Exposure to Abnormal Self Antigens During Non-Malignant Inflammatory Events Provides Immunological Defense Against Tumors

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Submitted to the Graduate Faculty of

The School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2013

UNIVERSITY OF PITTSBURGH

School of Medicine

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EXPOSURE TO ABNORMAL SELF ANTIGENS DURING NON-MALIGNANT INFLAMMATORY EVENTS PROVIDES IMMUNOLOGICAL DEFENSE AGAINST TUMORS

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University of Pittsburgh, 2013

ABSTRACT

Cancer immunosurveillance is the body's sentinel mechanism of recognizing and eliminating malignancy. Specifically, the immune system can mount a response against cancer through the recognition of tumor associated antigens (TAA). While studying two TAAs, MUC1 and cyclin B1, our group discovered T cell and antibody responses specific for these abnormal molecules not only in cancer patients but also in healthy individuals with no cancer history. While seeking to explain why TAA specific responses exist in healthy people, our group and others' epidemiologic studies revealed that individuals who had a history of febrile pathogenic infections had a lower risk of cancer development. These results led us to hypothesize that non-malignant events such as influenza infection, elicit abnormal expression of multiple self-antigens on infected cells and specific immune memory against those antigens. Abnormal expression of the same antigens on tumor cells triggers specific immune responses and provides adaptive immune memory to participate in tumor surveillance. Rather than classifying these abnormal molecules common to virus infected and malignant cells as TAAs, they should be recognized as disease associated antigens (DAA). I first tested this hypothesis in MUC1Tg mice and found that, influenza infection induces abnormal MUC1 expression in the lung, MUC1 specific $CD8^+$ T cells, and that influenza experienced mice control $MUC1^+$ tumor growth. I next addressed if this infection model could lead to the identification of other DAA. I modified the mouse model by using C57BL/6 mice, using two influenza virus strains as the stronger pathogenic insult to the lung and using a lung tumor Lewis Lung Carcinoma (3LL) as the tumor challenge. Through the use of 2D-Difference Gel Electrophoresis to resolve and identify tumor proteins detected deferentially by pre- and post-infection mouse sera, I selected from over 120 proteins five specific molecules (DAAs) for further

study: GAPDH, Histone H4, HSP90, Malate Dehydrogenase 2 and Annexin A2. Western blot analysis confirmed their overexpression in two mouse tumor cell lines and in flu-infected lungs compared to healthy lungs. Additionally I confirmed that antibodies and $CD8^+$ T cell specific responses were generated against these 5 DAAs following flu infection. Lastly, animals vaccinated with peptide derived from these candidate DAA demonstrated a prolonged delay in tumor growth. Better understanding of early life events that prepare the immune system to protect individuals against known and unknown pathogens as well as future malignancies will help direct vaccines towards strengthening life-long immunosurveillance. Importantly, these findings support the use of vaccines based on DAAs/TAAs for cancer prevention.

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PREFACE

Foremost, I would like to thank my wonderful mentor and thesis advisor, Dr. Olivera J. Finn. Olja, you have mentored and guided me through this tough yet exciting and fascinating journey of science. You taught me the skills to ask the right questions in experiment planning and the art of collaboration. You allowed me to explore my project with a lot of rope, yet reeled me back in when I was in need of help. You're always there for helpful tips and advice, hugs as well as celebrating our collective achievements. When I see the glass half empty, you taught me to see it as half full. For all your guidance I thank you.

I would like to thank members of the Finn Lab family, both past and present but especially the graduate students, Adam Farkas, Doug Marvel, and Dawn Reichenbach, with whom I spent the majority of my time in lab with. Between the late nights in the laboratory, lunchtime shenanigans, angry birds, juice boxes, Secret Santa, 'other place,' shoofly pie, and the wretched pickle dip, you guys made lab that much more enjoyable. I will always remember these times as you all go on to bigger and better things.

I would like to thank the University of Pittsburgh and UPMC, the Pittsburgh MSTP leaders and administration especially Richard Steinman MD, PhD (current director), Clayton Wiley MD, PhD (former director), the Department of Immunology administrative office, the graduate studies office, and last but not least, my thesis committee as well as Ted Ross, PhD and Jonathan Minden, PhD for the various roles they have played towards my achievements and goals.

I would like to thank Becky Bayer for her love and support. I truly appreciate all of our adventures together and look forward to many more. I would like to thank my friends both past and present and a special recognition of my high school Phillips Academy Andover. It is truly a

special place and I always keep the two lines ‘Non Sibi- Not for ones self’ and ‘Finis Origine Pendet- The end depends on the beginning,’ in my heart.

Last but not least, I would like to thank my family my father and mother Remy and Lawrencia Iheagwara, my sisters Chioma, Nnenna, and Tochi Iheagwara and our dog ‘Foxworth.’ You all are my rock. All my academic accomplishments are a direct reflection on your love and support. I know I can never repay you all for all that you have done for me, especially since I am not done with my training, but I will try my hardest to make you all proud that I’m your son and little brother.

1.0 INTRODUCTION

1.1 CURRENT VIEWS ON CANCER IMMUNOSURVEILLANCE

1.1.1 Development of the Tumor Immunosurveillance Concept

The idea that the immune system could recognize and eliminate tumors first came about during the early 1900s. Paul Ehrich proposed that tumor occurrence in organisms with long lifespans would be much higher if the immune system were not present to keep tumor outgrowth under control (1). Burnett and Thomas formulated this proposal into the concept of tumor immunosurveillance, which defines that adaptive immunity is the major mechanism responsible for protecting immunocompetent individuals against cancer (2-5). Not long after the proposal, the idea of cancer immunosurveillance came under heavy scrutiny. The biggest setback came from Stutman et al. who demonstrated that rates of carcinogen-induced or spontaneous tumors in immunocompetent wildtype mice were similar to immunocompromised nude mice (6). Nude mice lack a thymus and as a result have impairment in producing mature T cells. Therefore, it was expected that cancer rates in these immunocompromised mice would be higher than their immunocompetent counterparts. Subsequently the tumor immunosurveillance theory was discredited and abandoned for quite some time. It was not until the advent of gene knockout

technology that provided better mouse models and other new testing modalities that the immunosurveillance theory was revived and accepted.

In the 1990s, many experimental outcomes led to renewed interest in tumor immunosurveillance. Dighe et al. demonstrated that Interferon-γ (IFN-γ) plays an important role in the elimination of tumors. Specifically, they showed enhancement in tumor growth through the use of IFN-γ neutralizing antibodies in tumor bearing animals or challenging animals with an IFN-γ dominant-negative tumor (7). In studies using knockout technology, IFN-γ receptor knockout mice showed an increase in susceptibility to transplantable and spontaneous tumor growth. Downstream in the IFN-γ signaling cascade, molecules such as Signal Transducer and Activators of Transcription-1 (STAT-1) were tested as well. STAT-1 knockout mice were shown to be more susceptible to tumor growth (8). One of the biggest pieces of evidence supporting tumor immunosurveillance came from the use of $RAG2^{-/-}$ mice that lack T cells, B cells, and Natural Killer T (NKT) cells (9). $RAG2^{-/-}$ mice were highly susceptible to 3-methylcholanthrene (MCA)-induced sarcomas. Tumor growth was further enhanced in $RAG2^{-/-}$ and $STAT1^{-/-}$ (RkSk) double knockout mice. Additional studies using $Perforin^{-/-}$ mice (a cytolytic molecule involved in NK and $CD8^{+}$ T cell killing) or using mice depleted of NK or NKT cells showed that these cells participate in immunosurveillance (10, 11). These new data explained why Stutman et al. observed similar rates of carcinogenesis between nude and wildtype animals. Nude mice do not have T or B cells, but they do have NK cells capable of playing a critical role in immune surveillance. These data further strengthened the notion that tumor immunosurveillance is a real and necessary sentinel immune process.

1.1.2 Cancer Immunosurveillance Defined as Cancer Immunoediting

The concept of cancer immunosurveillance underwent modifications beginning in the early 2000s. Evidence began sprouting up that the immune system shapes and sculpts the immunogenicity of tumors. This process became known as tumor immunoediting (12). Three distinct and highly dynamic periods exist during tumor/immune system interaction: Elimination, Equilibrium, and Escape. The aforementioned periods called the 3E's can proceed sequentially or directly enter into any one of the periods at any point. The directionality of the phases can be affected by such external factors as aging, immunotherapies, and environmental stressors.

1.1.2.1 Elimination

The Elimination phase of immunoediting involves cells of both the innate and adaptive immune system recognizing and killing tumors prior to clinical manifestation. Cells of the innate immune system such as NK cells, macrophages, γδ T cells, and NKT cells are the first responders at the site of tumorigenesis and adaptive immune cells such as T and B cells are late responders (13). If the tumor is destroyed, elimination represents the endpoint of immunosurveillance. If the tumor is incompletely destroyed, remains dormant, or continues to grow, the tumor/immune system interaction may enter a state of equilibrium or escape. One way that the immune system recognizes tumors is through tumor associated antigens (TAA). TAAs are molecules that are abnormally expressed by tumor cells. $CD4^+$ and $CD8^+$ T cells can recognize processed tumor antigens presented in the context of Major Histocompatibility Complex (MHC) class II and I, respectively. B cells recognize cell surface tumor antigens or soluble TAA and trigger antigen specific antibody responses. TAA can be classified a number of different ways based on their characteristics. Many of the antigens discovered can belong to multiple categories. Categories include mutated proteins,

tissue specific or cell differentiation molecules, aberrantly processed molecules, embryonic molecules abnormally expressed in adult cells, overexpressed normal proteins and proteins derived from oncogenic viruses.

Mutated antigens are products of genes that are mutated through spontaneous mutations, chemical carcinogens, or physical carcinogens. Some of these antigens are derived from tumor suppressor genes or oncogenes. They contain substituted amino acids, truncated or lengthened amino acid sequence, or completely different sequences all together. Some examples of mutated tumor antigens include p53, EGFR, and K-ras (14, 15).

Differentiation antigens are specifically expressed by a particular cell type or cell differentiation stage. These antigens are expressed on both normal and malignant cells of the same lineage. Some of the best known differentiation antigens are from the melanocyte lineage and expressed on melanoma cells. They include tyrosinase, gp100 and Melan-A/MART-1 (16, 17).

Tumor antigens can also arise as a result of abnormal post-translational modifications including glycosylation, phosphorylation, and deamidation among others (18). An example of an antigen that is hypoglycosylated compared to its normal counterpart in healthy cells is MUC1 (19). Tumor antigens that are a result of abnormal phosphorylated are termed phosphoantigens. They can be presented on MHC class I or II in tumors as a result of dysregulation in protein kinases and other normal cellular pathways in cancer. This dysregulation commonly leads to an accumulation of phosphopeptides that may be aberrantly expressed antigens in MHC pathways. Examples of phosphoantigens are p-β-catenin (phospho-β catenin) (20) and p-MART-1 (phospho-MART-1) (21).

Cancer Testis (CT) antigens are examples of tumor antigens generated by unscheduled expression of developmentally related molecules. They are normally only expressed in germ-line

cells and trophoblasts and silent in all other normal tissue in adulthood. When malignant transformation occurs, tumors may begin to express these antigens. To date, these antigens have been found in an array of tumors including melanoma, breast, bladder, and lung cancer. Examples include New York Esophageal Squamous Cell Carcinoma-1 (NY-ESO1), synaptonemal complex protein 1 (SCP1), and melanoma antigen family A, 1-7 (MAGE1-7) (22).

Tumor antigens can also be generated by overexpression of normal molecules that have low or tightly regulated levels of expression in normal cells but have extremely high levels of expression in cancerous cells. Examples include Her-2, MUC1, CEA, and Cyclin B1 (16, 17).

Oncogenic viral antigens, that are perceived in a similar fashion as tumor antigens, are virus-encoded proteins utilized at some point in the virus life cycle in infected cells that overtime transform into malignant cells. Viruses such as Hepatitis B and C, which commonly result in hepatocellular carcinoma, and human papillomavirus (HPV), which is a driver of cervical and head and neck cancers, encode their viral antigens in the infection and oncogenic processes. Preventing these viral infections can reduce or eliminate the risk of certain cancers. Major targets in vaccine development against these oncogenic viruses currently include the L1 capsid protein and on going studies on developing vaccines based on the envelope proteins E6 and E7 from HPV and Hepatitis B surface antigen (HBsAg) from Hepatitis B Virus (16, 17).

Identification and subsequent classification of tumor antigens remain an important aspect in understanding and aiding in the attempts to strengthen immune responses against cancer. With greater understanding of the types of molecules tumors express or the viral proteins involved in virus pathogenesis, better and more targeted immunotherapies can be developed to promote antigen specific responses in vivo. Currently, Sipuleucel-T and the HPV vaccine, Gardasil, represent successful FDA approved therapies to elicit antigen specific immune responses against

cancer or viruses that lead to the development of cancer, respectively. In 2010, Sipuleucel -T was approved to treat hormone castration resistant metastatic prostate cancer (23). Sipuleucel-T consists of prostatic acid phosphatase (PAP), a prostate cancer antigen, fused to granulocyte-macrophage-colony stimulating factor (GM-CSF). It is used on autologous dendritic cells (DC) enriched from peripheral blood mononuclear cells (PBMCs) to stimulate an immune response against PAP. The treated DCs are then transfused back into the individual. This represented the first FDA approved cell based immunotherapy.

Gardasil is a HPV vaccine effective against HPV types six, 11, 16, and 18. These HPV types are responsible for 70% of cervical cancers and some vaginal and vulvar cancers (24). The vaccine is comprised of major capsid protein L1 from each of the aforementioned HPV types. Vaccination against these HPV subtypes leads to the induction of antibodies specific for L1. The antibodies can then neutralize HPV preventing the infection from taking place and essentially protecting from HPV induced cervical and head and neck cancers. Although this is a tremendous step towards cancer prevention, the vast majority cancers are of non-viral origin. Therefore there is still a need to advance not only therapeutic but also prophylactic cancer vaccinations to promote the elimination of tumors. Such studies are underway with the tumor antigen MUC1 (25).

1.1.2.2 Equilibrium

Equilibrium represents a state of functional inactivity of tumors. It is here that immune system and tumor cell growth are dynamically balanced. It is suggested that there are two types of equilibria. One type involves a cancer entering into a dormant quiescent state in which neither cell death or cell division is occurring (26). The second type is where tumor cell death and division occur at the same rate resulting in no net growth of the tumor (27, 28). Because the immune system cannot completely eliminate the tumor, immune selection occurs due to the accrual of mutations that can

eventually lead to tumor escape variants (29). For example, in a select number of melanoma patients who relapse following treatment with a NY-ESO-1 vaccine, histological analysis reveled loss of NY-ESO-1 and HLA expression. This observation demonstrated that immunoselection/immunosculpting occurred to make the tumor less detectable (30, 31). Empirical evidence in humans supports the idea of an equilibrium phase. For example, cancers were found in transplanted organs of immunosuppressed recipients when there was no evidence of malignancy in the original donors (32-34). It was assumed that these tumor cells were dormant in the immunocompetent donor but once the immune environment changed to an immunosuppressed recipient, tumor outgrowth occurred. An equilibrium state can also be inferred when examining the premalignant state of monoclonal gammopathy of undetermined significance (MGUS) and full blown malignancy of multiple myeloma. $CD4^+$ and $CD8^+$ T cell MGUS specific responses against autologous MGUS cells exist in MGUS patients yet T cell specific responses are non-existent in multiple myeloma patients against myeloma cells (35). MGUS could represent the equilibrium state in which the immune system has malignancy under control until other factors/instability leads to escape, which is represented as multiple myeloma. The first concrete evidence distinctly pointing out the existence of the equilibrium phase was in 2007 (36). Koebel et al. subjected wildtype mice to low dose treatments with MCA. A very small portion of these animals went on to develop tumors. However, when the same wildtype mice were depleted of adaptive immunity components, CD4, CD8 T cells, IL-12 and IFN-γ, sarcomas progressively grew out in roughly 50% of animals at the site of MCA injection compared to control treated group (29). This did not occur in mice depleted of NK cells, blocked for NK cell recognition via NKG2D, or disrupted in NK cell killing via TNF-related apoptosis inducing ligand (TRAIL). Koebel et al. also demonstrated that the tumors that developed were not from de novo cancer cells but instead, pre-

formed primary cancer cells that progressed from equilibrium to edited escape tumors. These pieces of evidence furthered the distinction between tumor elimination and equilibrium. Elimination needs both the innate and the adaptive immunity whereas equilibrium only requires adaptive immunity.

1.1.2.3 Escape

Tumor Escape is defined by the evasion of immune system recognition, control, and/or destruction, representing a failure of immunosurveillance. Escape occurs when the immune system loses recognition of the tumor through loss of tumor antigen or MHC, the immune system is suppressed by the tumor microenvironment, or there's an increase in the tumor's resistance to killing. The tumor microenvironment leads to the creation of extrinsic immunosuppressive mechanisms. Specifically, immunosuppressive cells such as T-regulatory cells (T_{regs}) and myeloid derived suppressor cells (MDSCs) are created, sustained, or recruited to the tumor. Natural and induced $CD4^+$ $CD25^+FoxP3^+T_{regs}$ can migrate to the site of the tumor. Once at the tumor site, T_{regs} can secrete inhibitory cytokines, TGF-β and IL-10, consume IL-2 preventing proper T effector cell function (37), kill effector T cells through TRAIL (38) and Granzyme B (39) mechanisms, and induce professional antigen presenting cells (APCs) such as DCs to become tolerogenic and express IL-10, IDO, and TGF-β (40). Myeloid Derived Suppressor Cells are a heterogeneous population of immature myeloid cells that have substantial suppressor function. MDSCs are found both in the periphery as well as at the site of the tumor. These cells secrete arginase I, which leads to depletion of L-arginine preventing proliferating effector T cells from using them, IL-10, TGF-β, and reactive oxygen species as well as induce T_{regs} (41). MDSCs also express galectin 9, which

binds to Tim-3 on T cells inducing apoptosis and have the ability to nitrate T cell receptors and chemokine receptors, making them dysfunctional (42, 43).

In addition to cellular mediators of immune suppression, tumors also constantly produce molecules that create an environment to ensure their survival. As tumors grow, they produce even more growth factors and other substances that are advantageous to the tumor and detrimental to the immune system. For instance vascular endothelial growth factor (VEGF) is commonly secreted by tumors for angiogenesis. VEGF secreted by tumors also induces programmed death ligand 1 (PD-L1) on dendritic cells leading to suppression of T cell activation (44). Other soluble immunosuppressive factors include TGF-β, galectin, Indolamine-2,3-Dioxygenase (IDO), and IL-10 (45). Inherent genomic instability in cancer can lead to antigenic loss variant cells and subsequent tumor outgrowth. Cancer cells that acquire defects in steps of the antigen presentation pathway escape immune cell recognition via MHC class I. For example, cancer cells that are defective in proteasome subunits LMP2 (46) and LMP7 (47), TAP1, β_2m, MHC class I itself (48), or responsiveness to IFN-γ can lead towards escape. Tumors can induce T cell anergy through lack of appropriate co-stimulatory signals. Lack of appropriate second signal co-stimulatory molecules prevents cytotoxic T-lymphocyte (CTL) mediated killing. Expression of negative co-stimulatory molecules such as PD-L1 (49), HLA-G (50), and HLA-E (51) prevents tumor killing by CTLs and NK cells. Tumors can escape NK cell recognition through upregulation of cellular microRNAs promoting the loss of NKG2D ligands (52). Escape can also be mediated by upregulation of anti-apoptotic molecules BCL-XL and FLIP (53, 54) or mutations of death receptors TRAIL-R (55) and Fas (56).

1.1.3 Tumor Associated Antigen Specific T cells and Antibodies in Cancer Patients and Healthy Individuals

Although cancer is mainly a disease caused by genetic and epigenetic changes, many of the identified antigen specific response are directed towards abnormally expressed self antigens rather than mutated self-antigens. These self-antigens expressed by tumors are classified as TAA. Antibodies and T-cell TAA specific responses are commonly found in cancer patients with a wide variety of malignancies. In many of these studies, immune responses are monitored to investigate the effectiveness of a therapeutic agent in inducing antigen specific responses. For instance, TAA specific antibodies and/or T-cell responses are assayed prior to therapeutic intervention in cancer patients as well as post treatment. In almost all instances, healthy donors who assumingly have no pre-existing TAA responses are used as a comparison.

One particular study examined the quantity and quality of the TAA glycoprotein 5T4 in colorecetal cancer patient. In this study, the group discovered that IFN-γ producing $CD4^+$ T cells not only existed in colorectal cancer patients but remarkably existed in 100% of healthy individuals tested. Furthermore, these 5T4 specific $CD4^+$ T cells were from the $CD45RO^+$ memory pool of T cells. This was not the only study that suggests the existence of TAA specific responses in healthy individuals (57).

In a study from our group examining the TAA cyclin B1, >40% of healthy individuals had cyclin B1 IgG antibodies above the average levels. The predominant IgG subclass was IgG3, which is strongly associated with T_h1 mediated immunity (58). Since age is associated with greater risk of malignancy, age was examined as a surrogate of underlying cancer. There was no correlation between age and cyclin B1 antibody levels, indirectly ruling out the possibility of undiagnosed cancers, which would not be expected to occur early in life, causing these elevated

antibody levels. Memory $CD8^+$ and $CD4^+$ T cells specific for cyclin B1 were also detected in healthy people (59).

Kallikrein 4 is a serine protease that is overexpressed in prostate cancer. One group discovered an immunogenic peptide in the signal sequence region of kallikrein 4 that was naturally processed and presented on HLA-A*0201. $CD8^+$ T cells recognizing the kallikrein 4 peptide were found in prostate cancer patients and healthy individuals. Clones of the kallikrein 4 specific $CD8^+$ T cells from both were able to kill CAOV3 ovarian cancer cells and weakly lyse LnCAP prostate cancer cell lines. The difference in $CD8^+$ mediated killing was likely due to MHC class I cell surface expression (60).

