppressive network in tumor bearing patients and support a robust anti-tumor immune response.

Outline of Thesis

There are different immunotherapeutic approaches that can augment ones immune response or components thereof to shift the balance of tumor immunosurveillance towards an environment that promotes tumors elimination. The focus of this work is to develop and implement novel vaccine-based immunotherapies that enhance tumor-specific immune responses.

This thesis examines two important components of immunotherapy related to tumor vaccines; the choice of antigen and the method of antigen delivery in a vaccination protocol.

The work presented in the second chapter addresses the following questions related to a novel reconstitution/vaccination protocol:

1. How is antigen-specific T cell proliferation or survival impacted by homeostasis-driven proliferation and antigen-driven proliferation in a lymphopenic host?
2. Can stimulation of toll-like receptor 4 specifically augment antigen-driven proliferation in reconstituted/vaccinated lymphopenic mice?
3. Can vaccination with peptide-pulsed splenocytes in combination with a TLR ligand stimulate a durable, non-tolerizing antigen-specific immune response?

The third chapter continues to address novel tumor vaccines but shifts focus from delivery of antigen towards the source and quality of the antigen itself. Specifically, this chapter asks the following questions related to vaccination with antigens associated with either an intact cell or cell-derived autophagosomes.

1. Is there a qualitative difference between a vaccine derived from an intact sarcoma versus one from tumor-derived autophagosomes?
2. Do short-lived proteins (SLiPs) accumulate in autophagosomes and can they drive antigen-specific T cell proliferation?
3. Can limiting the accumulation of SLiPs reduce the efficacy of an autophagosome vaccine?
4. How are SLiPs delivered to the autophagosomes?

CHAPTER TWO: Novel intravenous peptide vaccine strategy stimulates functional antigen-specific T-cells

ABSTRACT

Vaccination strategies have failed to significantly impact outcomes of cancer patients. We hypothesize that a primary reason for this failure is because the magnitude of the antitumor immune response is insufficient to mediate tumor regression. Recently, we described a novel strategy to augment priming of tumor-specific T cells by vaccinating lymphopenic mice that had been reconstituted with naïve spleen cells. Tumor vaccine-draining lymph nodes (TVDLN) of these reconstituted lymphopenic mice (RLM), vaccinated with a GM-CSF-secreting tumor vaccine, contained an increased number of activated tumor-specific $CD4^+$ and $CD8^+$ T cells. Based on these studies we hypothesized that this immunotherapeutic strategy could be further augmented if the vaccination could take advantage of the entire lymphopenic compartment rather than just the lymph nodes draining the subcutaneous vaccines. To test this idea, we developed a model wherein lymphopenic hosts were reconstituted with splenocytes that had been pulsed with antigen, thereby allowing for simultaneous vaccination and reconstitution. The reconstitution/vaccination pool consisted of a mixture of wild-type (wt) and gp100-specific TCR transgenic splenocytes pulsed with human gp100 peptide. While previous studies employing intravenous (i.v.) vaccination of intact hosts induced T cell tolerance to defined antigens, this strategy induced expansion and persistence of antigen-specific T cells in an antigen-dependent fashion.

Further, including a TLR4 (toll-like receptor 4) ligand during the vaccination/reconstitution, enhanced both the activation and frequency of gp100-specific T cells. These T cells were neither deleted nor likely anergic as they were able to produce peptide-specific IL-2 and IFN-γ as measured by both ELISA and intracellular cytokine staining (ICS). Antigen-responsive T cells could still be detected over 30 days post-vaccination by ICS. These preliminary studies suggest that i.v. vaccination at the time of reconstitution of lymphopenic patients or at the time of stem cell transplant may provide an opportunity to exploit the endogenous cytokine environment and lack of competition for space and cytokines to augment expansion of tumor-specific T cells.

INTRODUCTION

T-cell tolerance is a hurdle of tumor immunotherapy, while being a goal of many therapies against T-cell mediated autoimmune diseases (3). There are multiple factors that can augment or diminish a vaccine's ability to prime a robust immune response, one of which is the vaccine's route of administration (2, 80). Peptides synthesized from T-cell epitopes of allergens and autoantigens administered both intravenously (i.v.) and intraperitoneally (i.p.) induce antigen-specific tolerance *in vivo* in experimental models as well as in clinical studies (94). Using adoptively transferred antigen-specific T cells, researchers were able to monitor both a productive immune response resulting from a subcutaneous injection of antigen and a tolerizing immune response resulting from an intravenous injection of antigen (80). These studies demonstrated that the route of antigen administration influences priming or tolerance.

In addition to the route of administration, the complexity of the antigen is an important determinant of a vaccine's efficacy. This idea is exemplified with the comparison of a whole tumor cell vaccine or a tumor-associated peptide vaccine. The salient difference of the two types of vaccines is generally related to the antigenic diversity: a whole cell vaccine introduces a wide range of antigens and epitopes whereas a peptide vaccine has a defined repertoire of epitopes. Peptide vaccines have the advantage of controlling all of the vaccine components whereas a cellular vaccine may include immunosuppressive molecules such as TGF-β and prostaglandins or PD-L1 and TRAIL which have the potential to blunt immunity. Recent work from our laboratory suggests that multiple vaccinations with a GM-CSF-secreting whole cell vaccine can reduce the tumor-specific immune response (93). Futhermore, the epitopes used in peptide vaccines can be modified to increase TCR and/or MHC affinity producing a more robust immune response (14, 127).

The potential of peptide vaccines has been demonstrated with viral peptide immunizations that protected mice from a subsequent LCMV infection (2) and controlled viremia and prevented AIDS in SIV-infected non-human primates (24, 36). Additionally, peptide vaccines using the tumor-associated transcription factor, WT1, have demonstrated strong clinical responses in the treatment of various leukemias (122). In all of these diseases, the peptide vaccine strategy induced durable antigen-specific responses directed at specific viral or tumor-associated targets. Peptide vaccines applied to a tumor setting are not confined to generating a singular or defined immune response as multi-peptide vaccines (27) and epitope spreading (80, 177) can each broaden the repertoire of the reactive T cells. This

broadening of the repertoire may help to offset immune escape as well foster a more robust immune response by the inclusion of both $CD4^+$ and $CD8^+$ T cell epitopes.

Based on some of the promising data supporting peptide-based vaccines as well as data demonstrating the strong reactivity of T cells stimulated with the high affinity heteroclitic melanoma-associated epitope human (h)$gp100_{25\text{-}33}$ (58, 127), naïve splenocytes pulsed with hgp100 were used to vaccinate mice by an intravenous adoptive transfer. We hypothesized that this vaccine would be augmented in a lymphopenic setting as work from our lab (70) and others (41, 55) have established that vaccination of reconstituted lymphopenic hosts results in superior tumor-specific immune responses and tumor regression compared to an intact host. Additionally, pulsing the naïve splenocytes used to reconstitute the RLM with peptide would afford an opportunity to provide antigen directly to the APCs contained in the reconstituting population. This vaccine/reconstitution method could offset the inability of free peptide introduced systemically to bind APCs due to its rapid clearance *in vivo*, a potential mechanism for the induction of tolerance in an intravenous vaccine (80). We further hypothesized that reconstituting lymphopenic mice with $hgp100_{25\text{-}33}$ peptide-pulsed splenocytes will not limit the priming of antigen-specific T cells to specific draining lymph nodes but instead utilize the entire lymphopenic compartment, allowing for a systemic priming. The maturity or function of antigen-pulsed APCs in this vaccine is likely a critical factor as research has demonstrated that activated APCs efficiently prime immune responses whereas immature APCs can inhibit or tolerize immune responses (164). We therefore predicted that stimulatory signals provided to the APCs in the splenocyte population by toll-

like receptor (TLR) ligands during the *ex-vivo* pulse period would augment the vaccine's efficacy.

The research discussed in the following chapter examines the requirements of a successful vaccine while challenging the concept that an intravenous administration of antigen produces T cell tolerance. Specifically, the effects of a lymphopenic environment coupled with the inclusion of antigen and/or TLR agonist were addressed by characterizing the activation, frequency, and function of antigen-specific T cells at different time points in an effort to assess the vaccine's efficacy. The goal of this work was to use a clinically applicable novel tumor vaccine strategy to define critical parameters capable of redirecting a weak or tolerizing T cell response towards a robust immune response.

MATERIALS AND METHODS

Mice

Female C57BL/6J (B6) mice expressing either CD90.1 (Thy1.1) or CD90.2 (Thy1.2) or CD45.1 (Ly5.1) or CD45.2 (Ly5.2) congenic markers were purchased from Charles River (Wilmington, MA). Pmel breeders transgenic for the TCR that recognizes $gp100_{25\text{-}33}$ in the context of $H\text{-}2K^b$ were obtained from Dr. Nicholas P. Restifo (National Cancer Institute, NIH (126)). These pmel mice were on C57BL/6J background expressing either CD90.1, CD90.2, or GFP (under an actin promoter). All mice were maintained and used in accordance with the Earl A. Chiles Research Institute Animal Care and Use Committee and recognized

principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996).

Reconstitution/Vaccination

Spleens from naïve female C57BL/6J mice and pmel TCR transgenic were harvested and individually processed into a single cell suspension with HBSS (Lanza). $9x10^6$ wt "filler" splenocytes and $1x10^6$ pmel splenocytes were pulsed with hgp100 peptide (Anaspec) at a final concentration of 10μM in 0.5mL for 30-45 min at 37°C. TLR ligands were added at various concentrations during the pulse period but volumes remained constant. Without washing, the pulsed splenocytes were transferred intravenously to irradiated (500R) naïve female C57BL/6J mice.

Frequency and Activation Analysis

Spleens were removed from vaccinated mice at different time points and processed into a single cell suspension. Splenocytes were stained with antibody to determine frequency (anti-CD3, anti-CD8, and anti-CD90) or activation status (anti-CD3, anti-CD8, anti-CD44 and anti-CD62L). Absolute counts were determined by multiplying the number of splenocytes by the frequency of live-gated $CD3^+/CD8^+/CD90^+$ or GFP^+ cells. For functional assays, the splenocytes were stimulated with 10 μg of hgp100, mgp100, SIINFEKL, or immobilized anti-CD3 in 2 ml of CM in 24-well plates.

IL-2 and IFN-γ ELISA

Splenocytes were cultured at 37°C in 24-well plates for 24 h with 5 μg/mL of hgp100, mgp100, SIINFEKL, or immobilized anti-CD3 in 2 ml of CM. Supernatants were harvested and assayed for either IL-2 or IFN-γ using commercially available reagents (BD Biosciences Pharmingen, San Diego, CA). The concentration of cytokines in the supernatant was determined by regression analysis.

Intracellular Cytokine Staining with Flowcytometric Analysis.

Brefeldin A was added to splenocytes that were cultured with a total of 5 μg/mL of hgp100, mgp100, SIINFEKL, or immobilized anti-CD3 at 37°C for 8 h in 2 ml of CM. Cells were harvested and stained with FITC-labeled anti-CD90.1 antibody, PE-labeled anti-IFN-γ antibody, PE-Cy7-labeled anti-CD8 antibody, and APC-labeled anti-CD3 antibody after fixing and permeabilization (Cytofix/Cytoperm kit; Pharmingen, San Diego, CA). Fifty thousand gated events based on forward and light scatter were collected and analyzed, and all of the analyses were gated on $CD3^+$ and $CD8^+$ cells.

RESULTS

A reconstituted lymphopenic host promotes an increased number of antigen-specific T cells compared to an intact host.

Work from our institute and others has contributed to a greater understanding of the impact of lymphopenia on T cell immunobiology which has further been translated into human clinical trials (145). A lymphopenic setting can augment an immune response by enhancing survival, proliferation and function of T cells (55, 70, 172) and is a cornerstone of this vaccine strategy. A lymphopenic host provides an opportunity to not only infuse lymphocytes during reconstitution but also the means to deliver peptide-pulsed APCs that can prime an antigen-specific immune response.

To initially address the contribution of the lymphopenic environment to this vaccine, mice were either left intact or lymphodepleted with 500R of gamma irradiation before receiving $9x10^6$ wt "filler" naïve syngeneic splenocytes ($CD45.1^+/CD90.2^+$) and $1x10^6$ gp100-specific pmel cells ($CD45.1^-/CD90.2^+$) that had been pulsed with 10μM hgp100. Results described in Figure 2 demonstrate that on day 8, the frequency of antigen-specific $CD3^+/CD8^+$ pmel T cells was nearly 100-fold increased in a lymphopenic host compared to an intact host. This large increase in the frequency of pmel T cells in a lymphopenic host may be partly explained by the difference in the frequency of endogenous $CD3^+/CD8^+$ lymphocytes ($CD45.1^-/CD90.2^-$) in a lymphopenic versus lymphoreplete host on day 8. However, while the disproportional amount of endogenous lymphocytes may help account for the vast difference in the frequency of adoptively transferred T cells, it can't account for a

nearly 20-fold increase in the absolute number of antigen-specific T cells in a lymphopenic host (Figure 2). The clear benefit of a lymphopenic environment was seen with the increase in survival and/or proliferation of pmel T cells in the irradiated host, which may be partly due to homeostasis-driven proliferation (HDP) (173).

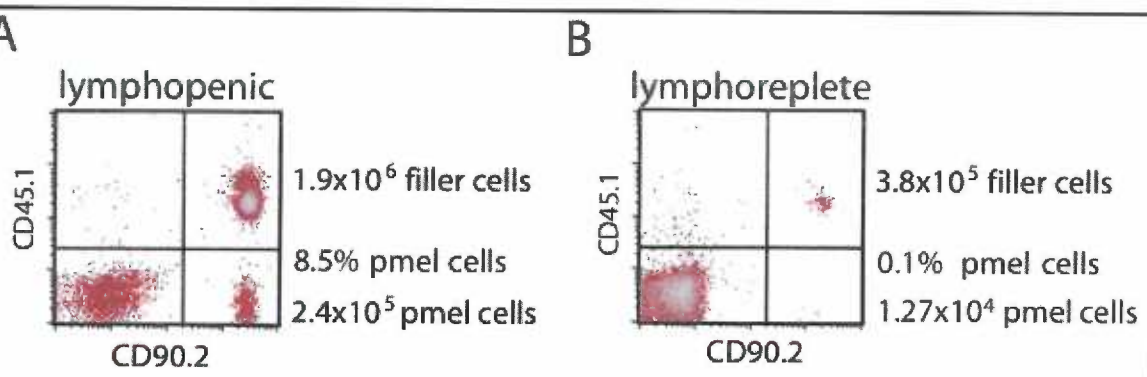
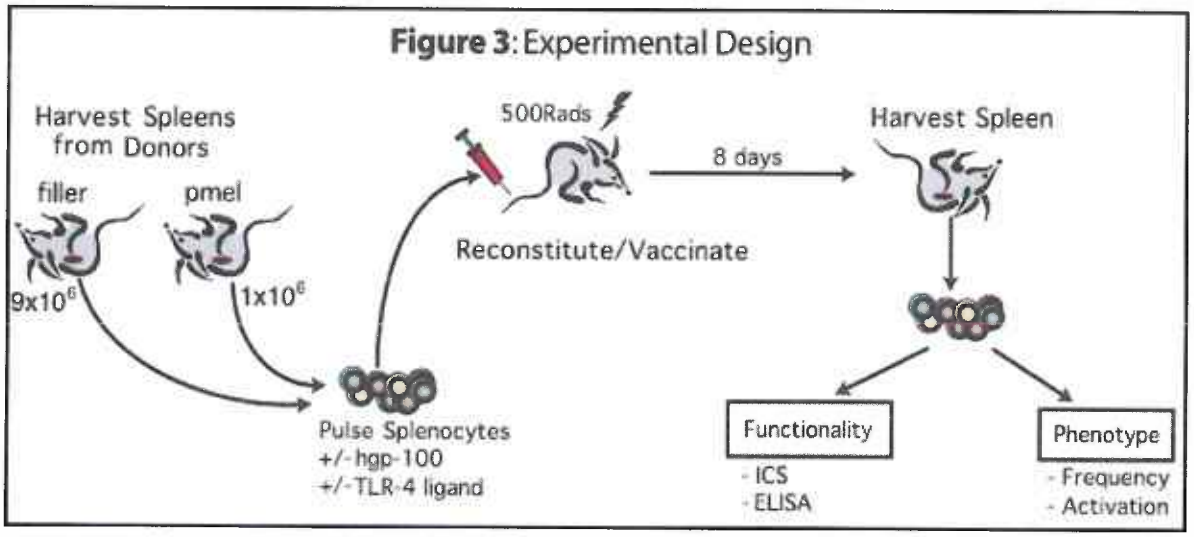


Figure 2: Increase in pmel frequency and absolute counts in lymphopenic vs. lymphoreplete host. $1x10^6$ pmel ($CD90.2^+/CD45.1^-$) and $9x10^6$ wt "filler" ($CD90.2^+/CD45.1^+$) splenocytes were mixed with 10μM hgp100 and adoptively transferred into (A) irradiated (500R) lymphopenic or (B) lymphoreplete wt hosts. Spleens were harvested on day 8 and stained for flow cytometric analysis. Numbers to the right of the panels indicate the absolute count of $CD90.2^+/CD45.1^-$ pmel cells or $CD90.2^+/CD45.1^+$ transferred cells or frequency of $CD90.2^+/CD45.1^-$ pmel cells. Dot blots were first gated through a $CD3^+/CD8^+$ gate. Representative of 2 mice.

A vaccinated reconstituted lymphopenic mouse promotes an increased number and frequency of antigen-specific T cells compared to a non-vaccinated reconstituted lymphopenic mouse.

T cell survival or proliferation in a vaccinated host was superior in a lymphopenic environment as the magnitude of adoptively transferred antigen-specific T cells recovered was substantially greater in a RLM compared to an intact host. To address the contribution of both antigen-driven proliferation (ADP) and homeostasis-driven proliferation (HDP) in the

context of a lymphopenic setting, irradiated mice were reconstituted with $9x10^6$ wt "filler" cells and $1x10^6$ antigen specific pmel cells with or without 10 μM hgp100 (Figure 3). The frequency of antigen-specific T cells in the spleens of the vaccinated mice harvested 8 days after the adoptive transfer was over twice the frequency of non-vaccinated mice (Figure 4). While proliferative and survival benefits of homeostasis-driven proliferation are well documented and may contribute to vaccine efficacy, Figure 4 demonstrates the dominance of antigen-driven proliferation as an intravenous vaccine increases the frequency of antigen-specific T cells in a RLM.



