The effects of host response and vaccine design on early signaling profiles of MUC1-specific T cells

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The effects of host response and vaccine design on early signaling profiles of MUC1-specific T cells

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Vaccines are beginning to be explored for cancer prevention. Since the time between vaccination and determination of efficacy is long, there is a need for predictive early biomarkers. Immunological endpoints that are determined weeks or months post vaccination are currently being evaluated as biomarkers of vaccine efficacy to replace disease outcome that could take years to assess. However, when multiple vaccines are available, waiting for the development of humoral and cellular immunity could still cause delays while an earlier assessment would allow a timely change to a more effective prevention modality. We hypothesize that early activation of signaling networks within T cells in response to immunization predicts the outcome of the immune response and may be used as a biomarker of vaccine efficacy. Applying the phospho-flow technique to primary T cells, we examined phosphorylation of proteins that shape activation, proliferation, and differentiation of MUC1 tumor antigen-specific CD4 T cells within the first 24 hours post immunization as candidate biomarkers. Previously, our lab has shown that a vaccine composed of a MUC1 peptide loaded on DC is more effective in eliciting T cell immunity than a vaccine composed of the same peptide plus adjuvant. Furthermore, both vaccines stimulate T cells more effectively in wildtype (WT) compared to MUC1.Tg mice. We examined if signaling events downstream of the TCR or through various cell proliferation and survival pathways could predict differential potential of these two vaccines in two different hosts as early as 3, 6, 12, and 24 hours post immunization. Signaling signatures obtained reflect

primarily differences between vaccines rather than between the hosts. We demonstrate the feasibility of using this approach to evaluate a vaccine's potential to elicit a desired response leading to better vaccine design.

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PREFACE

I would like to acknowledge the guidance and support given by Dr. Olivera Finn, my dissertation advisor and mentor. Olja, you gave me the freedom to explore and grow as a scientist, and confidence in myself and my abilities. Thank you for all your support in both my academic and personal endeavors.

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1.0 INTRODUCTION

The American Cancer Society expected over 1.6 million new cases of cancer to occur in 2012, and the mortality rate of those who suffer from the disease to be higher than 1,500 per day(2). For these patients, favorable prognostic outcome correlates with early detection of disease for a variety of reasons including smaller tumor burden and less immune suppression in an individual(3, 4). In efforts to better control the progression of cancer and eliminate tumors, oncology research is being directed at the discovery of mechanisms of disease, understanding the immune response to malignant cells, design and implementation of clinically beneficial therapeutic strategies, and preventing recurrence and even initial occurrence of cancer. Current therapeutic modalities are directed mainly at cellular processes that occur at an increased rate in cancer cells rather than specifically in malignant compared to healthy cells, resulting in a great deal of toxicity to the patient. Advancements in the field of immunotherapy may provide tumor specific approaches that will alleviate much of the damage incurred by the surrounding healthy tissue. The importance of the immune response in contributing to control of cancer has been well illustrated. Evidence from both clinical trials and preclinical models have shown that the potential of a cell that has undergone malignant changes to develop into a tumor is contingent mainly upon the host response as well as the tumor microenvironment.

1.1 CANCER IMMUNOEDITING: THE INTERPLAY OF THE IMMUNE RESPONSE AND TUMOR EVOLUTION

The relationship between the host immune system and tumor evolution is a dynamic process consisting of three levels of interplay: elimination, equilibrium, and escape. Initial theories, proposed by Burnet and Thomas(5-7), were focused mainly on immunosurveillance and elimination of tumor cells by the immune system. However, as our understanding of contributions of both innate and adaptive systems to tumor immunosurveillance advanced, so did our appreciation of the dynamic tumor microenvironment and the genetic instability of tumors that allow their evolution to hide from immune mechanisms of elimination. Tumor cells that persist through initial immune mechanisms of identification and destruction are shaped by the immune mechanisms in their environment(8). A state of equilibrium occurs, in which there is no clinical outgrowth of tumor yet the immune system isn't successful at eliminating all tumor cells due to successful evasion by mutation. During the equilibrium phase a number of things may happen. The immune system may be able to eliminate the transformed cells, the system could remain in a state of permanent flux in which the tumor burden is neither eliminated nor able to progress to a clinically detectable level, or the transformed cells may progress to a clinically detectable tumor burden known as the phase of tumor escape. Tumor cells that persist, leading to tumor outgrowth under heavy immune pressure of a robust immune response, will be less immunogenic and better designed to continue immune evasion than those tumors that progress in immune compromised environments(9, 10). This is important when considering therapeutic regimens to induce immune responses to existing tumor burden, especially metastases.