OCT-4 is an embryonic stem cell transcription factor that is overexpressed on many germ line tumors. Studies by Dhodapkar et al. investigated the presence of OCT-4 specific T cells. In their studies they defined OCT-4 reactive individuals as those who produce Interferon γ inducible protein-10 (IP10) in the supernatant of in vitro cultures. This study demonstrated the presence of OCT-4 specific $CD4^+$ T cells in 83% of healthy individuals assayed (N=30). These OCT-4 specific $CD4^+$ T cells were shown to be from the $CD45RO^+$memory pool (61).

The unexpected observation of responses to TAA in healthy individuals was made in other studies examining TAA such as MUC1 (62, 63), P53 (64-66), MAGE A10(67), Melan-A/MART1 (68), NY-ESO-1 (69), Cytochrome P450 1B1 (70), WT1(71), various phosphopeptides (72), and Telomerase (73). Although many of the T cell and/or antibody specific responses are low, they are not absent in healthy individuals. This likely indicates that some previous event(s) led to an immune response specific for the previously mentioned TAAs. While underlying neoplastic events that were eliminated by the immune system cannot be ruled out, our own studies and others point towards non-malignant events inducing immune responses against TAA.

1.1.4 Febrile Childhood Infections and Cancer Immunosurveillance

Modified from Iheagwara UK, Beatty PL, Chan S, Rigatti LH, Ross TM, and Finn OJ. MUC-1 as a therapeutic target in cancer: programming the immune system through childhood infections", In: Reese R, editor. Cancer Immunology and Immunotherapy. Oxford University Press; 2014. In press. Copyright permission is kept on file with Uzoma K. Iheagwara

The idea of febrile infections conferring protection against the development of tumors began to develop in the late 19th and early 20th century. Initially, anecdotal evidence based on physicians' observations and examination of medical histories of patients suggested beneficial effects of infections on cancer risk. More formalized data collection followed and in 1912 a study demonstrated that United States and Canadian Native American populations that had six times higher mortality rate from infectious disease had a lower rate of mortality from cancer (74). In 1916, another study examined mortality rates in New York, Boston, Philadelphia, and New Orleans from 1864-1888 and 1889-1913. Over these two time periods, deaths from infectious disease decreased while simultaneously cancer death rates increased by over 55%. In more contemporary times, a study examined the relationship between cancer and infectious disease rates in Italy (75). The authors proposed that there were four factors to consider when investigating possible causality in this relationship: 1. Consistency and strong associations among studies, 2. Temporality of the association, 3. True plausibility when considering the field of research, and 4. A dose response relationship between agent and disease. According to them, temporality was the

most important aspect in determining association. With this as a guide, they attempted to resolve the conflict between evidence that infections cause cancer versus evidence that infections can prevent cancer. Establishing a stricter temporal relationship between observations of a decrease in infections in Italy and observation of an increased cancer rate confirmed that in the first half of the 20th century the cancer increase indeed followed the decrease in infectious disease rates (75).

As evidence began to mount in favor of infections having a beneficial effect on preventing cancer, so did anecdotal evidence that concurrent febrile infection in cancer patients led to a better cancer control. Dr. William Coley was the first to administer infectious agents to a substantial number of cancer patients. Coley administered a mixture of heat killed *Streptococcus pyogenes* and *Serratia marrescens* to late-stage sarcoma patients. This mixture, known as Coley's Toxins, lead to cancer remissions in many sarcoma patients (76). Even today, infection of the bladder with *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG) is the treatment of choice for patients with non-muscle invasive bladder cancer (77). These bacterial infectious agents are thought to act as adjuvants enhancing ongoing anti-tumor immune responses. While this may be the case, infections might play other roles in the development of anti-tumor immune responses. These novel roles of infections will be investigated later in this dissertation.

In exploring relationships between childhood infections and cancer, the majority of studies focused on influenza, measles, mumps, pertussis/whooping cough, chicken pox, scarlet fever, rubella, and diphtheria. A study done in 1966 was designed to examine the relationship between exposure to X-ray radiation and hormonal therapy and increasing mortality rates from ovarian cancer. 97 patients with ovarian cancer and 97 controls with benign ovarian tumors were recruited and a thorough medical history was obtained. In the final analysis, X-ray radiation and hormone therapy were not found to have an impact on ovarian cancer mortality rates. Unexpectedly,

however, individuals who had reported a past history of mumps parotitis had a significantly reduced risk of ovarian cancer (p=0.0007)(78). This study was the first to suggest that mumps infection could lead to lower incidence of ovarian cancer. The mechanism, however, remained unknown until Cramer et al proposed an immune mechanism in 2010 (62).

In 1977, another study addressed possible causes for the rising incidence of ovarian cancer. 300 women with laparotomy-confirmed ovarian cancer were recruited in 17 medical centers. They were administered a questionnaire that included inquiries on X-ray exposure, pregnancies, smoking, chronic illness, history of acute infections, contraception use and prior surgeries, among others. The study included two control groups: 1. Gynecological patients in the hospital for reasons other than suspected ovarian cysts or tumors and 2. A group of women in the community provided from a list generated by general practitioners. These two control groups were provided with the same questionnaire as the case group. When the data was compiled, many women diagnosed with ovarian cancer reported fewer past bouts of mumps, measles, chicken pox, and rubella. The study also displayed a statistically significantly reduced relative risk of developing ovarian cancer in the second control group among women who had a history of measles (Relative Risk (RR)=0.47), mumps (RR=0.61), rubella (RR=0.62), and chicken pox (RR=0.66) (79). A recent retrospective review confirmed the outcome of reduced risk for ovarian cancer correlating with those four infections (74).

Kömel et al. performed a case control study in 1992 in support of his hypothesis that childhood and/or adulthood febrile infections influenced a higher risk of melanoma. The study was carried out at the University of Göttingen on 139 newly diagnosed melanoma patients whose primary tumor was removed between January 1988 and September 1991. The study also included 271 controls recruited from a pool of Ophthalmology and Dermatology clinic patients whose

diseases were not related to malignant melanoma. Both cases and controls were age and gender matched. These groups were administered questionnaires to collect information on frequency and temperature of febrile infections experienced as well as questions on childhood, adulthood infections, and common infections within the 5 years of melanoma diagnosis. Group one in the study consisted of individuals who had one or more of the following: measles, mumps, rubella, chicken pox, scarlet fever, diphtheria, whooping, and tonsillectomy performed prior to the age of 13 (assumed to have tonsillitis). Group two were individuals who had infections in adulthood associated with fever that included hepatitis, tuberculosis, erysipelas, chronic infectious diseases, febrile abscess, furunculosis, wound infections, tropical diseases, and other fever producing diseases. Common diseases accompanied by a fever within the five years of melanoma diagnosis (group three) included gastroenteritis, influenza/common cold, pneumonia, herpes simplex virus 1, and "other trivial diseases" associated with fever. Individuals in Group one were not found to have statistically significantly reduced risk of melanoma. However, Group two with the history of chronic infectious diseases (adjusted Odds Ratio (OR)=0.32) and febrile abscesses/furunculosis/wound infections (adjusted OR=0.21), were found to have a significantly reduced risk for melanoma. Individuals in group three, particularly with a history of influenza/common cold (adjusted OR=0.32) and HSV1 (adjusted OR=0.45) also had significant risk reduction for melanoma. There was also evidence of the cumulative effects of multiple febrile infections compared to never experiencing a serious febrile infection. The study showed that more fevers experienced in group two ($p=0.01$) and group three ($p=0.0001$) translated to a significant risk reduction. Although the calculated risk for melanoma was not significantly reduced in group one, childhood diseases such as chicken pox, measles and mumps had odds ratios <1 indicating a possible reduction of melanoma risk (80).

A repeat of this study on a larger number of cases included 603 melanoma patients from 11 medical centers linked via the European Organization for Research and Treatment of Cancer (EORTC) Melanoma Cooperative Group. The control group included 627 individuals who were age matched and selected from the same neighborhoods of the melanoma cases. Individuals in this study were grouped according to a history of severe diseases (group one- hepatitis, tuberculosis, *Staphylococcus aureus* infections, urinary tract infections, sepsis, meningitis, rheumatic fever, cholecystitis, and erysipelas), and less severe infections suffered five years prior to primary tumor removal (group two- influenza, infectious enteritis, bronchitis, pneumonia, and HSV). Results from this study confirmed the relationship between strong febrile infections accompanied by a body temperature above 38.5°C, and risk reduction for melanoma (81).

Association between gliomas and Varicella-Zoster Virus (VZV) was examined in the San Francisco Bay Area Glioma Study. Individuals were questioned on their shingles and chicken pox histories. These diseases caused by VZV were of particular interest for a link to glioma due to the virus having neurological sequela. The group found that glioma patients were less likely to have had a history of shingles or chicken pox compared to the control group. In order to have a more reliable proof of infection than self-reporting, a repeat of this study was performed on newly diagnosed glioma patients from whom the self-reported history of having chicken pox was obtained and confirmed serologically by positivity for anti-VZV antibodies. Data obtained on 462 glioma subjects and 443 controls matched for age, sex, and ethnicity demonstrated a reduced risk for developing glioma in individuals with a history of VZV (OR=0.4). The importance of having a more reliable measure than self-reporting, such as serologic markers for this type of studies was justified by the difference in this study between self-reported chicken pox infections and

seropositivity for VZV. Individuals who were IgG seropositive for VZV had a reduced risk (OR=0.6) for having a glioma (82).

Albonico et al. studied a cohort of cancer patients with solid epithelial tumors diagnosed by 35 general practitioners in Switzerland, matched them according to age, gender, and physician with a recruitment limit of 20 patients/physicians office. A total of 410 patients were administered questionnaires that requested information on age, gender, history of febrile childhood infections (in this study defined as measles, mumps, scarlet fever, pertussis, rubella, and chicken pox), and frequency of other infections that resulted in fever >39°C prior to the age of 21. Additionally, when answering questions specifically on febrile childhood infections; answering options were limited to "yes", "uncertain yes," "uncertain no," and "no" in order to reduce recall/memory bias. Almost 50% were breast cancer patients and therefore the final analysis was carried out on breast cancers vs. non-breast cancers. Analysis was also divided according to age ≤60 vs. >60. Ultimately the study found a statistically significant reduction of risk of solid malignancy in individuals who had a history of chickenpox (p=0.044) or rubella (p=0.0003) (83). The risk was further lowered with the increased number of infections. Curiously these results were obtained only in the 50% of individuals who had cancers other than breast cancer. Cancer risk reduction due to the history of febrile diseases did not hold for breast cancer patients, leading the authors to suggest that beneficial protection provided by childhood infections might be body site specific.

Additional studies on patients with many different cancers came up with similar results (74, 83, 84). One case-control study demonstrated that chicken pox and pertussis infections lowered risk for stomach, breast, colorectal, and ovarian cancer (84). Furthermore, an increase in frequency of cold and flu infections experienced also decreased risk for these cancers. We found only two studies that obtained opposite results (74, 85). Chickenpox (OR=2.09) and mumps

(OR=2.61) were shown to increase the risk of cancer. As a result, Hoffman et al proclaimed that 'no final statement could be made on the association of childhood diseases or fever and cancer should be made.' It is difficult to compare these two studies with the majority of studies described above and come up with a reasonable explanation for the different outcome. However the majority of studies published before and after Hoffman et al. findings support the link of a history of infections to decreased cancer risk.

Not all studies examining the relationship between infections and cancer deal with solid malignancies. Increasingly reports and studies are demonstrating an increased risk for acute lymphoblastic leukemia (ALL) with decreased exposure to childhood infections. Currently no specific pathogen has been implicated in lowering the risk of ALL development. Evidence continues to point towards a 'delayed infection hypothesis'- that ALL risk increases with the delay in exposure to certain infections early in life.

The study by Urayama et al. looked at different indicators in addition to infection in order to firmly establish that it is the early childhood infections that lower the risk of ALL. Specifically the study examined simultaneous effects of: 1. Birth order, 2. Day-care attendance, and 3. Common childhood infections. Subjects between the ages of 1-14 were enrolled in the Northern California Childhood Leukemia Study (NCCLS) conducted from 1995 to 2008. Approximately, 669 ALL subjects (284 non-Hispanic whites and 385 Hispanics) and 977 controls (458 non-Hispanic whites and 519 Hispanics) were selected to address the relationship to socio-demographic differences. ALL positive status was defined as a diagnosis of $CD10^{+}CD19^{+}$ALL between the ages of two to five. Cases and controls were compared separately for each ethnicity due to inherent differences in daycare utilization and family size. The group found that non-Hispanic white children who attended daycare by six months of age ($p=0.046$) and who had one or more, older siblings

(p=0.004) had lower risk for ALL. Also both Hispanic (OR: 0.48 [0.27-0.83]) and non-Hispanic white (OR: 0.39 [0.17-0.91]) children had a decreased odds ratio for having ALL if they had an ear infection before the age of six months (86). Additional social contact measures in Hispanic children did not demonstrate decreased risk for ALL. Other studies examining the traditional measures of childhood infections (measles, chickenpox, mumps, rubella, and pertussis) were also found to play a protective role against the development of other non-solid malignancies such AML and CLL in adulthood (87).

Anic et al. utilized similar indirect measures of infection risk with a study on adult glioma risk (88). This study examined glioma risk with birth order, family size, birth weight, season of birth, and breast-feeding history. The study recruited 889 adult glioma patients 18 years or older that were less than 3 months from glioma resection. All glioma case participants were recruited from neurosurgery and oncology clinics from southeastern universities and cancer center. About 903 non-blood related brain tumor free individuals from the same communities were used as case controls. Individuals were subjected to interviewer questionnaires to collect data on cancer risk. Anic et al. found that individuals who had any siblings (OR = 0.64; p = 0.020) or older siblings (OR = 0.75; p = 0.004) were at a lower risk of developing a glioma (88). All other risk factors tested were not significant.

Another study specifically dealing with Non-Hodgkin's Lymphoma (NHL), investigated the relationship between cancer and direct (i.e. self reported infection exposure) or indirect (i.e. family size, birth order, day care attendance etc.) measures of exposure to infection. NHL rates were rising by 3-4% in the developed world and it was hypothesized that this increase could be due to delayed infections leading to immune dysregulation (89). To test this, 1388 NHL patients, 354 Hodgkin's Lymphoma patients, and 1718 healthy controls were recruited in Italy and

questioned on their family size and history of acute and chronic infectious diseases as well as autoimmune disease. This particular study found that individuals were at an increased risk for NHL if exposure to their first bacterial or viral infection was delayed to after the age of four. Smaller family size also appeared to be greater risk factor for NHL (89).

Contracting a live pathogen is not the only way of providing protections against cancer. Vaccination with attenuated pathogens may also lower cancer risk. One group conducted a study on 542 malignant melanoma patients assessing the effect of vaccination practices on survival (90). The particular childhood vaccinations of interest, BCG and Vaccinia, were common until the late 1970s and 1980s. Although these patients already were dealing with cancer, valuable information was gathered. According to Kaplan-Meier analysis, melanoma patients immunized as children with BCG and/or Vaccinia survived much longer than their unvaccinated counterparts. At five years following malignant melanoma diagnosis, vaccinated group's survival was about 75% compared to approximately 50% in the unvaccinated group. Whether BCG and Vaccinia vaccination were considered separately or jointly, the hazard ratio for death in melanoma patients was decreased compared to unvaccinated patients. The study also analyzed the relationship between the number of reported bouts of infection and length of survival. As number of infections (that included osteomyelitis, mastitis, abscess, or furuncle) increased, the hazard ratio decreased yielding a significant difference in survival irrespective of whether the infections were accompanied by elevated temperature (p-value=0.004).

1.1.5 Summary

Cancer Immunosurveillance is real and accepted mechanism utilized by the immune system to kill tumor cells and restore normal tissue function. The adaptive immune system recognizes abnormal self molecules on tumors called TAA. In addition to cancer patients, TAA specific responses are also found in healthy individuals. In efforts to explain the presence of TAA specific responses in healthy people, epidemiology studies were queried and a large consensus of the studies indicated that a history of febrile infections correlates with decreased cancer risk. A formal biological link between febrile infections and cancer risk, first addressed by studies of Cramer, Finn and colleagues (62, 63, 91), needs to be explored further to provide additional explanation for the existence of TAA specific responses in healthy people and their role in tumor immunosurveillance.

1.2 INTRODUCTION TO THE PROJECT

Our lab previously demonstrated that healthy individuals have humoral and T-cell responses to various tumor associated antigens (TAA) such as cyclin B1 and MUC1. Currently it is unknown why there are memory responses to TAA in healthy individuals. This finding and other epidemiology studies suggested that early exposure to febrile infections decreases cancer risk. Currently there are no formal experimental studies or animal models that explain the mechanism by which prior infections can lead to a decrease in cancer risk. I therefore hypothesized that infections and accompanied acute inflammation early in life could cause abnormal expression of some self-antigens and induce specific immune memory against those antigens. Abnormal expression of the same antigens on tumor cells later in life could trigger infection-induced immune memory responses against these antigens and provide anti-tumor immunosurveillance. Rather than classifying these abnormally expressed molecules common to virus infected or malignant cells as TAA, they should be recognized as disease associated antigens (DAA). My thesis project was designed to test this hypothesis in a mouse model and provide evidence for an overlap between TAA and DAA.

The specific aims of my thesis work were:

Specific Aim 1: Validate influenza infection in mice as a disease model that will show changes in expression of self-antigens of interest and explore methodology that would allow identification of new antigens abnormally expressed during viral infection.

Specific Aim 2: Characterize molecularly and determine immunogenicity of epithelial cell proteins that are abnormally expressed in the lung during influenza infection.

- Sub-Aim 2.1: Identification of abnormally expressed, immunogenic self molecules.

- Sub-Aim 2.2: Determine if experiencing multiple influenza infection affects tumor growth kinetics

Specific Aim 3: Determine in vivo if immunization with one or more of the post-infection proteins confers protection against tumor challenge or influenza in vivo.

- Sub-Aim 3.1: Tumor immunosurveillance
- Sub-Aim 3.2: Virus immunosurveillance

2.0 METHODS FOR DISEASE ASSOCIATED ANTIGEN DISCOVERY

2.1 ESTABLISHING THE PATHOGEN AND TUMOR IMMUNOSURVEILLANCE MODEL

Numerous epidemiologic studies described above have suggested the link between the history of febrile infections and lowered cancer incidence. To date, there have been no experimental efforts to define the underlying mechanisms. What was lacking were appropriate animal models that would allow mechanistic studies. As part of my thesis project, I set out to test and validate a mouse model in which these studies could be pursued. My initial focus was on a single TAA, MUC1, for which there was sufficient evidence from both epidemiologic studies as well as in vitro immunologic testing, that it satisfies the criteria for a DAA due to changes of its expression not only on human epithelial tumors but also during acute infections. The ability to recreate these changes in a mouse model as part of specific aim one, was intended to serve as a validation that a similar mouse model might reveal other DAA. I chose either MUC1 transgenic (MUC1Tg) mice (on a C57BL/6 background) or C57BL/6 WT mice and for a pathogenic insult I selected murine adapted strains of influenza virus were. The influenza virus is a cytopathic virus that infects lung epithelium. Once infected, mice become feverish and lethargic, clear the virus, and recover. In our model, we planned to infected animals twice with two different strains of influenza to provide both the priming event and a boosting event to generate a maximal immune response against infection-induced abnormal self antigens. In the MUC1 antigen specific model we used, as a tumor challenge, mouse lymphoma cell line RMA stably transfected with MUC1 (RMA-MUC1).

For the continuation of the studies designed to detect other DAA, we focused on antigenic changes in flu-infected lung epithelial cells that could be expected to generate immune responses to abnormally expressed epithelial cell antigens preferentially expressed on epithelial cell tumors. So for the tumor challenge in those studies I selected two mouse epithelial tumor cell lines, the Lewis Lung Carcinoma (3LL) line and the IG-10 line. In order to identify abnormally expressed disease associated antigens following influenza infection, we planned to collect mouse sera pre- and post-influenza infection to determine what antigens were newly recognized by post-infection antibodies. In the following chapter, methods for antigen identification are reviewed and the rationale/methodology for DAA discovery discussed.

2.2 REVIEW OF ESTABLISHED TAA DISCOVERY METHODS AND SELECTION OF BEST METHOD FOR DAA IDENTIFICATION

Discovery and screening for potential tumor antigens is pivotal for producing targeted immunotherapies and increasing our knowledge of tumor immunosurveillance. Most techniques involve use of T cells and antibodies as screening tools. Several variations of these tools currently exist. Options include tumor cell line/autologous T cell screening, serological analysis of recombinant cDNA expression libraries (SEREX), biochemical approaches (with mild acid elution of peptides bound on MHC class I), and computer algorithms from reverse immunology (defined as predictive identification of possible immunogenic peptides via gene or protein sequences).

Tumor cell line and autologous T cell screening involve multiple steps. Tumor cells are co-cultured with autologous T cells. Once stimulated, the T cells are used to screen target cells that are transfected with tumor cell cDNA. Killing of the target cells indicates tumor antigen production

and processing in the target cell. Subsequently overlapping synthetic peptides can be then employed to better characterize the antigenic peptide region. SEREX involves using cancer patient sera to screen proteins from cDNA expression libraries derived from tumors. Common biological chemical approaches involve taking tumor cells derived from cell lines or cancer patients and eluting peptides bound on MHC class I molecules with a mild acid. These peptides are then subjected to liquid chromatography and mass spectrometry to identify the eluted antigens. In another approach, reverse immunology can be used to identify candidate antigens. In order to make use of this technique, one must already have a protein of interest. The protein is then analyzed through predictive computer algorithms such as the Immune Epitope Database (IEDB) or SYFPEITHI database to identify peptide sequences that might bind to MHC class I or II. High affinity peptides can then be synthesized, loaded onto dendritic cells for in-vitro priming of autologous T cells. Once these T-cells are primed, they can be co-cultured with tumor cells to look for immunogenicity.