Since intravenous administration of antigen has been shown to lead to a tolerized T cell population, the question of the quality of T cells primed with an intravenous reconstitution/vaccination remained. To first address this, RLM were reconstituted/vaccinated as in Figure 3 and after 8 days, antigen-specific pmel T cells ($CD3^+/CD8^+/GFP^+$) from the spleen were assessed for cell surface expression of the

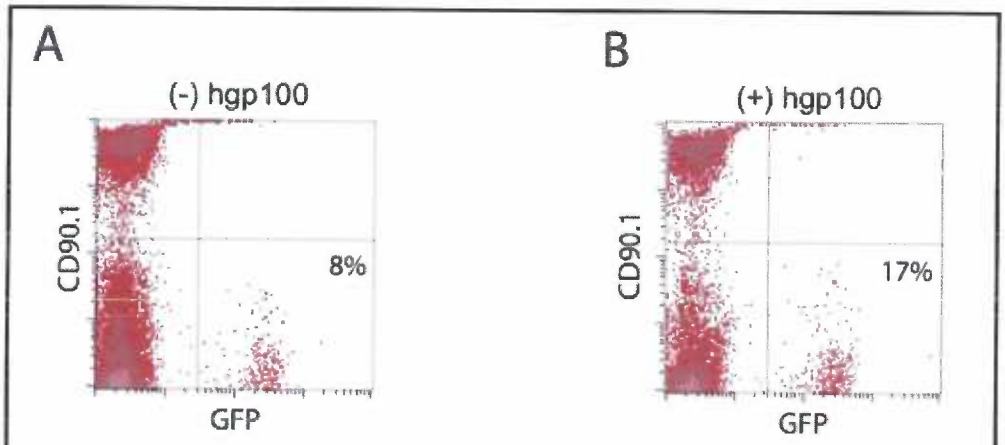


Figure 4: Vaccination of RLM with hgp100 increases frequency of pmel T cells. 1X10^6 pmel (CD90.1$^-$/GFP$^+$) and 9x10^6 wt (CD90.1$^+$/GFP$^-$) splenocytes were pulsed (A) without or (B) with 10μM hgp100 in 0.5mL of HBSS for 30 minutes at 37°C and used to reconstitute/vaccinate a lymphopenic mouse (500R). Spleens were harvested on day 8. Numbers in the lower right quadrant of the panels indicate the percent of CD90.1$^-$/GFP$^+$ pmel cells compared to CD90.1$^+$/GFP$^-$ transferred cells. Dot blots were first gated through a CD3$^+$/CD8$^+$ gate. Representative of 2 mice.

activation markers, CD62L and CD44. Activated T cells have previously been defined as having an elevated expression of CD44 while maintaining a low expression of CD62L (47). Antigen-specific T cells that were not vaccinated and only underwent homeostasis-driven proliferation displayed a very limited activation phenotype with only 11% of the pmel T cells being CD62L^{low}/CD44^{high} (Figure 5A, left plot). When RLM were reconstituted/vaccinated with splenocytes that had been pulsed with hgp100 peptide, there was a marked increase in activated antigen-specific T cells to 24% CD62L^{low}/CD44^{high} (Figure 5A, middle plot), suggesting that inclusion of antigen enhanced the stimulation of antigen specific T cells.

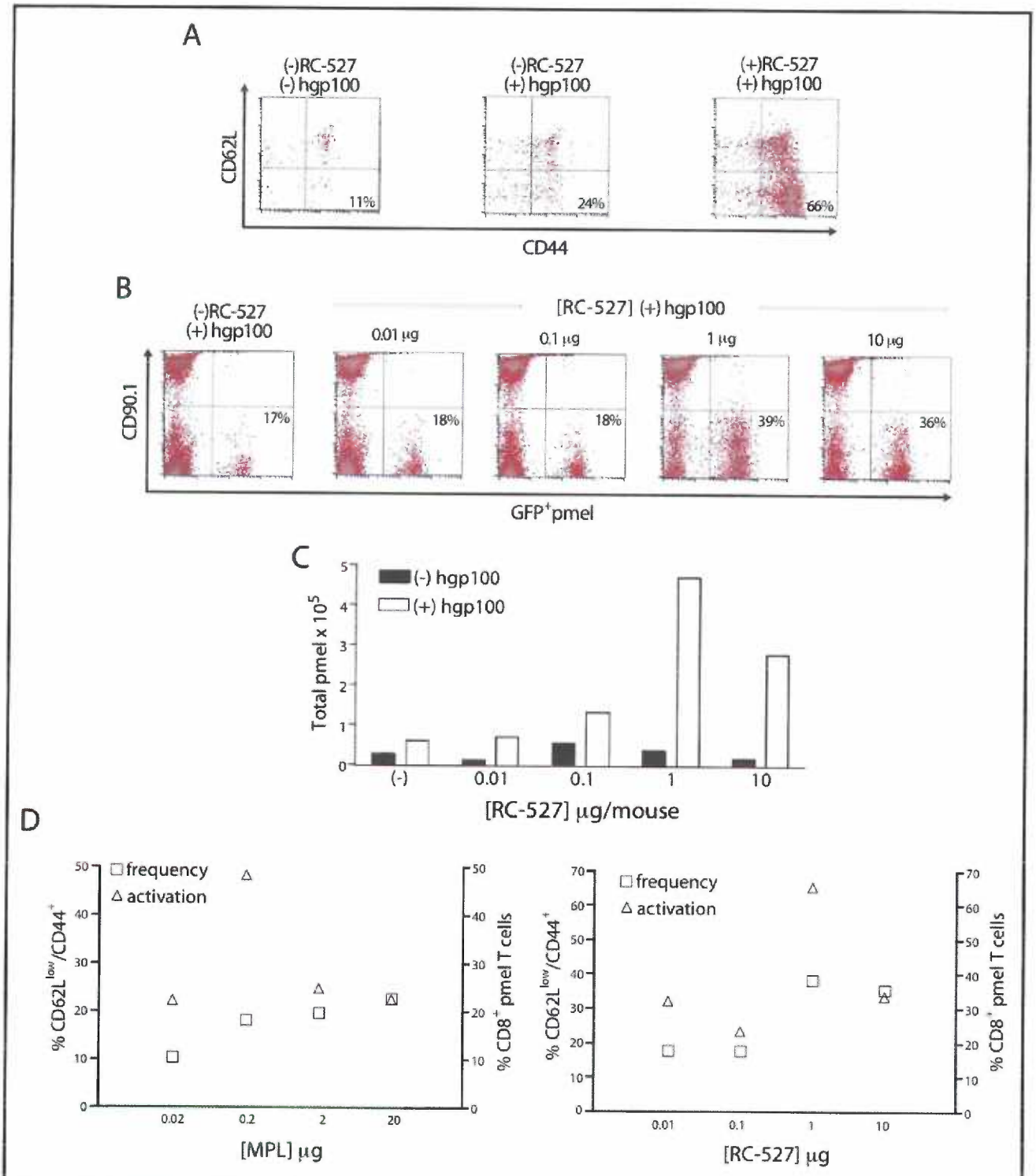


Figure 5: Vaccination of RLM with hgp100 and RC-527 increases activation phenotype, frequency and absolute count of pmel T cells. 1X10^6 pmel and 9x10^6 wt splenocytes were pulsed with or without 10µM hgp100 and (A) 1µg or (B and C) a titration of RC-527 in 0.5mL of HBSS for 30 minutes at 37°C and used to reconstitute/vaccinate a lymphopenic host. Spleens were harvested on day 10 and stained for flow cytometric analysis. All dot blots were first gated through (A) a CD3$^+$/GFP$^+$ gate or (B and C) a CD3$^+$/CD8$^+$ gate. (A) Numbers in the lower right quadrant indicate the percent of activated (CD62L^{low}/CD44$^+$) pmel T cells. (B) Numbers in the lower right quadrant indicate the frequency of GFP$^+$/CD90.1$^-$ pmel cells compared to GFP$^-$/CD90.1$^+$ transferred cells. (C) Total number of pmel T cells was determined by multiplying the frequency of pmel T cells (as done in (B)) by the number of splenocytes recovered. (D) Mice were vaccinated as in (B) with 10µM hgp100 and a titration of RC-527 or MPL. Spleens were harvested on day 10 and stained for flow cytometric analysis . Activation was based on the percentage of CD3$^+$/GFP$^+$/CD62L^{low}/CD44$^+$ splenocytes and frequency was based on the percentage of CD3$^+$/GFP$^+$/CD8$^+$ splenocytes. (A-D) representative of 2 mice.

Addition of the TLR4 ligand, RC-527, specifically augments antigen-specific T cell frequency and activation phenotype.

While 24% of T cells with an activated phenotype would be an expected frequency of $CD62L^{low}/CD44^{high}$ polyclonal T cells found in a draining lymph node after a whole cell vaccine (105), activation of the pmel TCR transgenic T cells has been reported much higher (179). The relatively low frequency of activated antigen-specific T cells was likely the result of inefficient priming. A lack of activated APCs in the reconstituting splenocytes could be responsible for the inefficient priming as immature DC have been implicated in suppressing immune responses. (164). Alternatively or in conjunction, endogenous APCs may augment the priming event but may be unable to capture and cross-present antigen in an i.v. setting due to the peptide's rapid clearance (80). Based on these possibilities, it was hypothesized that the addition of a TLR agonist during both the *ex-vivo* pulse and the reconstitution would help activate transferred and endogenous APCs leading to increased numbers and a larger frequency of activated antigen-specific T cells.

The TLR4 agonist RC-527, a modified component of the immunostimulatory molecule lipopolysaccharide capable of generating MyD88 dependent and independent signals (68), was added during the *ex-vivo* pulse period and transferred i.v. along with reconstituting splenocytes. A titration of RC-527 demonstrated that the higher doses of this compound were associated with a substantial increase in the frequency of antigen-specific T cells (Figure 5B). This RC-527-mediated increase in pmel T cells was antigen-dependent, as the absolute counts of pmel T cells greatly increased with both antigen and TLR ligand

(Figure 5C). However, without inclusion of hgp100, the absolute counts remained low regardless of the concentration of RC-527. A productive priming event should yield not only an increased frequency or absolute count of antigen specific T cells, but these cells should display an augmented activation phenotype. The modest increase in frequency of activated pmel T cells with the addition of antigen was dwarfed by the increase in frequency of activated pmel T cells with the inclusion of RC-527 plus antigen (Figure 5A). Moreover, the 1μg dose provided not only the greatest frequency and number of gp100 specific T cells, but also the greatest frequency of activated antigen-specific T cells, even when compared to the similar TLR4 ligand MPL (Figure 5D). Addition of MPL did enhance the activation phenotype at a lower dose but did not increase the frequency of antigen-specific T cells at the concentrations used for this assay.

Intravenous reconstitution/vaccination with both hgp100 and the TLR4 ligand results in functional antigen-specific T cells at day 8.

To understand better the antigenic requirements and the adjuvant effect incorporated into this novel vaccine strategy, further functional analysis of stimulated lymphocytes was performed. Initially, spleens were harvested from RLM that were reconstituted/vaccinated with or without hgp100 and with or without RC-527 and restimulated with different targets. In agreement with the previous data that demonstrated greater numbers, enhanced frequency, and more strongly activated antigen-specific T cells, only splenocytes primed with a vaccine consisting of hgp100 and RC-527 secreted hgp100 specific IFN-γ as measured by ELISA

(Figure 6). The addition of the TLR4 ligand to the vaccine did not promote a non-specific immune response, as the primed splenocytes did not respond to stimulation with the H2-K^b restricted irrelevant peptide SIINFEKL. An important determinant of a productive immune response is the ability of stimulated T cells to produce IL-2. Additionally, T cell anergy has been defined by the lack of IL-2 production when a T cell is (re)stimulated with its cognate antigen (28). Although CD4 T cells can make more IL-2 than CD8 T cells, the production of this cytokine by splenocytes of vaccinated mice was evaluated to gain a better understanding of the T cell biology related to this i.v. strategy. There was no measurable IL-2 secreted in response to hgp100 from T cells stimulated without antigen or without TLR ligand, suggestive of a poor immune response.

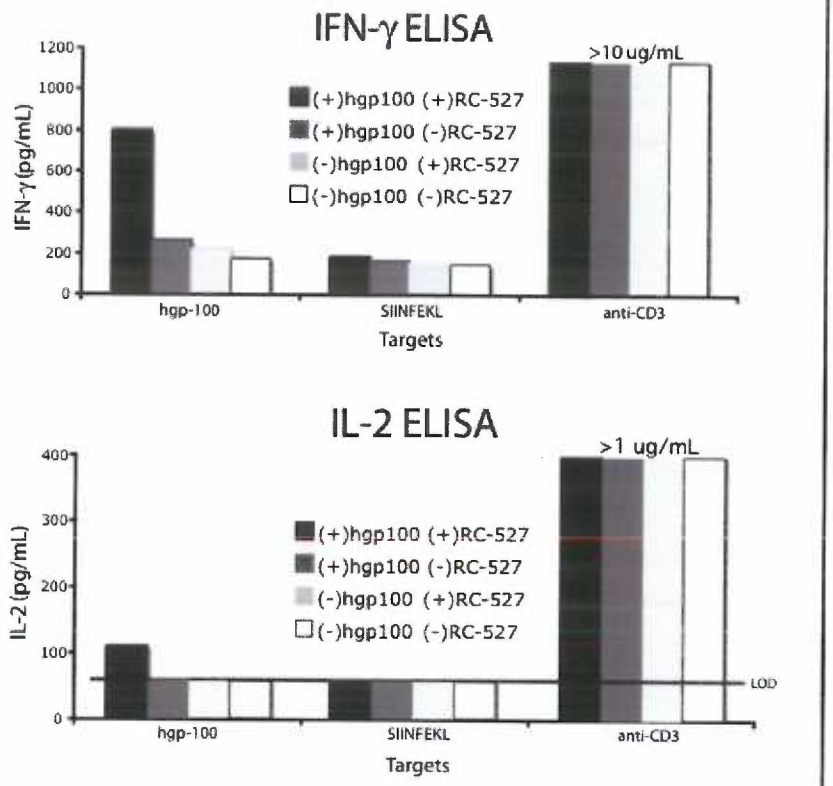


Figure 6: Peptide-specific responses measured by IFN-γ and IL-2. 1X10^6 pmel and 9x10^6 wt splenocytes were pulsed with 10μM hgp100 and 1μg of a RC-527 in 0.5mL of HBSS for 30 minutes at 37°C and used to reconstitute/vaccinate a lymphopenic host. On day 8 splenocytes (combination of 3 mice per group or 2 mice per group for (-)hgp100 (-)RC-527)) were stimulated with 10μg of hgp100, SIINFEKL or soluble anti-CD3 for 24 hours at 37°C. Supernatants were collected and used for both IFN-γ and IL-2 ELISAs. n=1 experiment. LOD = limit of detection for this assay.

However, the same vaccine conditions that yielded the largest IFN-γ response (inclusion of both antigen and RC-527) also primed T cells to secrete IL-2 in response to hgp100. It is important to note that the low level of IL-2 secreted was a meaningful result as it was above the limit of detection, a blocking antibody against the IL-2 receptor was absent during the stimulation period, and the freshly isolated splenocytes were stimulated without prior sensitization. Antigen-specific release of both cytokines as measured by ELISA supports the necessity of the TLR4 agonist in combination with the hgp100 peptide to stimulate functional T cells with an intravenous vaccine.

The function of T cells isolated from the vaccinated RLM was additionally examined using an intracellular cytokine release (ICS) assay. While this assay shares the same endpoint (IFN-γ production) as the ELISA assay described above, production of the cytokine can be traced on a per cell basis, allowing for the direct assessment of antigen-specific T cell function. Accordingly, the antigen-specific IFN-γ T cell response was present at a low frequency (22%) when RLM was vaccinated without antigen or TLR4 ligand (Figure 7). This frequency increased modestly to 26% when RC-527 was added. Inclusion of antigen in a vaccine lacking RC-527 created a further increase in the frequency of functional antigen-specific T cells to 31%. Similar to the ELISA results, the vaccine that consisted of both the antigen and the TLR4 ligand stimulated nearly half of the antigen-specific pmel T cells to produce hgp100-specific IFN-γ. Also similar to the ELISA results was the lack of cross-

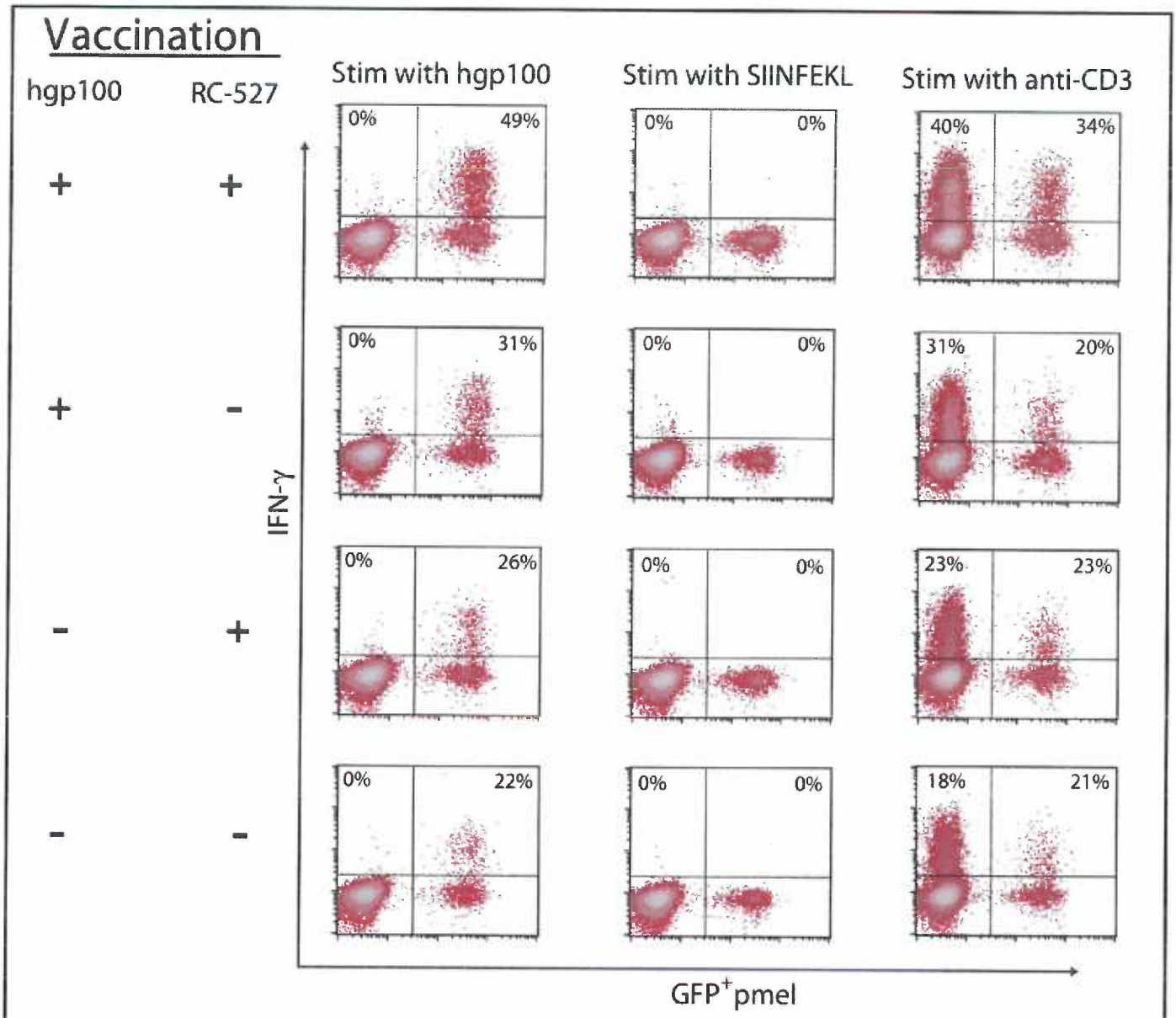


Figure 7: i.v. vaccination with RC-527 augments peptide-specific IFN-γ response. $1X10^6$ pmel and $9x10^6$ wt splenocytes were pulsed with or without 10μM hgp100 and with or without 1μg of RC-527 and used to reconstitute/vaccinate a lymphopenic host. On day 8 splenocytes were stimulated with 10μg of hgp100, SIINFEKL or soluble anti-CD3 in the prescence of 10μg of Brefeldin A for 8 hours at 37°C. Numbers in the upper left or right quadrant indicate the percent of $CD3^+/CD8^+/GFP^-$ transferred and endogenous cells or $CD3^+/CD8^+/GFP^+$ pmel cells, respectively, producing IFN-γ vs. not producing IFN-γ. Representative of 2 independent experiments with 2-3 pooled spleens per group.

reactivity with the irrelevant peptide. This ICS data further demonstrates the specificity of vaccine-induced immune response, as the contribution of the endogenous or wt "filler" cells that produced IFN-γ in response to hgp100 stimulation was absent (Figure 7). Combined with the ELISA data, these results illustrate that this intravenous vaccine delivered with both a reconstituting population of lymphocytes and antigen-pulsed APCs as well as a potent TLR4 agonist can prime a productive immune response as determined by the frequency and absolute count, phenotype, and function of antigen-specific T cells.