1.1.1 *Induction of the immune response*

Immunosurveillance begins with immature DC sampling antigen from the host environment. Peptide fragments, generated from antigen breakdown in the proteasomes, are presented and cross-presented in the context of MHC II and I to CD4 and CD8 T cells, respectively. At the time of antigen uptake a DC receives environmental signals of maturation, such as ATP, TLR ligands, and other danger signals. Appropriate maturation signals will result in upregulation of costimulation, such as B7 ligands and OX40L, and immunity promoting cytokine production, IL-12, by DC. However if DC encounter insufficient maturation signals, tolerance to the antigen presented will be promoted.

Mature DC travel to the draining lymph node where initiation of T and B cell responses occur. The induction of a robust protective T cell response requires 3 signals: stable TCR:MHC-peptide complex interactions dictated largely by TCR affinity for the antigen, strong costimulation, and IL-12 cytokine production by the DC to promote Th1 development. DC with low or no levels of costimulation or producing IL-6 and TNF will induce more tolerant T and B cell responses.

1.1.1.1 *Protective immune responses*

Both the adaptive and innate responses contribute to protection from tumor formation, control of tumor growth, and prevention of disease progression. Mice deficient in the RAG2 gene, necessary for somatic rearrangement of antigen receptor gene segments and generation of T and B cells, are more susceptible to spontaneous and chemically induced tumor formation than wildtype counterparts(9). There is evidence that γδ T cells and αβ T cells play a role in immune mediated protection(11, 12). CD8 cytotoxic lymphocytes and CD4 effector T cells protect

against tumorigenesis by CTL perforin-mediated elimination of tumor cells(13, 14) and production of IFNγ(9). IFNγ promotes Th1 development, CTL function, and induces cytotoxic functions of macrophages. Preclinical studies have shown mice deficient in IFNγ are more susceptible to tumor induction(10, 15). Clinical studies have demonstrated that the presence of IFNγ producing T cells in the tumor environment is correlated to prolonged survival in patients(16, 17).

Activation of NK and NKT cells diminishes the occurrence and growth of tumors(18-21). Tumor cells may induce the perforin and granzyme cytotoxic functions of these cells by recognition of missing self or tumor expression of ligands such as NKG2D and MICA/B(8). In addition, the production of Type I interferons by innate cells has been shown to be protective, in part by inducing activation of NK and NKT cells in responses to tumors(22, 23).

1.1.1.2 *Immune tolerance*

Tolerant responses, such as T cell anergy or induction of regulatory T cells, occur when DC are not sufficiently matured, lack costimulatory molecules, or increase regulatory molecules such as PDL1 while interacting with T cells. In addition, the production of TNF and IL-6 cytokines by DC can skew differentiation of CD4 T helper cells in the lymph node towards Th2 lineage cells that can produce IL-13 and promote growth of some tumor cells. These T cells then home to the tumor bed and exert their immunosuppressive mechanisms within the tumor microenvironment.

Preclinical mouse models demonstrate slowed tumor growth upon Treg depletion, establishing the immunosuppressive function of Treg on the immune response to tumors(24). Treg production of suppressive cytokines, IL-10 and TGFβ, can downregulate MHC and costimulatory molecules on DC, resulting in the priming of anergic T cells(25). Treg also prevent effector T cell function directly by acting as an IL-2 sink, due to constituently high levels

of expression of IL-2 receptor on their cell surface, as well as direct killing mediated by granzyme and perforin(26). Treg have many additional mechanisms that can decrease effector T cell function, including the blocking of productive costimulatory interaction due to CTL4 binding(27) and stimulation of IDO production, resulting in less tryptophan(28).

1.1.2 *Tumor evasion of the immune response*

The induction of tolerance by improper activation of antigen presenting cells and induction of anergic or immunosuppressive T cells are not the only ways that tumors avoid elimination. Tumors employ their own immune evasion techniques in order to avoid detection and deletion, allowing progression to clinically detectable levels. Tumor cells can develop defects in HLA expression(29) and TAP activity(30), leading to deficiencies in antigen processing and presentation pathways, as well as downregulate the expression of costimulatory molecules in order to decrease activation of effector T cells. Expression of surface molecules that prevent activation or induce T cell apoptosis: CTLA-4, FasL, TRAIL, and PDL1 are often seen upregulated in tumors. Creation of an immunosuppressive microenvironment is furthered by the secretion of IL-10 and TGFβ cytokines(31), the production of IDO(32) and prostaglandins (PGE2), and the release of adenosine; together these promote Treg function while inhibiting effector T cell function. Some tumors have demonstrated the ability to evade the effector functions of T cells by developing defects in the IFNγ receptor signaling pathway(15), as well as the loss of targeted tumor associated antigens (TAA)(33).