Two other methods have been described for identifying novel TAA. The first method involves the use of 2D-SDS PAGE Gel Electrophoresis followed by western blotting. In this method, tumor proteins derived from whole cell lysates are separated according to isoelectric point (pI) in the first dimension on the gel followed by molecular weight separation in the second dimension. A second gel is run concurrently with the same sample. Proteins from the gel are immunoblotted with sera under study. Spots appearing in the blot that differentiate one set of sera from another can be either sent for proteomics identification or used as a guide in selecting protein spots for mass spectrometry analysis from concurrently run parallel gels. In other methods, sera can be used in immunoprecipitation experiments to pull down bound proteins that can then be run on 2D gels for further characterization.

Figure one shows an example of this technique used to identify differences in tumor cell lysate recognition between sera pre and post infection. From previous projects in the lab, our group had sera from two groups of animals: 1. Mice immunized against ultraviolet inactivated modified vaccinia ankara (UV-MVA/UV-MVA) (control group) and 2. Mice infected with a mouse pox virus, ectromelia (UV-MVA/Ectromelia). UV-MVA vaccination was used to ensure that mice from those experiments survived the ectromelia infection. LO2, a murine lymphoma cell line derived from a C56Bl/6 p53-/- mouse, tumor lysate was run on two 2D gels. LO2 tumor was used to keep consistency with previous projects from the lab. Once proteins were transferred onto PVDF membranes, each set of pooled sera was immuno-blotted onto the membranes. Although detectable differences were found between each set of sera, 2D western blotting proved to be too inconsistent between gels and blots to confidently compare and identify true differences in spots.

In subsequent sections, I describe the history and modification of the above 2D gel technique for use in our mouse model for identification of new DAA.

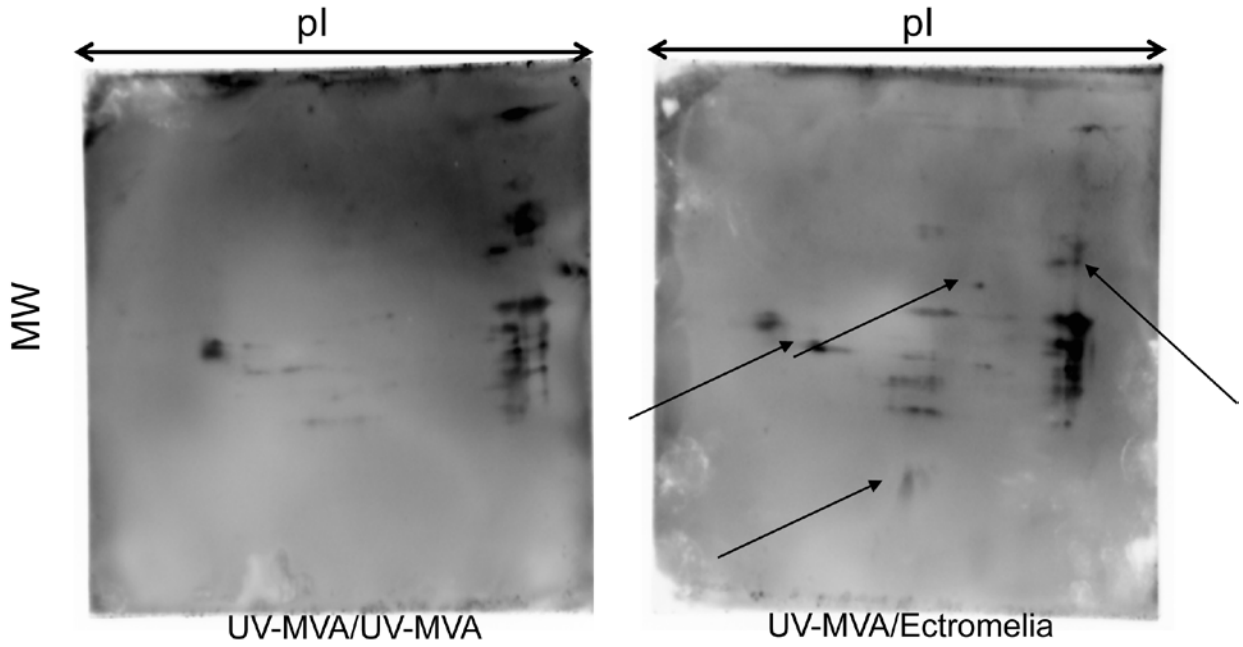

Figure 1 2D-blot of LO2 lysate.

Sera were obtained from mice immunized against Ultraviolet Inactivated Modified Vaccinia Ankara (UV-MVA/UV-MVA) (control group) or from mice infected with a mouse pox virus, ectromelia (UV-MVA/Ectromelia). Two sets of sera were isolated and pooled together and blotted against the LO2 tumor lysate. 1st dimension run on 7cm IPG strip pH 3-10 immobilized non-linear gradient (NL). Black arrows indicate new spots recognized following ectromelia virus exposure.

2.2.1 History of Difference Gel Electrophoresis (DIGE)

The convention for comparing two sets of proteins in the 1980s was to run parallel two-dimensional gels (2D-gels). Issues with consistency proved to be problematic. Recognizing this problem, Dr. Jonathan Minden, Carnegie Mellon University, came up with the idea to label each set of samples through a chemical reaction and combine the two samples to be run on one 2D-gel (92).

Three types of labels were tested: 1. Radioactive probes, 2. Color probes, 3. Fluorescent probes. Radioactive probes were too difficult to differentiate between various radiolabels. Additionally these probes required long exposure times. Color probes only worked when copious amounts of proteins were available. This left fluorescent tags as the best choice for labeling proteins especially in low concentrations. Advantages included being able to synthesize multiple probes with different spectra and only small amounts of the probes were needed in labeling reactions (92).

The next issue was the selection and coupling of fluorescent tags. Minden created a set of criteria needed to execute his idea. The tags needed to be: 1. Similar in size and charge, 2. pH insensitive due to the wide range of pH during the isoelectric separation step in 2D-gels, and 3. Should not alter the intrinsic charge of the proteins. Amino acids lysine and cysteine were chosen as possible coupling sites on protein. These two amino acids were the most reactive of all the other amino acids. Minden initially focused on lysine since it was not known at the time how many proteins would contain at a minimum one cysteine. Lysine contained an attractive amino group amenable to coupling reactions. However, coupling reactions would leave the lysine with a neutral charge as opposed to its natural positive charge. Therefore Minden decided that the probe needed to have a quaternary amine that would leave all protein's lysine's with a normal net positive charge

therefore not disrupting protein migration patterns during isoelectric focusing in 2D Electrophoresis (92). These decisions among other creations by Jonathan Minden would set the stage for the establishment of Difference in Gel Electrophoresis (DIGE) technique (93).

2.2.2 Labeling

2.2.2.1 Minimal Labeling

There are two primary ways to label protein samples: minimal labeling and saturation labeling. Here we will discuss the former. Cyanine (Cy) dyes have distinct wavelengths. Cy3 has an excitation maximum of 553 and an emission wavelength of 569. Cy5 has an excitation maximum of 645 and emission max of 664. Minimal labeling involves using cyanine dyes (Cy3 N-hydroxysuccinimidyl (Cy3-NHS), and Cy5 N-hydroxysuccinimidyl (Cy5-NHS) DIGE fluors) to label cysteines in the protein sample. Specifically N-hydroxysuccinimidyl (NHS) ester group on the dye reacts to form a covalent bond with the epsilon amino group of lysine in the sample protein (Fig. 2). The amino acid lysine has an inherent +1 charge. Therefore, to keep the overall charge at +1 following amide linkage, the CyDyes have a +1 charge. This prevents any potential change in natural protein migration during isoelectric focusing 1^{st} dimension separation in 2D-gel electrophoresis. It is important to note that all protein samples to be tested must be between pH 8-9. The dyes will not bind below pH 8 and multiple labelings may occur with a pH over 9.

An important aspect of minimal labeling is keeping the sample to dye ratios constant. As a result, only 1-2% of the available lysines on proteins are labeled and only single lysine per protein molecule. Increasing the amount of dye may cause multiple labels on one protein and thus altering their migration during 2D gel electrophoresis and prevent appropriate protein identification in downstream applications.

In our studies we chose against using minimal labeling as our approach to discovering abnormally expressed antigens during viral infection. For our purposes, we needed a labeling method that would label virtually all of our immunoprecipitated protein sample. Differences in our samples may be so small that we would miss them using this minimal labeling. Therefore we turned our attention to an alternative labeling method, saturation labeling.

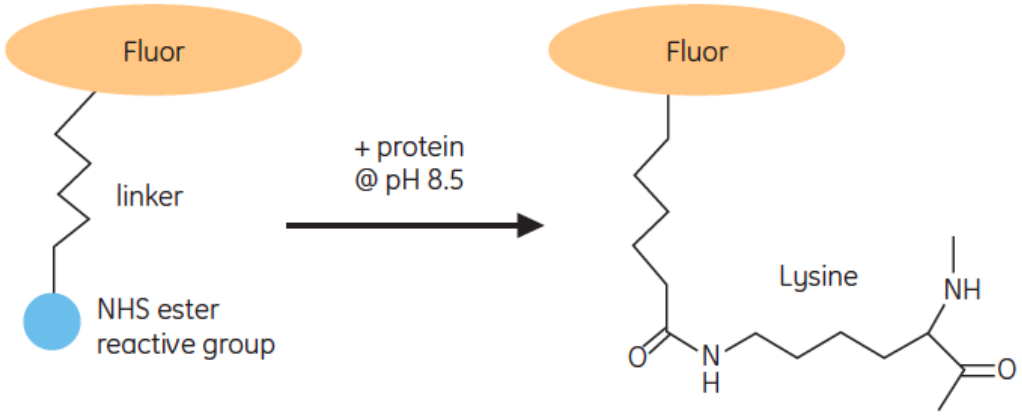

Figure 2: Minimal labeling reaction

Fluorescent cyanine dyes (Cy3-NHS and Cy5-NHS) containing an ester reactive group can react with the epsilon amino group on lysine to form a covalent amide bond to proteins of interest. This results in the fluorescent labeling of proteins.

2.2.2.2 Saturation Labeling

Saturation labeling is used to label samples that are scarce or precious. Saturation labeling can be applied to protein samples as low as 5ug. This labeling involves CyDyes that have been modified to contain maleimide group. Cy3-maleimide (Cy3-mal) and Cy5-maleimide (Cy5-mal) saturation dyes covalently bind to the thiol group on cysteine residues of the protein sample (Fig. 3). This thioester linkage is applied to all cysteine groups in the protein samples. Both Cy3-mal and Cy5-mal are relatively small at 680Da and have a neutral charge meaning that both sets of protein samples can successfully co-migrate and differential spot analysis can still be performed, although the pattern will be different compared to Cy3-NHS and Cy5-NHS samples. Saturation labeling was appropriate for our purposes due to our limited samples.

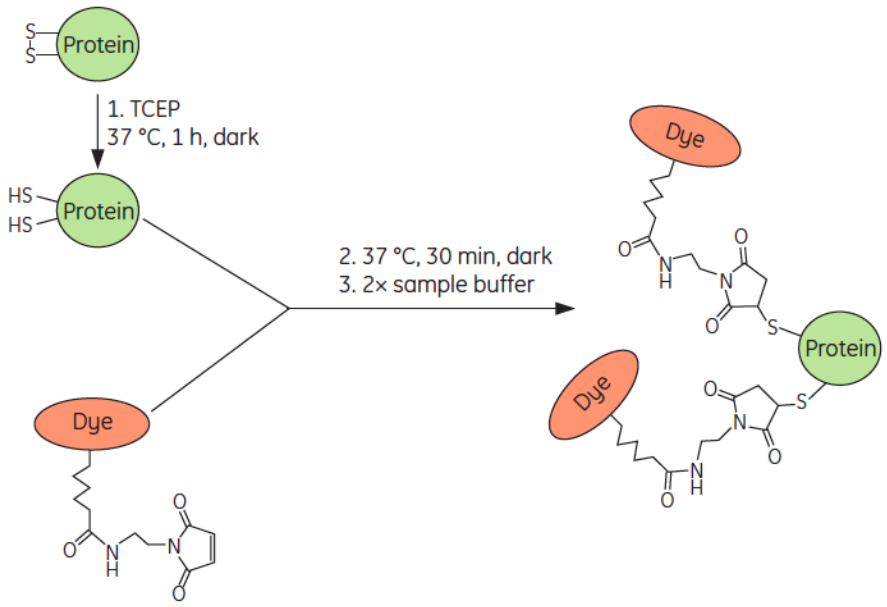

Figure 3: Saturation labeling reaction

Disulfide bonds on proteins of interest are first denatured with TCEP. The denatured proteins can now react with fluorescent cyanine dyes (Cy3-mal and Cy5-mal) containing an malimide reactive group can react with the cysteines to form a covalent thioester bonds to proteins of interest. This results in the fluorescent labeling of proteins.

3.0 TUMOR ASSOCIATED ANTIGEN MUC1 IS A DISEASE ASSOCIATED ANTIGEN IMPORTANT FOR CANCER IMMUNOSURVEILLANCE

Modified from Iheagwara UK, Beatty PL, Chan S, Rigatti LH, Ross TM, and Finn OJ. MUC-1 as a therapeutic target in cancer: programming the immune system through childhood infections", In: Reese R, editor. Cancer Immunology and Immunotherapy. Oxford University Press; 2014. In press. Copyright permission is kept on file with Uzoma K. Iheagwara

3.1 ABSTRACT

There is ample evidence that the immune system recognizes cancer as abnormal and halts tumor growth or eliminates tumors through recognition of tumor antigens, abnormally expressed self molecules. MUC1 tumor antigen is abnormally expressed on mouse tumors and in different human cancers. The same abnormal expression (overexpression, hypoglycosylation, loss of polarization) of MUC1 is found in non-malignant conditions, such as viral and bacterial infections and chronic inflammation. Epidemiological studies that identify cancer risk have demonstrated that a history of febrile childhood diseases lowers lifetime risk for a variety of cancers. We propose that these diseases enhance cancer immunosurveillance through generating immune responses and immune memory for abnormally expressed self antigens. In the current study we tested this hypothesis in a mouse model of cancer immunosurveillance by studying the effect of a repeated infection of MUC1 transgenic mice with influenza virus on the growth of a MUC1 expressing tumor. MUC1

transgenic mice infected with influenza virus transiently express abnormal MUC1 in their lungs and develop immune responses and immune memory. When challenged with a MUC1+ tumor, influenza experienced mice control MUC1+ tumor much better than influenza naïve mice. We also show that eliciting anti-MUC1 immunity with a vaccine lessens the intensity and pathology of influenza infection.

3.2 INTRODUCTION

Cancer takes several years and sometime decades to develop. Studies in animal models and cancer patients have provided evidence that the immune system can recognize cancer cells and halt their growth or eliminate them through a complex and varied process of immunosurveillance (94). This process depends on the specific recognition of abnormal molecules (tumor antigens) expressed by cancer cells and on appropriate activation and balance between innate and adaptive immunity. This occurs both systemically and in the tumor microenvironment. Therefore, in some instances, an anti-tumor response can result in increased T cell infiltration into a tumor and control of its progression, resulting in increased survival. Alternatively, tumor growth continues and coexists with evidence of adaptive anti-tumor immunity that is affected by profound adaptive and innate immune suppression mechanisms such as regulatory T cells (T_{reg}) and myeloid derived suppressor cells (MDSC) (95-97). The difference between these two outcomes can be a result of many individual differences between tumors (even of the same tissue type) and between patients. One of the important differences that could determine the cancer outcome is the individual's history of pathogenic events, especially those occurring in early life. Such events may expose the immune system to some of the same abnormal molecules, which are classically characterized as

tumor associated antigens (TAA) that occur in cancer. The immune memory of such molecules could later in life contribute to elimination of tumor or control of tumor growth (Fig. 4).

MUC1 is a glycoprotein that is expressed at low levels on the apical surface of normal epithelial cells (19). The extracellular domain of MUC1 is characterized by a variable number of tandem repeats (VNTR) region consisting of a tandemly repeated 20-amino acid sequence PDTRPAPGSTAPPAHGVTSA. In healthy epithelia the VNTR is highly glycosylated on serines and threonines with long and branched O-linked carbohydrates and the molecule is localized to the apical surface of ductal epithelial cells. In ductal epithelial adenocarcinomas, such as those of the breast, pancreas, ovary, colon and lung, MUC1 loses its apical polarization and becomes overexpressed and hypoglycosylated thus taking on a very different appearance and function than on normal cells (19). The reduced glycosylation exposes the peptide backbone that can then be processed by antigen presenting cells in the draining lymph nodes into peptide epitopes as well as glycopeptide epitopes with truncated glycans and presented to T cells (98, 99). T cells and antibodies specific for these epitopes can be found in cancer patients (100-103). MUC1 specific antibody and T cells effect on cancer development prior to diagnosis can only be postulated, however, their presence at diagnosis has been associated with better disease outcome (104, 105).

While studying MUC1-specific immunity in cancer, we made an important observations that the same hypoglycosylated ‘tumor’ form of MUC1 is expressed also at sites of chronic inflammation of epithelia, such as inflammatory bowel disease in the colon and mastitis in the breast, or endometriosis in the uterus (101, 106, 107). We also found expression of the tumor form of MUC1 in the salivary gland during mumps infection (62). Individuals with a history of having one or more of these non-malignant but inflammatory conditions also had MUC1-specific immune memory. Most importantly, these events and anti-MUC1 immunity correlated with a significant

reduction of risk for developing ovarian cancer (representative epithelial adenocarcinoma) (63). Based on these data we hypothesized that individuals with an immune memory for abnormally expressed MUC1 on cancer and other pathologies that affect various tissues, might be those with evidence of successful immunosurveillance (cancer elimination or cancer that is infiltrated with T cells that control its growth or metastasis). We wanted to test this hypothesis in a mouse model of cancer immunosurveillance by studying the effect of a repeated infection of MUC1 transgenic mice with influenza virus on the growth of a MUC1 expressing tumor. More specifically, we asked if influenza infection would lead to abnormal expression (non-polarized overexpression and hypoglycosylation) of MUC1 in the affected lung and if it would elicit MUC1-specific immune memory that would control growth of MUC1 positive tumors.

We show that influenza infection leads to changes in MUC1 in the lungs of infected mice and induction of MUC1-specific immune response that can delay MUC1+ tumor growth. We also show that eliciting anti-MUC1 immunity with a vaccine can lessen the intensity and pathology of influenza infection.

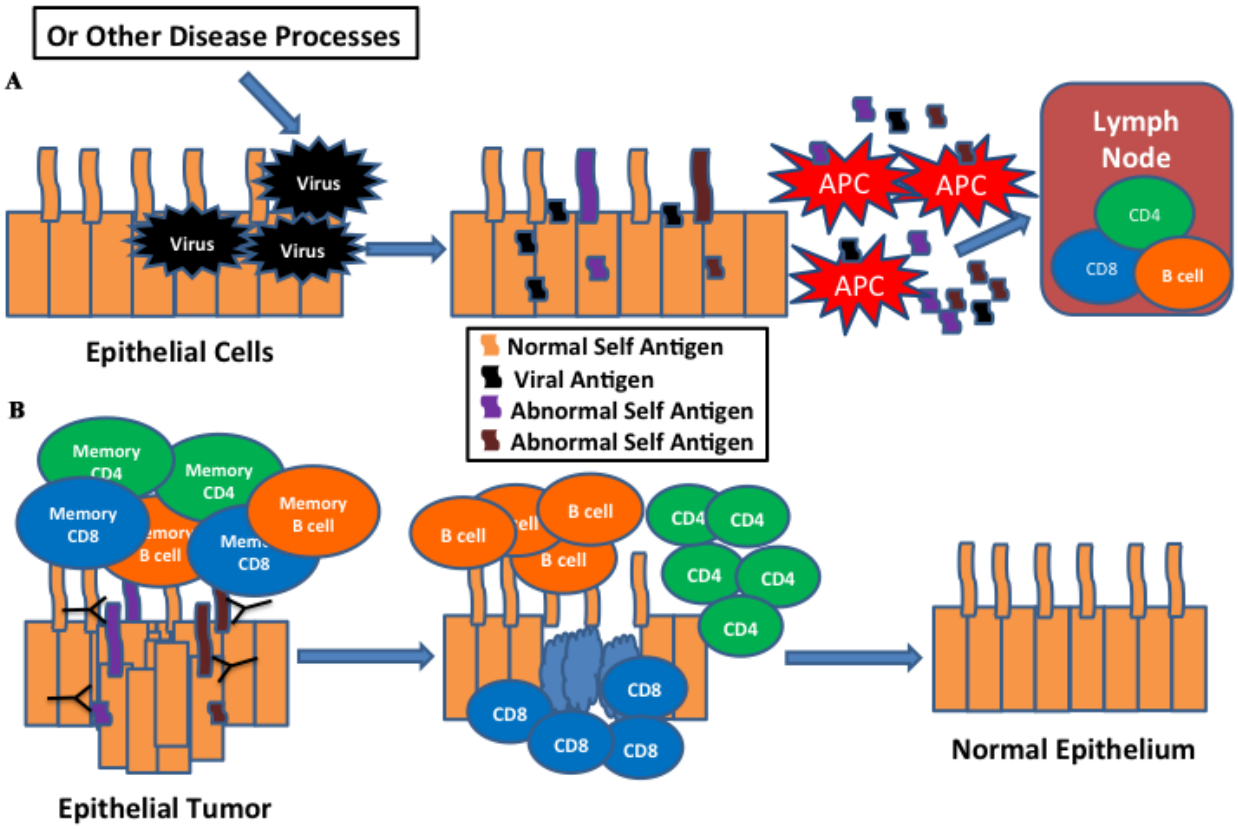

Figure 4: A new model of tumor immunosurveillance

(A) Epithelial cells that are infected by a virus express viral antigens and also self antigens abnormally expressed as a consequence of viral infection. These antigens are released into the lymphatics as soluble proteins or through tumor cell apoptosis and necrosis and picked up by antigen presenting cells (APC) that prime naïve $CD4^+$ and $CD8^+$ lymphocytes in the draining lymph node. Antibodies recognizing these antigens are also produced by B cells. Memory $CD4^+$ and $CD8^+$ T cells, memory B cells and newly formed antibodies are generated specific for viral antigens and for abnormal self antigens. (B) A tumor expressing abnormal self antigens can be bound by the previously formed antibodies as well as activate the memory T and B cells leading to its elimination or better control of its growth.