Antigen-specific T cells are present and functional 34 days after intravenous vaccination.

Based on absolute cell counts, phenotype and function, the above data suggests that antigen-specific T cell deletion or anergy has not occurred 8 days after the reconstitution and vaccination of RLM. However, others have shown that the peripheral tolerance associated with delivery of systemic antigen can occur later, as antigen-specific T cells numbers can remain relatively high at days 3 and 5 but by day 17 the T cell population can be reduced to the background levels of a non-vaccinated animal (80). To examine this possibility, the absolute cell count and function of antigen-specific T cells isolated from the spleens of lymphopenic mice reconstituted/vaccinated with hgp100 and RC-527 was assessed on days 0, 8 and 34. Irradiated were mice reconstituted with $0.24x10^6$ $CD8^+$ pmel T cells on day 0 and by day 8 there was an average of $0.26x10^6$ $CD8^+$ pmel T cells in the spleens of 3 different mice (Figure 8). While the absolute number pmel T cells used for reconstitution (day 0) appears to be the same as on day 8, proliferation likely occurred as many of the

transferred T cells do not make it out the lung and/or traffic to somewhere in the periphery other than the spleen. Deletion of the antigen-specific T cells was likely not occuring at the later time point as the absolute number of $CD8^+$ pmel T cells in 2 of 3 mice was the same or had even increased on day 34 compared to day 8. To address whether the antigen-specific T cells retained their function 34 days after the transfer, fresh isolated splenocytes were stimulated with various targets and IFN-γ production was measured by ICS (Figure 9). While the average absolute count of pmel T cells remained constant on day 34 relative to day 8 (Figure 8), there was a diminished frequency of IFN-γ-producing pmel T cells (Figure 9). However, over 25% of the pmel T cells were still functional even with the lower affinity murine gp100 (mgp100) peptide. This response was antigen specific as splenocytes made no IFN-γ when stimulated with the irrelevant peptide. The presence of a functional antigen-specific T cell population at this later time point suggests that reconstitution of lymphopenic mice with peptide pulsed splenocytes in combination with a

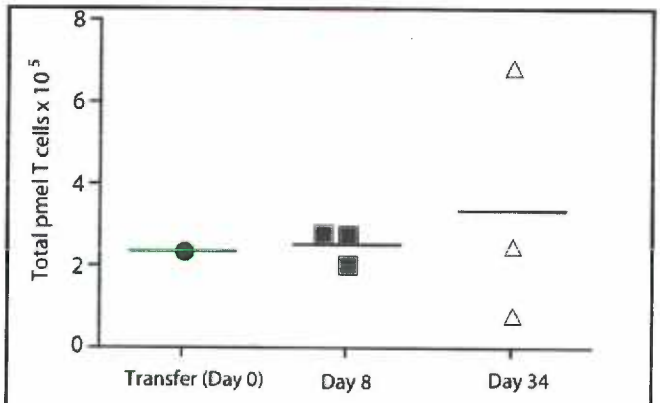


Figure 8: Vaccination of RLM with hgp100 and RC-527 maintains pmel T cell numbers. $1X10^6$ pmel and $9x10^6$ wt splenocytes were pulsed with 10μM hgp100 and 1μg of a RC-527 and used to reconstitute/vaccinate a lymphopenic host. Before transfer (day 0) or on days 8 and 34 splenocytes were stained for cytometric analysis with anti-CD8, anti-CD3, and anti-CD90.1 antibodies. Total number of pmel cells was determined by multiplying the frequency of $CD3^+/CD8^+/CD90.1^+$ pmel cells by the number of splenocytes recovered. 3 mice per group (days 8 and 34).

potent TLR4 ligand does not promote a tolerized immune response but sustains a modest yet functional population of antigen-specific T cells.

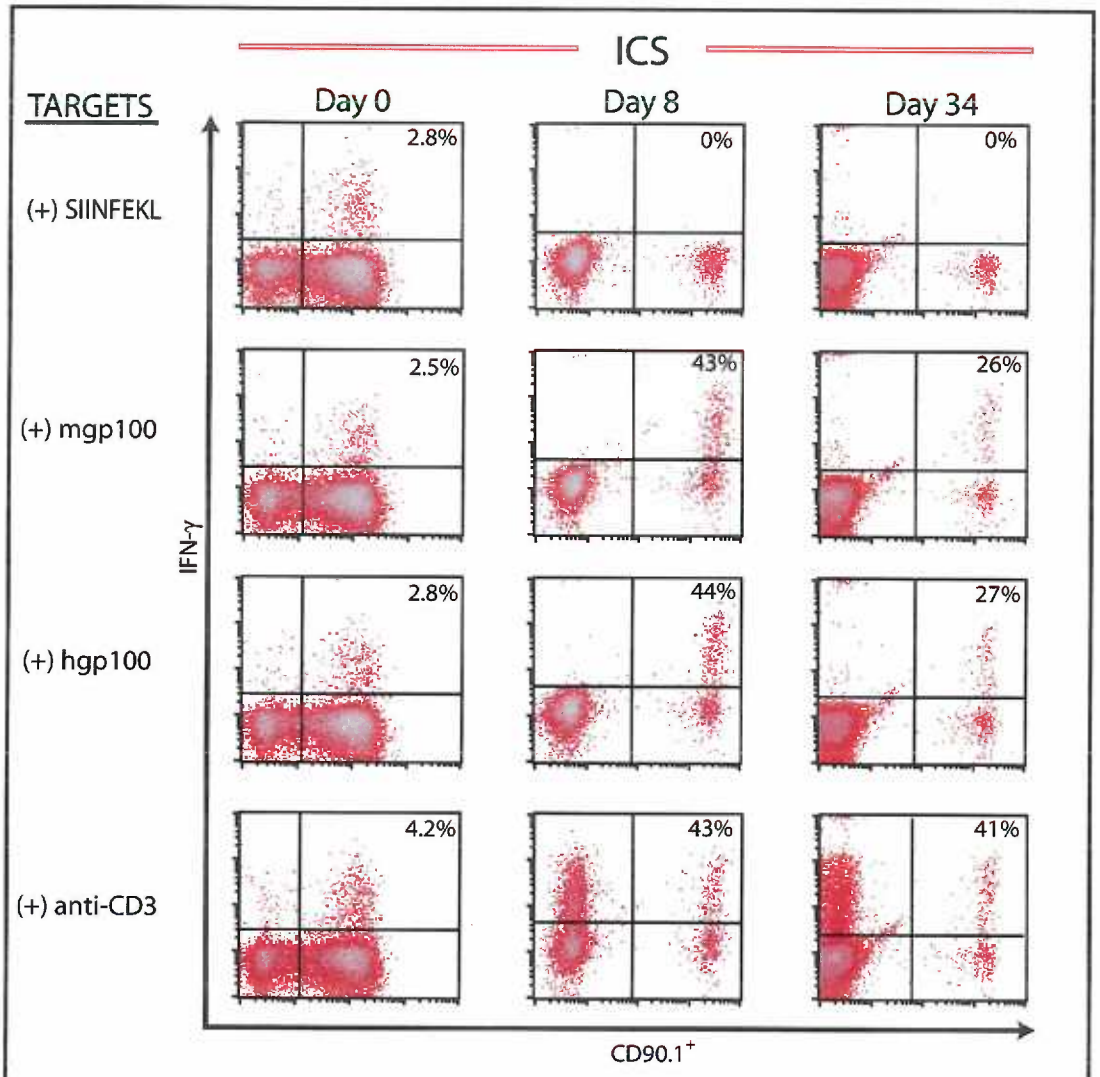


Figure 9: Vaccination of RLM with hgp100 and RC-527 sustains a functional population of pmel T cells. 1X10^6 CD90.1$^+$ pmel cells and 9x10^6 CD90.1$^-$ wt splenocytes were pulsed with 10μM hgp100 and 1μg of RC-527 and used to reconstitute/vaccinate a lymphopenic host. Splenocytes from either day 0, 8 or 34 were stimulated with 10μg of SIINFEKL, mgp-100, hgp100, or soluble anti-CD3 in the prescence of Brefeldin A for 8 hours at 37°C. Intracellular cytokine staining and flow cytometric analysis was performed by staining the cells with anti-CD90.1, anti-CD8, anti-CD3, and anti-IFN-γ antibodies. Numbers in the upper right quadrant indicate the percent of CD3$^+$/CD8$^+$/CD90.1$^+$ pmel cells producing IFN-γ vs. not producing IFN-γ. Representative of 3 mice

DISCUSSION

In trying to understand and positively impact tumor immunity, our laboratory has focused its research on multiple facets of the immune response including Th1 and Th2 polarization (185), CD4 T cell help (71, 72) (Friedman *et al.*, manuscript submitted), co-stimulatory molecules (71), effector molecules (135), peripheral tolerance mechanisms (131, 134), lymphopenia (70, 184) and whole cell vaccine requirements (93). While this study is partly an extension of nearly all of this research, the vaccine design was greatly influenced by research that exploited the lymphopenic environment to enhance tumor-specific T cell responses in tumor-bearing mice. Hu and colleagues demonstrated in an adoptive transfer model that T cells primed in a lymphopenic host could mediate tumor regression more efficiently than those primed in a lymphoreplete host (70). This observation coupled with compelling data from HIV immunotherapy studies where reinfusion of autologous peripheral blood mononuclear cells pulsed with viral peptides induced robust T cell-mediated viral responses (36) lead to the hypothesis that simultaneous reconstitution and vaccination with peptide-pulsed splenocytes in combination with a TLR ligand would stimulate a durable non-tolerizing antigen-specific immune response.

Initial experiments addressed the advantage of using a lymphopenic host as a recipient of adoptively transferred antigen-specific $CD8^+$ T cells. The impact of a lymphopenic environment was demonstrable by a large increase in the frequency and absolute counts of transferred T cells recovered from irradiated hosts versus an intact host (Figure 2). Homeostasis-driven proliferation likely contributed to the exceptional difference

of pmel T cell numbers recovered from the lymphopenic host relative to the lymphoreplete host as evidence by the effect of homeostasis-driven proliferation on the endogenous "filler" lymphocytes which had an increased absolute cell count in irradiated hosts versus intact host (Figure 2). However, both homeostasis-driven proliferation and antigen-driven proliferation are likely augmented in an environment where lymphocytes have a reduced signaling threshold, greater access to the co-stimulatory molecules as well as MHC Class I and II molecules of APCs, and increased availability of leukocytotropic cytokines such as IL-7 and IL-2 (55, 172, 173).

To specifically address the contribution of antigen-driven proliferation in the RLM, the reconstituting pool of splenocytes was pulsed with or without antigen to allow the adoptive transfer to act as a vaccine or not. The incorporation of peptide into the reconstituting splenocytes resulted in greater than a doubling of antigen-specific T cell frequency relative to mice reconstituted without the peptide (Figure 4). While this experiment was not designed to specifically address T cell tolerance, the increase of antigen specific T cells with vaccination does suggest that a major mechanism of tolerance, deletion, has not occurred by day 8. Additionally, the ability of antigen to drive a greater frequency of pmel T cells than simply the lymphopenic condition (Figure 4) supports the conclusion that antigen-driven proliferation and not homeostasis-driven proliferation was primarily responsible for the increase in pmel T cell numbers in Figure 2. This should not imply that homeostasis-driven proliferation does not positively contribute to the vaccine strategy, as antigen-specific T cells that do not initially encounter antigen will have the benefit of the

lymphopenic condition to increase their likelihood of survival until they are properly stimulated.

Since both the dose and route of antigen administration used in this vaccine have been implicated in the induction of T cell tolerance (80), it was important to determine whether the vaccine was triggering an activated T cell phenotype. As expected, the addition of hgp100 increased the frequency of $CD62L^{low}/CD44^{high}$ antigen-specific T cells over two-fold (Figure 5A) but less than has been reported using a subcutaneous DC vaccination (179). While a variety of scenarios could explain the sub par activation of antigen-specific T cells using this vaccine, the focus was narrowed to related mechanisms of tolerance. One possibility is the rapid clearance of intravenously delivered peptides, leading to reduced availability of antigen compared with emulsified peptides delivered subcutaneously (80). While this possibility cannot be ruled out, in a macaque SIV model, peptide pulsed PBMCs transferred i.v. produced a strong virus-specific T cell response, suggesting that a peptide-pulsed reconstitution approach can provide a sufficient dose of antigen (36). The peptide-pulsed vaccine/reconstitution approach incorporates an *ex vivo* culturing of splenocytes with peptide which was designed to encourage antigen uptake and thus provide a depot. Immature or quiescent DCs and/or non-professional APCs capable of presenting the peptide may play a role in the poor activation as stimulation of T cells by non-professional APCs (10, 164) and even quiescent DCs (18) results in insufficient activation and tolerance. Since antigen presentation in the steady state is critical to tolerance (164), we hypothesized that by inducing an inflammatory environment in the *ex-vivo* splenocyte-peptide culture as well as

endogenously during reconstitution/vaccination, a stronger antigen-specific immune response would result.

Inclusion of the TLR4 ligand RC-527 into the vaccine was able to strongly activate pmel T cells as indicated by the superior activation phenotype in Figure 5A, supporting the above hypothesis. The criteria for using the TLR agonists were that they had to be tolerated by the reconstituted lymphopenic mouse and enhance the T cell immune response in an antigen-specific manner. The TLR4 agonist RC-527 was chosen not only because the maximal dose of 10 μg was well tolerated by the irradiated mice but because a dose titration demonstrated that it was able to enhance antigen-specific T cell activation and frequency without non-specific activation of the "filler" T cells (Figure 5B-D and data not shown). By directly comparing the vaccine conditions, the additive benefit of including RC-527 into the vaccine was apparent in the increased frequency of IFN-γ^+ pmel T cells measured by ICS (Figure 7) as well as with release of both IFN-γ and IL-2 measured by ELISA (Figure 6). Based on the frequency, activation phenotype and function of the antigen-specific pmel T cells, the combination of TLR4 ligand and hgp100 peptide in the reconstitution/vaccination was able to stimulate a strong T cell response and limit tolerance. Antigen-specific T cell counts and function were measured on day 34 to gauge the efficacy of this vaccine strategy at a later time point. The average number of pmel T cells was not reduced relative to the day 8 time point, suggesting that antigen-specific T cells were not deleted in at least two of three mice (Figure 8). On day 34 there was a reduced frequency of antigen-specific IFN-γ^+ T cells,

yet over 25% of the transferred pmel cells were still functional when stimulated with human or murine gp100 after 34 days (Figure 9).

Collectively, the data describes a novel vaccine strategy cable of stimulating a durable, antigen-specific immune response. While the simplicity of this approach has clinical relevance, the advantage of this vaccine needs to be compared to a peptide-pulsed dendritic cell vaccine or the "golden standard" of a whole cell vaccine protocol and be evaluated in the context of a prophylactic or therapeutic tumor model. Further, there are still critical parameters of this reconstitution/vaccination that need to be addressed. Specifically, $CD4^+$ T cell help, which can be critical to T cell priming during active-specific immunity (72), memory formation (170, 171), and trafficking of $CD8^+$ T cells (118) was not addressed with these experiments and may significantly augment the vaccination. Inclusion of multiple gp100 epitopes was initially tested with polyclonal splenocytes with limited success in preliminary experiments (data not shown). However, based on the results presented above, additional experiments using this model with the multiple peptide epitopes capable of priming both $CD4^+$ T cells and $CD8^+$ T cells warrants further testing. While there are clearly variables that need further definition, the work presented in this chapter demonstrates the feasibility of a novel vaccine strategy that may be combined with or serve as an alternative to whole cell vaccines.

CHAPTER THREE: Vaccination with short-lived proteins incorporated into autophagosomes isolated from bortezomib-treated sarcomas induces cross-protective immune responses

ABSTRACT

Tumor-specific antigens of 3-methylcholanthrene (MCA)-induced sarcomas were defined by the narrow immune responses they elicited, which uniquely rejected the homologous tumor, with no reproducible cross-reactions between different MCA-induced tumors. Here, we have demonstrated that in contrast to a whole cell tumor vaccine, autophagosomes isolated from MCA-induced sarcomas treated with a proteasome inhibitor prime T cells that cross-react with different sarcomas and protect a significant proportion of vaccinated hosts from a non-homologous tumor challenge. Our data suggests that ubiquitinylated short-lived proteins (SLiPs), which are stabilized by proteasome blockade and delivered to autophagosomes in a p62/sequestosome-dependent fashion, are a critical component of the autophagosome vaccine as their depletion limits the survival of vaccinated mice challenged with either a homologous or a non-homologous tumor. Collectively, these data fundamentally redefine the MCA-induced tumor paradigm of antigenically distinct sarcomas. This work suggests that common short-lived sarcoma-specific antigens are sequestered in autophagosomes and can provide cross-protection against independently-derived sarcomas.

INTRODUCTION

Cross-presentation was identified as a means by which antigens can be presented by cells in which they were not synthesized, thus obviating direct presentation as the sole mechanism to prime an immune response (15). These findings were expanded by the demonstration that cross-presentation of melanoma antigens during vaccination was essential for the generation of an effective anti-tumor immune response (73). One component of cross-presentation that has been debated and still remains unknown is the source of antigen and the method of its delivery to pAPC. While some groups have shown that the source of antigen is cellular protein, others argue that it is peptides chaperoned by heat-shock proteins (HSPs) (92, 153). To complicate the debate further, Johanthan Yewdell's group demonstrated that cross-priming results from both the donation of proteasome substrates as well as stable cytosolic peptides in conjunction with HSP90 (97, 121). Recently, we described a pool of antigen used for cross presentation that is dependant on macroautophagy (hereafter referred to as autophagy).

We have previously shown that autophagy in tumor cells is essential for efficient cross-presentation and subsequent induction of tumor immunity (99). Cross-presentation, which was measured by proliferation of CFSE-labeled antigen-specific T cells, was significantly inhibited when autophagy was blocked and increased when autophagy was promoted. Interestingly, when cell lysates were fractionated and used as an antigen source, the fraction with the greatest cross-presentation activity also had the highest level of the specific autophagosome marker LC3. By treating cells with the proteasome inhibitor,

bortezomib, and the lysosomotropic agent, NH_4Cl, which prevents fusion of autophagosomes with lysosomes, autophagosome-containing vesicles could be isolated. These isolated autophagosome-containing vesicles, termed DRibbles (98) served as a potent antigen source in cross-presentation assays and in *in vivo* vaccine studies. In combination with the results of two recent publications, which demonstrated enhanced antigen presentation related to autophagy (44, 76), our work has further defined the function of autophagy as a means of sequestering antigen for cross-presentation.