Furthermore, the tumor stroma secretes chemoattractants for the recruitment of suppressive immune cells: Treg, myeloid derived suppressor cells (MDSC), and anti-inflammatory macrophages (M2)(34). The activation of MDSC and M2 macrophages has been

shown in preclinical mouse models to correlate with increased tumor growth, disease progression, and the development of metastases. MDSCs release arginase, nitric oxide synthase, and reactive oxygen species, suppressing CTL function(35). Macrophages have also been shown to suppress T cell function by increasing angiogenesis by release of matrix metalloprotease-9 (MMP9)(36, 37). Treg mediate immune suppression as previously discussed.

1.2 TUMOR IMMUNOTHERAPY

There are two types of tumor immunotherapy: the transfer of passive immunity and the induction of active immunity. Passive immunity is the transfer of antibodies or ex vivo generated CTL and effector cells into the host. Active immunity is the induction of the host immune response through vaccination or other therapeutic intervention. Antibodies and vaccines can each function to control tumor progression; however, vaccination is advantageous in control of cancer before clinical disease starts.

Most established FDA approved immune therapies (antibodies, recombinant cytokines, or adoptive T cell transfer) confer passive immunity to the patient. These immunotherapeutic approaches have generated clinically relevant anti-tumor effects. Antibodies can induce anti-tumor immunity by activating complement-mediated cytotoxicity, direct killing of the tumor cells by antibody-dependent cellular cytotoxicity, or tumor specific delivery of radioactive elements or other cytotoxic substances(38, 39). The first attempt to induce active immunity in cancer patients via vaccination was conducted by William B. Coley. While treating patients with sarcoma, Coley observed that those patients whose tumors were in remission had suffered from a severe bacterial infection of the skin. He tried to recreate this event by injecting patients with a

combination of live or attenuated *Streptococcus* and *Serratia marcescens*(40, 41). Coley's attempts were met with little success. However, today we do mimic the danger signals these infections were producing to boost the immune response by our use of adjuvants with anti-tumor vaccines.

Our understanding of immunotherapy has advanced significantly since Coley's first experiments to induce a protective immune response, as is evident in our current understanding of vaccine design. As we expand our knowledge of interactions of the immune response, the parameters we use as biomarkers of immunogenicity and correlates predictive of disease outcome are refined. This leads to improvement in vaccine design and with it our ability to modulate the immune response.

1.2.1 *Tumor vaccines*

A tumor's immunosuppressive microenvironment can impede the induction of a protective immune response as well as hinder and suppress the effector arm of the immune response. However, clinical evidence has demonstrated a strong association of the presence and type of T cells in a tumor to outcome of disease(16, 17). Therefore, considerable effort has been placed on developing vaccines and other immunotherapies to boost pre-existing CTL activity, decrease mediators of suppression in the tumor microenvironment, and induce the activation and proliferation of new effector T cells. There are three main places for therapeutic intervention in the immune response to tumors (**Figure 1-1**)(1). Manipulation of the antigen presenting function of DC is one place of improvement. This can include increasing antigen presentation and cross-presentation, the maturation of DC with adjuvants, the quality of antigen (choice of antigen that is recognized with high affinity by T cell receptors), and the quantity of antigen processed. The

second area is induction of a protective T and B cell response in the lymph node, including increasing costimulatory molecules and appropriate cytokine stimulus(1). Lastly, decreasing immunosuppressive functions, whether mediated by the tumor or immune cells, is an area of great therapeutic importance in mounting a productive immune response(1). Currently, most antibody therapy addresses immune suppression while vaccine therapy focuses on induction of an immune response. Tumor vaccines are advantageous in that they not only induce effector responses but long term memory responses that are important in disease-free patient survival.

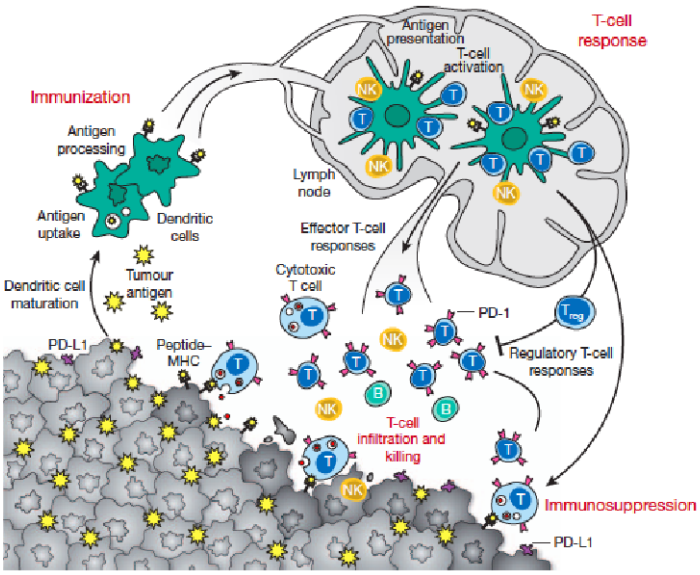


Figure 1-1. Generation and regulation of antitumor immunity.