3.3 METHODS AND MATERIALS

Mice

Wild type C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). MUC1 transgenic mice on a C57Bl/6 background were developed by Dr. S.J. Gendler (The Mayo Clinic, Scottsdale, AZ). Both were bred at the University of Pittsburgh and treated according to guidelines from the Institutional Animal Care and Use Committee (IACUC). All experiments were approved by the IACUC.

Virus Infection

Mice were anesthetized using a mixture of Ketamine and Xylazine and intranasally inoculated with mouse-adapted H1N1, A//PuertoRico/8/1934 virus (PR8) at 15-18 weeks of age. At three weeks post-PR8 infection, mice were infected the second time with H1N1 A/NewCaledonia/20/1999 virus (NC). Virus titration was performed on lungs of Wt mice infected with different concentrations of PR8 and NC viral strains according to published protocol (108).

Peptide synthesis and in vitro glycosylation

The TnMUC1 100-mer peptide used for immunization corresponds to five tandem repeats of the 20–amino acid sequence HGVTSAPDTRPAPGSTAPPA from the extracellular VNTR region of MUC1. Enzymatic addition of GalNAc to the peptide was done using recombinant UDP-GalNAc polypeptide N-acetyl-galactosaminyltransferases rGalNAc-T1 as previously described (109). MUC1 glycopeptide MUC1-10-5GalNAc is from the same VNTR region but 10aa in length (SAPDTRPAPG) with the glycosylated T residue in the five positions. The peptides were synthesized at the University of Pittsburgh Genomics and Proteomics Core Laboratories.

Vaccine protocol

Mice in the vaccine group received 20 μL intranasally (10 μL/nare) of 30 μg of TnMUC1 100-mer peptide mixed with three μg of adjuvant E6020 (synthetic, attenuated Toll-like receptor-4 agonist provided by Eisai Research Institute, Andover, MA) (110, 111).

Mice were boosted twice at two-week intervals.

Immunohistochemistry

Tissue paraffin sections (5μm thick) were deparaffinized by baking overnight at 59°C. Endogenous peroxidase activity was eliminated by treatment with 30% H_2O_2 for 15 min at room temperature. Antigen retrieval was done by microwave heating in 0.1% citrate buffer. Nonspecific binding sites were blocked with Protein Blocking Agent (Thermo-Shandon). The anti-MUC1 antibody HMPV (BD Pharmingen) recognizes all forms of MUC1 by binding the epitope APDTR in the VNTR region in a glycosylation-independent manner. The anti-MUC1 antibody VU-4H5 (Santa Cruz Biotechnology) recognizes the epitope APDTRPAP in the VNTR region of hypoglycosylated MUC1. Staining was done by the avidin-biotinperoxidase method with a commercial kit (Vectastain ABC kit, Vector Laboratories). Color development was done using a 3,3′-diaminobenzidine kit (BD Pharmingen).

Flow cytometry

Isolated cells from spleens were washed and resuspended in fluorescence-activated cell sorting buffer (2% fetal bovine serum in PBS) and plated at 0.5×10^6 to 1×10^6 per well. Surface Fc receptors were blocked with the addition of anti-CD16 (BD Biosciences) and incubated for 20

min. Cells were stained with anti-CD3, anti-CD4, anti-CD8, (BD Biosciences). DimerX, Soluble Dimeric Mouse H- 2Kb:Ig Fusion Protein (BD Biosciences) was used according to the manufacturer's protocol to detect MUC1-specific CD8 T cells. Cells were analyzed on a LSR II flow cytometer (BD Biosciences), running FACSDiva software.

Microscopy and image acquisition

Histology sections were observed using an Olympus BX40 microscope. Images were acquired using a Leica DFC420 camera and Leica Application Suite version 2.7.1 R1.

3.4 RESULTS AND DISCUSSION

Abnormal expression of MUC1 in flu-infected lungs

Human MUC1 Tg mice express low levels of the fully glycosylated (normal) MUC1 on the surface of lung epithelial cells (112). We examined the effect of an acute viral infection on MUC1 expression in the lung as well as the effect of MUC1 expression on the pathology accompanying lung infection. We infected MUC1Tg mice with influenza virus and compared the results of the infection with flu-infected wild type (Wt) mice that do not express human MUC1. Both strains of mice were intranasally inoculated with mouse-adapted H1N1 A/PuertoRico/8/1934 (PR8) virus. All mice had body weight recorded and were inspected for general health condition following PR8 infection. We observed that both groups began to lose weight between days four and six post-infection; however, Wt mice lost significantly more weight at days four, six, and ten compared to MUC1 Tg mice (Fig. 5A) and this resulted in several Wt mice being removed from the protocol and sacrificed early. Wt mice experienced maximum weight loss at day ten and started to rebound

at day 12 but did not recover to their original body weight until day 30. MUC1 Tg mice experienced maximum weight loss at day eight and recovered to their original body weight by day 14.

Given the significant difference in weight loss that suggested milder infection in MUC1 Tg mice, we questioned whether the presence of MUC1 on the surface of the lung epithelia might have simply interfered with virus entry into the cell. We had previously published that this indeed occurs during adenovirus infection and that MUC1 can hamper adenovirus-mediated gene therapy (113). Infected mice were sacrificed at day three post infection and virus titers in mouse lungs were determined in both groups. We found little difference in the virus titer between MUC1 Tg and Wt mice at that time point (Fig. 5B). We then sacrificed mice at day eight post-infection to determine if both groups were clearing the virus at similar rates. Four of four Wt mice and two of four of MUC1 Tg mice had cleared the infection by that time (Fig. 5B). These results suggested that MUC1 was not playing a role in either the initial infection or in virus clearance.

To look at potential changes in MUC1 expression, especially the hypothesized increase in expression of the abnormal hypoglycosylated form, we examined by immunochemistry lung tissue sections from flu-infected and from age-matched uninfected MUC1 Tg mice. We used two different anti-MUC1 antibodies to detect different forms of MUC1: anti-MUC1 HMPV that recognizes all forms of MUC1 regardless of the glycosylation pattern and antibody VU-4H5 that recognizes only the hypoglycosylated ‘tumor’ form of MUC1. Figure 5C shows representative examples from both groups. MUC1 expression in uninfected MUC1 Tg mice is restricted to a thin brown line on the apical surface of bronchiolar epithelial cells (top left) with no expression of the hypoglycosylated ‘tumor’ MUC1 (top right). In contrast, MUC1 expression was dramatically increased in acutely infected bronchiolar epithelial cells (bottom left). Higher magnification shows

loss of apical polarization and high level of cytoplasmic MUC1 that is in the hypoglycosylated ‘tumor’ form (bottom right). This staining pattern is consistent with the pattern observed in neoplastic lung tissue.

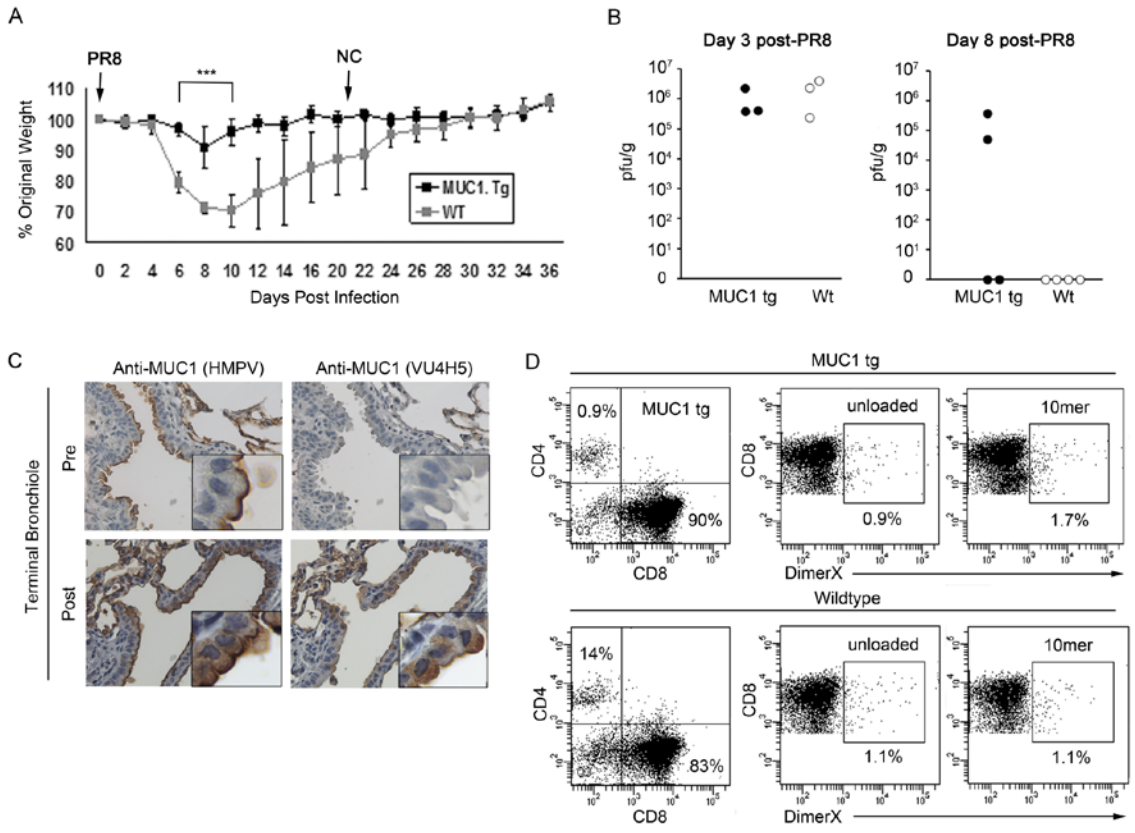

Figure 5: Influenza infection in MUC1Tg and WT mice

(A) Following infection with a sublethal dose of PR8 virus, mice were evaluated for weight loss. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (B) Influenza virus titer was evaluated day three post-PR8 and day eight post-PR8 infection in lung supernatants of MUC1 Tg (black circles) and Wt (open circles) mice by plaquing on MDCK cells. (C) Immunostaining of pre- and post-PR8 infected lung tissue. HMPV Ab is glycosylation independent and VU-4H5 is glycosylation dependent. (D) Splenocytes from post-PR8 infected MUC1 Tg and Wt mice were stained with MUC1 peptide-loaded H2Kb-Ig dimer. Figure based on experiments performed by Dr. Pam Beatty with assistance by the author.

Flu infection elicits MUC1-specific CD8 T cells and delays MUC1+ tumor growth

We next wanted to examine if the difference in expression and glycosylation of MUC1 in infected lungs during flu infection could lead to MUC1-specific immunity. To answer this question we looked for MUC1-specific $CD8^+$ T cells in spleens of infected MUC1 Tg mice. We isolated spleen cells from both MUC1 Tg and Wt mouse at day 12 post-PR8 infection and stained them with anti-CD3, anti-CD4 and anti-CD8 antibodies and with MUC1 peptides-loaded MHC class I H-$2K^b$ DimerX (BD Biosciences). DimerX is MHC-Ig fusion protein that when loaded with peptide can bind to antigen specific T cell receptors on antigen-specific $CD8^+$ T cells (114). DimerX was loaded with either the MUC1–8mer peptide (SAPDTRPA) or MUC1–10mer (SAPDTRPAPG) glycopeptide, both derived from the VNTR region. The MUC1–10mer glycopeptide has the GalNAc glycan attached to the threonine at position five. Unloaded DimerX and peptide loaded DimerX staining of T cells from infected Wt mice were used as negative controls. We detected a two-fold increase in MUC1-10mer specific $CD8^+$ T cells compared to unloaded DimerX background staining in flu infected MUC1 Tg mice (Fig 5D). No difference was detected between MUC1-10mer loaded DimerX and unloaded DimerX background staining in Wt mice. No MUC1-8mer-specific $CD8^+$ T cells were detected in either group.

Next we asked whether this MUC1-specific immune response could suppress MUC1 positive tumor growth. Our expectation was that a MUC1 expressing tumor would reactivate the MUC1-specific memory response that had been generated during the influenza infection, which might result in some control of tumor growth. Wt and MUC1 Tg mice were inoculated with PR8 virus followed by NC virus three weeks later. We used repeated infection to better mimic what

might occur in people who are exposed to multiple infections and acute inflammatory events over a life time, each serving as a booster of immune memory. Mice were allowed to fully recover from the second infection and rested for 80 days to allow for the development of immunological memory. Mice were then challenged with the MUC1-transfected lymphoma cell line RMA-MUC1 and the parental line RMA and tumor growth compared between previously infected and uninfected mice. We observed little difference in the growth of the $MUC1^-$ tumor RMA between infected and uninfected Wt mice (Fig. 6A). There was also no difference in RMA/MUC1 tumor growth between infected and uninfected Wt mice (Fig. 6C). In addition, we compared RMA tumor size (Fig, 6E) and RMA/MUC1 tumor size (Fig. 6F) at day 16 post-tumor challenge between infected and uninfected Wt mice and found no differences. Influenza's lack of an effect on tumor growth in wildtype animals may be due to a couple of factors. Influenza infection may not have been severe enough due to the second influenza exposure being of the H1N1 subtype. Also the use of a lymphoma cell line may not be optimal in the wildtype mouse setting due to the possibility that lymphoma antigens might be different enough from an epithelial tumor thus preventing any immunological memory developed following influenza exposure from being effective. In MUC1 Tg mice, however, we observed delayed growth of the RMA tumor in three out of five influenza infected MUC1 Tg mice compared to uninfected MUC1, especially evident between days eight and 12 (Fig. 6B). The comparison of tumor size at day 15 was not significantly different between infected and uninfected MUC1 Tg mice (Fig. 6G). In contrast, we observed a dramatic difference in RMA/MUC1 tumor growth (Fig. 6D) and statistically significant difference in RMA/MUC1 tumor size at day 15 between influenza infected and uninfected MUC1 Tg mice (Fig. 6H).

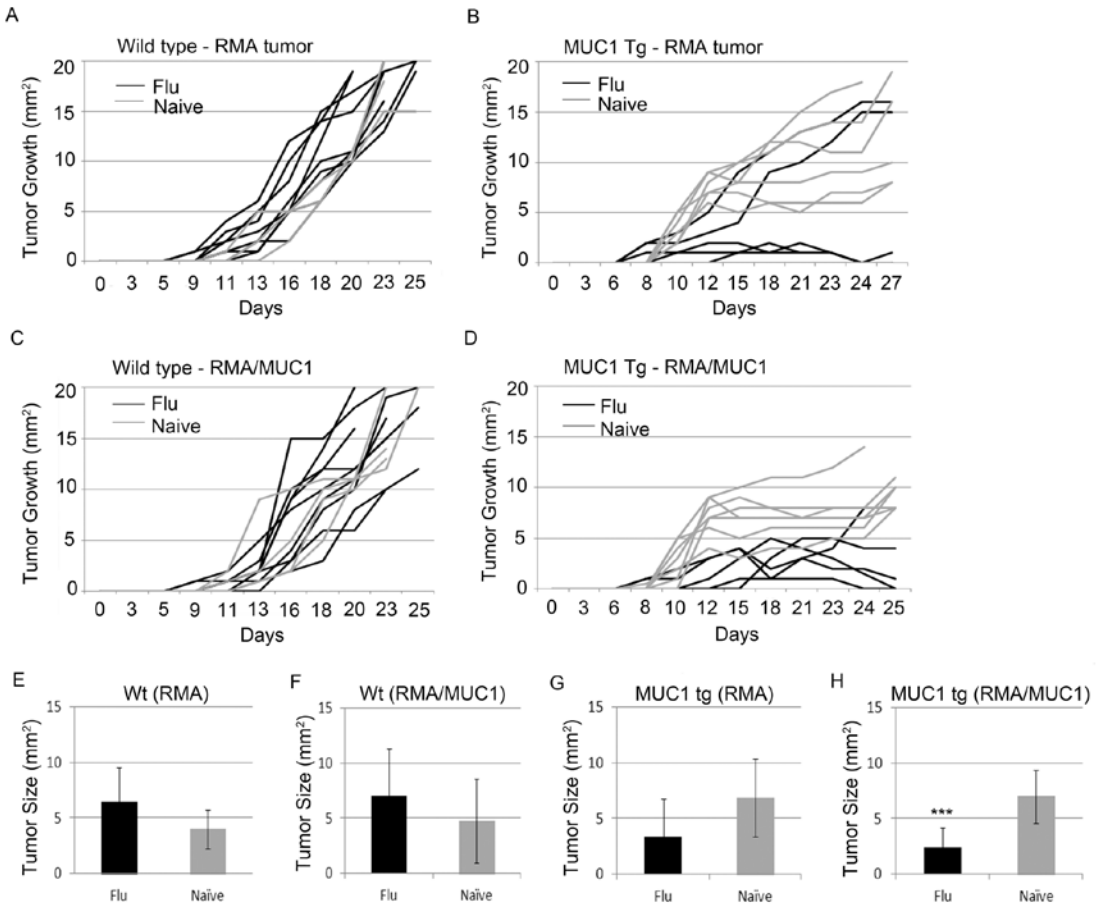

Figure 6: Tumor growth in post-PR8 infected MUC1Tg and Wt mice

(A-D) Tumor growth curves in MUC1 Tg and Wt mice. (E-F) Tumor size in Wt mice at day 16 post-tumor challenge. (G-H) Tumor size in MUC1 Tg. mice at day 15 post-tumor challenge.* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Figure based on experiments performed by Dr. Pam Beatty with assistance by the author.

Anti-MUC1 immunity elicited by vaccination attenuates flu virus infection

We have previously tested the efficacy of MUC1 vaccines for protection from MUC1 positive tumor challenge (115). Given that we observed that influenza infection changes MUC1 expression and elicits MUC1 immunity we wanted to know if a similar anti-MUC1 immune response induced through vaccination would have an effect on the extent and duration of influenza infection in MUC1 Tg mice. The assumption was that influenza virus infected cells would express abnormal MUC1 and be targeted for destruction by vaccine-induced MUC1-specific T cells. MUC1 vaccine consisted of a synthetic 100-mer glycopeptide TnMUC1 corresponding to five 20–amino acid tandem repeats of MUC1, glycosylated in vitro with the tumor-associated glycan GalNAc, plus E6020 adjuvant, a synthetic, attenuated Toll-like receptor-4 agonist that promotes both systemic and mucosal immunity (110, 111). The vaccine group received nasal administration of the MUC1 vaccine on day zero followed by a booster two weeks later. Mice were inoculated with PR8 virus three weeks following the booster. Age-matched non-vaccinated MUC1 Tg mice were also inoculated with PR8 virus and both groups were monitored for weight loss and flu symptoms. We observed that both groups began to lose weight at the same time point between days four and six post-PR8 infection, however, weight loss was dramatically different between the two groups. Non-vaccinated mice lost significantly more weight by day eight compared to vaccinated mice (Fig. 7A). Both groups began to rebound between days eight and ten and both groups recovered to their original body weight at day 30. We compared lung pathology between the two groups and found less pathology in lungs from the vaccinated group compared to the non-vaccinated group (Fig. 7B). In addition, the inflammatory infiltrate into the lung was dramatically different between the two groups. Lungs from the vaccinated group had a predominant $CD3^+$ T

cell population and foamy macrophages within alveolar spaces. In comparison, the untreated group had a more intense mixed inflammatory cell infiltrate with hemorrhage, large areas of atelectasis, and regions with multifocal type II pneumocyte hyperplasia (Fig. 7C).

Several mechanisms may be postulated to explain our findings: 1) infections may provide adjuvant/immunostimulatory effects to increase recognition of abnormally expressed self molecules (disease associated antigens, DAA) and generate adaptive immunity that can later assist in tumor elimination via the same antigens (a.k.a. tumor associated antigens, TAA); 2) there may also be antigenic cross-reactivity between antigens derived from pathogens and TAA on tumor cells; and 3) previous infections may influence types of immunity elicited by subsequent infections, e.g. Th1 vs. Th2, as proposed by the Hygiene Hypothesis (116). The work done by our group favors the first scenario in support of the hypothesis that immune responses generated early in life against abnormal self antigens encountered during febrile viral or bacterial infections and transient acute inflammations carry out cancer immunosurveillance (Fig. 4).

The published studies by us and others and the new data we presented are consistent with our hypothesis that an important general mechanism of immunosurveillance against cancer and other diseases is through generation of immune memory early in life against abnormal self molecules generated during pathogenic events in a particular organ or tissue. During the early process of malignant transformation similar changes are recapitulated leading to an anti-tumor immune response that is actually a memory response to abnormal self.

Collectively, these studies have far reaching implications on the way we view tumor antigens and anti-tumor immunity. If the above hypothesis is supported by future studies, it would not only establish the importance of acute infections in boosting tumor immunosurveillance, but would also represent a paradigm shift in the way we think about infections and cancer. This may

lead to the development of new methods to elicit and boost an anti-tumor immune response. It is likely that further studies will reveal additional associations between cancer and many diseases and the antigens connecting them. Prophylactic vaccines against these disease associated antigens that are also tumor associated antigens could be expected to generate immune memory early in life for a broad protection from all diseases, including cancer.