To understand better the function of autophagy in cross-presentation, we developed a model that incorporates the DRiP hypothesis (191). A significant proportion of MHC class I binding peptides originate from defective ribosomal products (DRiPs), including misfolded and truncated polypeptides, which are degraded by the proteasome shortly after their translation and loaded onto MHC class I molecules (121). Since DRiPs, as well as other short-lived proteins (SLiPs), are stabilized by proteasome inhibition, we hypothesized that autophagosome-containing vesicles isolated from bortezomib-treated cells would contain DRiPs and SLiPs and thereby provide a unique spectrum of potential tumor rejection antigens. We further hypothesized that using these vesicles to prime an immune response will generate a broader T-cell response.

To test these hypotheses, two different model systems were used: stably transduced cell lines expressing either short-lived or more stable model antigens and the well-established chemically induced sarcoma tumor model (137). Prehn and Main established the unique specificity of chemically induced 3-methycholanthrene (MCA) sarcomas, whereby sarcomas

generated in genetically identical mice with similar morphology and growth characteristics would only protect vaccinated mice from a challenge with the immunizing tumor but not other syngeneic sarcomas. While there has been a paucity of antigens associated with the unique specificity of this tumor model (75), genetic analysis of a MCA-induced sarcoma after CTL immunoselection revealed a deletion in a region rich with oncogenes and tumor suppressor genes (4). Even though a unique immunodominant antigen results from each MCA treatment, this data demonstrates that specific loci or chromosomal regions are more susceptible to the mutating effects of MCA. Moreover, using CTL immunoselection, a secondary tumor antigen shared by an independent sarcoma cell line was uncovered, demonstrating that the unique rejection antigen is only part of the tumor antigen profile (39). Others have demonstrated cross-reactivity among heterogenic clones of the MCA-106 sarcoma using effector cells primed with the parental MCA-106 line but no cross-reactivity between an "antigenically" distinct MCA-205 sarcoma (110). There are therefore limited examples of common antigens among the MCA-induced sarcomas in the few publications reported.

In this thesis, we examine the role of autophagy in tumor immunity by focusing on autophagosomes as the source of antigen for cross-presentation. We have demonstrated that vaccination with antigens derived from autophagosomes can broaden the T-cell response beyond that seen following whole cell vaccination. Studies using MCA sarcomas as well as HEK 293T cells stably expressing a short-lived model antigen both indicate that short-lived proteins are necessary for this unique autophagosome-mediated immune response. Further,

we demonstrate that the ubiquitin/LC3-binding protein sequestosome (p62) may be a key regulator of selective autophagy, as it associates with both ubiquitinylated antigen and LC3 and is needed for the sequestration of SLiPs into the autophagosomes. Based on these findings, we propose that the broad array of tumor antigens contained in autophagosomes is dependent upon ubiquitinylated SLiPs incorporated by the sequestosome.

MATERIALS AND METHODS

Mice and cell lines

Female C57BL/6J (B6) mice were purchased from Charles River (Wilmington, MA). OT-1 breeders transgenic for the TCR that recognizes chicken ovalbumin (peptide sequence SIINFEKL) in the context of H-2K^b were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were maintained and used in accordance with the Earl A. Chiles Research Institute Animal Care and Use Committee and recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996).

Human embryonic kidney 293T cells were cultured in complete medium (CM), which consisted of DMEM (Lanza) supplemented with 10% FBS (Life Technologies, Grand Island, New York). Cell lines were maintained in T-75 or T-150 culture flasks in a 5% CO_2 incubator at 37 °C. Stable expression of both the stable OVA (mOVA) and short-lived OVA

(rOVA) in HEK 293T cells was achieved with lentiviral transduction as described earlier (99). For the generation of recombinant lentiviruses, HEK 293T cells were transiently transfected with vector plasmid pWPT or pGIPz, virus packaging plasmid pPAX2, envelope plasmid VSV-G MD2, and helper plasmid pAdv. Viral supernatant was used to infect HEK 293T cells and transduced cells were sorted based on GFP expression by flow cytometry.

3-Methylcholanthrene (MCA)-induced sarcomas were previously generated in our laboratory (185, 186). Unique MCA sarcomas were passaged *in vivo* by excising a tumor from a female C57BL/6J mouse followed by triple enzyme digestion of the tumor (incubation at room temperature with hyaluronidase, collagenase and DNAse for 1-3 hours with agitation) and subcutaneous injection into a naïve mouse.

B16BL/6J-D5 (D5), a poorly immunogenic subclone of the spontaneously arising B16BL6 melanoma, was kindly provided by Dr. S. Shu (Earle A. Chiles Research Institute, Portland, Oregon). D5 cells were cultured in complete medium (CM), which consisted of RPMI 1640 (BioWhittaker,Walkersville, Maryland) supplemented with 10% FBS (Life Technologies, Grand Island, New York), 50μM 2-mercaptoethanol (Aldrich, Milwaukee, Wisconsin), 0.1mM non-essential amino acids, 1 mM sodium pyruvate, 2mM L-glutamine and 50μg/mL gentamicin sulfate. Cell lines were maintained in T-75 or T-150 culture flasks in a 5% CO_2 incubator at 37 °C.

Antigen presenting cells (APC) were generated in the spleens of C57BL6 mice via hydrodynamic gene transfer (169). Female C57BL/6J (B6) mice were sequentially injected ~2 mL i.v. with 2μg of plasmid DNA encoding murine Flt3 ligand and granulocyte macrophage colony stimulating factor. The spleens are typically enlarged and enriched for $CD8^+/CD11c^+$ cells (26, 64, 99). Spleens were harvested and processed into a single cell suspension and frozen.

Tumor vaccine and challenge

Female C57BL/6J mice were vaccinated in the lower right flank with a subcutaneous injection of $5x10^6$ irradiated tumor cells from a freshly digested sarcoma or with $3x10^6$ APCs pulsed with autophagosomes ($3x10^6$ cell equivalents) for 6-8 hours. To control for the use of dendritic cells in the autophagosome vaccine, $3x10^6$ APCs pulsed with $5x10^6$ irradiated tumor cells for 6 hours, were washed and used as a vaccine as described above. For the cyclohexamide experiments, $5x10^6$ frozen tumor cells were thawed, irradiated and used as vaccine. Mice were challenged 2 weeks after vaccination by subcutaneous injection in the lower left (opposite) flank with $2\text{-}3x10^4$ viable digested MCA tumor cells. Tumor growth was assessed by measuring the perpendicular diameters of the sarcoma. Mice were sacrificed when the area of the tumor, determined by the product of the perpendicular diameters, reached 150 mm^2 or greater.

Isolation of autophagosome-containing vesicles

Autophagosome-containing vesicles (DRibbles) were harvested from HEK 293 cells or tumor cells after treating the cells with 100nM bortezomib (Velcade) and 10 mM of NH_4Cl in CM for 18-24 hours in a 5% CO_2 incubator at 37°C. The cells and the supernatant were harvested and spun at 480 x g, as described by Stromhaug *et al.* (166). The supernatant was then spun at 12,000 x g to harvest the autophagosome-containing pellet. Based on the amount of cells originally seeded, the autophagosome pellet was resuspended at 10^8 cell equivalents (CE) per mL (typically in the range of 0.5-1 mg/mL total protein by BCA).

DNA Construction and Transfection

Plasmid DNA vector cloning was described earlier (99). Briefly, Ub-X-GFP-expressing plasmids were fused with an OVA antigen by PCR with Vent polymerase and cloned into the lentiviral vector pWPT (kindly provided by Dr. D Trono at the Department of Microbiology, Geneva School of Medicine, Geneva, Switzerland). The LC3 fusion plasmid, pCMV-GFP-LC3 and the p62 fusion plasmid, pCMV-tdTomato-p62 were kindly provided Dr. T Johansen (Biochemistry Department, Institute of Medical Biology, University of Tromso, Tromso, Norway). Both the LC3 and p62 constructs were sub-cloned into the pWPT vector using PCR with Vent polymerase. Transient transfections of HEK 293FT cells with LC3, p-62 or OVA expressing vectors were performed using metafectene pro (Biontex Laboratories GmbH).

Measurement of cytokine production by primed LN T cells from vaccinated mice

To measure the priming of naïve lymphocytes, mice were vaccinated in both the fore and hind flanks by subcutaneous injection of $1x10^6$ irradiated sarcoma cells or $2x10^6$ dendritic cells pulsed with $3x10^6$ cell equivalents of autophagosomes. Draining lymph nodes (DLN) were harvested after 11 days and cultured with soluble anti-CD3 (5ug/mL) for 48 hours and then expanded with IL-2 (60 IU/mL) for 72 hours. After this *in vitro* activation and expansion, effector T cells were washed, resuspended in CM and IL-2 (60 IU/ml), and seeded at $2x10^6$/2 ml/well in a 24-well plate. The cells were either cultured without further stimulation or stimulated with $2x10^5$ D5, primary (triple enzyme digested) MCA-304, MCA-309, MCA-310, MCA-311 tumor cells, primary kidney cells or immobilized anti-CD3 (positive control). Supernatants were harvested after 24 h and assayed for IFN-γ by ELISA using commercially available reagents (IFN-γ, BD Biosciences Pharmingen, San Diego, CA). The concentration of cytokines in the supernatant was determined by regression analysis.

siRNA knockdown of sequestosome (p62)

siGENOME SMARTpool M-010230-00-0005 against human SQSTM1 (p62) was purchased from Dharmacon (Thermo Scientific). Non-specific control siRNA sc-36869 was purchased from Santa Cruz Biotechnology. 50 nM of either siRNA was transfected into HEK 293 T cells using Invitrogen's lipofectamine 2000.

Immunoprecipitation/Western blotting

Radioimmunoprecipitation assay (RIPA) buffer with a protease inhibitor cocktail (Roche) was used to lyse autophagosomes isolated from treated 293rOVA-GFP or parental HEK 293T cells. The lysate (50 μg) was initially incubated with 100 μL of protein A/G agarose (Thermo scientific). The pre-cleared lysate (20 μg) was then incubated overnight with 1 μg of a goat anti-GFP antibody (Rockland). Fresh protein A/G agarose was added to the anti-GFP/lysate mixture and incubated for 4 hours. 60uL of 2X NuPAGE LDS buffer were added directly to beads. The beads were subjected SDS PAGE and western blotting as described below.

For western blots, HEK 293T cells ($1x10^6$) or autophagosomes ($10x10^6$ cell equivalents) were lysed in 100 μL of radioimmunoprecipitation assay (RIPA) buffer with a protease inhibitor cocktail (Roche). The lysates were mixed with 4X NuPAGE LDS sample buffer and samples were resolved by 4% to 20% SDSPAGE (Invitrogen). Proteins were transferred to a nitrocellulose membrane, incubated with primary antibodies, diluted in blocking buffer (5% dry milk) overnight, and then incubated with horseradish peroxidase (HRP)–conjugated secondary antibodies for 1 h. Protein bands were revealed by using chemiluminescent reagents (Pierce). The primary antibodies included rabbit anti-actin (1:2,000; Sigma), goat anti-GFP (1:1,000; Rockland), rabbit anti-ubiquitin (1:1,000; Upstate), rabbit anti-LC3 (1:1,000; MBL) and goat anti-sequestosome (1:700; santa cruz biotechnology). The secondary antibodies were goat anti-rabbit HRP (1:10,000; Jackson ImmunoResearch), and donkey anti-goat HRP (1:10,000; Jackson ImmunoResearch).

CFSE proliferation assay

CFSE-labeled naïve T cells from ovalbumin-specific OT-1 TCR transgenic mice were added to APCs pulsed with autophagosomes, SIINFEKL peptide (10 μg) or whole soluble ovalbumin (50 μg). Cross-presentation of antigens to labeled OT-I T cells was assessed by measuring the dilution of CFSE by flow cytometry. Splenocytes from OT-I mice were labeled with 5 μmol/L of CFSE according to the manufacturer's protocol (Invitrogen). T-cell proliferation was measured as loss of CFSE intensity after 3 or 4 days of APC and T-cell co-incubation.

Confocal Microscopy

The images were acquired at the Advanced Light Microscopy Core at The Jungers Center of Oregon Health and Science University on a high resolution wide field Core DV system (Applied Precision™). This system is an Olympus IX71 inverted microscope with a proprietary XYZ stage enclosed in a controlled environment chamber; differential interference contrast (DIC) transmitted light and a short arc 250W Xenon lamp for fluorescence. The camera is a Nikon Coolsnap ES2 HQ. Each image was acquired as Z-stacks in a 1024x1024 format with a 60x1.42 NA Plan Apo N objective in 3 colors: green, red and blue. The pixel size was 0.107 x 0.107 x 0.5 microns. The images were deconvolved with the appropriate OTF (optical transfer function) using an iterative algorithm of 10 iterations. The histogram was optimized for the most positive image and applied to all

the other images for consistency before saving the images as 24 bit merged TIFF. A reference DIC image was acquired from the middle of the Z-stack.

Statistical Methods

For the time-to-death endpoint, the survival distribution was estimated using the Kaplan-Meier method. The log-rank test was used to compare the hazard rates of the vaccines.

For ELISA data, the $\log_{10}$-transformed IFN-γ values were analyzed using a mixed effects model that appropriately accounted for different sources of variability introduced by the experimental design. This model had fixed effects for the vaccine group, stimulator, and their interaction, and random effects for the experiment (nested within vaccine group), the stimulator pool interaction, and the assay replicate. Different variances were allowed for each vaccine group. Contrasts using least squares means for each stimulator-vaccine group combination were used to test for mean differences. A significance level of α=0.05 between different vaccine groups was limited to individual stimulators. The significance level was α=0.05. All analyses were done using SAS v9.2 using either PROC LIFETEST or PROC MIXED.

RESULTS

Vaccination with autophagosome-containing vesicles derived from 3-MCA-induced sarcomas generates cross-reactive T cells.

Previously generated MCA sarcomas were irradiated and used as a subcutaneous vaccination to protect mice from independently generated tumors. Similar to what has been described (137), irradiated MCA-310 cells protected mice against a MCA-310 tumor challenge but not a MCA-311 tumor challenge. Conversely, irradiated MCA-311 cells protected mice against a MCA-311 tumor challenge but not against MCA-310 (Figure 10A). To understand better the lack of immunity against a related but non-vaccinating tumor such as illustrated in Figure 10A, MCA-304 and MCA-310 tumor-draining lymph nodes were harvested, activated in vitro with anti-CD3 and expanded with IL-2. The resultant effector T cells were assayed for their ability to secrete IFN-γ when cultured with different tumor targets. Both MCA-304 and MCA-310 primed effectors secreted significant amounts of tumor-specific IFN-γ when stimulated with the immunizing tumor (Figure 10B, black bars). Both groups of effector T cells failed to secrete significant IFN-γ in response to all other targets, including the non-vaccinating MCA-induced sarcomas. Based on our hypothesis that SLiPs or DRiPs incorporated into autophagosomes will provide unique antigens that will broaden the T-cell response, autophagosome-containing vesicles were isolated from intact sarcomas treated overnight with the bortezomib and used as a subcutaneous vaccine. Surprisingly, the autophagosome vaccine derived from MCA-304 tumors primed an immune

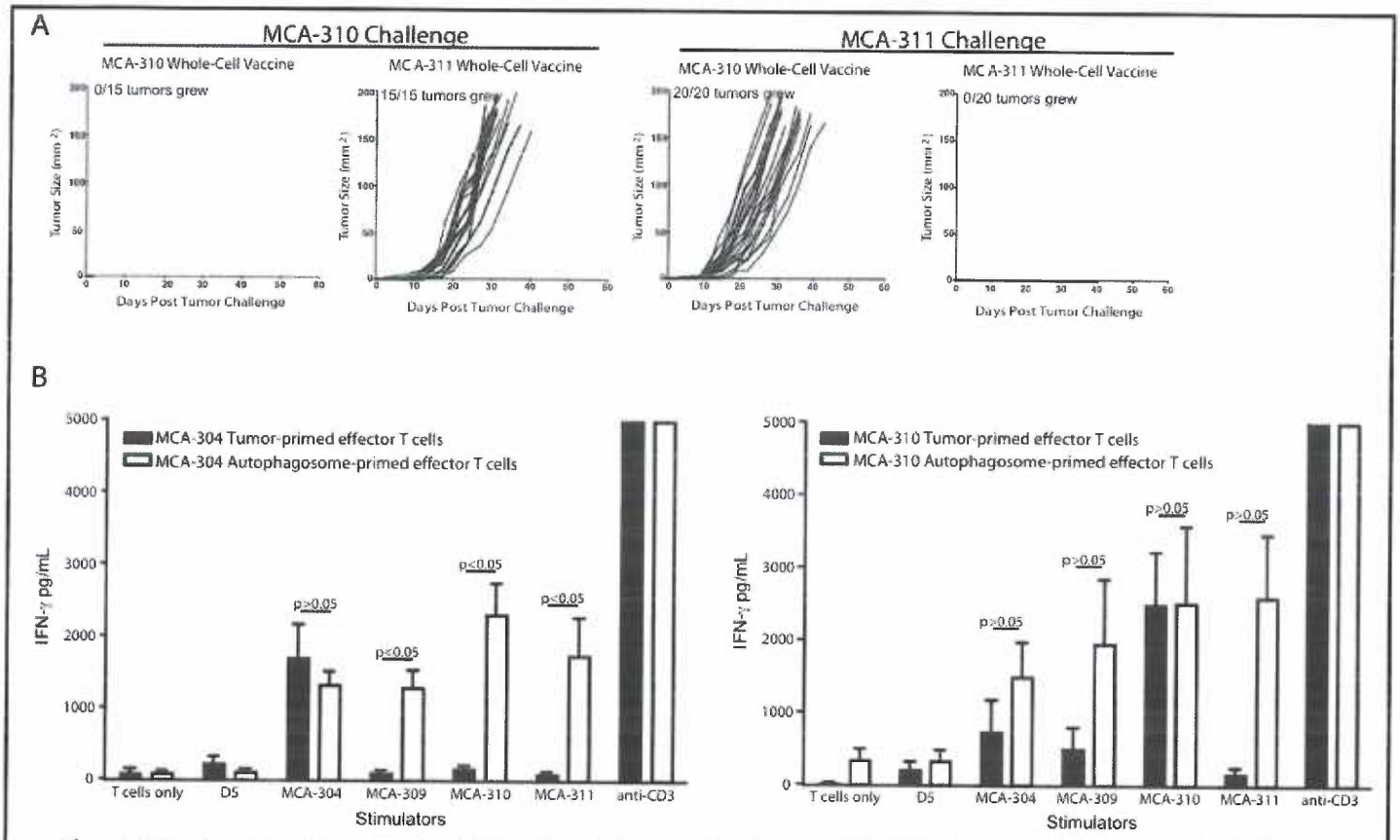


Figure 10: Isolated autophagosomes prime a broader array of T cells. (A) Tumor growth curves of mice vaccinated with $5x10^6$ irradiated sarcoma cells and challenged 14 days later with 30,000 sarcoma cells. Tumor growth was monitored twice weekly. Each growth curve represents either 3 independent experiments with 5 mice per group (n=15 mice) or 4 independent experiments with 5 mice per group (n=20). (B) ELISA measuring IFN-γ secreted by effector T cells primed with either a whole cell or an autophagosome vaccine. T cells were stimulated with targets and supernatants were harvested after 24 h (n=3 independent experiments except for MCA-310 autophagosome-primed effectors stimulated with MCA-311 and MCA-309 sarcomas (n=2 independent experiments)).

response to not only MCA-304 but also to other independently derived sarcomas (Figure 10B, grey bars). A similar trend was observed with an autophagosome vaccine derived from MCA-310 sarcomas. These responses were specific because the autophagosome vaccine did not prime T cells to react with the syngeneic, but unrelated, B16 melanoma subclone (D5) or a primary kidney cell line (Figure 10B and data not shown). This suggests that an autophagosome vaccine may prime T cells to a more diverse sarcoma-related antigenic repertoire.