Understanding the events in generating and regulating antitumor immunity suggests at least three sites for therapeutic intervention: promoting the antigen presentation functions of dendritic cells, promoting the protective T cell responses and overcoming immunosuppression in the tumour bed. From Mellman I., Coukos G., and Dranoff, G. 2011. Nature. 480: 480-489. (1)

Vaccines can be designed as either prophylactic or therapeutic regimens. Evidence suggests that a prophylactic vaccine would induce a more robust immune response. It has been

established that the best protection against infectious disease occurs before the host encounters the infectious agent, and reasonably the same would hold true against cancerous cells. Initiating an immune response before tumorigenesis, would allow for the identification and elimination of the neoplastic cells before the tumor burden becomes large, thus preventing much of the immunosuppressive microenvironment associated with established tumor beds(1). In addition, the immune response may be weakened by radiation and/or chemotherapy treatments. However, the vast majority of vaccines approved for anti-tumor therapy in clinical trials are therapeutic and will remain as such until safety and efficacy are proven. The basis of their therapeutic value lies in the ability to stimulate pre-existing CD4 and CD8 tumor specific cells residing in the tumors(1).

Although the stage of the immune response targeted as well as the state of the immune response (whether given prophylactic or therapeutic treatment) play very important roles in the immunogenicity of a vaccine, vaccine design also plays a major role. There are three major facets that comprise vaccine design: route of administration, choice of adjuvant, and choice of antigen(42). The augmentation of a more local, versus systemic, response can vary dependent on route of administration, and may affect the ability to mount an effective primary and memory response. The choice of adjuvant is of equal if not greater importance. A suitable adjuvant can vary widely, from GMCSF or TLR ligands which induce strong maturation signals in DC, cytokines such as IL-2 to promote T cell activation, vaccine virus vectors to induce strong immune responses to viral products, coupling to monoclonal antibodies for more specific targeting or to induce specific signaling, matured DC themselves, and many others. Common to all these is the ability to induce protective T cell responses, often through the strong maturation of DC. Lastly, but very important in the manipulation of the immune response is the choice of

antigen. The immune response is dependent on the ability of a DC to process the antigen, present the antigen and costimulatory molecules, and the affinity of the TCR for the antigen presented. As with adjuvants, there are numerous possible cancer-associated antigens that may stimulate an immune response. Suitable antigen candidates for tumor vaccines must not only elicit a strong immune response, but must meet certain criteria in the host response to ensure safety as well as criteria in tumors to ensure widespread applicability. It is important for immunogenicity in patients that the antigen not be subjected to self-tolerance and imperative for safety that intensifying the immune response to the antigen does not lead to autoimmunity. The widespread and stable expression of an antigen on a variety of tumors, throughout progression of disease, is an important factor in the ability of the immune response to mount and eliminate cancerous cells. A favorable antigen would be one that is essential for persistence of the tumor, preventing the possibility of immunoediting(8).

1.2.2 *Tumor antigens*

1.2.2.1 *Tumor specific antigens*

Some tumors may express mutated or viral genes that are not expressed in normal cells. There are several known viruses associated with tumorigenesis: Human papilloma virus (HPV), Merkel cell polyomavirus (MCV), Epstein Barr virus (EBV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), human T lymphotrophic virus 1 (HTLV-1) and type 2 (HTLV-2), and human herpes virus 8 (HHV8)(34, 43). Viral proteins are processed and presented in MHC on the surface of the transformed cells, however sometimes not in an adequate concentration to mount an effective T cell response. These tumor specific proteins are very good candidates for vaccines in that a

more robust T cell response could provide better elimination of infected cells, the viral antigens are not subject to self-tolerance, and there is no risk of autoimmune disease.

1.2.2.2 *Tumor-associated antigens (TAA)*

Tumor-associated antigens (TAA) comprise the majority of tumor antigens. These are self-antigens that are expressed on healthy cells, but are aberrantly expressed or have undergone posttranslational modifications in tumor cells. For instance, the well-known molecules HER2/neu and Cyclin B1 are overexpressed in breast cancer. The aberrant glycosylation and overexpression are well characterized in the abnormal tumor form of the protein MUC1 in a variety of cancers. Other tumor antigens have been identified and are being used as biomarkers of disease. These antigens may be subject to central or peripheral tolerance in order to prevent damage to healthy tissues. Several of these TAAs have been well characterized and are in vaccine development in clinical trials.

Human epidermal growth factor receptor 2 (HER2/neu) is a transmembrane glycoprotein expressed at low levels on the surface of normal cells. This receptor tyrosine kinase is part of the epidermal growth factor family and is normally involved in regulation of the cell cycle. Overexpression of HER2/neu is seen a variety of adenocarcinomas including that of breast, ovarian, and uterine tissues(44), is associated with aggressive disease and has been indicated to play a role in disease progression. Detection of aberrant expression of HER2/neu in patients is correlated with poor prognosis and poor survival(45-47).