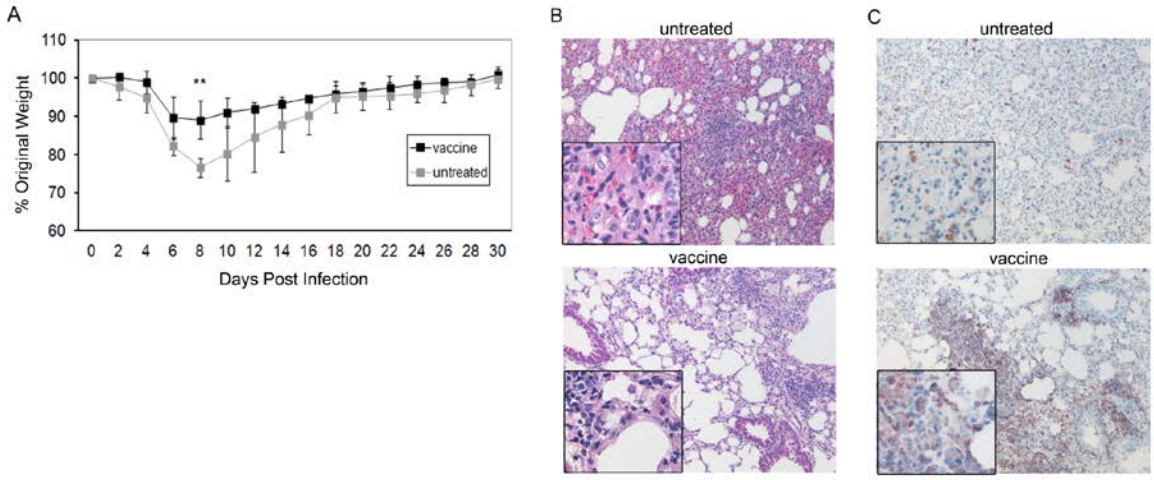

Figure 7: Anti-MUC1 immunity elicited by vaccination attenuated flu virus infection

A) Vaccinated MUC1 Tg. mice were evaluated for weight loss following a sublethal dose of PR8 virus. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (B) H&E stained lung tissue from untreated or vaccinated MUC1 Tg. mice following MUC1 Tg. PR-8 infection. (C) Immunostained lung tissue from untreated or vaccinated MUC1 Tg. mice following MUC1 Tg. PR-8 infection. Figure based on experiments performed by Dr. Pam Beatty with assistance by the author.

4.0 INFLUENZA VIRUS INFECTION ELICITS ANTIBODIES AND T CELLS TO HOST CELL ANTIGENS PREVIOUSLY IDENTIFIED AS TUMOR ASSOCIATED ANTIGENS

Data in this chapter are adapted from Iheagwara, UK, Beatty, PL, Van, PT, Ross TM, Minden, JS, Finn, OJ. 2013. "Influenza virus infection elicits antibodies and T cells to host cell antigens previously identified as tumor associated antigens: a new view of cancer immunosurveillance", *Cancer Immunology Research.*

4.1 ABSTRACT

Most TAAs are self-molecules that are abnormally expressed in cancer cells and thus become targets of anti-tumor immune responses. Antibodies and T cells specific for some TAA have also been found in healthy individuals and associated with lowered lifetime risk for developing cancer. Lower risk for cancer has also been associated with a history of febrile viral diseases. We hypothesized that virus infections could lead to transient expression of abnormal forms of self molecules, some of which are TAAs, and facilitated by the adjuvant effects of infection and inflammation, elicit specific antibodies and T cells and lasting immune memory against those antigens simultaneously with immunity against viral antigens. Such infection-induced immune memory for TAA would be expected to provide life-long immune surveillance of cancer. Using

influenza virus infection in mice as a model system, we tested this hypothesis and demonstrated that influenza experienced mice control 3LL mouse lung tumor challenge better then infection naive control mice. Using 2D-Difference Gel Electrophoresis (2D-DIGE) and mass spectrometry, we identified numerous molecules on the 3LL tumor cells recognized by antibodies elicited by two successive influenza infections, some of which were already known tumor antigens. We studied in more detail immune responses against GAPDH, Histone H4, HSP90, Malate Dehydrogenase 2 and Annexin A2, which were all overexpressed in influenza infected lungs compared to normal lungs, as well as in tumor cells. Lastly, we show that immune responses generated through vaccination against peptides derived from these antigens correlated with improved initial tumor growth control.

4.2 INTRODUCTION

Many cancers are a result of genetic mutations and epigenetic modifications leading to cellular transformation (117). One of the important cancer cell extrinsic mechanisms that allows or prevents tumor outgrowth is tumor immunosurveillance, the ability of the immune system to recognize abnormal cells (12). Successful tumor immunosurveillance leading to tumor elimination involves, among other immune mechanisms, recognition by B and T cells of tumor-specific or tumor associated antigens. T cells recognize tumor antigens presented on Major Histocompatibility Complex (MHC) class I and II molecules and kill tumor cells via lytic granule release (CD8+ T cells) or promote cellular and humoral responses through production of cytokines (CD4+ T cells). TAA specific antibodies can bind to tumor cells and lyse them with the help of complement or facilitate their killing by T cells and natural killer (NK) cells through antibody

dependent cellular cytotoxicity (ADCC) (118, 119). Molecular and biochemical characterization of numerous tumor antigens has led to a better understanding of targets that are especially important for immunosurveillance and facilitated development of immunotherapeutic strategies directed against those targets.

Cancer patients have circulating tumor specific antibodies and T cells that have been used as reagents to characterize individual tumor antigens (120-123). Immune responses to several TAA, when present at diagnosis, have been correlated with a favorable prognosis. Even when target antigens are not known, infiltration of tumors by activated T cells has correlated with better prognosis and longer disease free and overall survival (124). Nevertheless, a diagnosis of cancer is a sign that the cancer has escaped immune control. The newest approaches in cancer immunotherapy are directed towards regaining immune control by drug-targeting both the cancer and the immune system (125).

DNA sequencing has shown that tumors have on average a dozen or more individual mutations that could generate new epitopes highly specific for each individual's tumor, known as tumor-specific antigens (126). While tumors could express these epitopes as targets and adoptive transfer of T cells or antibodies could lead to their recognition and elimination, spontaneous immune responses to such epitopes (e.g. mutated Kras, EGFR or p53) have not been found with the regularity that could be expected from the frequency of these mutations (127). Instead, the majority of anti-tumor immune responses are against non-mutated self antigens, which are in various ways abnormally expressed on tumor cells and thus named TAA. These include molecules that on tumors compared to normal cells are either overexpressed [e.g. Her-2neu (128), MUC1 (19), CEA (129), Cyclin B1 (120)], show unscheduled expression [e.g. oncofetal antigens α-fetoprotein (130), cancer-testis antigens NY-ESO1 (131), Mage 1-7 (132)], or undergo different

posttranslational modifications (glycosylation or phosphorylation)[e.g. hypoglycosylation of MUC1 (19) or aberrant phosphorylation of β-catenin (21)]. Abnormal expression of many of these antigens can be seen early in the process of tumor development on premalignant precursors of various cancers (106, 121).

Several large epidemiological studies have been published showing that a history of febrile childhood infections is correlated with a reduced life-time risk of many different cancers (74, 81, 86). The mechanisms underlying this protective function were unknown. At the same time, observations were being made that healthy individuals with no previous history of cancer can have antibodies and/or T cells specific for several well-known TAA (59, 133). For example, we found that the TAA MUC1 was expressed in the tumor form (overexpressed and hypoglycosylated) on salivary gland ducts during mumps parotitis infection (62), on breast ducts during lactation and in lactational mastitis (101), and in inflammatory bowel disease (106). We further showed that the presence of anti-MUC1 IgG in women that experienced early in life one or more of these events, correlated with a significantly lower ovarian cancer risk (91).

In this study we present the first attempt to recapitulate these observations in a mouse model. Having an experimental model allowed us to start testing our hypothesis that immunity and immune memory against abnormal self antigens, known as TAA, is not elicited in response to their *de novo* expression on tumor cells or premalignant lesions that develop late in life, but rather it is elicited early in life in response to their expression during acute inflammations accompanying viral and other infections. When some of the same self antigens are abnormally expressed on premalignant lesions or tumor cells, they can be recognized by infection-primed immune memory responses leading to tumor elimination or enhanced tumor control. We show that mice that experience two infections with two different influenza viruses develop immunity to self antigens

abnormally expressed on infected lungs, which correlates with their ability to control growth of a transplantable lung tumor that abnormally expresses those same self antigens. We analyzed in detail infection elicited immune responses to five such antigens: Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), Histone H4, Malate Dehydrogenase 2 (MDH2), Annexin A2, and Heat Shock Protein 90 (HSP90). They were all recognized in tumor cell lysates by post-infection sera. We show that they are all overexpressed in tumor cells, as well as in influenza virus-infected lungs compared to healthy lungs, and that influenza virus infection generates antibody and $CD8^+$T cells specific for these antigens. We demonstrate that immunization of mice with peptides derived from these antigens protects them effectively against a tumor challenge.

4.3 METHODS AND MATERIALS

Mice, tumor cell lines, and influenza virus

Six to eight week old female C57BL/6 wildtype (WT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the University of Pittsburgh Animal Facility. All animal protocols were in accordance with IUCAC guidelines at the University of Pittsburgh. Lewis Lung Carcinoma cell line (3LL), a murine lung epithelial tumor, was maintained in c-DMEM media containing 10% heat inactivated fetal calf serum (FCS), 1% Non-essential Amino Acid, 1% Penicillin/Streptomycin, 1% Sodium Pyruvate, 1% L-glutamine, 0.1% 2-mercaptoethanol. IG10, an epithelial tumor cell line derived from mouse ovarian epithelium, was cultured as previously described (134).

Influenza Virus Infection and Tumor Challenge

All mice were anesthetized with Ketamine (100mg/mL)/Xylazine (20mg/mL) solution. Mice were infected intranasally with $1.25x10^3$ pfu of H1N1 Influenza A/Puerto Rico/8/34 (PR8) virus and re-infected 35 days later with $1.25x10^3$ pfu of H3N2 Influenza A/Aichi/2/68 (Aichi) X-31 virus. Percent weight loss was classified as a measure of successful infection. Subsequently, mice were weighed at two-day intervals. On day 60 following the first infection, mice were injected subcutaneously in the right hind flank with $1x10^5$ 3LL tumor cells. Tumor length and width were measured every two days using calipers. Mice were kept until tumor diameter reached 20 mm, tumors became severely ulcerated, or otherwise advised by the University of Pittsburgh animal facility.

Staining of tumor cells with pre and post infection sera

Four days prior to primary influenza infection, mice were bled to obtain their pre-infection sera antibody repertoire. Ten days following secondary infection, mice were bled to obtain post-infection sera antibodies. Prior to staining, both sets of sera were diluted 1:62.5 in PBS. $2x10^5$ 3LL and IG10 tumor cells were plated in a 96 well plate and stained with 100uL of pre or post-infection sera on ice for one hour. Cells were then stained with FITC conjugated Rat anti-mouse IgG2a (BD Bioscience) as the secondary antibody on ice for 30 minutes. Cells were fixed thereafter in 1.6% paraformaldehyde and samples were run on LSRII flow cytometer.

Affinity purification of 3LL antigens

Total cell lysate was generated from $50x10^6$ 3LL cells in 300uL NP-40 lysis buffer (0.5% NP40, 0.5% Mega 9 (octylglucoside), 150 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.5, 2 mM PMSF, 5

mM iodoacetamide, and Protease Inhibitor (Roche)). Lysates were subsequently pre-cleared by adding Protein G Sepharose beads (Sigma-Aldrich, Inc, St. Louis, MO) and incubating the mixture for one hour at 4° C on an orbital shaker. Protein G beads were removed by centrifugation at 1200 rpm prior to affinity purification. Protein G HP Spin Trap Columns and Buffer Kits (GE Healthcare UK) were used following manufacturer's protocol with the following modifications. In brief, pre-infection and post-infection sera were pooled separately from mice (n=6) and each set of sera was poured over multiple protein G columns (100 µL per column). Columns were washed with the provided wash buffer and 50 mM dimethyl pimelimidate dihydrochloride (DMP) was added to covalently cross-link bound antibodies from each set of sera to the protein G columns, as described in the manufacturer's protocol. This was done to ensure that only bound protein fractions were eluted from the columns and not the antibodies. 400 µL of 3LL tumor lysate was then added to both pre-and post-infection sera columns and incubated overnight at 4° C on an orbital shaker. The following day columns were washed with TBS (50 mM Tris, 150 mM NaCl, pH 7.5) and bound proteins were eluted off the columns with 0.1 M glycine provided from the kit with 2 M urea, pH 2.9. Pooled proteins from pre-infection antibody columns and post-infection antibody columns were concentrated and buffers were exchanged from the elution buffer to 2D-gel buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT, 10m M HEPES, pH 8.0) using 5000 MWCO Vivaspin columns (Sartorious Stedim Biotech, Goettingen, Germany).

2D-DIGE and Liquid Chromatography/Mass Spectrometry (LC/MS) analysis

The immunoprecipitated proteins were subjected to Difference Gel Electrophoresis (DIGE) (93) to identify proteins largely or uniquely precipitated by post-infection sera. Protein labeling, Isoelectric Point Focusing (IEF), and 2nd dimension SDS-PAGE were conducted as previously

described (135) with some modifications. In brief, 2.5µg of pre-infection proteins and post-infection proteins were reduced in 10 mM Tris(2-carboxyethyl)phosphine (TCEP, Sigma) for 60 minutes in the dark at 37°C. 10 mM CyDye DIGE Fluor Cy3 or Cy5-maleimide saturation dyes (GE Healthcare, Uppsala, Sweden) diluted in Dimethylformamide (DMF) (Sigma), which label all available TCEP reduced cysteines on all proteins, were added to each sample for 30 minutes at 37° C. Labeling was quenched with 7M Dithiothreitol (DTT). Samples were then combined and immobilized pH gradient (IPG) buffer (GE Healthcare) was added at 1µL/40µL of sample. Labeling of the two samples was reversed (reciprocal labeling) and run concurrently on a 2nd gel to eliminate dye-dependent differences. Proteins were separated in the 1st dimension on 13cm pH3-10NL IPG strips on an IPGphor apparatus (GE Healthcare) for 35000 Volt-hours. The samples were then separated on 2nd dimension SDS-PAGE in pre-cast 10-20% gradient polyacrylamide gels encased in low fluorescent glass (www.precastgels.com) in standard Tris-Glycine-SDS running buffer. Fluorescent images of reciprocal gels were taken as previously described (135). The Bioinformatics Analysis Core of the University of Pittsburgh Genomics and Proteomics Core Laboratories analyzed resultant fluorescent images and selected spots that were then cut from the gels and identified via Nano LC-ESI-MS/MS, as previously described (136).

Western blot and densitometry analysis

Lung tissues were homogenized with a two mL dounce homogenizer and total lysates were obtained in NP-40 lysis buffer. The same procedure was applied to generate total cell lysates from 3LL and IG-10 tumor cells. Prior to Western blotting, protein amounts were determined via Bradford assay. 50 µg of protein from various groups were separated on 10% TGX pre-cast gels (Bio-Rad) and immunoblotted onto PVDF membranes. The following antibodies were used to

probe for their respective proteins on separate blots: anti-HSP 90α/β (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Annexin II (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Histone H4 (1:1000, Abcam, Cambridge, MA), anti-GAPDH (1:1000, Abcam, Cambridge, MA), anti-Malate Dehydrogenase 2 (1:100, Abcam, Cambridge, MA), anti-Actin (1:15000, Sigma-Aldrich, Inc, St. Louis, MO), goat anti-mouse HRP (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-rabbit HRP (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA). All Western blots were scanned on Kodak Image Station 4000MM and band densitometry analysis was performed on all blots using Image J (NIH, Bethesda, MD). All bands were normalized according to their actin control. Once normalized, all experimental bands lanes were directly compared to a normal uninfected mouse lung.

ELISA

15 μg/mL of one of the following proteins were coated on Immulon 4HBX ELISA plates (Thermo scientific) in duplicate wells to examine differences in antibody recognition between pre- and post-infection sera: MDH2 (Novus Biologicals, Littleton, CO), GAPDH (Abcam, Cambridge, MA), Histone H4 (New England Biolabs, Ipswich, MA), HSP90a (Abcam, Cambridge, MA), Annexin A2 (Novoprotein, Summit, NJ). Human proteins were used due to their high conservation between mouse and human. Duplicate wells that were not coated with antigen served as controls for non-specific binding. Plates were then placed on an orbital shaker overnight at 4° C. The next day pre- and post-infection sera were diluted (1:62.5) in PBS, added to ELISA plates, and placed on an orbital shaker for two hours at room temperature. Plates were washed and 0.3% Hydrogen Peroxide was added wells to block background peroxidase activity. Plates were washed and rat anti-mouse IgG-HRP (1:500) was added. Plates were again washed and TMB substrate was added

for 15 minutes and 2N Sulfuric Acid was added to stop the developing signal. ELISA plates were then read at 450 nm on a Gen 5 plate reader. Data were represented using the average of duplicate antigen-coated wells after subtracting out the no antigen control wells.

Peptide identification and MHC-I binding assays

Candidate peptide sequences were identified using the Immune Epitope Database (IEDB) MHC-I binding predictor program with a percentile rank of five or less (137). $MDH_{251-260}$ (MAYAGARFVF), $GAPDH_{300-310}$ (ALNDNFVKLIS), Annexin $A2_{184-191}$ (SVIDYELI), $H4_{87-95}$ (VVYALKRQG), Histone H4 with an amino acid substitution (H4-sub VVYAFKRQG) peptides were synthesized by the Peptide Synthesis Core of the University of Pittsburgh Genomics and Proteomics Core Laboratories as previously described (138). Peptide binding to MHC class I was verified by performing RMA-S MHC-Class I stabilization assays. In short, $2x10^5$ RMA-S cells, a TAP deficient cell line, were plated in a 96 well plate. The cells were cultured overnight at 29° C. Unloaded RMA-S cells served as controls. Each peptide candidate was added in triplicate to the plate from 10^{-4} M to 10^{-7} M for two hours and 30 minutes in a 29° C incubator. Cells were placed in a 37° C incubator for one hour and 30 minutes. RMA-S cells were fixed in 1.6% Paraformaldehyde and then stained with anti-H2-K^b or anti-H2-D^b (BD Bioscience). Samples were run on the LSR II Flow Cytometer (BD Bioscience) and analyzed using FACS Diva Software (Fig. 12).

Antigen specific T cell detection

Animals were sacrificed six days following the second influenza infection. Lungs and spleens were harvested and cells isolated as previously described (139). $1x10^6$ cells from each set of tissues

were stained with anti-CD3, anti-CD4, and anti-CD8 antibodies (BD Bioscience). Dimer-X soluble dimeric mouse H-2K^{b}:Ig Fusion Protein and H-2D^{b}:Ig Fusion Protein (BD Bioscience) were used according to the manufacturers protocol to detect $MDH_{251-260}$, $GAPDH_{300-310}$, Annexin $A2_{184-191}$, $H4_{87-95}$, $PA_{224-233}$ (GenScript USA, Piscataway, NJ), and $NP_{147-155}$ (GenScript USA, Piscataway, NJ) antigen specific $CD8^{+}$ T cells. 100,000 events were collected and samples were run on the LSR II Flow Cytometer (BD Bioscience), gated (Fig. 13) and analyzed using FACS Diva Software.

Vaccination and Tumor Challenge

D1 dendritic cells (DC) are an established growth factor dependent immature DC line (140). D1 DC were grown and maintained as previously described and used in all vaccinations (140). $1.25x10^{6}$ D1 DCs/ mouse were cultured in six well plates, loaded separately with 100 µg of $MDH_{251-260}$, $GAPDH_{300-310}$, Annexin $A2_{184-191}$, or $H4_{87-95,}$ and matured with 12.5 µg/mL of Poly IC:LC adjuvant. $0.25x10^{6}$ D1 DCs loaded with individual peptides were pooled together for a total number of $1x10^{6}$D1 DCs. An additional 50 µg of each soluble peptide per mouse was added to the mixture and injected into mice in the right hind flank. Unloaded, Poly IC:LC matured D1 DCs were injected in control mice. Animals were vaccinated at week zero, week two, and week six. They were challenged with $1x10^{5}$ 3LL cells in the right hind flank two days following week 6 vaccination. Tumor length and width were measured at two-day intervals using calipers.

Data Analysis

Statistical analysis was performed using GraphPad Prism v6.0 software (GraphPad Inc. San Diego, CA). Results were represented as means ± standard error of the mean (SEM). Statistical means

and significance were analyzed using unpaired two-tailed student's t test. Kaplan-Meier survival curves were analyzed with the log rank test. Significance for all experiments was defined as the following: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

4.4 RESULTS

Influenza virus infection induces antibodies to multiple host cell antigens, some of which are known TAA

Mice were infected with influenza virus PR8 and then 35 days later with the second influenza strain Aichi, as described in Materials and Methods. Mice experienced general signs of malaise and lost close to 20% of starting body weight in the first week following each infection. By seven to eight days post infection, mice began to recover and by days 16-18 their weight returned to baseline (Fig. 8A). Day 25 post second infection, animals were injected subcutaneously with 3LL tumor cells. Tumors became palpable eight days post injection (Fig. 8B). At day 14, tumor growth kinetics between influenza experienced animals and the naïve mock infected group began to diverge. Tumors in influenza-experienced animals grew slower from days 14 to 22. On day 16 the average tumor size in influenza-experienced mice was 20.67 mm^2 versus the size in the control group of 51.63 mm^2. The size difference was still significant at day 18 when the average tumor size in the influenza-experienced group was 31.78 mm^2 compared to 73.25 mm^2 in the control mice.

We examined the post-infection sera for the ability to stain 3LL tumor cells, which would suggest that flu infections generated antibodies against, among others, tumor cell surface proteins.

In the experiment illustrated in Figure 8C, we obtained sera from mice (n=7) pre and post influenza infection. Tumor cell surface staining was performed with individual sera and the results were pooled into pre and post infection groups and represented as an average value. Average Mean Fluorescence Intensity (MFI) of staining of 3LL tumor cells with post-infection sera was significantly higher compared to pre-infection sera. The same result was obtained staining another mouse epithelial tumor cell line IG10 with the same sera.