Autophagosome vaccination protects against multiple independently derived sarcomas.

To determine if the autophagosome vaccine would not only prime T cells to a broader array of tumor or tumor-associated antigens, but would also protect the vaccinated host from different sarcomas, we vaccinated groups of mice with irradiated MCA-induced tumors or autophagosomes derived from these tumors before tumor challenge. As in Figure 10A, vaccination with irradiated whole tumor cells provided complete protection against a challenge with the homologous (immunizing) tumor. Although the irradiated whole cell vaccine protected more mice than an autophagosome vaccine derived from the same tumor, the autophagosome vaccine provided immunity against not only the homologous sarcoma, but also against other independently derived sarcomas (Figure 11A-F). This autophagosome-mediated cross-protection was evident in 8 of the 9 combinations tested whereas the whole cell vaccine provided cross-protection in 0 of 9 combinations tested (Figure 12). These results coupled with the previous experiment in Figure 10B demonstrate that autophagosome-containing vesicles derived from MCA-induced sarcomas induce the cross-presentation of additional epitopes that can prime a T-cell population capable of cross-reacting and protecting the host from a challenge with sarcomas other than the one used during vaccination.

To control for the possibility that the addition of antigen-presenting cells (APCs) potentiates the cross-protection observed with the autophagosomal vaccination, APCs were pulsed with irradiated whole cells and used as a vaccine. Similar to what was observed in

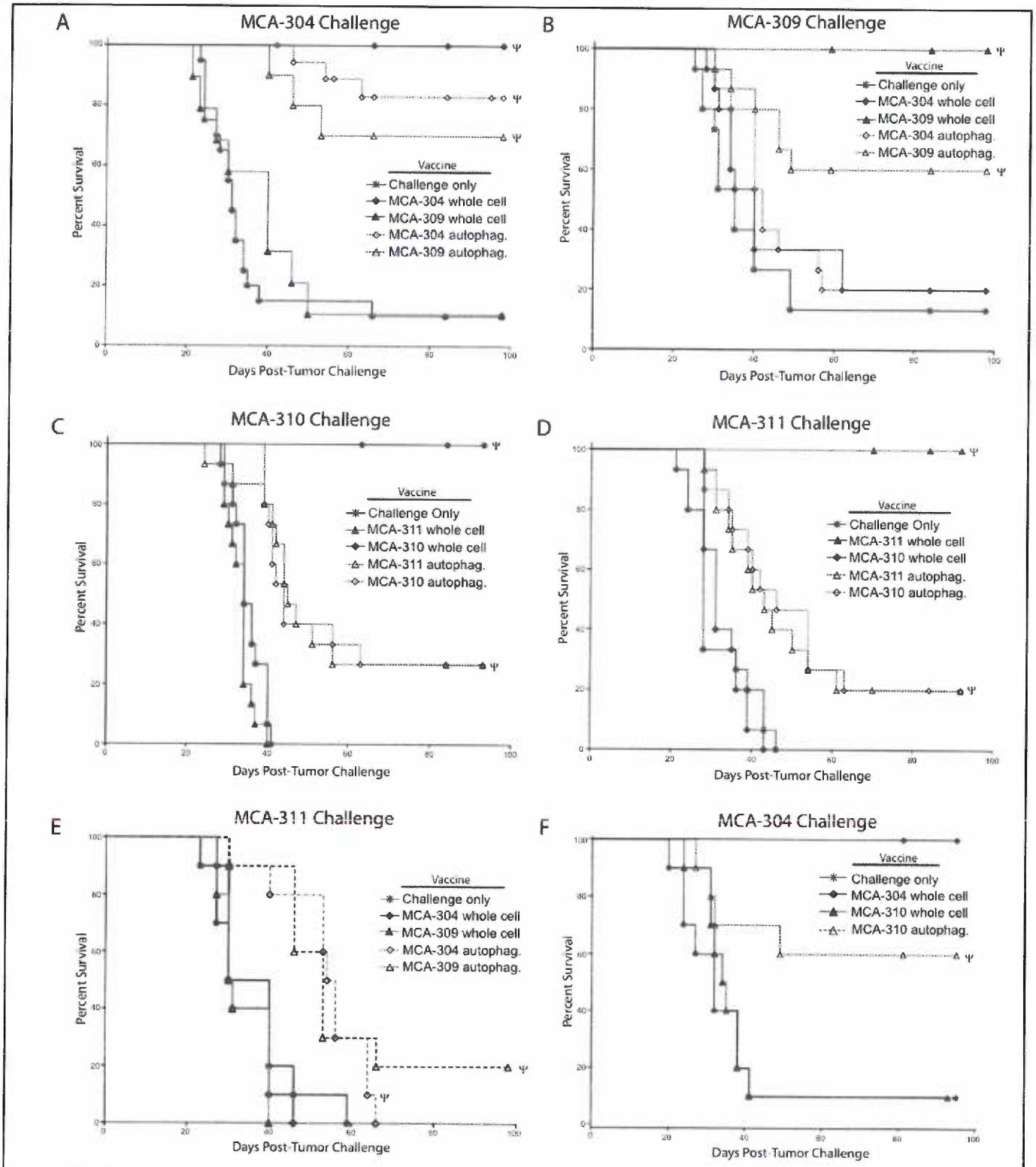


Figure 11: Autophagosome vaccination protects mice from a challenge against non-homologous sarcomas. (A-F) Kaplan-Meier survival plots comparing protection of mice vaccinated with an irradiated whole cell sarcoma or autophagosomes derived from the sarcomas. Autophagosome vaccination consisted of $3x10^6$ APCs pulsed with $3x10^6$ CE of autophagosomes and was subcutaneously injected into the lower right flank of the mouse. Whole cell vaccinations were performed as above in Figure 10A. Vaccinated mice were challenged with 30,000 viable (A and F) MCA-304, (B) MCA-309, (C) MCA-310 or (D and E) MCA-311 tumors. Survival curves denoted with Ψ were significantly different than the corresponding no vaccine group of mice ($p<0.05$). Each survival plot represents (A-D) 3 independent experiments with 5 mice per group (n=15) or (E and F) 2 independent experiments with 5 mice per group (n=10) .

experiments without APCs, whole tumor cell vaccination with APCs was able to protect vaccinated mice from a challenge with the immunizing tumor (Figure 13). In contrast to the autophagosomal vaccination, which was able to cross-protect vaccinated mice, whole cell vaccination with APCs could not cross-protect mice from a challenge with a closely related but independently derived sarcoma in the four combinations tested (Figure 13).

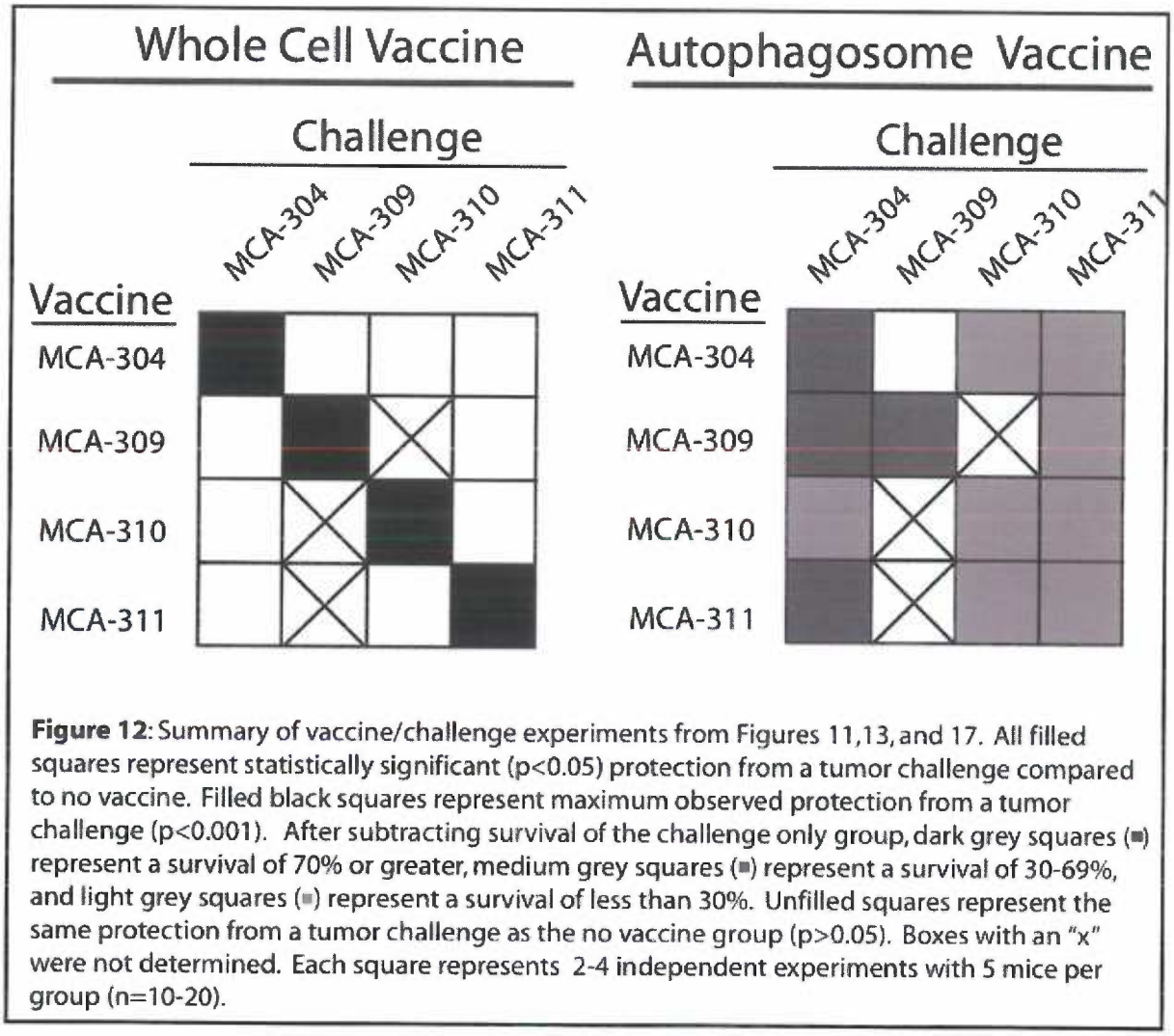


Figure 12: Summary of vaccine/challenge experiments from Figures 11,13, and 17. All filled squares represent statistically significant ($p<0.05$) protection from a tumor challenge compared to no vaccine. Filled black squares represent maximum observed protection from a tumor challenge ($p<0.001$). After subtracting survival of the challenge only group, dark grey squares (■) represent a survival of 70% or greater, medium grey squares (■) represent a survival of 30-69%, and light grey squares (■) represent a survival of less than 30%. Unfilled squares represent the same protection from a tumor challenge as the no vaccine group ($p>0.05$). Boxes with an "x" were not determined. Each square represents 2-4 independent experiments with 5 mice per group (n=10-20).

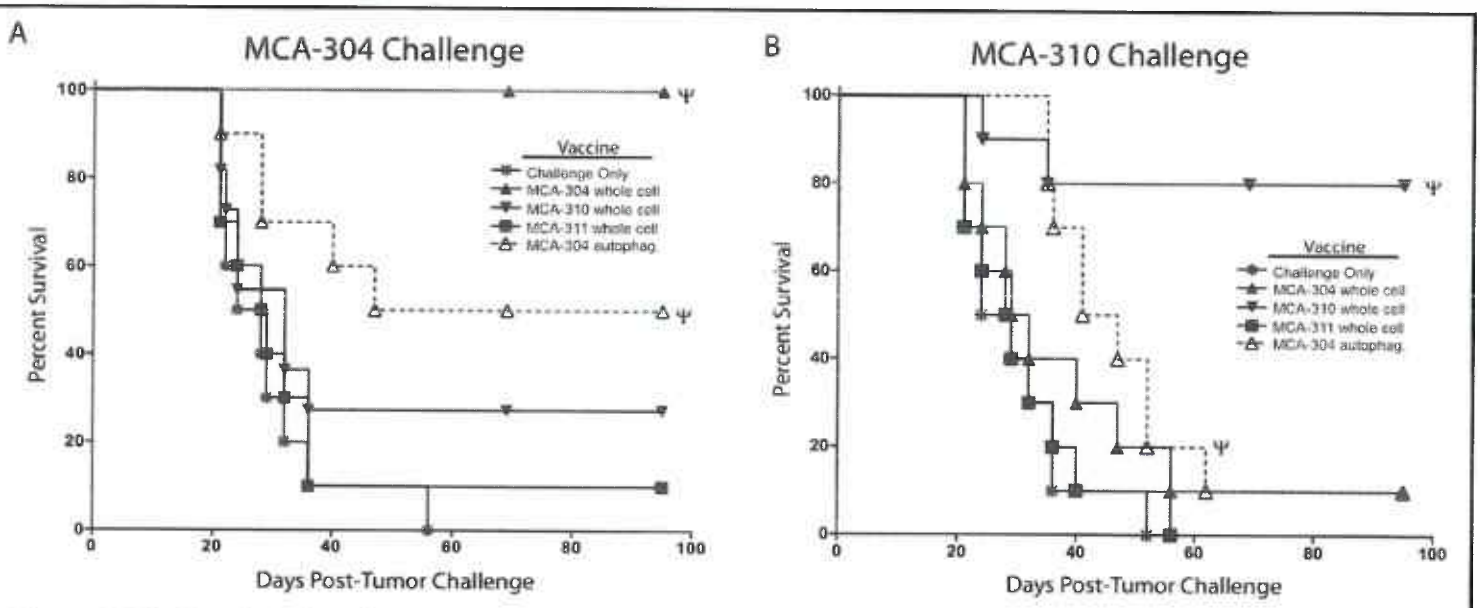


Figure 13: Irradiated whole cell sarcoma-pulsed APCs do not protect mice from a challenge against non-homologous sacomas. Kaplan-Meier survival curves of mice vaccinated with irradiated whole cell sarcoma-pulsed APCs or autophagosome-pulsed APCs. Autophagosome vaccination was performed as in figure 11. Whole cell vaccinations consisted of $3x10^6$ antigen presenting cells pulsed with $5x10^6$ irradiated tumor cells that were subcutaneously injected into the lower right flank of the mouse. Mice were challenged with 30,000 viable (A) MCA-304 or (B) MCA-310 sarcomas. Tumor growth was monitored at least twice weekly. Each survival plot represents 2 independent experiments with 5 mice per group (n=10). Survival curves denoted with Ψ represent statistically significant ($p>0.05$) protection from a tumor challenge compared to no vaccine.

Autophagosomes contain short-lived proteins that can induce proliferation of naive antigen-specific T cells

To understand how this cross-protection with the autophagosome vaccine may work, we used a model antigen system to test the hypothesis that short-lived proteins were necessary for autophagosome-induced immunity. HEK 293T cells were transduced to stably express the model antigen ovalbumin (OVA) with a moderate half-life (approximately 8-10 hours) or a shorter half-life (approximately 20 minutes) (99). The half-life of the model proteins was controlled by inserting either a stabilizing methionine residue (mOVA) or a

destabilizing arginine residue (rOVA) at the N-terminus of the protein as determined by the N-end rule (9). Additionally, to detect the model antigen more easily, the OVA proteins were fused with an enhanced green fluorescent protein (eGFP) (99). The accumulation of fusion proteins in the HEK 293 T cells was measured by flow cytometry for the presence of eGFP (Figure 14A). The amount of the stable model protein mGFP-OVA remained nearly constant when cultured overnight with or without proteasome inhibition. In contrast, rapid turnover of the short-lived model antigen (rGFP-OVA) was proteasome-dependent; blockade of the proteasome with bortezomib increased the expression of the rGFP-OVA protein.

Autophagosomes ($3x10^6$ CE) isolated from both 293rGFP-OVA and 293mGFP-OVA cells treated with or without the proteasome inhibitor, bortezomib, were pulsed onto antigen-presenting cells for 6 hours and used to

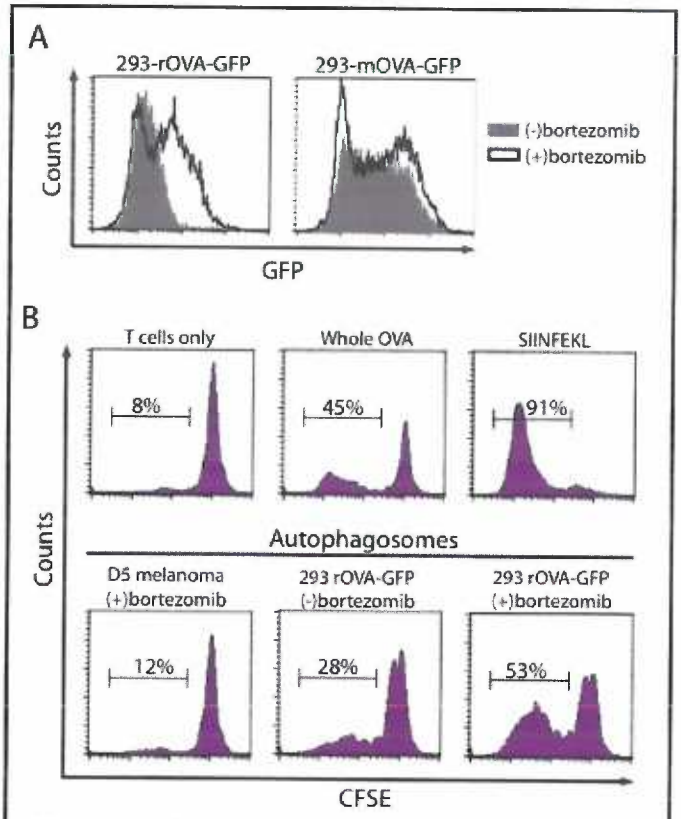


Figure 14: Short-lived proteins found in autophagosomes isolated from bortezomib-treated cells stimulate antigen-specific T cells. (A) Expression of the short-lived GFP-OVA fusion protein (rGFP-OVA) or the stable GFP-OVA fusion protein (mGFP-OVA) in HEK 293T cells treated with or without bortezomib was measured by flow cytometry (solid line and filled plot respectively). (B) Proliferation of naïve antigen-specific OT-1 cells measured by CFSE dilution. CFSE-labeled naïve T cells from ovalbumin-specific OT-1 TCR transgenic mice were added to an autophagosome-pulsed APC culture for 3-4 days at 37°C. n=2 experiments.

stimulate naïve antigen-specific CFSE-labeled T cells. Proliferation of the stimulated T cells was measured by dilution of CFSE. Autophagosomes isolated from 293rGFP-OVA cells treated with bortezomib caused a substantial increase in T-cell proliferation compared to autophagosomes isolated from untreated cells (Figure 14B). This stimulation was not only antigen specific, as autophagosomes generated in an unrelated cell line could not drive T-cell proliferation, but also dependent on a short-lived protein.