Cyclin B1 is another example of a self-antigen that can be recognized by the immune system after abnormal expression upon malignant changes in a cell. Normally cyclin B1 is expressed transiently in small amount in the nucleus of dividing cells to promote transition from the G2 to M phase of the cell cycle. A constitutive expression of cyclin B1 leading to its

detection in the cytoplasm has been seen in tumor cells as a result of dysfunction of the well-known tumor suppressor, p53(48, 49). This overexpression and cytoplasmic localization allows for initiation of an immune response that wouldn't normally occur.

Mucin1 (MUC1) is a transmembrane glycoprotein expressed on the apical surface of secretory epithelial cells. Its extracellular domain is comprised largely of a VNTR (variable number tandem repeat) region of 20 amino acids repeated in tandem 20-120 times. Two serine and three threonine residues per 20 amino acid sequence binds highly glycosylated o-linked carbohydrates. Upon malignant transformation, MUC1 loses polarity and is overexpressed. The VNTR region becomes hypoglycosylated and the carbohydrate chains present are less branched with only one or two sugars (**Figure 1-2**). This exposes the peptide backbone for antibody recognition as well as resulting in differences in the way the molecule is processed for antigen presentation.

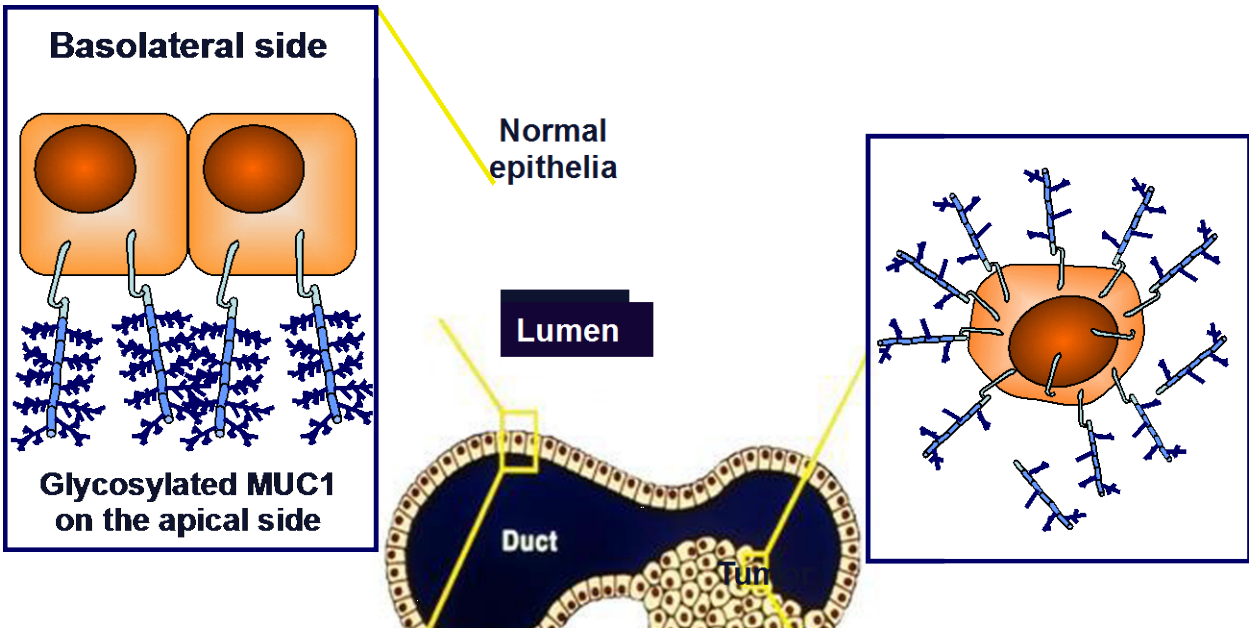


Figure 1-2. Normal and abnormal MUC1

In healthy normal epithelia, MUC1 is highly glycosylated and expressed on the apical surface. When a cell undergoes neoplastic events, MUC1 becomes hypoglycosylated, loses polarity, and is overexpressed. Figure courtesy of Pamela Beatty.

1.2.2.3 *Tissue-restricted antigens*

Malignant cells can also express proteins that are usually restricted, in healthy cells, to expression in a specific type of tissue, a specific lineage of cells, or during a particular phase of development, such as during embryonic development. Frequently these antigens are restricted to sites of immune privilege, such as the testis or placenta(50). These otherwise tissue-restricted antigens are expressed in many adenocarcinomas, melanomas, and a wide variety of other cancers. For instance, numerous adenocarcinomas express carcinoembryonic antigen (CEA), normally only expressed or secreted during fetal development(51, 52). Another example, NY-ESO-1 is normally restricted to expression in germ cells and trophoblasts; however this testis antigen is expressed in a large array of tumors(53). Melanomas often express cancer/testis antigens such as MAGE and gp100. These otherwise tissue-restricted antigens are thought to have considerably less risk of inducing autoimmunity due to their normal expression in immune privileged sites, making them attractive antigens for tumor vaccines in clinical trials(54).