To identify molecules specifically recognized by post-infection sera, pre- and post-infection antibodies were bound to Protein G columns for affinity purification of proteins from 3LL tumor cell lysates. Tumor proteins bound to the antibody columns were eluted, labeled with two different Cyanine-based saturation dyes and resolved by 2D-DIGE as described in Materials and Methods. In a concurrently-run reciprocal gel, labeling was reversed such that the sample previously labeled with Cy3 was labeled with Cy5 and *vice versa*. Gel images were false-colored for analysis: green for Cy3, red for Cy5. Overlays were then created using Delta2D software, where proteins unique to one sample appeared either green or red, while proteins common to both samples appeared yellow (green and red combined). Figure 9 shows the gel used to identify antigens described below. Green dots belong to proteins eluted from pre-infection antibody columns while red dots represent proteins eluted from post-infection antibody columns. Spots that were deemed to be most remarkable (yellow circles) were cut out and subjected to mass spectrometry analysis and protein sequencing. Many proteins with a wide variety of functions were identified. Those included voltage dependent ion channels, proteasome subunits, mitochondrial and cytosolic enzymes, heat shock proteins and structural proteins (Table 1). We selected four identified proteins: Histone H4, Malate Dehydrogenase 2 (MDH2), Annexin A2, Glyceraldhyde-3-Phosphate Dehydrogenase (GAPDH) and Heat Shock Protein 90 (HSP90) for further study (Table

2). Several previous reports based on methods and approaches unrelated to ours had already identified these molecules either in tumor bearing mice or in cancer patients as tumor associated antigens (141-145).

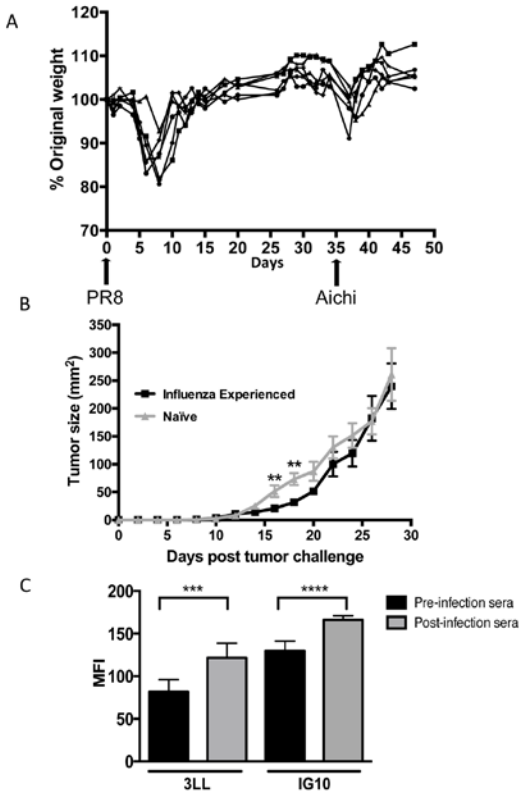

Figure 8: Influenza virus infection delays tumor growth at early time points.

(A) Animals were intranasally infected with PR8 and Aichi influenza viruses on day zero and day 35 respectively. Mice were weighed every two days. (B) Influenza experienced animals and naïve animals were challenged subcutaneously with $1x10^5$ 3LL tumor cells in the right hind flank. Tumor length and width were measured every two days using calipers. Data are representative of two experiments with at least 8 mice per group and are expressed as means ± SEM. (C) Sera from animals (n=7) four days prior to PR8 infection (pre-infection) and ten days after Aichi infection (post-infection) were diluted 1:62.5 and used to stain 3LL and IG10 tumor cells. Cells were subsequently stained with FITC conjugated goat anti-mouse IgG2a antibody and analyzed on the LSRII flow cytometer. Results are shown as Mean Fluorescent Intensity (MFI). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

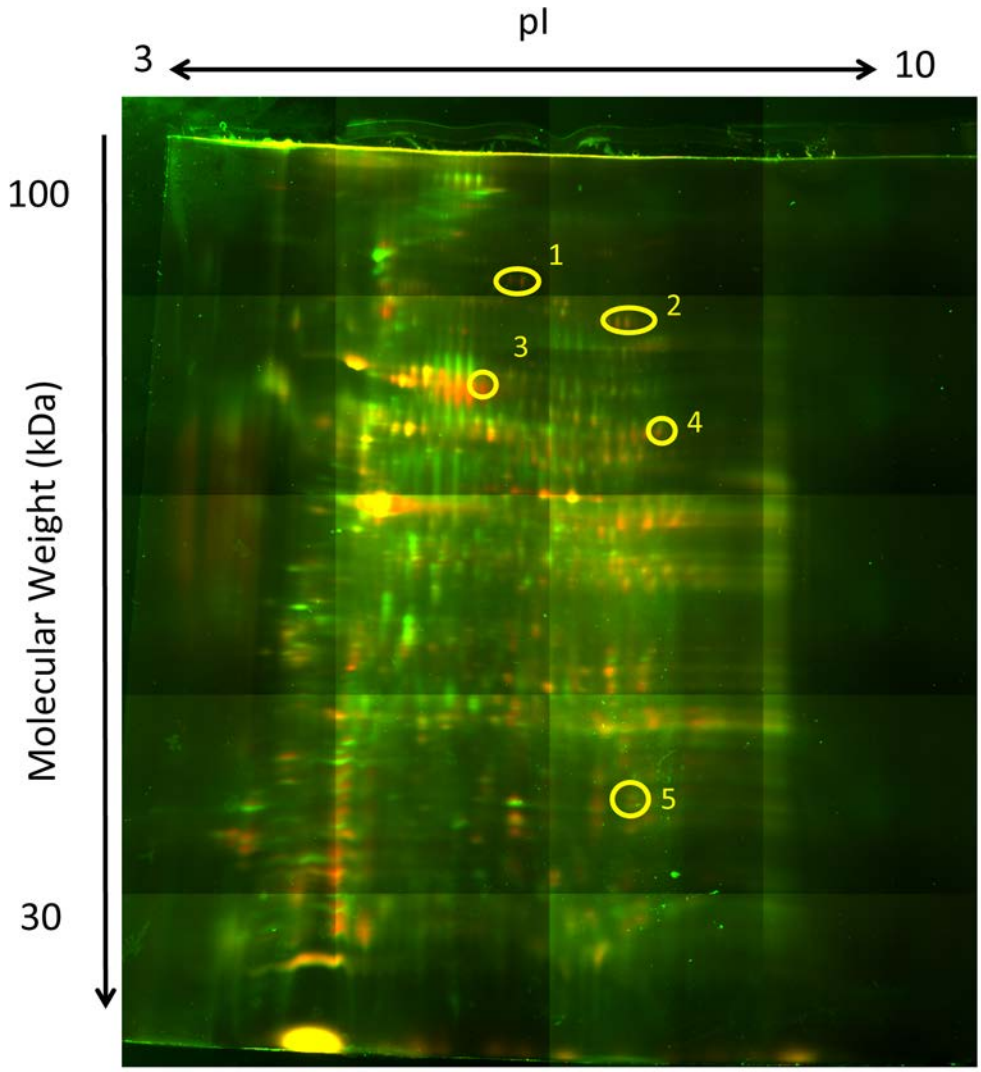

Figure 9: **Influenza infection induces antibodies against specific proteins of tumor cells.**

3LL tumor lysates were affinity purified on protein G columns to which post-infection and pre-infection antibodies were covalently bound and eluted proteins compared by 2D-DIGE gels. Green spots belong to proteins eluted from pre-infection antibody columns and red spots from post-infection antibody columns. Yellow color marks overlapping spots. Yellow circles indicate spots picked for sequencing. Spots were chosen according to image analysis provided by the University of Pittsburgh Bioinformatics Analysis Core (BAC) of the Genomics and Proteomics Core Laboratories.

Table 1: List of identified proteins from selected post-precipitate spots.

Candidate proteins from spots, selected according to image were sequenced via liquid chromatography/mass spectrometry analysis. Protein sequences were identified using SEQUEST by the University of Pittsburgh Bioinformatics Analysis Core (BAC) of the Genomics and Proteomics Core Laboratories

Description
Putative uncharacterized protein Krt5
Putative uncharacterized protein Krt6a
similar to desmoplakin
71 kDa protein
Peptidyl-prolyl cis-trans isomerase
Nucleoside diphosphate kinase B
Junction plakoglobin
Putative uncharacterized protein
Cofilin-1
Peroxiredoxin 1
Elongation factor 1-alpha 1
protease, serine, 1
Putative uncharacterized protein Snx3
Annexin A2
Putative uncharacterized protein
Beta-actin FE-3 (Fragment)
Putative uncharacterized protein Krt35
Ubiquitin thioesterase Zranb1
Putative uncharacterized protein Krt5
similar to desmoplakin
Junction plakoglobin
Putative uncharacterized protein Krt6a
71 kDa protein
Isoform C of Prelamin-A/C
hypothetical protein LOC239673
Putative uncharacterized protein
Isoform PLEC-1H of Plectin
Myosin-9
35 kDa protein
Isoform 1 of Tropomyosin alpha-3 chain
Putative uncharacterized protein Tpm3
Annexin A2
AHNAK nucleoprotein isoform 1
Actin, cytoplasmic 1
Insulin-degrading enzyme
Elongation factor 1-alpha 1
Isoform 2 of Myosin-14
Plakophilin-1
Putative uncharacterized protein (Fragment)
Isoform M2 of Pyruvate kinase isozymes M1/M2
Alpha-actinin-4
Protein-glutamine gamma-glutamyltransferase K
Putative uncharacterized protein Krt17
Histidine ammonia-lyase
Desmoglein-1-alpha
Histone H4
78 kDa glucose-regulated protein
Heat shock 70 kDa protein 1A
Enolase
protease, serine, 1
Ras-related protein Rab-14
Peroxiredoxin 1
Bleomycin hydrolase
protein POF1B
non-specific cytotoxic cell receptor protein 1 homolog
similar to valosin isoform 1
Periplakin
Cytoplasmic dynein 1 heavy chain 1
Putative uncharacterized protein
14-3-3 protein sigma
similar to Eukaryotic translation elongation factor 2

Description
Putative uncharacterized protein Krt5
71 kDa protein
Junction plakoglobin
protease, serine, 1
Annexin A2
Proteasome subunit beta type-1
Protein S100-A14
Proteasome subunit alpha type-3
Guanine nucleotide-binding protein subunit beta-2-like 1
Isoform 1 of Transmembrane emp24 domain-containing protein 10
Isoform 1 of Tropomyosin beta chain
Putative uncharacterized protein
Gasdermin-A
Zinc finger protein 212 isoform 2
similar to S-adenosylhomocysteine hydrolase
Actin-related protein 2/3 complex subunit 4
similar to adenine nucleotide translocase
Nascent polypeptide-associated complex subunit alpha
Isoform 1 of Geranylgeranyl transferase type-2 subunit alpha
Isoform Mt-VDAC1 of Voltage-dependent anion-selective channel protein 1
similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isoform 1
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit
Putative uncharacterized protein
RAB5C, member RAS oncogene family
Proteasome subunit alpha type-7
similar to Synaptotagmin-like 3
Vinculin
Proteasome subunit alpha type
Proteasome subunit alpha type-4
Plastin-3
Malate dehydrogenase, mitochondrial
Actin-related protein 2/3 complex subunit 2
ADP/ATP translocase 1
Isoform 2 of Probable JmjC domain-containing histone demethylation protein 2C
Actin-related protein 3
Arginase-1
Voltage-dependent anion-selective channel protein 3
Actin-related protein 2/3 complex subunit 5-like protein
Isoform 2 of Rab GDP dissociation inhibitor beta
T-complex protein 1 subunit gamma
Putative uncharacterized protein Ywhaz
Envoplakin
ATP synthase subunit beta, mitochondrial
Histidyl-tRNA synthetase, cytoplasmic
similar to LOC645974 protein
Putative uncharacterized protein
Serum albumin
Lysozyme C-1
Fc receptor-like protein 6
Delta-aminolevulinic acid dehydratase
43 kDa protein
Acyl-Coenzyme A dehydrogenase, very long chain
Proteasome subunit beta type-2
similar to tubulin beta-3
Lysosome-associated membrane glycoprotein 1
Proteasome subunit beta type
Proteasome subunit alpha type-6
Structural maintenance of chromosomes flexible hinge domain-containing protein 1
Voltage-dependent anion-selective channel protein 2
Protein
40S ribosomal protein SA

Table 2: Biological characterization of selected tumor antigens detected by post infection antibodies

Spot Number	Accession Number	Protein Name	Peptide Sequence	X-Corr	ΔScore	Charge	M/Z[Da]	MH+[Da]	ΔM [ppm]	MW [kDa]	pI	% Sequence Coverage	Ions Matched
2	IPI00885367.1	Annexin A2	TNQELQEINR QDIAFAYQR	2.74 2.55	0.36 1.00	2	622.81384 556.28143	1244.62041 1111.55559	-2.08 2.22	19.6	5.96	10.80	14/18 14/16
3	IPI00468203.3	Annexin A2	TPAQYDASELK AEDGSVIDYELIDQDAR SLYYYIQQDTK TNQELQEINR WISIMTER DIISDTSGDFR QDIAFAYQR	3.52 3.46 3.30 3.08 2.67 2.53 2.41	0.65 0.68 0.47 0.47 1.00 1.00 0.60	2 2 2 2 2 2 2	611.80090 954.94586 711.35215 622.81384 518.26892 613.28820 556.28027	1222.59453 1908.88445 1421.69704 1244.62041 1035.53057 1225.56914 1111.55327	-0.43 1.16 1.62 -2.08 1.29 -0.36 0.13	38.7	7.69	22.71	15/20 17/32 16/20 15/18 13/14 16/20 15/16
4	IPI00407339.7	Histone H4	ISGLIYEETR TVTAMDVVYALK TVTAmDVVYALK DAVTYTEHAK VFLENVIR	3.39 3.22 2.88 2.61 2.56	0.68 0.74 0.42 0.52 0.36	2 2 2 2 2	590.81536 655.85559 663.85327 567.77410 495.29345	1180.62346 1310.70391 1326.69927 1134.54094 989.57964	2.19 1.06 1.39 -1.47 1.76	11.4	11.36	38.83	16/18 16/22 14/22 15/18 12/14
4	IPI00622795.2	GAPDH	LENPAKYDDIK	2.36	8.03	2	653.33881	1305.67035	0.20	35.8	8.03	3.30	10/20
4	IPI00323592.2	Malate Dehydrogenase 2	IFGVTTLDIVR	2.82	0.41	2	617.36487	1233.72246	0.41	35.6	8.68	3.25	14/20
5	IPI00885367.1	Annexin A2	TNQELQEINR	3.06	0.47	2	622.81384	1244.62041	-2.08	19.6	5.96	5.68	14/18

Protein Accessions- Protein Accessions in IPI mouse database
Protein- Protein name
Peptide sequences- Primary amino acid sequence for identified peptides
Xcorr- XCorr is the cross-correlation of the experimental and theoretical spectra from SEQUEST search
Δ Score- A measure of the difference between the top two scores for the peptides identified by that spectrum
Charge- Charge stage of precursor ion
M/Z [Da]- Observed mass to charge ratio of precursor ion
MH+ [Da]- Monoisotopic mass for protonated molecular ion MH calculated based on observed m/z
ΔM [ppm]- Deviation from expected MH
MW-Molecular Weight
pI- Isoelectric Point
% Sequence Coverage- percentage of the database protein sequence covered by matching peptides
Ions Matched- The number of fragment ions matched out of the total number of fragment masses for the peptide

Overexpression of GAPDH, Histone H4, MDH2, Annexin A2, and HSP90 in tumor cells and influenza virus infected lungs leads to specific immunity

We hypothesized that post-infection antibodies against these proteins were elicited due to differences in their expression in infected versus normal lungs, which would be consistent with their abnormal expression in tumors. Western blot analysis showed that these proteins were constitutively overexpressed in both epithelial tumor cell lines, 3LL and IG10, and also at various time points in flu-infected lungs (2-7.5-fold higher compared to healthy lungs) (Fig. 10). Expression of GAPDH in influenza-infected lungs appeared to be the highest at day three post infection. Histone H4 protein levels were constitutively elevated in tumor cells and at all time points post flu infection by 5 to 7-fold higher than in normal lung. MDH2 levels were elevated at 12 hours post infection and decreased to normal levels at day three post infection. Annexin A2 remained three-fold higher than in normal lung at all time points. HSP90 protein level was the highest at day two post infection.

Even though these antigens were identified by affinity purification on post-infection sera bound to Protein G columns, we wanted to confirm in another assay the specificity of post-infection antibodies for each of these individual molecules. Figure 11 shows that in most mice there was an increase post-infection in IgG specific for GAPDH, Histone H4, MDH2, Annexin A2 and HSP90α as determined by ELISA.

Influenza virus infection also induced an increase in $CD8^+$ T cells for all of these antigens. Spleens and lungs were harvested from mice six days after the second influenza infection and from uninfected control mice. Peptides $GAPDH_{300-310}$ (ALNDNFVKLIS), Annexin $A2_{184-191}$ (SVIDYELI), $MDH_{251-260}$ (MAYAGARFVF), Histone $H4_{87-95}$ (VVYALKRQG), Histone H4 with an amino acid substitution (H4-sub VVYAFKRQG) (142), were selected from the Immune

Epitope Database (IEDB) and confirmed to bind to MHC-I in RMA-S stabilization assays (Fig. 12). Each peptide was loaded onto DimerX H-2K^b or H-2D^b molecules and used to detect specific CD3$^+$CD8$^+$ T cells. There were no CD8 T cells specific for these peptides in lungs and spleens of uninfected control mice but in flu-experienced animals they were present in similar numbers to the flu-specific T cells (Fig. 14). The highest numbers both in the lungs and in the spleens were H2-K^b restricted GAPDH specific and H2-D^b restricted MDH2 specific T-cells.

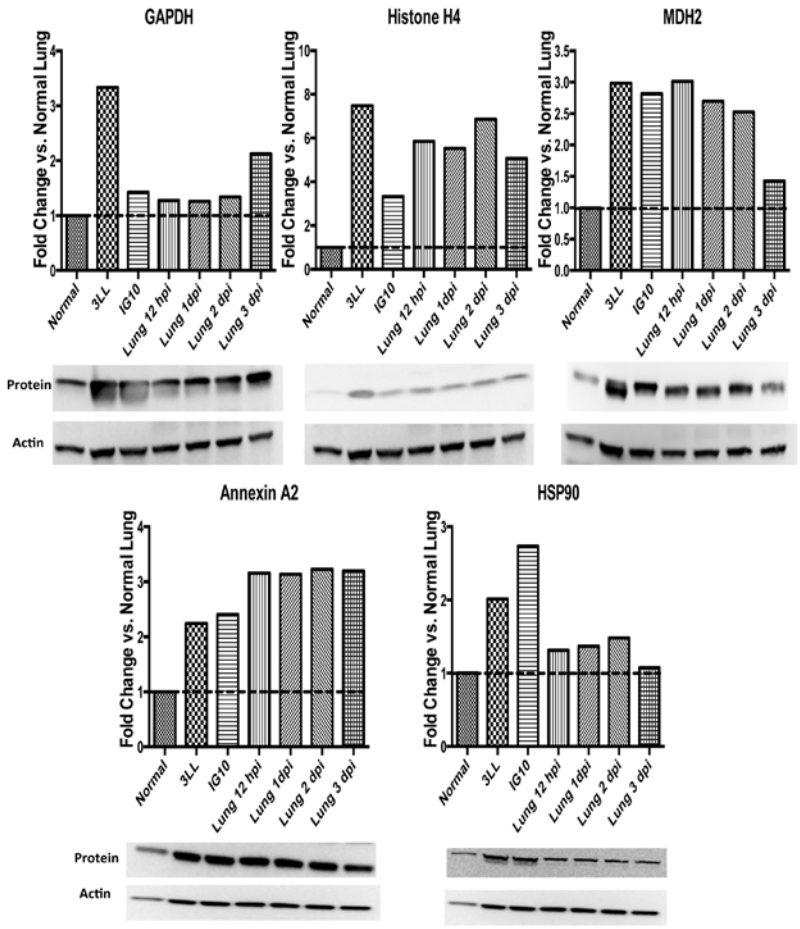

Figure 10: **Histone H4, MDH2, GAPDH and HSP90 are elevated in epithelial tumor cells lines and influenza infected lungs.**

Histone H4, MDH2, GAPDH and HSP90 are elevated in epithelial tumor cells lines and influenza infected lungs. Whole cell lysates were generated from 3LL and IG10 cell lines and from normal and influenza infected lungs. 50µg of protein was loaded on each gel, resolved by electrophoresis and transferred onto PVDF membranes. Membranes were blotted using antibodies against GAPDH, Histone H4, MDH2, Annexin A2, and HSP90. Actin loading controls were used to normalize each band. Densitometry analysis was performed using ImageJ. All lanes were compared to Normal Lung. Data are representative of two experiments.