Inhibition of protein synthesis before proteasomal blockade prevents accumulation of short-lived proteins.

To further define the relationship of short-lived proteins and autophagosomes, cells were pretreated with the protein synthesis inhibitor cyclohexamide (CHX) before the addition of bortezomib to prevent the expression and therefore inclusion of short-lived proteins into autophagosomes. To assess the effects of CHX, HEK 293T cells were depleted of all methionine in culture before being treated with CHX at 10 μg/mL or at 50 μg/mL or with vehicle alone for 4 hours. During this time, the cells were pulsed with a non-radioactive labeled methionine (L-azidohomoalanine) and visualized using a streptavidin-HRP-enzymatic reaction. This detection reaction can react with endogenous biotin, such as the biotin carboxylase subunits of acetyl-CoA (39 KDa band in Figure 15A). As little as 10ug/mL in 4 hours of cyclohexamide prevented synthesis of all proteins (Figure 15A).

We hypothesized that since mOVA is a stable protein, its expression would be detected after a 2 hour pretreatment with CHX prior to either a 4-hour or overnight culture with bortezomib whereas the short half-life of rOVA would lead to its diminished expression. Expression of GFP in HEK 293mGFP-OVA cells was essentially unchanged after 4 hours regardless of whether the cells were treated with bortezomib or CHX (Figure 15B, left panel). However, the expression of GFP in HEK 293rGFP-OVA cells was diminished in the absence of proteasome inhibition and absent following CHX pretreatment (Figure 15B, right panel). This reduced expression of the model short-lived protein was not due to decreased cell viability or a secondary effect of bortezomib or CHX as both 293rGFP-OVA cells and the 293mGFP-OVA cells treated with CHX and bortezomib or bortezomib alone had similar morphology (slightly more rounded cells with CHX), growth patterns and ability to exclude trypan blue even after an overnight culture (data not shown).

The expression of both mGFP-OVA and rGFP-OVA in HEK 293T cell lysates was additionally examined by western blot. Similar to the expression of GFP measured by flow cytometry, bortezomib had a modest effect on the presence of the more stable mOVA protein. However HEK 293mGFP-OVA cells cultured overnight with CHX and bortezomib did have a substantial reduction in the levels of the fusion protein (Figure 15C). Bortezomib was able to stabilize the rGFP-OVA fusion protein in HEK 293rGFP-OVA cells but not if the cells were pretreated with CHX before the addition of bortezomib.

Expression of GFP-OVA in autophagosomes isolated from both the mGFP-OVA and rGFP-OVA cells described above was also examined by western blot. Autophagosomes

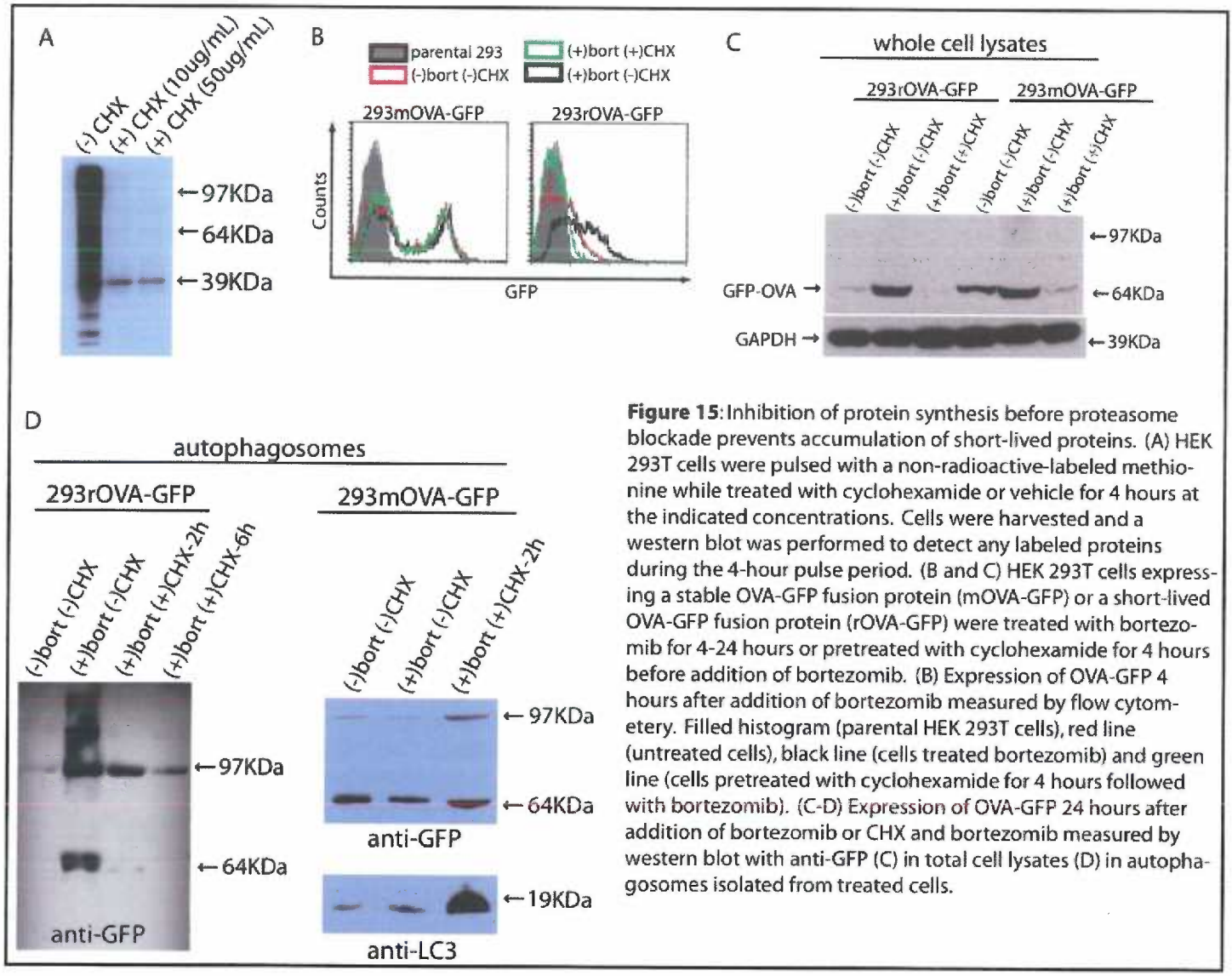


Figure 15: Inhibition of protein synthesis before proteasome blockade prevents accumulation of short-lived proteins. (A) HEK 293T cells were pulsed with a non-radioactive-labeled methionine while treated with cyclohexamide or vehicle for 4 hours at the indicated concentrations. Cells were harvested and a western blot was performed to detect any labeled proteins during the 4-hour pulse period. (B and C) HEK 293T cells expressing a stable OVA-GFP fusion protein (mOVA-GFP) or a short-lived OVA-GFP fusion protein (rOVA-GFP) were treated with bortezomib for 4-24 hours or pretreated with cyclohexamide for 4 hours before addition of bortezomib. (B) Expression of OVA-GFP 4 hours after addition of bortezomib measured by flow cytometery. Filled histogram (parental HEK 293T cells), red line (untreated cells), black line (cells treated bortezomib) and green line (cells pretreated with cyclohexamide for 4 hours followed with bortezomib). (C-D) Expression of OVA-GFP 24 hours after addition of bortezomib or CHX and bortezomib measured by western blot with anti-GFP (C) in total cell lysates (D) in autophagosomes isolated from treated cells.

isolated from HEK 293mGFP-OVA cells had nearly the same expression of mGFP-OVA regardless of treatment with bortezomib or CHX. Interestingly, the autophagosomal marker LC3 was overexpressed in the CHX treated cells relative to the +/- bortezomib groups (Figure 15D). This increase in LC3 has been demonstrated to occur when cells undergo stress, such as inhibition of protein synthesis (1). It is also of note that while the cellular

expression of mGFP-OVA pre-treated with CHX was reduced (Figure 15C), its expression in the autophagosome-containing vesicles was not (Figure 15D). This suggests that mOVA-containing autophagosomes blebbed from the cells, reducing the cellular expression of this fusion protein while maintaining its expression in the autophagosomes. The model short-lived protein, rGFP-OVA was absent in autophagosomes following CHX pretreatement, mirroring what was observed in the whole cell lysate. Therefore, by inhibiting protein synthesis before blocking the proteasome, autophagosome-containing vesicles have minimal amounts of short-lived protein.

Autophagosomes produced from cells that lack short-lived proteins have diminished capacity to stimulate antigen-specific T cells.

Autophagosomes both sufficient and deficient of short-lived proteins derived from cells expressing the model GFP-OVA protein were isolated according to the methods of Figure 15 and used as an antigen source to drive naïve antigen-specific OT-1 T-cell proliferation. Results from the CFSE proliferation assay demonstrated that autophagosomes from cells expressing the stable mGFP-OVA protein caused OT-1 T cells to proliferate at nearly the same level whether they were generated in the presence or absence of bortezomib, or with CHX (Figure 16A). These data provide evidence that CHX pretreatment does not prevent stable proteins from being sequestered in autophagosomes nor does it prevent the formation of "functional" autophagosomes. However, when HEK 293rGFP-OVA cells were treated with CHX, the resultant autophagosomes were unable to induce antigen-specific T-

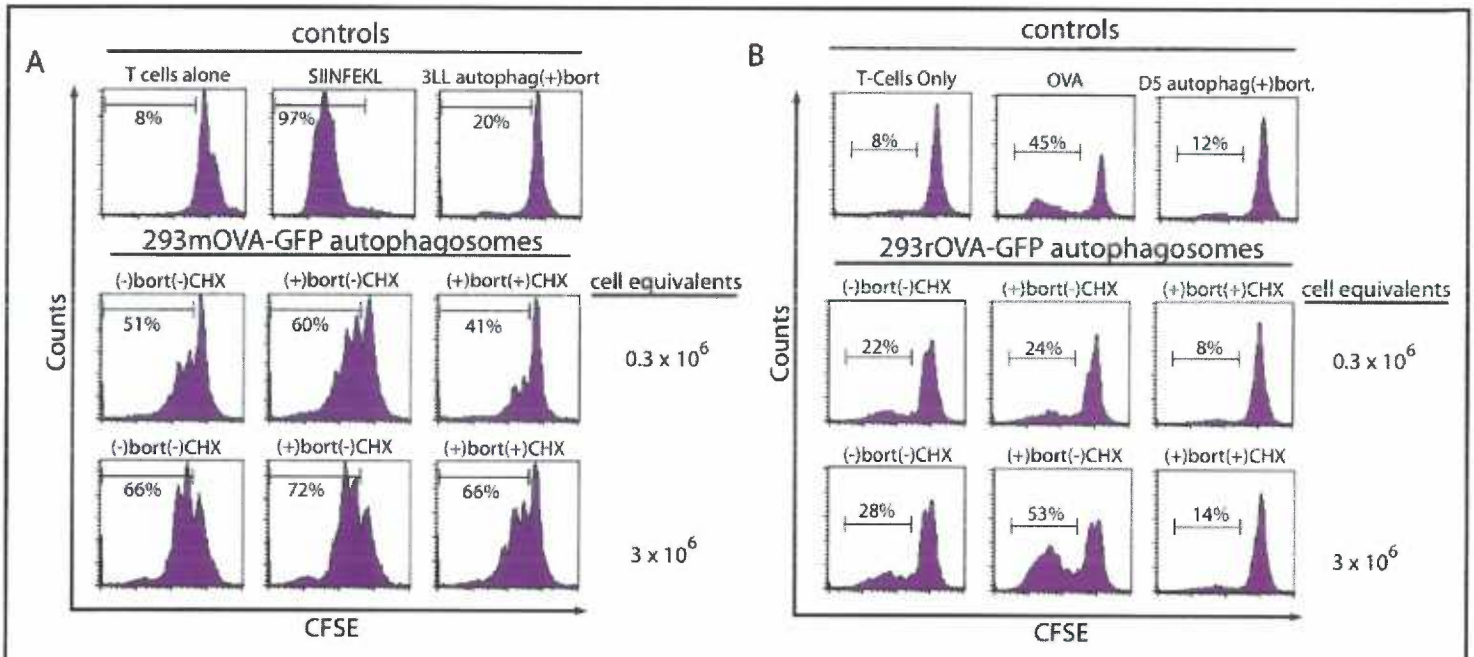


Figure 16: Antigen-specific T-cells stimulated with autophagosomes produced without short-lived proteins show diminished proliferation. (A-B) 293rOVA-GFP or 293mOVA-GFP cells treated with or without bortezomib or pretreated with cyclohexamide before addition of bortezomib as in Figure 15. Non-specific control autophagosomes were isolated from (A) 3LL carcinoma or (B) D5 melanoma. Autophagosomes were isolated, pulsed onto APCs and used to stimulate naïve antigen-specific OT-1 T cells. Proliferation was measured by CFSE dilution. (A) 293mOVA-GFP derived autophagosomes (B) 293rOVA-GFP derived autophagosomes. (A) n=2 experiments. (B) n=3 experiments.

cell proliferation (Figure 16B). This diminished proliferation was more apparent when higher doses of autophagosomes were used to stimulate the naïve T cells. These experiments demonstrate that stable proteins are incorporated into autophagosomes when protein synthesis is inhibited, whereas short-lived proteins are absent from autophagosomes, thus narrowing the effects of CHX pretreatment to short-lived proteins. This observation provides a means of testing the hypothesis that short-lived proteins are necessary for autophagosome-mediated MCA sarcoma cross-protection observed in Figure 16.

Autophagosomes produced from MCA tumor cells lacking short-lived proteins cannot protect hosts from tumor challenge.

Based on the data from the HEK 293 model antigen system which suggests that SLiPs are incorporated into autophagosomes and may determine their utility as a vaccine, MCA sarcomas were treated with CHX before addition of bortezomib during the production of autophagosomes. Autophagosomes isolated from MCA-311 cells treated with or without CHX were used to vaccinate mice, which were then challenged with MCA-304 to assess the ability of MCA-311 autophagosomes to cross-protect (Figure 17A). While the "complete" autophagosome vaccine protected 85% (17 of 20) of the mice, vaccination with CHX pretreated autophagosomes, which lacked protein synthesis during autophagosome production, protected only 47% (9 of 19 mice) of the mice. This reduced protection in the CHX treated cells was nearly the same as MCA-304 challenge alone (8 of 20 mice protected) or MCA-311 whole vaccine (4 of 10 mice protected). These results suggest that the short-lived proteins in autophagosomes are vital for generating cross-protection to other unique sarcomas.

To understand further the role that SLiPs may play in protecting vaccinated hosts from an homologous tumor challenge, autophagosomes isolated from CHX-treated MCA-304 cells were pulsed onto APCs and used to vaccinate groups of mice. The MCA-304 whole cell vaccine protected 80% (16 of 20) of mice while the MCA-304 autophagosome vaccine protected 68% (17 of 25) of mice (Figure 17B). Interestingly, the CHX-treated MCA-304 autophagosome vaccine protected only 36% (9 of 25) of vaccinated mice, nearly

the same limited protection as the non-vaccinated mice (8 of 25), suggesting that SLiPs also play a roll in the autophagosome vaccine-mediated protection against a homologous tumor challenge.

While CHX can have dramatic biological effects, MCA-304 cells treated overnight with bortezomib and with or without CHX displayed similar viability and autophagosomes isolated from these treated tumors had similar protein concentrations (1.97 mg/mL and 2.15 mg/mL respectively). To additionally control for the potential negative effects of CHX on the cellular processes of autophagy, which could limit more than the incorporation of SLiPs

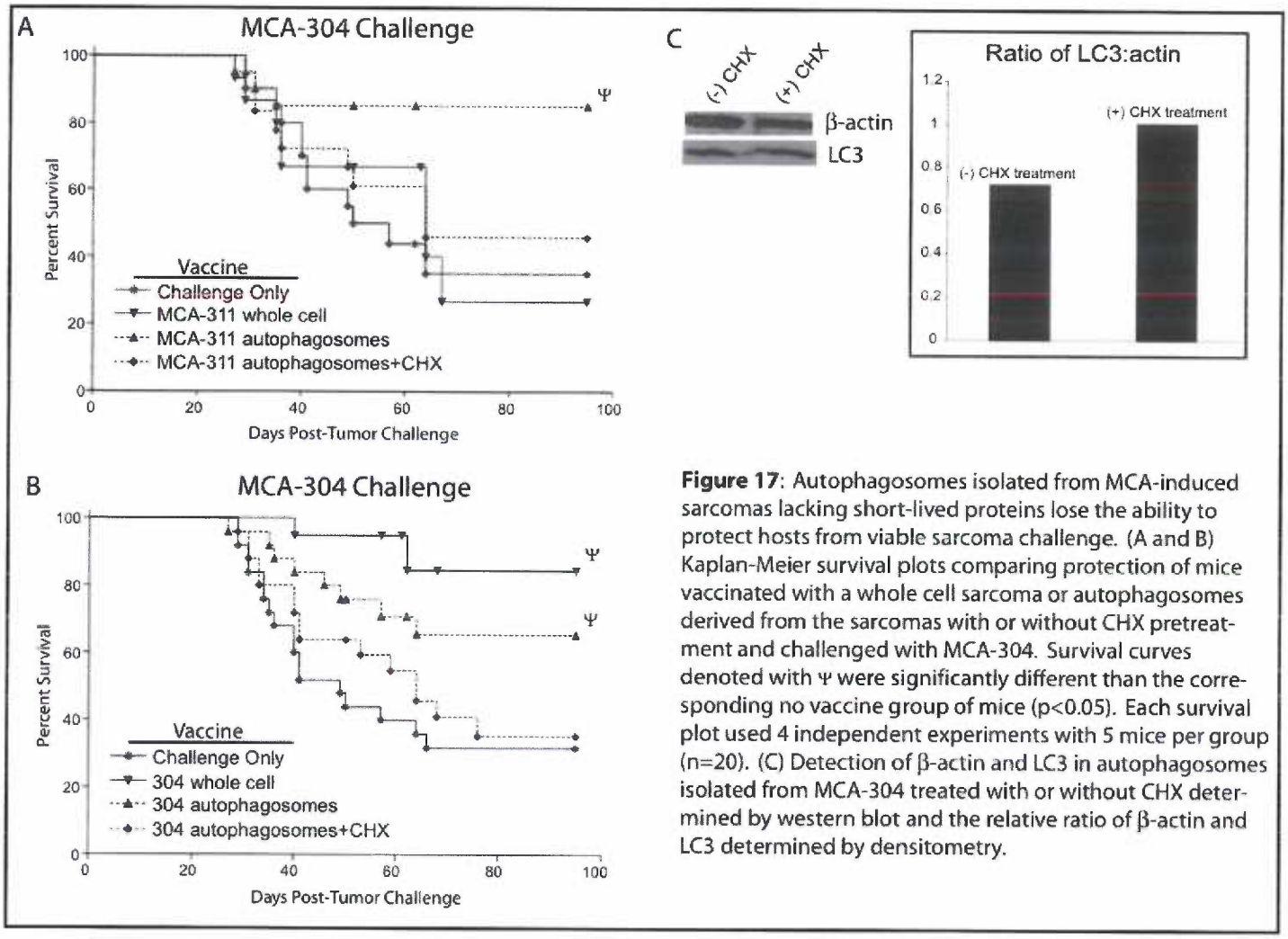


Figure 17: Autophagosomes isolated from MCA-induced sarcomas lacking short-lived proteins lose the ability to protect hosts from viable sarcoma challenge. (A and B) Kaplan-Meier survival plots comparing protection of mice vaccinated with a whole cell sarcoma or autophagosomes derived from the sarcomas with or without CHX pretreatment and challenged with MCA-304. Survival curves denoted with Ψ were significantly different than the corresponding no vaccine group of mice ($p<0.05$). Each survival plot used 4 independent experiments with 5 mice per group ($n=20$). (C) Detection of β-actin and LC3 in autophagosomes isolated from MCA-304 treated with or without CHX determined by western blot and the relative ratio of β-actin and LC3 determined by densitometry.

and DRiPs into autophagosomes, the presence of LC3, a critical component necessary for the induction of autophagy and thus autophagosome formation, was assessed by western blot. The ratio of LC3 to total protein was the same or even greater with CHX pretreatment for both HEK293mGFP-OVA and MCA-304 autophagosomes respectively (Figures 15D and 17C). This suggests that while the SLiPs were eliminated from cells treated with CHX, the production of autophagosomes were not grossly inhibited.