1.2.3 *Current status of approved immunotherapies to treat cancer*

Despite each of their individual expression on a large percentage and a broad range of malignancies, there are few Food and Drug Administration (FDA) approved treatments directed against tumor antigens. Currently, there are several clinically effective, FDA-approved,

monoclonal antibody therapies against proteins associated with cancer, including growth factor receptors, B cell antigens, a T cell regulatory molecule, and tumor antigens(1, 55). Antibody against the negative immune regulator CTLA-4 has shown promise for activation and proliferation of pre-existing tumor specific T cells.. Although there has been evidence of significant levels of autoimmunity in some patients(56), disease stabilization and a distinct increase in survival have been seen in a subset of metastatic melanoma patients treated with anti-CTLA4 in multiple clinical trials(57, 58). Another promising therapy, Herceptin® (trastuzumab), is a monoclonal antibody that targets the HER2/neu receptor, a tumor-associated antigen. Herceptin is being used to control HER2/neu positive tumor growth after chemotherapy, reduce disease recurrence, and improve survival in breast cancer patients (59-61). In addition to antibodies, there are also several FDA approved recombinant cytokine therapies, such as IL-2 and interferon-α, demonstrating some clinical benefit for cancer patients.

Cancer immunotherapy has seen some recent breakthroughs with two FDA approved vaccines. The FDA has approved the first prophylactic cancer vaccine to a human papilloma virus (HPV) antigen. A multivalent L1-virus like particle vaccine to the L1 capsid protein of HPV has been shown to be effective in prevention of infection, including type 16 and 18, that is associated with cervical cancer(62-64). This vaccine could prevent the 70% of cervical cancer associated with HPV infection. Of late, the FDA approved the first active therapeutic for late stage cancer. Provenge® (sipuleucel-T) is a cellular therapy based on an individual patient's antigen presenting cells harvested from peripheral blood mononuclear cells (PBMC) and incubated with a fusion protein of GMCSF and the TAA prostatic acid phosphatase (PAP). Clinical trials reported no evidence of tumor control or stable disease by standard parameter of

tumor growth; however patients receiving the treatment had an increased median survival time of 4 months(65).

Recently, there has been a push to develop effective vaccines targeted against non-viral tumor-associated antigens, with many treatments showing promise in clinical trials. There are countless potential TAA to be used as immunotherapeutic targets, with new TAAs still being discovered. In the attempt to accelerate the development of a successful cancer vaccine targeting these antigens, the National Cancer Institute (NCI) and experts in the field have developed weighted criteria to prioritize research on tumor-associated antigens. Several promising candidates have been identified and among the highest in priority is the antigen MUC1(66).

1.3 MUC1

1.3.1 *Functions of MUC1*

Mucins, including MUC1, serve as lubricants and physical barriers for epithelial cells for protection from infectious microbes. Normally, MUC1 is located on the luminal surface, initiating signaling in response to environmental stimuli such as changes in pH or physical stimulation. However, loss of polarity in a cell leads to differential expression and glycosylation of these molecules and changes in their functions as well. On a cellular and molecular level the abnormal form of MUC1 has been implicated in multiple facets of tumorigenesis, tumor growth, and survival. On a tumor cell, the abnormal form of MUC1 is no longer segregated from receptor tyrosine kinases or other receptors usually expressed on the basal surface of the cell. Abnormal MUC1 has been shown to serve as an oncogene by modulating the activation of

multiple intracellular signaling molecules (67, 68). It has been found to bind chaperone molecules and increase activation of Ras/Raf pathway signaling downstream of epidermal growth factor receptor (EGFR) in an EGF-dependent manner(69). The tumor form of MUC1 can bind NF-κB blocking association with its inhibitor IκBα leading to activation of NF-κB mediated transcription(70, 71). In addition, abnormal MUC1 can prevent apoptosis of cancer cells by modulating activity of tumor suppressor genes, such as p53, by binding regulatory domains affecting transcription(72, 73).

The tumor form of MUC1 promotes metastasis of tumor cells in several ways. Intracellular junctions are created by E-cadherin binding with members of the catenin family of proteins. MUC1 causes destabilization of intracellular junctions by by binding and sequestering β-catenin, thereby disrupting the association of β -catenin and E cadherin(74). This stabilization of β -catenin in the cytoplasm weakens cell adhesion and promotes activation of the Wnt signaling pathway which is associated with tumor growth(75). In addition, the VNTR region of abnormal MUC1 can serve as a ligand to intracellular adhesion molecule 1(ICAM-1) and may play a role in metastasis(76).