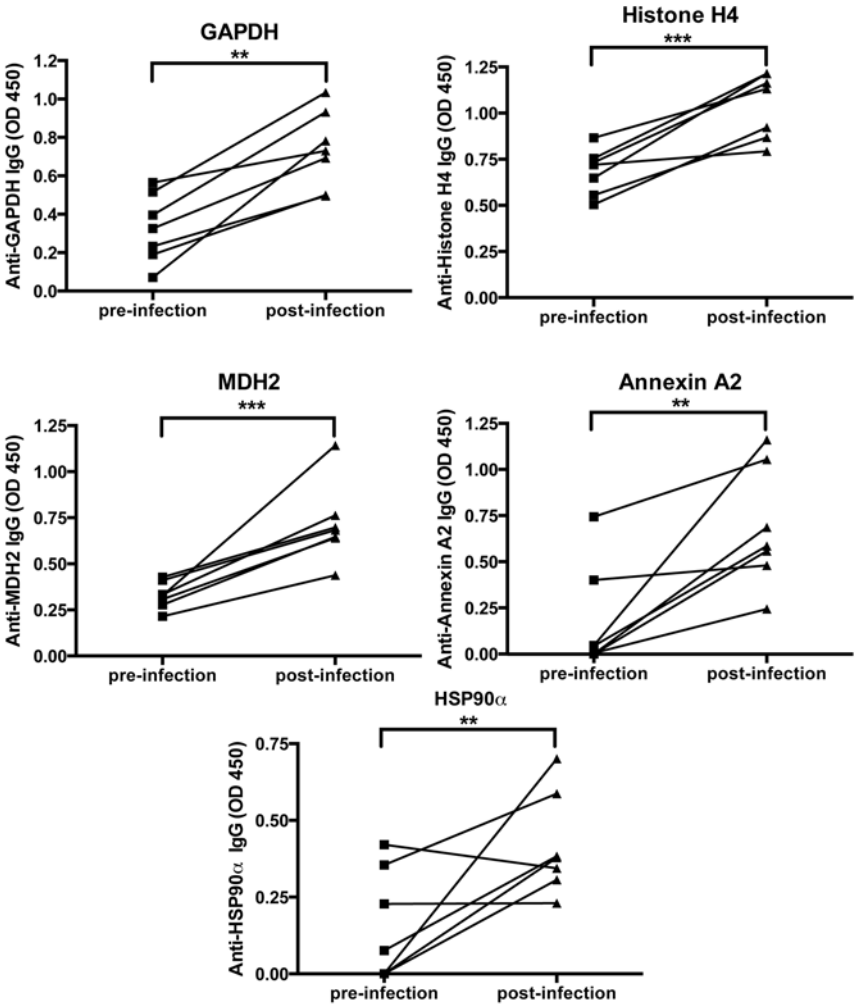

Figure 11: **Antibodies specific for GAPDH, Histone H4, MDH2, Annexin A2 and HSP90α increase following influenza infection.**

Pre-infection sera (four days prior to the first infection) and post-infection sera (ten days post second infection) were assayed on ELISA plates in duplicate wells coated with individual proteins. Uncoated wells served as non-specific binding controls and their values were subtracted from values in matching antigen-coated wells. Results are represented as mean optical density (OD) ± SEM of two experimental repeats with an n=7.

A

Protein	Peptide Position	Peptide Sequence	IEDB[1] MHC-I binding prediction H-2K^b	IEDB[1] MHC-I binding prediction H-2D^b
Histone H4	$H4_{87-95}$	VVYALKRQG	4.90	30.30
Annexin A2	Annexin $A2_{184-191}$	SVIDYELI	3.35	23.20
MDH	$MDH_{251-260}$	MAYAGARFVF	2.85	14.30
GAPDH	$GAPDH_{300-310}$	ALNDNFVKLIS	1.60	2.80
Ovalbumin	$OVA_{257-264}$	SIINFEKL	0.20	0.30
Influenza PA	$PA_{224-233}$	SSLENFRAYV	0.10	3.30
Influenza NP	$NP_{147-155}$	TYQRTRALV	12.55	40.30

[1]IEDB=Immune Epitope Database

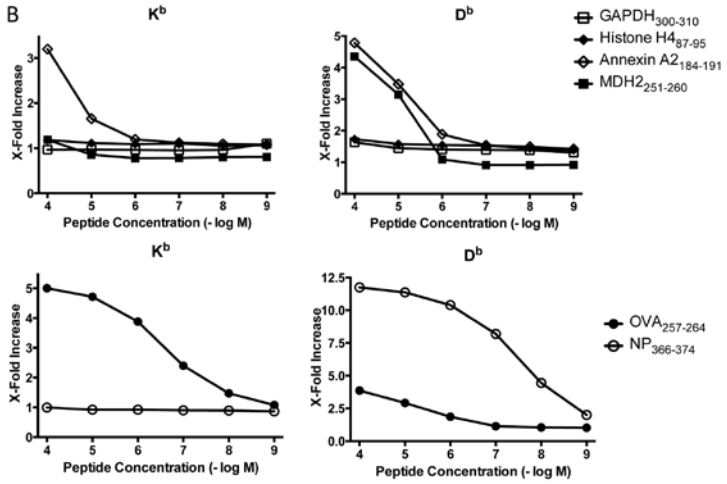

Figure 12: TAA peptides bind to MHC-I

(A) Peptide position and sequence as well as H-2K^b and H-2D^b binding prediction value. (B) RMA-S cells were incubated overnight at 29°C in 96-well microtiter plates. The next day, peptides were added to cells in triplicate wells in concentrations ranging from 10^{-4}M to 10^{-7}M and incubated at 29°C for 1 hour and 30 minutes. Unloaded RMA-S cells served as negative controls and $OVA_{257-264}$ and $NP_{366-374}$ loaded RMA-S cells served as positive controls. Plates were subsequently placed at 37°C for two hours and 30 minutes. Expression of Kb and Db was analyzed by flow cytometer. X-fold increase was calculated as follows: peptide loaded RMA-S cell median fluorescent intensity/unloaded RMA-S cell median fluorescent intensity.

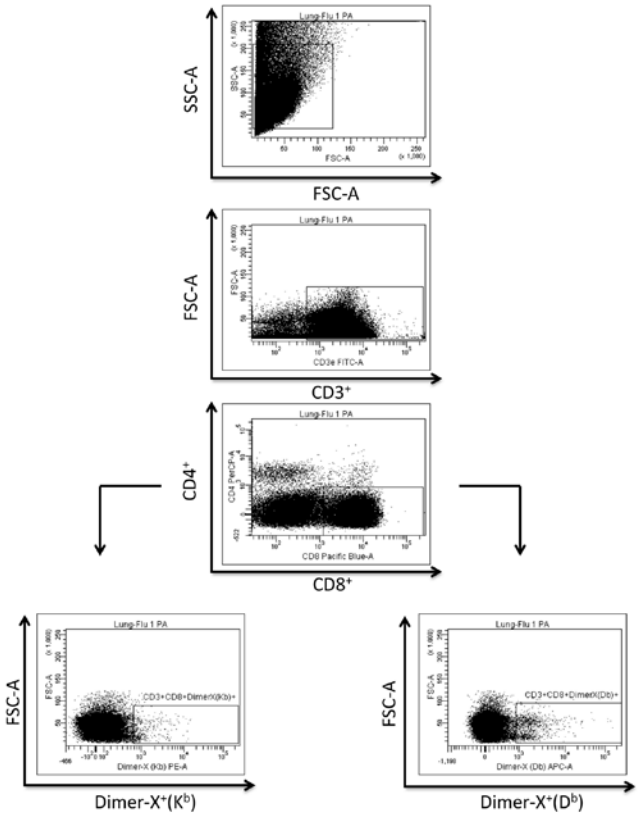

Figure 13: Example gating strategy of antigen specific $CD3^+CD8^+$Dimer-X^+ T cells.

Splenocytes and lung cells were processed and stained with anti-CD3, anti-CD4, and anti-CD8 antibodies (BD Bioscience). Dimer-X soluble dimeric mouse H-$2K^b$:Ig Fusion Protein and H-$2D^b$:Ig Fusion Protein (BD Bioscience) were used according to the manufacturers protocol to detect $MDH_{251-260}$, $GAPDH_{300-310}$, Annexin $A2_{184-191}$, $H4_{87-95}$, $PA_{224-233}$ (GenScript USA, Piscataway, NJ), and $NP_{147-155}$ (GenScript USA, Piscataway, NJ) antigen specific $CD8^+$ T cells. 100,000 events were collected and samples were run on the LSR II Flow Cytometer (BD Bioscience). Cells were first gated on SSC-A and FSC-A. Cells within this gate were analyzed for $CD3^+$ cells and then for $CD8^+$ cells. $CD3^+$ and $CD8^+$ T cells staining with either Dimer-X (K^b) or Dimer-X (D^b) loaded with various peptides were then gated and analyzed using FACS Diva Software.

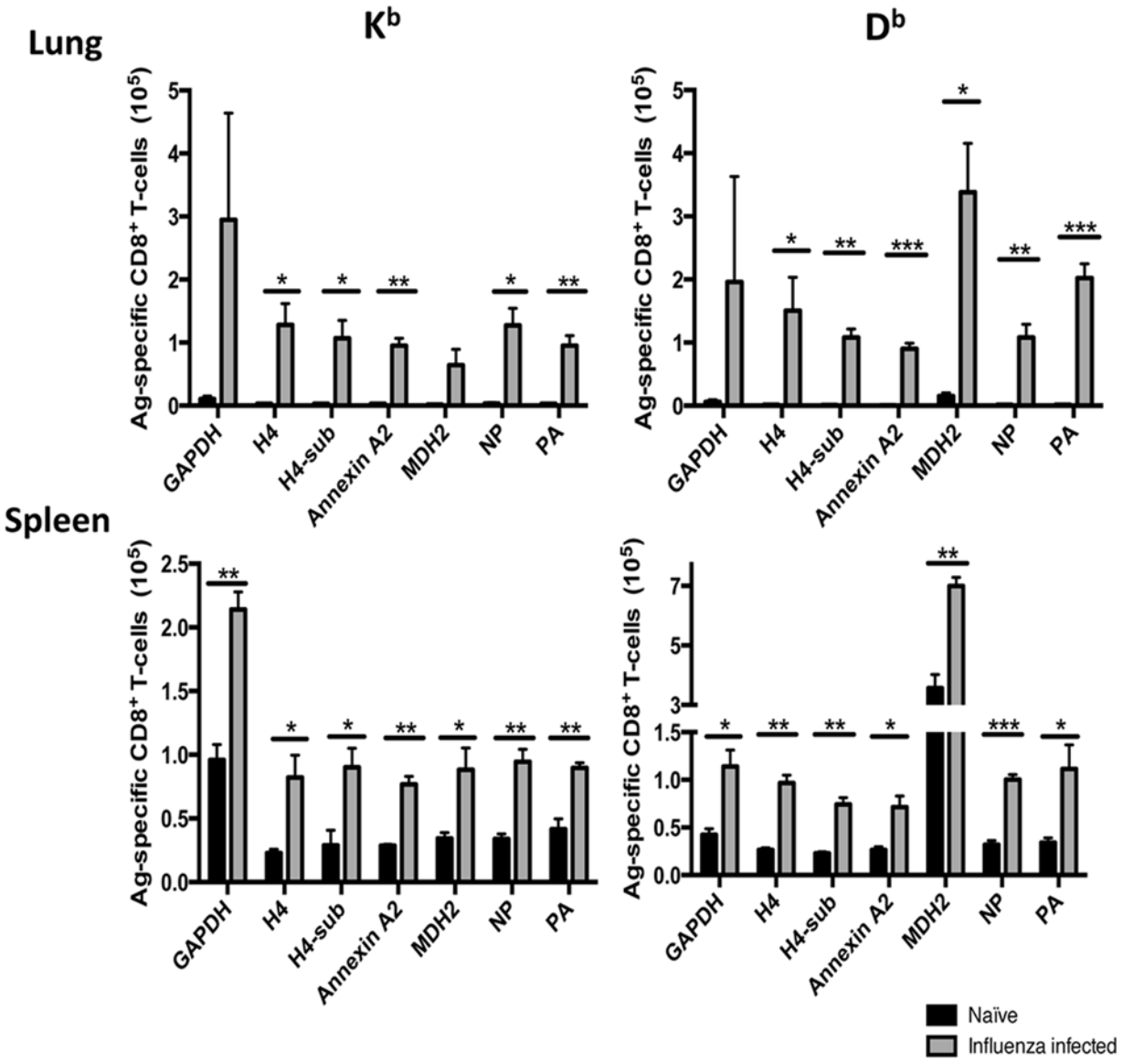

Figure 14: **Antigen-specific CD8$^+$ T cells increase in lungs and spleens following influenza infection.**

Mice were infected with PR8 and Aichi Influenza viruses or mock PBS infected on day zero and day 35 respectively. Six days after the second infection spleens and lungs were harvested and analyzed by Flow Cytometry for the presence of T cells staining with the Dimer-X reagent (as described in Methods and Materials). Data are representative of two experimental repeats of least n=3 animals per group and are expressed as means ± SEM.

Vaccination with DC loaded with the new TAA peptides delays tumor growth and promotes survival

We loaded the D1 dendritic cells with $MDH2_{251-260}$, $GAPDH_{300-310}$, Annexin $A2_{184-191}$, and $H4_{87-95}$ and vaccinated mice as described in Materials and Methods. Control mice were vaccinated with unloaded D1 cells. Two days following the second boost, mice were challenged subcutaneously with 3LL tumor cells. In vaccinated animals tumor growth began to slow down significantly by day twelve (Fig. 15A) resulting in all the vaccinated animals still surviving at day 40, compared to only two animals surviving in the unloaded DC vaccinated controls (Fig. 15B). On day twelve, average tumor size in peptide loaded DC vaccinated animals was 10.67 mm^2 compared to 30.67 mm^2 for the controls.

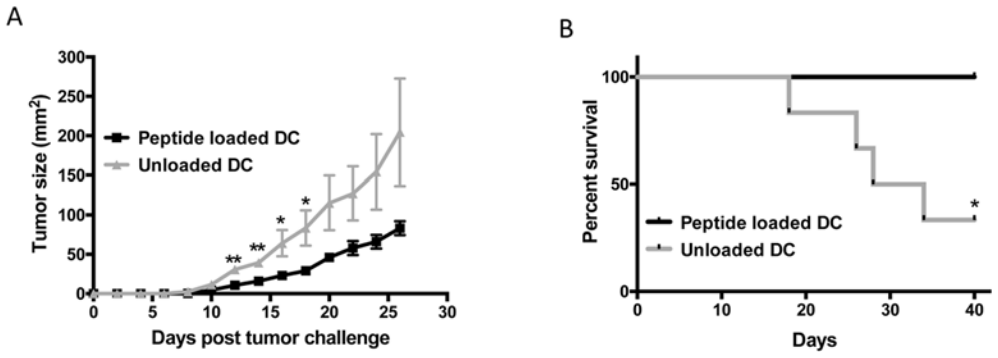

Figure 15: Vaccination with GAPDH, Histone H4, MDH2, and Annexin A2 peptides loaded on DC confers early protection and prolonged survival against 3LL tumor challenge.

(A) Mice were vaccinated in the right hind flank with $1x10^6$ D1 cells/mouse (a pool of $2.5x10^5$ D1 dendritic cells/peptide) at week zero week two, and week six. Control mice received the same number of unloaded D1 DC at the same time points. Two days after the week six booster, animals were challenged subcutaneously with $1x10^5$ 3LL tumor cells in the right hind flank. Tumor growth was measured with calipers at two-day intervals and expressed as length x width (mm^2). (B) Survival post tumor challenge. Data are representative of two experiments with at least n=8 mice per group and are expressed as means ± SEM.

4.5 DISCUSSION

The data we present here, adds a new dimension to our understanding of the process of cancer immunosurveillance and its targets. We show that immune responses against abnormally expressed self antigens, many of which have been characterized as TAAs, are generated during non-malignant infectious inflammatory events that occur much earlier in life than malignancies. We propose that immune memory for these antigens is later recruited for cancer immunosurveillance.

We used a mouse model to demonstrate that two bouts with influenza virus infection led to the ability to slow down tumor growth. This effect was small and transient, which may be all that could be expected from a limited exposure of the mice to this one type of infection and no other pathogens prior to that due to the pathogen free conditions that they are kept in. The significance of this small delay, however, was confirmed by our ability to show induction of antibodies against multiple molecules in the tumor cell lysate. Focusing on five antigens identified by infection-elicited antibodies, GAPDH, Histone H4, MDH2, Annexin A2, and HSP90, we showed that they were abnormally expressed (overexpressed) in flu infected lungs and in mouse epithelial tumor cell lines, and that in addition to IgG the flu infection induced antigen-specific CD8 T cells against these molecules. As predicted by our hypothesis, vaccination with peptides derived from GAPDH, H4, MDH2, and Annexin A2 led to a much more profound slowing down of tumor growth compared to the infection, and to prolonged survival of tumor bearing animals.

Other viral infections may be capable of inducing TAA specific antibodies and T cells. Vaccinia Virus (VV) and Lymphocytic Choriomeningitis Virus (LCMV) infected mice were previously reported to develop antibodies against many host cell antigens, some of which are orthologues of human TAAs (146). Human fibroblasts infected with varicella-zoster virus (VZV)

or human cytomegalovirus (CMV) overexpress cyclin B1 in the cytoplasm in a similar fashion to tumor cells where cyclin B1 was identified as a TAA (147, 148). Cyclin B1 has been found in VZV virions as well (149). Many healthy individuals, presumably having experienced these infections, have cyclin B1-specific IgG and memory T cells (59). It has been reported that GAPDH and Annexin A2 are found in influenza virions produced by infected epithelial cell lines Vero and A549 (150). HSP90, Annexin A2, and GAPDH were also found within human CMV particles (151). A study examining the measles virus effect on presentation of self-peptides on MHC class I during infection showed that two abundant self-peptides on HLA-A*0201 measles infected cells could induce auto-reactive $CD8^+$ T cells. One of the peptides identified was $HSP90\beta_{570-578}$ (ILDKKVEKV)(152). $HSP90\beta_{570-578}$ peptide has been found to be expressed in melanoma cell lines as well (153). It is possible that these "auto-reactive" T cells contribute to increased tumor immune surveillance. None of these observations were followed by experiments to test the potential anti-tumor effects of either the viral infections or the immune responses against the identified molecules, with the exception of cyclin B1 that we showed was a target of anti-tumor immune responses (59).

The same protective effect of influenza virus primed immunity specific for abnormally expressed self-antigens that we showed here, could be a collateral benefit of other viral, bacterial and parasitic infection or various acute inflammatory conditions of unknown etiologies. Therefore, we propose that the molecules abnormally expressed in these different disease states and also in cancer cells, that are currently referred to as TAAs, should be renamed disease associated antigens (DAA) (154). Pre-existing immune responses to several known tumor antigens that are candidate DAAs have been reported to increase the odds of successfully eliminating spontaneously arising tumors (91). The arrival of memory DAA-specific T cells to the site of the tumor as a secondary

immune response could promote priming of tumor-specific responses directed against individual mutations and epitope spreading, adding to the efficacy of immunosurveillance. If DAAs specific immune memory is lacking or weak due to limited early exposures to infections, this may lead to establishment of chronic inflammation at the tumor site due to unopposed innate immune responses, which is likely to promote tumor development. Better understanding of events early in life that prepare the immune system to protect an individual against known and unknown pathogens as well as future malignancies, will help direct vaccines and other immune manipulation towards strengthening rather than impairing the establishment of life-long immunosurveillance. In addition, these findings support the use of vaccines based on DAAs/TAAs for cancer prevention.

5.0 SIGNIFICANCE OF THIS NEW VIEW OF CANCER IMMUNOSURVEILLANCE

My thesis work provides experimental support to the epidemiological evidence that experiencing infections early in life provides protection from cancer later in life. Understanding the basic mechanisms of the formation of tumor immunosurveillance is of public health importance. For example, as tumors progress and grow out, they tend to create pro tumor growth and immunosuppressive environment. Currently, most antigen based immunotherapeutic interventions are tested in individuals diagnosed with late stage cancers. Additionally, many of these individuals have failed all other interventions. By this time, the immunotherapy is largely ineffective. Concentrating on prophylactic vaccinations against the most important or relevant DAAs in a tumor free or premalignant state may prove to be the more effective cancer control strategy. The most important DAAs might be defined as: 1. DAA that are expressed on the most common cancers, 2. Antigens that are expressed by cells affected by various pathogens and not just one particular antigen, and 3. DAAs that elicit the most robust T cell and antibody memory responses.

Experiencing certain pathogenic events might be predictive of future protection from cancer of a certain cell type. For example, individuals who self report infections or have serum antibodies specific for varicella zoster virus (VZV) have a lower incidence of gliomas (82). Individuals who have never experienced VZV may not have this same protection against gliomas or other brain cancers. Preventative vaccination of VZV inexperienced people with DAAs associated with specific with VZV could provide same beneficial protective coverage of gliomas. Also tumor cell origin may be a factor as well. A pathogen infecting cells of epithelial origin may provide increased tumor immunosurveillance for epithelial tumors.

Cancer incidence in the United States between 1974 and 1992 appeared to increase annually (155). Although incidence has since stabilized or decreased for cancer overall, incidence of certain cancers (liver, kidney, thyroid, pancreas, and melanoma) (156-158) continue to rise. These trends likely could be due to factors such as improved detection modalities, general increase in cancer awareness, and an increase in the lifespan of the population; other explanations cannot be ruled out. One explanation for increased incidence might be due to the implementation of childhood vaccination programs and resultant decrease of classical childhood illnesses such as measles, mumps, and rubella (91, 159). While my work should not be used to suggest stopping vaccinations against dangerous pathogens, it does suggest the possibility of vaccinating against DAA instead of specific pathogens for a broad protection against multiple diseases.

5.1 REMAINING QUESTIONS

In my model system, I examined the effects on having experienced influenza infection on tumor growth at an uninfected subcutaneous site in the animal. The site of prior infections might prove to be the most protected site as a preferred target of tumor immunosurveillance. Presumably since influenza infection induced overexpression of DAA in the lung, immune memory cells may preferentially migrate there and responses to tumor growth in the lung may be greater. One way I have begun to address this question is by using luciferase expressing Lewis Lung Carcinoma cells (LLC-luc) in a metastatic lung model. Tumor burden in lungs of naïve or influenza experienced animals can be assayed using an In Vivo Imaging System (IVIS) to measure bioluminescence. I expect that metastatic tumor burden in the lungs of flu experienced animals will be lower than naïve control animals. Initial results of this approach are shown in the Appendix.