Autophagosomes contain ubiquitinylated proteins and the sequestosome/p62, both of which co-localize with LC3.

Higher molecular weight bands in HEK 293rGFP-OVA autophagosomes were detected with anti-GFP in all groups treated with bortezomib (Figure 15D), but not in the cells used to produce the autophagosomes (Figure 15C). Although these bands were also present in autophagosomes isolated from cells expressing the more stable mGFP-OVA protein, they were less abundant (Figure 15). The N-end rule predicts that the model short-lived protein will be quickly degraded in a ubiquitin-proteasome-dependent fashion (9). We therefore hypothesized that these higher weight proteins were a ubiquitinylated species of the fusion protein that escaped degradation by the proteasome and was enriched in the autophagosomes isolated from the cells. To test this hypothesis, autophagosomes from HEK 293rGFP-OVA cells were immunoprecipitated with anti-GFP and western blotted with anti-ubiquitin. The anti-ubiquitin antibody bound only to the higher weight proteins found in autophagosomes isolated from cells treated with bortezomib (+/- CHX) (Figure 18A) but not

in autophagosomes from untreated 293rOVA-GFP cells or bortezomib-treated parental HEK 293T cells. This observation demonstrates that ubiquitinylated short-lived proteins were present in autophagosomes.

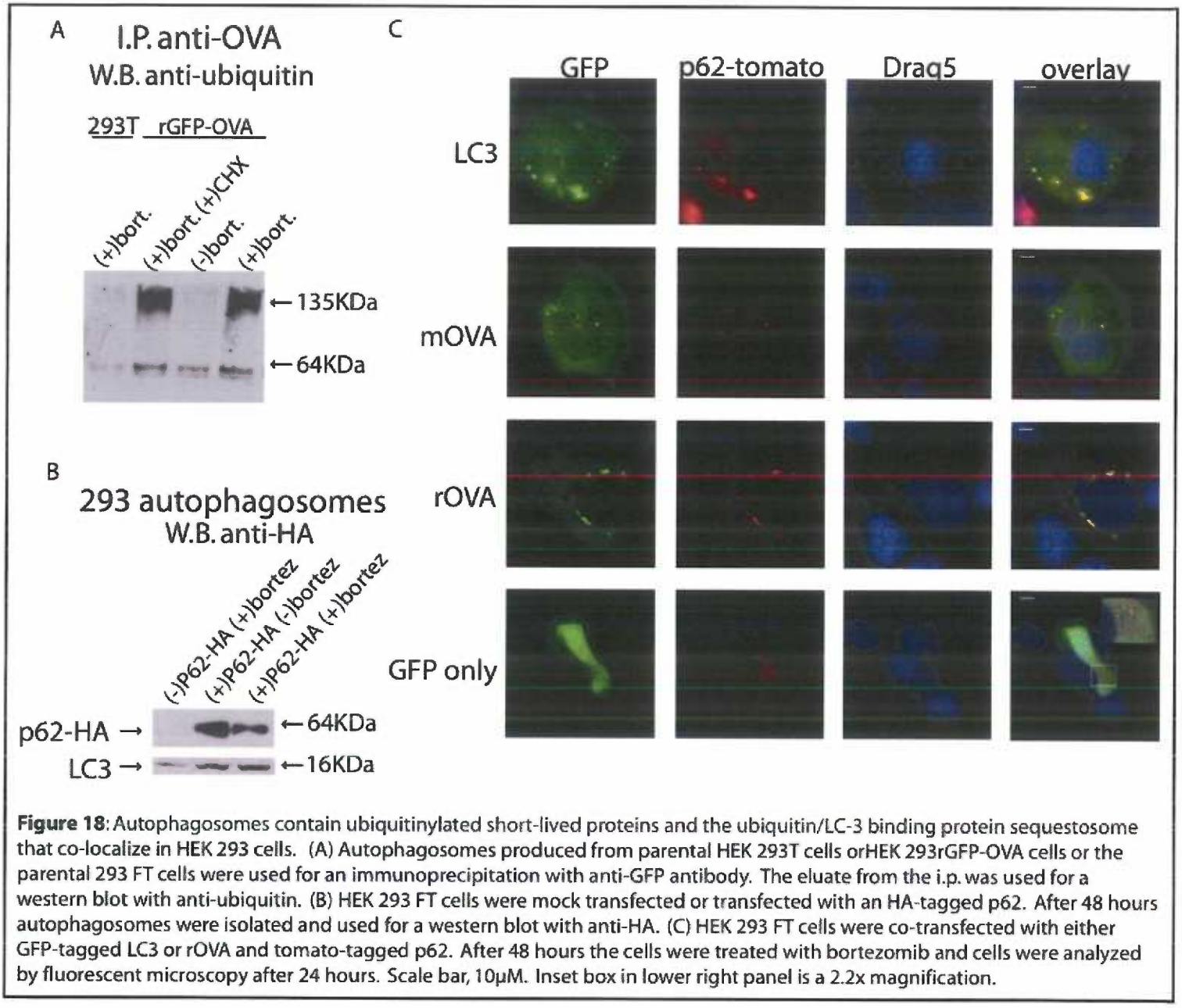


Figure 18: Autophagosomes contain ubiquitinylated short-lived proteins and the ubiquitin/LC-3 binding protein sequestosome that co-localize in HEK 293 cells. (A) Autophagosomes produced from parental HEK 293T cells orHEK 293rGFP-OVA cells or the parental 293 FT cells were used for an immunoprecipitation with anti-GFP antibody. The eluate from the i.p. was used for a western blot with anti-ubiquitin. (B) HEK 293 FT cells were mock transfected or transfected with an HA-tagged p62. After 48 hours autophagosomes were isolated and used for a western blot with anti-HA. (C) HEK 293 FT cells were co-transfected with either GFP-tagged LC3 or rOVA and tomato-tagged p62. After 48 hours the cells were treated with bortezomib and cells were analyzed by fluorescent microscopy after 24 hours. Scale bar, 10μM. Inset box in lower right panel is a 2.2x magnification.

Recent work has demonstrated that the ubiquitin binding protein sequestosome/p62 can interact with both ubiquitinylated proteins and autophagy-related initiator LC3, potentially delivering these proteins to the autophagy pathway (16, 82, 128). The possibility that p62 could interact with ubiquitinylated short-lived proteins as well as the LC3 was tested initially by transfecting HEK 293T cells with HA-tagged p62 and isolating autophagosomes as in Figure 14. Western blot clearly detected p62 in the autophagosomes (Figure 18B).

Additionally, co-localization of LC3 and p62 in HEK 293 T cells was assessed with confocal microscopy using GFP-tagged LC3 and tomato-tagged p62. The short-lived protein, rGFP-OVA, also co-localized with tomato-tagged p62 in transfected HEK 293 T cells but not with a control GFP vector (Figure 18C). These results demonstrate that ubiquitinylated proteins are packaged into autophagosomes, especially when cells are treated with bortezomib. Further, the ubiquitin/LC3 binding protein p62 which accumulates in the autophagosomes as well as associates with the model short-lived protein and LC3 intracellularly, could likely be a key player in delivery of ubiquitinylated proteins to autophagosomes.

P62 is necessary for the delivery of ubiquitinylated proteins to autophagosomes.

To understand how the sequestosome may influence trafficking of specific proteins to autophagosomes, p62 expression was knocked down in HEK 293T cells. A titration of p62 siRNA showed that protein expression was greatly reduced with 50 nM of the p62 smart pool (Figure 19A). HEK 293T cells were then transfected simultaneously with both p62 siRNA

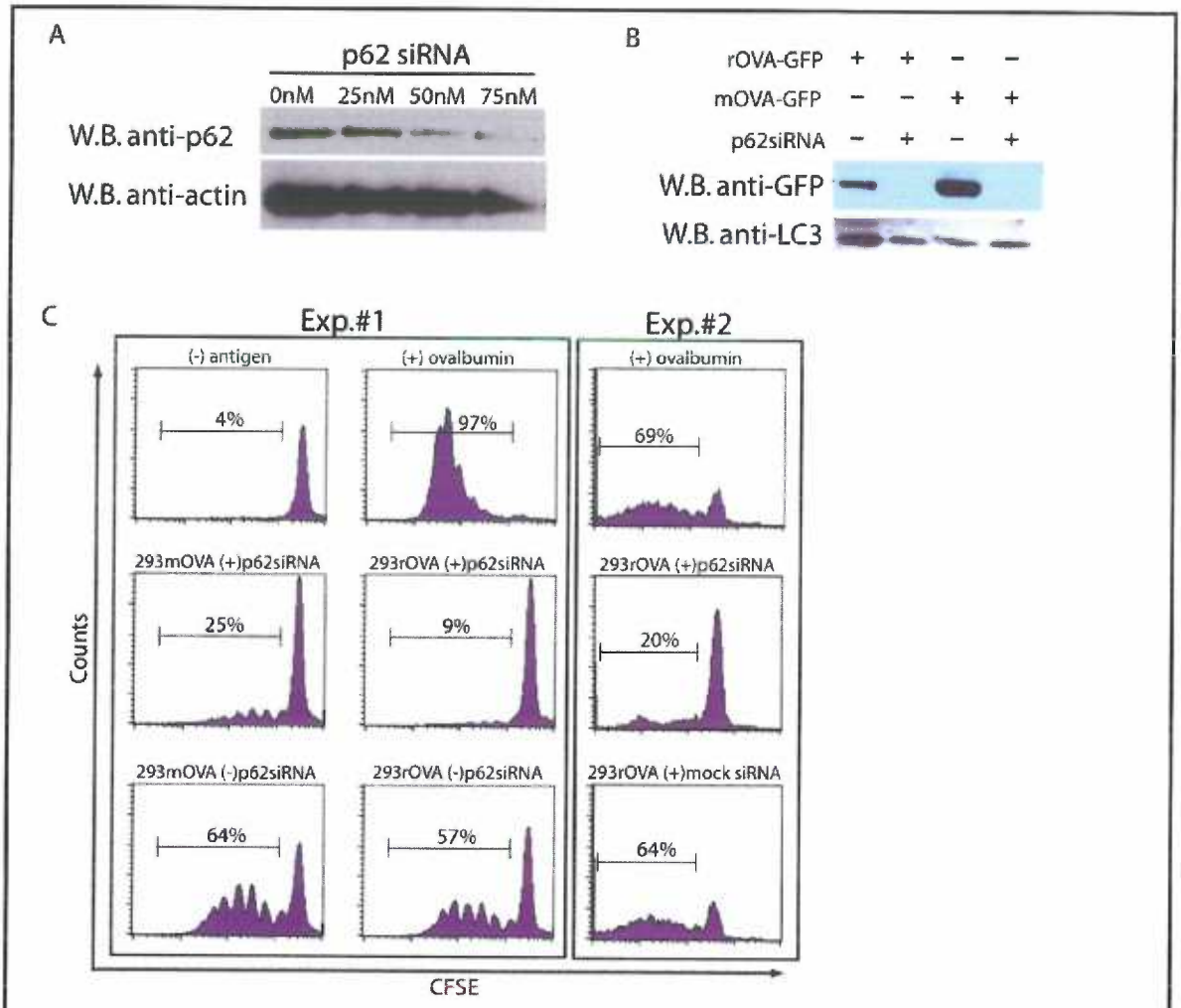


Figure 19: p62 in necessary for delivery of ubquitinylated proteins to autophagosomes. (A) HEK 293T cells were transfected with a pool of p62 siRNA at various concentrations. After 48 hours the cells were collected and lysates were probed for the presence of p62 by western blot. (B and C) HEK 293T cells were cotransfected with either mGFP-OVA or rGFP-OVA and p62siRNA or control siRNA. After 24 hours bortezomib was added to the cells and cultured for an additional 24 hours before harvesting the autophagosomes. (B) An anti-GFP and anti-LC3 western blot of the autophagosome preparation. (C) Autophagosome-pulsed APCs were washed and added to CFSE-labeled naïve OT-1 cells to measure T-cell proliferation as in figure 16. (B and C) n=2 experiments.

and a plasmid that expressed either rGFP-OVA or mGFP-OVA. After 24-hours, bortezomib was added and the cells were cultured for an additional 24 hours before isolating the autophagosome-containing vesicles. A portion of the autophagosomes isolated from the

transfected cells was used to detect the fusion protein by western blot (Figure 19B). The knockdown of p62 in both rGFP-OVA and mGFP-OVA transfected cells greatly reduced the level of the GFP-OVA. This knockdown did not affect the levels of LC3 in the autophagosomes, suggesting that autophagosome formation was independent of p62 expression (Figure 19B).

Based on the co-localization experiments and the presence of the ubiquitinylated protein species in isolated autophagosomes, we hypothesized that knockdown of the sequestosome would greatly diminish the ability of autophagosomes to stimulate naive T cell proliferation. To test this hypothesis, autophagosomes generated from the co-transfection described above were pulsed onto APCs and used to stimulate CFSE-labeled naive antigen specific T cells as previously described. T cells stimulated with autophagosomes generated from 293 cells with the p62 knockdown proliferated less than those stimulated without the knockdown. While the p62 knockdown diminished the proliferation of T cells stimulated with mGFP-OVA autophagosomes, the knockdown strongly inhibited the proliferation of T cells stimulated with rGFP-OVA autophagosomes (Figure 19C). These results coupled with the presence of ubiquitinylated proteins in the rGFP-OVA autophagosomes and the co-localization of the LC3 and p62 strongly suggests that delivery of short-lived proteins to autophagosomes is sequestosome dependent.

DISCUSSION

We have demonstrated that an autophagosome vaccine can prime a repertoire of T cells with a broader tumor-specific reactivity than the intact sarcoma from which it was derived. In contrast to the irradiated whole cell vaccine, vaccination with tumor-derived autophagosomes protected mice from a challenge with independently-derived MCA sarcomas. Experiments using MCA sarcomas, or the OVA model antigen system, both suggest that short-lived proteins incorporated into autophagosomes are necessary in generating a robust immune response. Additionally, these data suggest that the sequestering of SLiPs into autophagosomes is dependent on the ubiquitin binding protein sequestosome/p62. This novel vaccine strategy has redefined the tumor rejection antigens associated with MCA-induced tumors and has created the potential for new vaccine strategies.

While MCA-induced sarcomas can share similar histology, growth patterns, sensitivity to chemotherapeutics and are generated in syngeneic mice using the same carcinogen, each sarcoma induces a unique tumor-specific immune response. This observation has been made by many researchers (13, 85, 124, 137, 193), and it is also evident in our studies by the failure of our MCA-induced sarcoma whole cell vaccine to cross-protect against independently derived but related MCA-induced sarcomas (Figure 10A). While this failure to cross-protect has been attributed to each sarcoma's unique antigenic profile (13), it seems unlikely that a large panel of sarcomas of similar etiology will not have common antigens. A more plausible description of this unique specificity could be described by the

concept of a tumor rejection antigen, which functionally relates to how well an immune response directed against a tumor antigen can limit tumor growth (56, 176). The hierarchy of a tumor rejection antigen, which ranges from poor to strong, can be influenced by the ability of a specific antigen to prime an immune response. Thus while there may be a common pool of antigens shared between independently-derived sarcomas, individual tumors can express unique antigen(s) that may dominate the immune response, effectively limiting the priming of shared epitopes. This immune dominance may relate to multiple factors. Pion and colleagues have demonstrated that immune dominance is determined by not only the affinity of the TCR and peptide-MHC complex (p:MHC) but also the surface density of p:MHC on the APC (133), which is directly proportional to an epitope's MHC binding affinity, linking the establishment of immune dominance to antigen presentation. This observation coupled with previous findings which demonstrate the necessity of cross-presentation during tumor immunity (73) illustrates how cross-presentation may determine immune dominance and consequently the tumor specificity seen with MCA-induced sarcomas. Thus a possible tumor rejection antigen must not only be stable enough to be transferred to an APC, it must also possess an epitope that can compete for binding of the APC's MHC molecules as well as have an affinity for the TCR. Due to their transient nature, SLiPs are poorly cross-presented and are therefore removed from the pool of possible rejection antigens.

The rules governing cross-presentation of tumor or tumor-associated antigens with a cellular vaccine may not apply to an autophagosome vaccine as short-lived proteins can be stabilized by proteasome blockade, isolated in autophagosomes and pulsed onto APCs *ex*

vivo. Consequently, we have focused on the repertoire of antigen, specifically the short-lived proteins sequestered, as a means of altering the tumor specificity with an autophagosome vaccine. Based on the observation that both protection and cross protection from an MCA sarcoma challenge were reduced with elimination of SLiPs from the autophagosome vaccine (Figure 17), antigens common to independently derived MCA-induced sarcomas are likely to be SLiPs. These SLiPs may provide epitopes with a high affinity for MHC molecules allowing them to compete with the dominant whole cell rejection antigen for surface density on the APC. Alternatively, the dominant rejection antigen may not be sequestered into autophagosomes, providing sub-dominant, neo or cryptic antigens access to MHC molecules for cross presentation.