The hypoglycosylated form of MUC1 has roles in various immune functions. It serves as a chemoattractant for DCs, providing maturation stimuli upon their arrival(77). The maturation signals encountered in the MUC1 tumor environment promotes the generation of IL-6 and TNF producing DC that fail to make IL-12. Priming of naïve T cells by these DC skews T cell differentiation towards IL-5 and IL-13 producing Th2 effector cells(77). IL-13 can activate NKT cell mediated suppression of immunosurveillance as well as promote tumor growth directly(78).

The role MUC1 plays in cancer as well as its TAA properties, make MUC1 an attractive candidate and target for immunotherapy as well as other novel therapeutic interventions. Here,

our interest in MUC1 is solely as a tumor-associated antigen that can be targeted to enhance protective immunity against tumors.

1.3.2 *DC processing of MUC1*

Over expression and hypoglycosylation are important in initiating a different immune response to self versus abnormal self. The normal heavily glycosylated and abnormally hypoglycosylated forms of MUC1 can both be readily taken up by dendritic cells. However, heavily glycosylated proteins are not transported into late endosomes for antigen processing but are retained for long periods of time in the early endosome without processing(79). As a result there is very little presentation of processed antigen from the heavily glycosylated form on surface MHC II or I. However, abnormal MUC1 molecules are trafficked through antigen processing compartments for presentation in Class I and II molecules on the surface(79). Degradation and processing of abnormal MUC1 yields new peptides and glycopeptides capable of eliciting a T cell response. The degree of the CTL response has been shown to be inversely correlated to the degree of glycosylation of the molecule(80).

1.3.3 *Immune response to abnormal MUC1*

Expression of abnormal MUC1 has been established in many adenocarcinomas including those originating in the breast, colon, pancreas, ovaries and other tissues(81), as well as in non-small cell lung carcinomas (NSCLC)(82), multiple myelomas(83), and head and neck squamous cell carcinomas (HNSCC)(84). Patients with MUC1 positive tumors have been reported to exhibit a weak immune response to MUC1. Low titers of MUC1 reactive antibodies, predominately of the

IgM isotype can be detected in their sera(85, 86). Low frequencies of CD8 CTL that are specific to the MUC1 polypeptide core can also be found(87-89). However, in vitro expansion of these CTL have shown that the majority of the response is MHC unrestricted(89, 90) with only a few MHC-restricted epitopes identified(91, 92). In addition, there has been little evidence of a MUC1-specific helper T cell response in cancer patients. The lack of a CD4 helper T cell response is a likely reason for the weak CTL response and absence of isotype switching of antibodies to IgG in these patients. Hiltbold et al showed that MUC1 specific CD4 T cells were not being deleted and consequentially not subject to central tolerance(93). MUC1-specific CD4 T cells could be generated from PBMC of healthy controls in vitro in response to MUC1 peptide. These observations suggest that eliciting a robust CD4 T cell response may lead to the generation of therapeutically relevant levels of CTL and antibodies.

The immune response to aberrant expression and hypoglycosylation is clinically relevant. Detection of abnormal MUC1 on tissue or in circulation is a strong correlate with poor prognosis and poor patient survival(94, 95). Conversely, detection of naturally occurring MUC1-specific antibodies in the sera is indicative of a more favorable prognosis. MUC1-specific antibodies in breast, pancreatic, and gastric cancer patients were correlated to prolonged survival and reduced incidence of metastasis(96-98). The existence of circulating MUC1 antibodies has even been shown to be protective, lowering the risk of cancer(99).

1.3.4 *MUC1 vaccines in clinical trials*

The protection afforded by natural induction of MUC1-specific antibodies in conjunction with the known deficiencies in the immune response to MUC1, suggest it would be advantageous to boost the endogenous antibody and T cell response through vaccination. Vaccine trials for

numerous TAAs, including MUC1, are ongoing in the clinic. These trials vary vaccine formulation (including peptides and adjuvants), modes of delivery, and vaccination schedules in efforts to create the best possible therapy providing a combination of low toxicity and high immunogenicity. Many clinical trials testing MUC1 vaccines have been conducted in patients suffering from breast, pancreatic, lung, prostrate, ovarian, and renal cancer in the past two decades(27, 81).

Pioneering the field with the first synthetic TAA vaccine, a Phase I trial of a MUC1 vaccine began in 1993(100). Sixty-three late stage breast, colon, and pancreatic cancer patients were enrolled post chemotherapy treatment to receive a vaccine of MUC1 105 amino acid peptide admixed with BCG. Importantly, the vaccine showed no signs of treatment related toxicity or development of autoimmunity. Seven of 22 patients tested were found to have a small increase in MUC1 specific CTL, however there was no clinical benefit or memory response observed. This study helped to establish the safety of the vaccine but potential to stimulate a strong response to MUC1 was difficult to assess in patients with impaired immune function and advanced disease.