My model system currently involves the transplantable 3LL tumor model. It is unknown if previous flu exposure has an effect on spontaneous tumor growth. To address this, our group and others have developed a colitis associated-colorectal cancer model with MUC1Tg animals where inflammation can be induced by dextran sulfate sodium (DSS) (160) and colorectal cancer developed with azoxymethane (AOM) (161). Therefore, colons from flu experienced animals and naïve animals can be histologically examined and quantified for the presence of tumor. It is expected that previous viral infections will yield early protection and prevent tumor outgrowth.

It is not known which immune effectors, antibodies or T cells, are most critical to initial protection against tumor growth. This question can be addressed by adoptively transferring either post-infection sera, B cells, or T cells from a flu experienced animal into a flu naïve animal, challenging that animal with 3LL tumor and measure tumor growth. Alternatively the role of CD4 or CD8 T cells in flu-infected animals can be examined using CD4 or CD8 T cell depleting antibodies prior to and during tumor challenge.

I demonstrated that there was an increase in antibodies specific for selected DAA. I also demonstrated an increase in post infection sera antibodies recognizing tumor cell surface antigens compared to pre infection. I did not examine these antibodies for their ability to induce antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC).

It would also be of interest to look at circulating inflammatory cytokines such as IFN-γ and TNF-α following multiple exposures to influenza. Some studies examining herpes viral latency providing protection from bacterial infection demonstrated that protection was mediated through elevated levels of inflammatory mediators rather than $CD8^+$ T cells(162, 163). Although our systems are quite different, this could be an important mechanism of increasing general tumor

immune surveillance or providing enough of an immunostimulatory environment to induce stronger immune response against DAA.

Antibodies and $CD8^+$ T cells specific for GAPDH, Histone H4, MDH2, HSP90, and Annexin A2 were generated in mice following influenza infection. The exact mechanism for how immune responses against these DAA are formed remains to be determined. One possibility could be through lytic release of these molecules in the setting of inflammation during viral infection. Another possibility could be by incorporation of these DAA in virus particles and subsequent infection of APCs (151, 164-169). Annexin A2 aids influenza viral replication through converting plasminogen to plasmin that in turn promotes the cleavage of HA0 to HA1 and HA2 (170, 171). HSP90 promotes virus propagation thorough assisting the nuclear import and assembly of influenza polymerase protein (172). In fact HSP90 and Annexin A2 may have to be recruited to virions for the virus to make use of them. Passenger antigens might also find there way into the virus particles by chance. Influenza virions commonly bud from lipid rafts (173, 174). Annexin A2 as well as GAPDH and other molecules can be found at these lipid rafts (175-177). Therefore as the virus buds off the infected cell, they take along passenger antigens or even peptide-MHC complexes that could be available for cross presentation (178, 179). This might also explain why the majority of antigens we identified and/or studied are intracellular molecules; although there is evidence of cell surface expression of Annexin A2 and GAPDH, (143, 180).

One of the biggest challenges will be to design appropriate models to test other non-malignant inflammatory events such as bacterial or fungal infections and chronic inflammations for induction of DAA and increase in tumor immunosurveillance. Exploring these different pathologic non-malignant conditions in appropriate model systems would strengthen our hypothesis. In addition, DAA prophylactic vaccination effects on viral immunosurveillance have

not been adequately addressed (Appendix B). It is interesting to note that viral infections can affect self peptide presentation on MHC class I. In one study using, influenza infected HeLa cells, many of the peptide repertoire presented on MHC class I either increased or uniquely presented several peptides compared to non-infected cells (181). Annexin A2 and HSP90 peptides in particular were uniquely presented on MHC class I molecules on flu-infected cells. Influenza virus is not the only virus capable of altering proteome expression. HIV virus also induces increased expression of several self peptides and CTL activity against cells expressing the self antigen (182, 183). Expression of DAA on virally infected cells may mark cells to be destroyed by the immune system in the proper setting. The ultimate outcome of confirming this new immune surveillance hypothesis is that it might lead to a universal vaccine providing protection against cancer and against pathogens thus eliminating the need for pathogen specific or cancer specific vaccinations (see model in Fig.16).

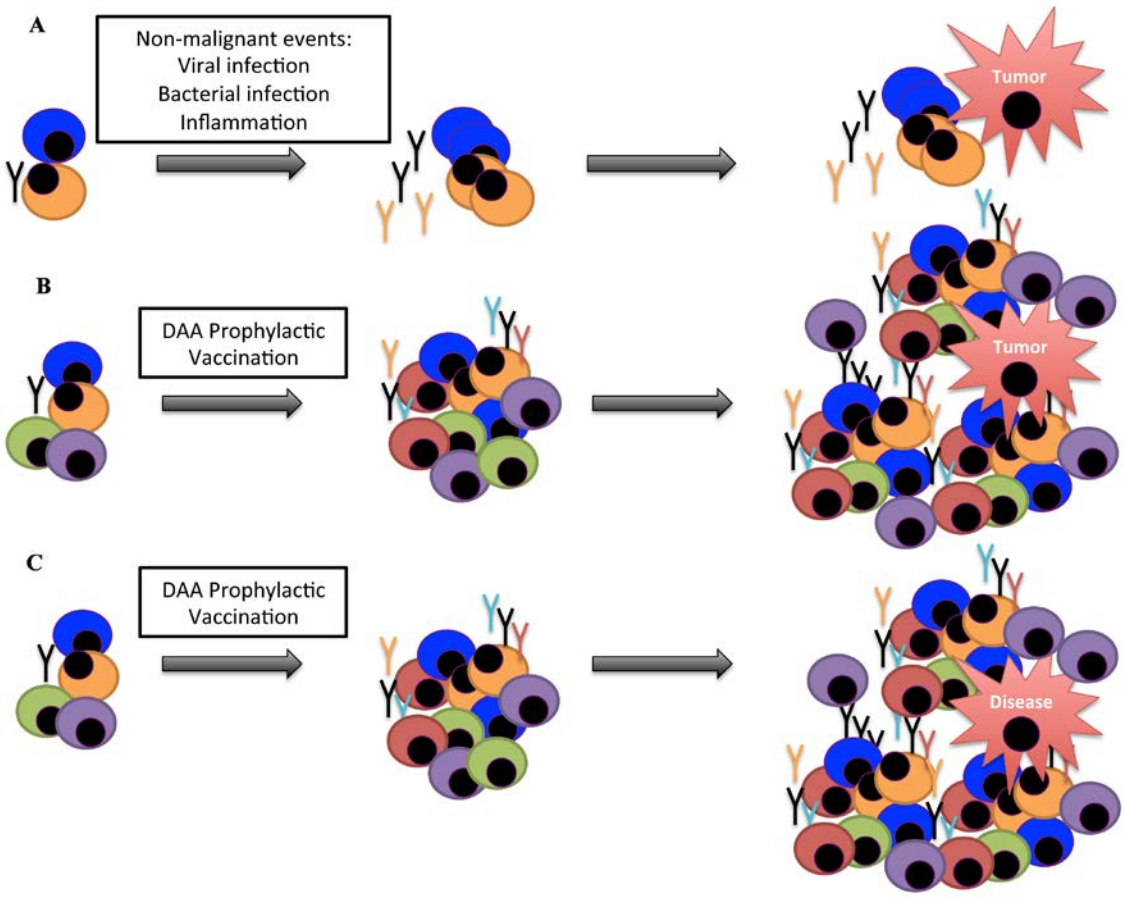

Figure 16: Proposed impact of DAA-specific immunity on immunosurveillance.

Naïve T and B cell clones, each capable of recognizing one of a millions different antigens by their unique antigen receptors, become primed, expand in number and generate immune memory specific for DAA transiently expressed during various acute inflammatory non-malignant events. (A)When a tumor begins to grow and express some of the same DAA, the immune memory response kicks into an effector response, memory T and B cells proliferate much faster than naïve cells and the tumor is eliminated. (B) The same result as in (A) can be achieved by prophylactically vaccinating individuals with the best or most commonly expressed DAA in lieu of an exposure to pathogens in order to produce immune memory against DAA for effective tumor immunosurveillance. (C) Vaccination against DA can also generate effective immunosurveillance against pathogen-infected cells.

APPENDIX A: Influenza Effects on Tumor Metastasis to the Lung

We have demonstrated that influenza infection delays tumor growth at a subcutaneous site. Immune responses to DAA may have the strongest impact at the site of infection. We therefore sought to address if prior influenza exposure impacts metastatic tumor growth in the lung. To answer the question we flu infected animals twice at day zero and day 35 after initial exposure. PBS mocked infected animals served as naïve controls. 25 days after second influenza infection, intravenous (I.V.) tail vein injections were performed with $5x10^5$ luciferase expressing Lewis Lung Carcinoma (LLC-luc) cells. Imaging the dorsal aspect of the thorax reveled a trend of less tumor burden in lungs of flu experienced animals (Fig 17A). Closer examination at day 28 post tumor challenge (Fig 17C) revealed a significant difference between groups. Although imaging the ventral side of the thorax did not reveal any differences, dorsal imaging is likely more accurate in terms of signal specificity to the lung due to the lungs being more superficial in the dorsal position (Fig 17B). These results are consistent with our previous findings using the subcutaneous tumor challenge. Although we cannot say definitively that lung metastasis (due to initial lung seeding at the time of intravenous challenge or secondary tumor metastasis from tumors seeded from another organ) was different between groups because of DAA immune responses due to flu exposure, our previous studies strongly suggest that DAA immunity was likely the protective factor.

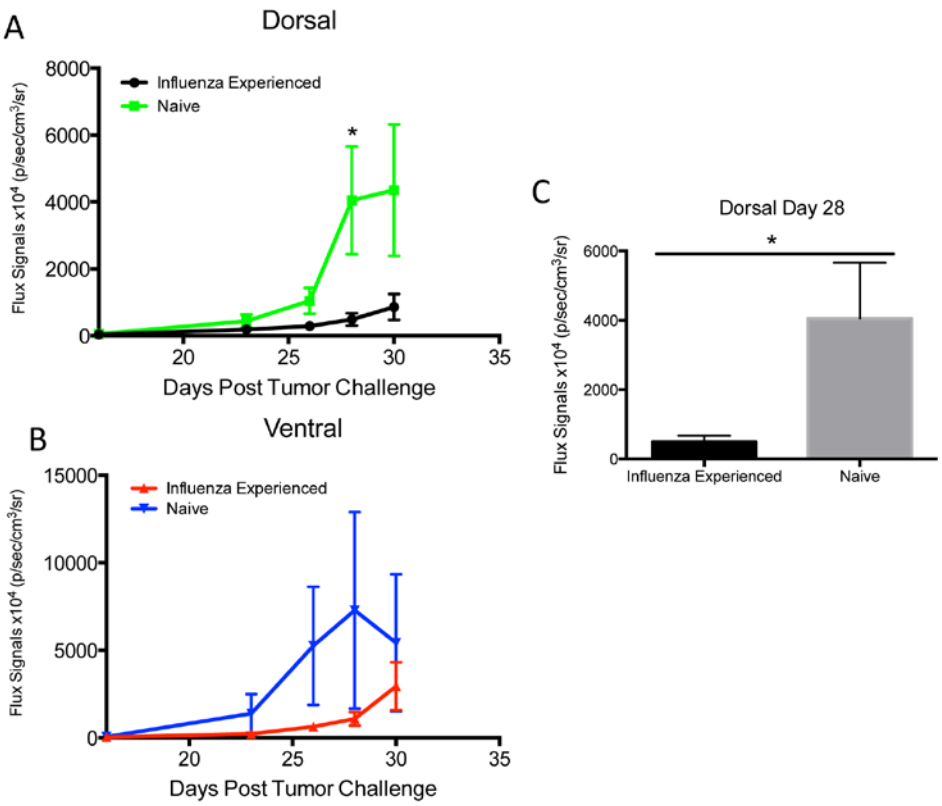

Figure 17: Influenza experienced animals have less lung metastases compared to naïve controls

(A-C) Animals were intranasally infected with PR8 and Aichi influenza viruses on day zero and day 35 respectively. Influenza experienced animals and naïve animals were challenged intravenous with $5x10^5$ 3LL tumor cells via tail vein injection. Tumor lung metastasis was measured Imaging of animals began 16 days after tumor challenge. Ten minutes prior to In Vivo Imaging System 200 (IVIS 200) imaging animals injected with D-luciferin (Caliper Life Sciences) (150mg/kg). Animals were imaged and analyzed with the living image software (Xenogen). Bioluminescence (Flux Signal in p/sec/cm^3/sr) was measured by drawing ROI on the dorsal (A, C) or ventral (B) sides of the thorax for lung metastasis. Data are representative of one experiments with at least 10 mice per group and are expressed as means ± SEM. Bioluminescence is measured as Flux Signal (p/sec/cm^3/sr). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

APPENDIX B: Viral Immunosurveillance

B.1.1 Tumor lysate and DAA pulsed DC vaccination affects viral infection kinetics

We have previously established that viral infection can induce the abnormal expression of DAA and immune responses against those same DAA. Since immune responses against DAA are important in bolstering tumor immunosurveillance, we hypothesized that immune responses against DAA are an important mechanism in bolstering viral immunosurveillance by targeting and eliminating virus infected cells. To test this hypothesis we vaccinated animals with DCs pulsed with 3LL tumor lysate (Fig 18) or DAA peptides previously described in chapter four (Fig 18) and intranasally infected animals with PR8 influenza virus. There was no difference in viral replication between lysate vaccinated or unvaccinated groups at day 3 post flu infection (Fig 18A). This result was only a snapshot of the virus infection therefore we believed it was more prudent to follow flu infection over a two to three week period. As an indirect measure of influenza infection severity, we measured percent weight loss in three groups of animals: PBS, Unloaded DC, and loaded DC (pulsed with 3LL lysate) (Fig 18B,C). Kinetics of influenza disease severity appears to be accelerated in the lysate loaded DC group. There was a significant difference in weight loss between PBS and tumor lysate pulsed DC groups as well as Unloaded DC and PBS groups (Fig 18D). Additionally, lysate loaded DC vaccinated group appears to recover to their baseline weight at an earlier time point than the other groups. We then decided to assay the effect of vaccinating animals with DC's pulsed with DAA previously described in chapter four. We saw a significant difference in weight loss between DCs pulsed with DAA and unloaded DC groups (Fig 19A,B). At this point it is difficult to say with certainty that lysate pulsed DC or DAA pulsed DC

vaccination is protecting animals from flu infection. In fact disease severity may be worse. However weight loss may be attributed to increased immunopathology caused by memory responses mounted against antigens in the lysate/selected DAA to eliminate virus infected cells rather than virus infection itself. More studies need to be done specifically looking at lung histology sections as well as virus plaque assays at various early time points.

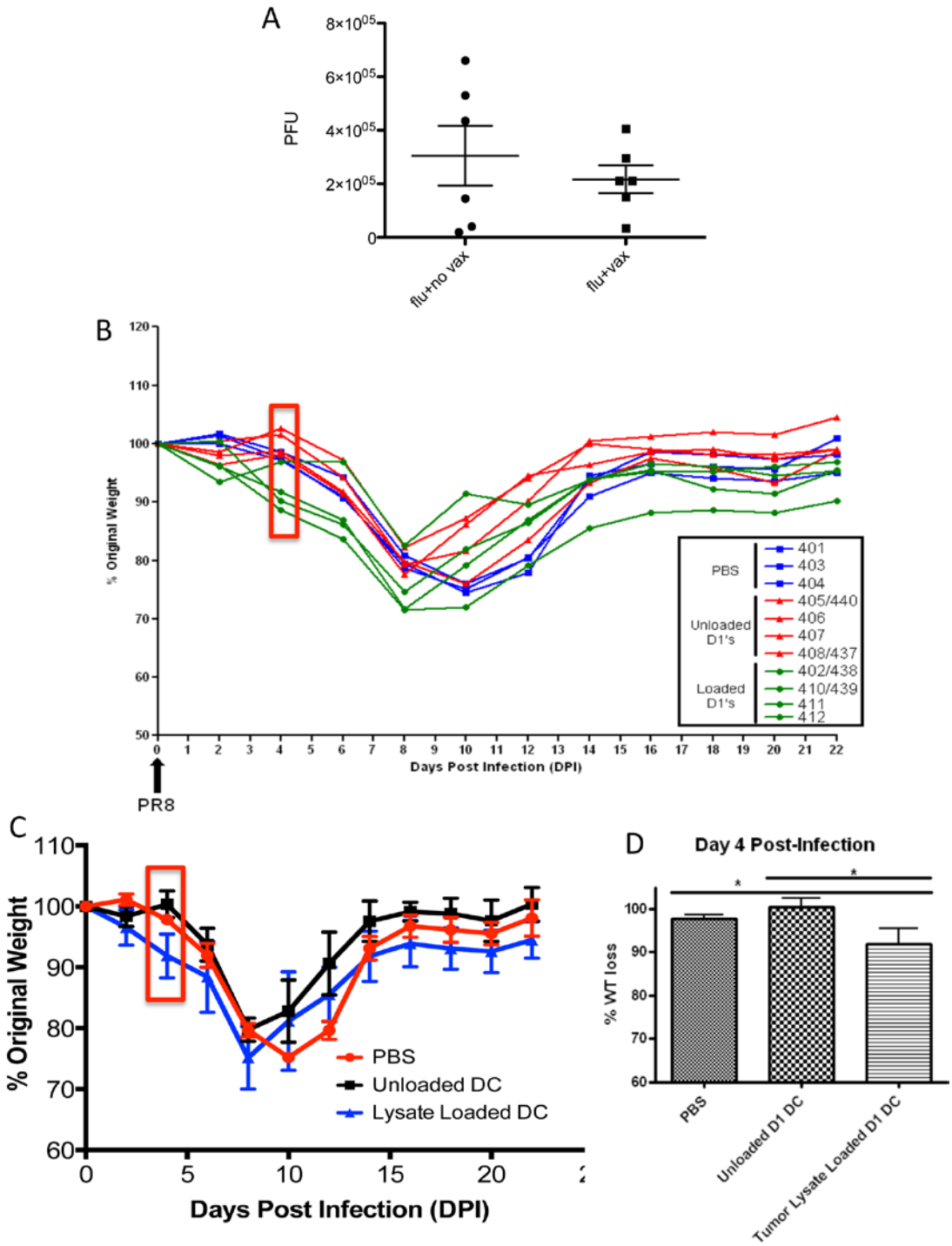

Figure 18: Vaccination with 3LL lysate loaded DC effects viral infection dynamics and accelerated weight loss in flu infected animals

(A) Mice were vaccinated in the right hind flank with $1.5x10^6$ D1 cells/mouse pulsed with 3LL lysate from $3x10^6$ at week zero, week two, and week six. Control mice received the same number

of unloaded D1 DC at the same time points. Two days after the week six booster, animals were infected with $1.25x10^4$ pfu PR8 influenza virus. Animals were sacrificed 3 days following flu infection and viral plaque assays were performed. (N=6) (B,C) Animals were vaccinated as described above. Two days after the week six booster, animals were infected with $1.25x10^4$ pfu PR8 influenza virus. Mouse weight was measured at two day intervals. (B) Individual mouse weight loss. (C) Pooled mouse weight loss. (D) Graph of weight loss at day 4 post infection. Data are representative of two experiments with at least n=8 mice per group and are expressed as means ± SEM.

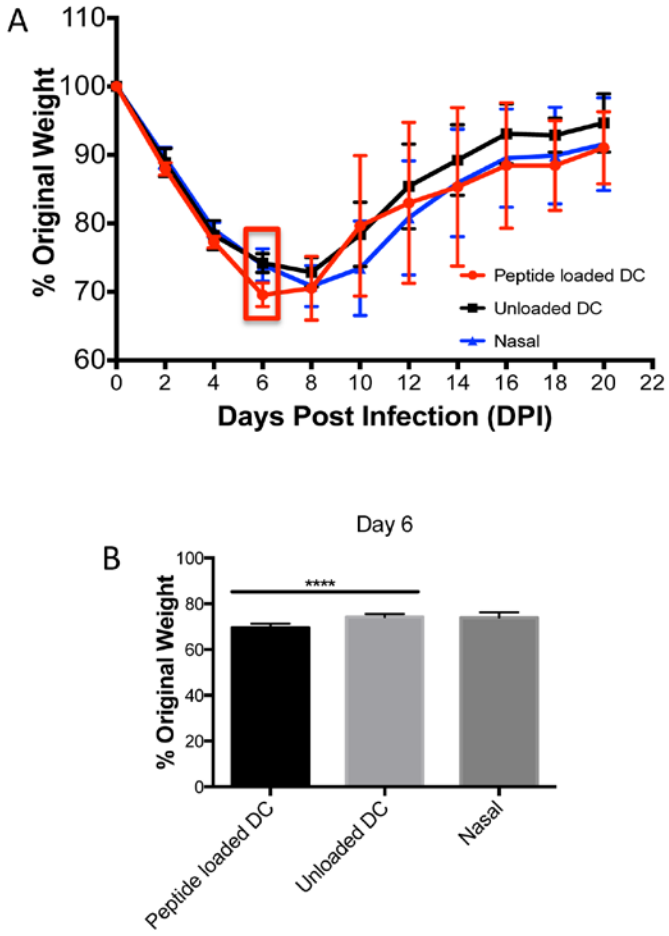

Figure 19: Vacation with GAPDH, Histone H4, MDH2, and Annexin A2 peptides loaded on DC accelerated weight loss in flu infected animals.

(A) Mice were vaccinated in the right hind flank with $1x10^6$ D1 cells/mouse (a pool of $2.5x10^5$ D1 dendritic cells/peptide) or intranasal with 2.5ug/protein with 12.5ug Poly IC:LC at week zero, week two, and week six. Control mice received the same number of unloaded D1 DC at the same time points. Two days after the week six booster, animals were challenged intranasal with $1.25x10^4$ pfu PR8 influenza virus. Mouse weight was measured at two day intervals. (B) % Original weight on day six post infection was measured and compared across groups. Data are representative of two experiments with at least n=8 mice per group and are expressed as means ± SEM.

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