The targeting of SLiPs into autophagosomes appeared to be coordinated, as cell lysates could not stimulate antigen specific T cells as effectively as could autophagosomes, suggesting a relative concentration of SLiPs in autophagosomes. This observation was supported by the remarkable increase in the proliferation of antigen-specific T cells when simulated with isolated autophagosomes versus whole cell lysate (Figure 20). Western blot analysis suggests that the short-lived OVA protein was readily detected at much lower total protein concentrations in autophagosomes than in cell lysates (Figures 15C and 15D and data not shown). Additionally, higher molecular weight fusion proteins were detected only in autophagosomes and not in cell lysates. These higher molecular weight proteins were more obvious in autophagosomes derived from bortezomib treated cells and especially those containing the model SLiP antigen which is rapidly ubiquitinylated versus the more stable

antigen (Figures 15D). The higher weight band, confirmed to be ubiquitinylated by western blot analysis after co-immunoprecipitation (Figure 18A), was likely responsible for directing SLiPs into autophagosomes based on recent publications

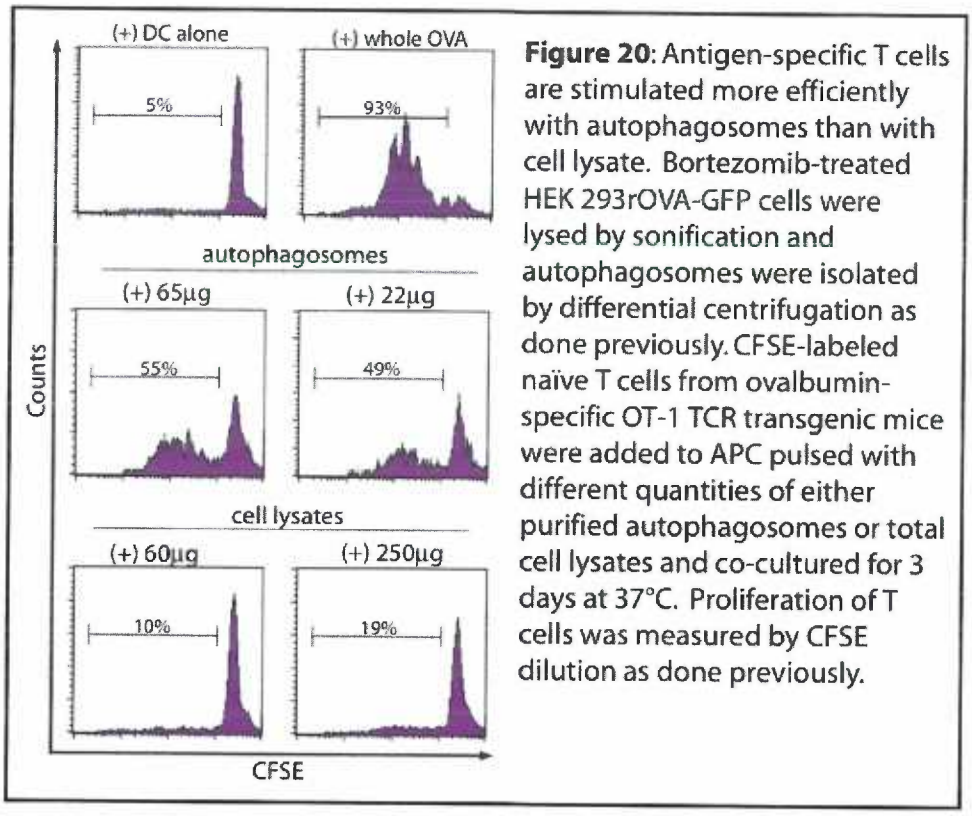


Figure 20: Antigen-specific T cells are stimulated more efficiently with autophagosomes than with cell lysate. Bortezomib-treated HEK 293rOVA-GFP cells were lysed by sonification and autophagosomes were isolated by differential centrifugation as done previously. CFSE-labeled naïve T cells from ovalbumin-specific OT-1 TCR transgenic mice were added to APC pulsed with different quantities of either purified autophagosomes or total cell lysates and co-cultured for 3 days at 37°C. Proliferation of T cells was measured by CFSE dilution as done previously.

that established a link between autophagy and ubiquitinylated proteins via the LC3 and ubiquitin binding protein p62/sequestosome (83, 87, 109). Indeed HA-tagged p62 was readily detected in autophagosomes isolated from transfected cells and fluorescent microscopy confirmed the interaction of p62 with both the model SLiP as well as LC3, all supporting the hypothesis that p62 regulates the sequestering of short-lived or ubiquitinylated proteins into autophagosomes (Figures 18B and 18C). Using siRNA to knockdown expression of p62 further confirmed the sequestosome's role in antigen delivery to autophagosomes as reduced expression of p62 limited the model antigen found in the autophagosomes and diminished proliferation of antigen-specific T cell stimulated with these

autophagosomes. Autophagosomes isolated from HEK293rGFP-OVA cells treated with p62 siRNA had a greater impairment in their ability to stimulate T-cell proliferation than autophagosomes isolated from p62 siRNA-treated cells expressing the more stable mGFP-OVA antigen (Figure 19C), suggesting a correlation between the stability or state of ubiquitinylation of the protein and the necessity of p62 to transfer the protein into the autophagosome. Collectively, our findings suggest that the sequestosome helps direct short-lived proteins into autophagosomes via their ubiquitinylation status (Figure 21). In contrast to cellular vaccines, these ubiquitinylated SLiPs found in purified autophagosomes are capable of generating a broad anti-tumor immune response.

It is interesting that despite the unique cross-protection afforded by the autophagosome vaccine, a diminished protection relative to the irradiated whole cell was observed when mice were challenged with the homologous sarcoma. There are many possible explanations for this observation including the possibility that some tumors can stimulate an immune response via direct-presentation (190). Alternatively, autophagosomes may provide epitopes with a lower affinity for the TCR than epitopes derived from a whole cell vaccine, thus limiting the highly reactive dominant T cell clones. By priming a broader repertoire of T cells with a lower avidity for tumor-associated p:MHC, the autophagosome vaccine could provide a more diverse yet less reactive population of effector T cells. Regardless of the level of protection afforded to vaccinated mice, the advantage of this vaccine strategy and the important finding of the thesis is the inclusion of common tumor antigen(s) in tumor-derived autophagosomes. It remains possible to exploit the advantage of

a more diverse T cell population by combining the vaccine strategy with OX-40, 4-1BB, anti-CTLA4 or another immunostimulatory molecule to augment the reactivity of autophagosome-primed effector T cells. If proven effective, the broad and robust immune response driven by a combinational strategy would have a strong clinical appeal.

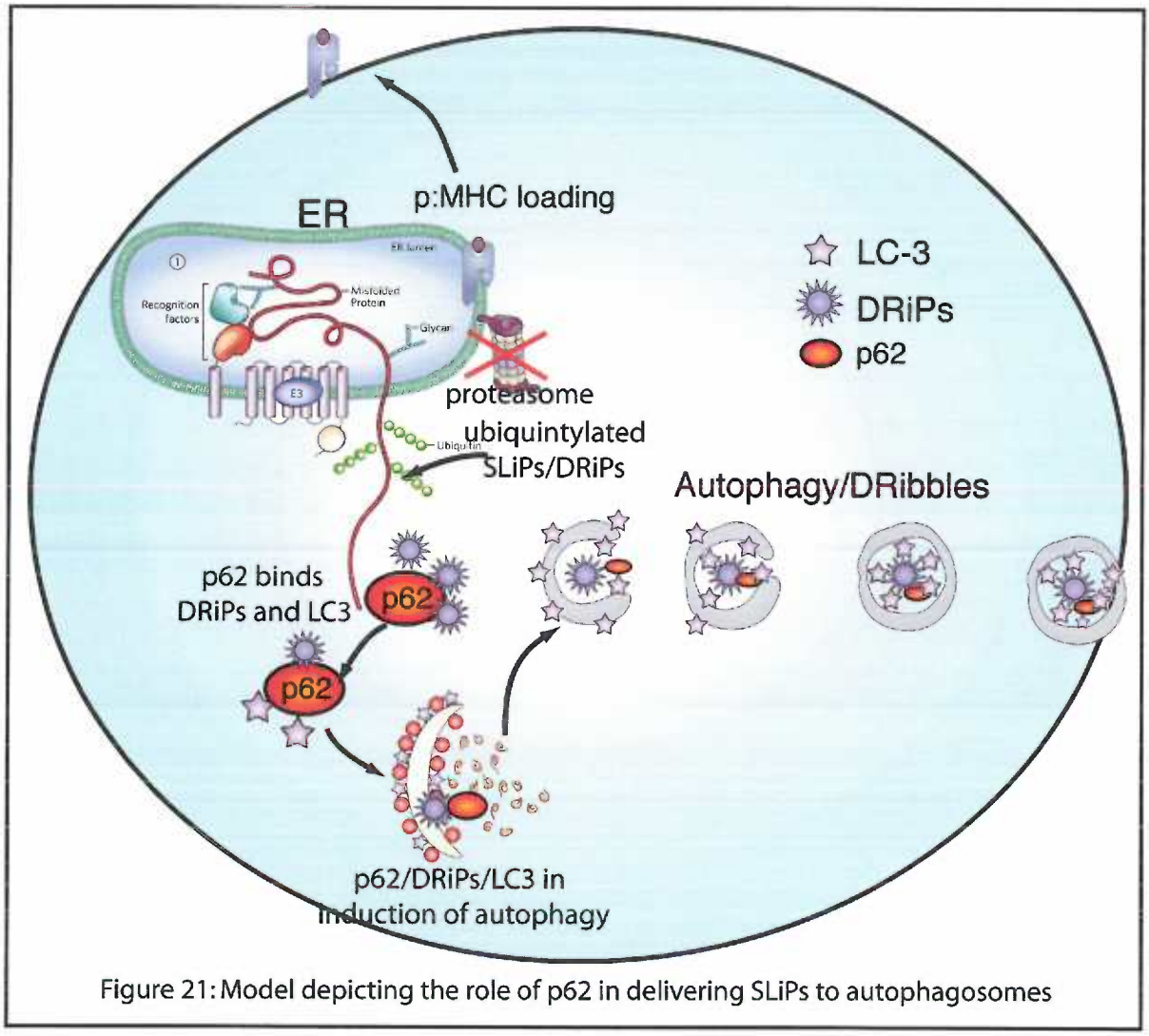


Figure 21: Model depicting the role of p62 in delivering SLiPs to autophagosomes

CONCLUDING REMARKS

The goal of many tumor immunologists is to discover and understand mechanisms that can shift the balance of tumor immunoediting towards tumor elimination. The various approaches to achieving this goal range from emphasizing early detection in combination with treatment (45) to the targeted biochemical approaches of small molecule inhibitors (38). While the success of cancer treatment may reasonably involve multiple scientific disciplines, our strong opinion remains that tumor immunotherapy holds the greatest promise to eliminate cancer. Tumor antigens play a fundamental role in the relationship between a tumor and the immune system as they can direct and shape the immune response. Towards this end, the scope of this work has focused on exploiting autophagy or lymphopenia to augment tumor-specific immune responses using two different vaccine strategies.

Recent studies reported strong immune responses and significant control of SIV viremia using a peptide-pulsed autologous PBMC vaccine (24, 36), demonstrating that an intravenous vaccine could drive a productive immune response in a viral model. The concept of this viral vaccine strategy was congruent with our lymphopenic model, which promotes enhanced tumor-specific immune responses relative to an intact host (70, 179). The lymphopenic environment was in fact vital to the i.v. peptide vaccine approach as the frequency and absolute numbers of antigen-specific T cells were severely reduced in an intact versus lymphopenic hosts (Figure 2). Based on the frequency of pmel T cells, the addition of antigen was also important since the vaccine without peptide was clearly less effective than the vaccine with peptide (Figure 4). The third and vital component of the

vaccine strategy was the inclusion of the TLR4 ligand, RC-527. Vaccination with hgp100 peptide and RC-527 drove a stronger immune response than the same vaccine without RC-527. This observation is based on antigen-specific T cell activation markers, frequency, absolute counts, and function (Figures 5-7). This TLR ligand was able to augment the T cell response in an antigen-specific fashion, as a titration of RC-527 without hgp100 did not appreciably stimulate pmel T cells (Figure 5C). Additionally, both the increased activation phenotype and frequency of antigen-specific T cells were coordinated with the addition of RC-527 (Figure 5D). Although the frequency of functional pmel cells was diminished at day 34, a functional population of antigen specific T cells was observed greater than 30 days after transfer, suggesting that this strategy can produce a durable response.

These studies provide proof that adoptively transferred peptide-pulsed splenocytes can both reconstitute a lymphopenic host while generating a productive immune response. However, many questions need to be addressed. The role of CD4 T cells was initially addressed in a preliminary experiment (data not shown) but the dose of CD4 T cells was inappropriate and overwhelmed the antigen-specific CD8 T cell response. However, based on data from our lab ((72), Friedman *et al*, manuscript submitted) as well as others (118, 170, 171), the addition of CD4 T cell help will likely increase memory formation and should be addressed. Other basic questions remain such as the necessity of the transferred APCs. Similarly, it is not clear if RC-527 needs to activate both endogenous and transferred APCs. These questions are clinically relevant as one of advantages of this strategy is to include antigen-pulsed APCs in the reconstitution and thereby eliminate the time and resources

needed for generation and *ex-vivo* expansion of APCs. Additionally, if the TLR ligand could be removed after the pulse period, it would streamline the translation of the vaccine towards the clinic.

Testing the melanoma-associated peptide vaccine in a tumor model will give another means of assessing this strategy and allow for a comparison with other types of vaccines. The i.v. vaccine may prove to be less efficacious than a whole cell or even an emulsified s.c. peptide vaccine but because of its simplicity, this strategy could be combined and potentially synergize with other vaccines strategies that incorporate a reconstitution step. The studies presented in the second chapter of this thesis, provide a different perspective of what a vaccine can be. By integrating ideas from two different vaccine strategies, an immunogenic vaccine with the potential to become effective immunotherapy was demonstrated.

Similar to the second chapter, the studies reported in the third chapter of the thesis examined vaccines and specifically tumor antigens. Work done at our institute established that autophagosomes are efficient at mediating cross-presentation of tumor antigens and that a tumor-derived autophagosome vaccine could mediate a strong anti-tumor response (98, 99). The application of this autophagosome-based vaccine to the MCA-induced tumor model generated an immune response that could cross-react with different independently-derived sarcoma targets (Figure 10B). This cross-reactive response did not occur with a whole cell vaccine (Figures 10A and 10B). Further, the autophagosome vaccines cross-protected mice from a non-homologous tumor challenge in nearly all the combinations tested (Figure 12). The whole cell tumor vaccine only protected vaccinated mice from a homologous tumor

challenge and demonstrated no cross-protection in any of the combination tested (Figure 12). The use of APCs in the autophagosome vaccine was suspected of providing a means of cross-protection since APCs were only used in the autophagosome vaccines. However, the results from Figure 13, rule out this possibility, as the whole cell tumor vaccine with APCs still did not cross-protect in any of the 4 combinations tested whereas the autophagosomes did show a statistically significant difference compared to the no vaccine control.

It was hypothesized that inhibition of the proteasome during the isolation of tumor-derived autophagosomes (99) created a pool of SLiPs responsible for the cross-protective immune responses seen in the MCA-induced tumor model. A cell line expressing either a short-lived protein or a stable protein was used to address this hypothesis and to gain a better mechanistic understanding of this phenomenon. Stimulation of naïve antigen-specific T cells with autophagosomes isolated from 293rOVA, which expresses the model SLiP ovalbumin, indicate that SLiPs were preferentially incorporated into autophagosomes when treated with bortezomib as measured by T cell proliferation (Figure 14B). The ability of SLiPs in autophagosomes to stimulate antigen-specific T cells was clearly demonstrated as T cell proliferation was greatly reduced when the SLiPs were eliminated from the autophagosomes by CHX pretreatment (Figure 16B). In contrast, autophagosomes isolated from cells expressing a more stable model antigen that were treated with or without CHX stimulated antigen-specific T cell proliferation (Figure 16A).

The findings of these experiments were applied to the MCA-induced tumor model to test the hypothesis that SLiPs are responsible for the autophagosome induced cross-

protection previously observed. The diminished protection of mice vaccinated with autophagosomes derived from MCA-induced sarcomas treated with CHX versus autophagosomes isolated without CHX treatment, strongly supports the hypothesis that SLiPs are responsible for not only the cross-protection but even direct protection against the homologous tumor (Figure 17). The potent effects of CHX treatment create the potential for it to alter more than just the presence of SLiPs in the autophagosomes. However, the presence of the autophagosome-specific marker LC3 in both 293 and MCA cells treated with CHX (Figures 15D and 17C) and the similar viability of cells treated overnight with or with CHX reduces this possibility and supports the above conclusion.

An OVA/GFP western blot of autophagosomes revealed the presence of higher molecular weight bands that were not seen with cell lysates (Figures 15C and 15D). These higher molecular weight bands were more evident with bortezomib-treated cells expressing the short-lived protein, suggesting that a modified short-lived protein was preferentially accumulating in autophagosomes from bortezomib-treated cells. We hypothesized that by blocking the proteasome, ubiquitinylated SLiPs would be spared degradation and instead accumulate predominately in autophagosomes.

By immunoprecipitating with an anti-GFP antibody and performing a western blot with an anti-ubiquitin antibody, these higher molecular weight bands were shown to be ubiquitinylated (Figure 18A). This observation coupled with a report describing a protein (p62) capable of interacting with both ubiquitinylated proteins and the autophagy-specific protein LC3 (128), created the possibility that p62 could be the link between the

ubiquitinylated proteins and autophagosomes. Ectopic expression demonstrated that p62 readily accumulates in autophagosomes (Figure 18B) and immunofluorescence microscopy confirmed the interaction of p62 with LC3 and short-lived rOVA but had a more limited interaction with the stable mOVA and even less with the control GFP (Figure 18C). The necessity of p62 in delivering antigen, particularly SLiPs, to autophagosomes was addressed by stimulating antigen-specific T cells with autophagosomes isolated from cells with p62 expression knocked down using siRNA. Expression of both mOVA and rOVA in isolated autophagosomes was eliminated with p62 knockdown as determined by western blot (Figure 19B) and T cells stimulated with these autophagosomes showed severely diminished proliferation, especially with the autophagosomes containing the SLiP (Figure 19B).

Collectively the data points to a model whereby SLiPs or other ubiquitinylated proteins are sequestered into autophagosomes in a p62 dependent fashion when the cell is treated with a proteasome inhibitor (Figure 21). Furthermore, data from the MCA-induced sarcoma cross-protection experiments suggest that these SLiPs include antigenic determinants common to multiple independently-derived sarcomas. Several possibilities may explain how a whole cell vaccine does not afford the cross-reactivity observed with an isolated autophagosome vaccine. The most simple explanation is that tumors rapidly turn-over SLiPs in a proteasome-dependant fashion thereby preventing their accumulation. This explanation makes the assumption that MCA-induced tumors do not directly prime an immune response since rapidly degraded proteins fill a large proportion of MHC class I molecules on the cell surface (191). Alternatively, SLiPs may accumulate at low levels but

are not stable enough to be efficiently cross-presented which is supported by the observation that superior T cell proliferation occurs with much less autophagosomes than cell lysates (Figure 20). If both SLiPs and whole cell tumor rejection antigens are equally cross-presented, the rejection antigens may assert immune dominance by binding MHC class I more efficiently or in the context of MHC class I, have a higher affinity for the TCR (133). Addressing these possibilities will provide a greater understanding of not only this novel finding but also a potential class of novel tumor antigens.

The implication of these studies from a translational point of view is that autophagosomes may be a way to uncover common antigenic determinants created during the transformation process. Evidence supporting this concept in this model comes from two studies. The first study identified a region of the mouse genome rich in tumor-modifier genes and associated with MCA-induced tumor antigens (4). A second study showed that mice vaccinated with a MCA-induced tumor that had lost the dominant antigen could generate CTL, which recognized a shared antigen expressed not only by the immunizing cell line, but also by independent sarcoma cell lines (39). The idea that tumor-derived autophagosomes may contain common tumor-specific or tumor-associated antigens is clinically appealing. These autophagosomes could have the potential of an "off the shelf" vaccine in an allogeneic setting, particularly if an autologous tumor is unavailable to the patient. Additionally, this strategy could be useful in priming immune responses against tumor-antigen loss variants seen with a progressively growing tumor and may even serve as an alternative vaccine to treat patients coming out of remission. This strategy has the

potential to augment immunity in clinical applications and positively contribute to the field of immunotherapy.

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