In 2004, a Phase I trial of sixteen pancreatic cancer patients addressed not only safety but also immunogenicity in a group having no chemotherapeutic intervention. After tumor resection, patients were treated with MUC1 100mer (a 100 amino acid sequence from the VNTR region) vaccine admixed with SB-AS2 adjuvant (monophosphoryl A, purified saponin and an oil-water immersion)(101). These patients exhibited a low but measurable increase in humoral response with five patients exhibiting MUC1 IgG antibodies, and an increase in CD8 T cells with no autoimmunity detected. A 32 and 61 month follow up proved two of fifteen patients to be free of disease. Although the elicited response was not as robust or therapeutic as desired, the

improvement seen was further evidence of a correlation of immune state and the ability of a vaccine to impact development of the immune response.

Several MUC1 vaccine trials in ovarian cancer have also shown safety of the vaccines and evidence of humoral and cellular responses to MUC1(102-104). In addition, MUC1 vaccines either conjugated to keyhole limpet hemocyanin or delivered in a vaccina virus were well tolerated, and antibody and CTL responses were attained in clinical trials of patients suffering from breast cancer(105-108). Evidence of clinical benefit of MUC1 immunotherapy has been seen in three renal cancer trials, in which not only was no toxicity associated with treatment and immunogenicity to MUC1 induced, there was some success achieving stable disease in multiple patients(109-111). Promising results have also been achieved in two Phase II clinical trials using MUC1 peptide in a liposome vector (BLP25) admixed with a monophosphoryl lipid A adjuvant(112, 113) and a recombinant vaccina virus as a MUC1 vehicle with IL-2(114, 115) to treat non-small cell lung carcinomas. Patients vaccinated with MUC1 had prolonged survival compared to non-vaccinated groups. Phase III large randomized trials are now underway for both MUC1 vaccines. Interestingly, only two of the sixteen patients exhibiting prolonged survival demonstrated an expansion of CTL as a predictor of immunogenicity. This underscores the importance of having reliable correlatives of protection while assessing vaccine efficacy that are both predictive of immunogenicity and outcome of disease.

There are other clinical trials that demonstrate the need for better biomarkers and parameters of vaccine efficacy. A Phase I/II trial of MUC1 vaccine efficacy in pancreatic patients displayed promising yet unclear results initially. Twelve pancreatic patients were treated with a MUC1 peptide loaded DC vaccine after resection of their tumors. The vaccine proved to be safe however due to an increase in the presence of Treg the ability to effectively

prime a protective T cell responsive was not distinguishable. Interestingly, long term assessment four years post vaccination revealed four of twelve patients without reoccurrence of disease(116). This study demonstrated a need for improved parameters of detection of vaccine efficacy that will correlate early immune responses with later immune function in controlling disease.

As demonstrated by the above studies, the use of TAA vaccines as a therapeutic measure to control cancer can lead to underestimation of the true capacity of a vaccine to induce a protective immune response. Prevention of infectious disease with the use of vaccines as a prophylactic regimen has proven to be quite successful(117). The same will most likely hold true for cancer, where priming can happen in an immune competent environment, leading to a more efficient immune response when cancer first arises and the tumor burden will be low. The first prophylactic cancer vaccine trial that used tumor antigens instead of viral antigens was recently conducted(118). Patients with premalignant colon polyps, at a high risk for developing colon cancer, were enrolled and given MUC1 100mer peptide admixed with Hiltonol (a TLR 3 ligand known as poly-ICLC). Patients exhibited high levels of IgG production (17/39) with a robust memory response upon boost. The lack of a humoral response (22/39) was correlated with a high frequency of myeloid derived suppressor cells (MDSC). This data suggests that a more robust immune response is generated in the prophylactic phase and in environments in which immune suppression is low leading to more effective prevention and control of disease. Determining the efficacy of a vaccine used as a prophylatically requires larger test groups and a long duration of study, illustrating the need for early detection of immunogenicity parameters that are predictive of disease outcome.

1.4 PRECLINICAL MODELS

Prior to clinical trials in patients, treatment regimens must pass through extensive rigorous investigations in multiple preclinical models. Preclinical models allow for an examination of treatment toxicity, several parameters of efficacy, and disease outcome, often more thoroughly then can be achieved in clinical trials. The most widely used preclinical model is the mouse. The advent of genetic engineering has allowed for the generation of many strains of specialized mice to hone in on specific functions of molecules, modulation of specific cells and entire systems, as well as both focused and systemic effects of treatments such as vaccination.

These mouse models have been the route of discovery for immune functions, key to understanding interactions in the immune response, and the ability to examine the effects of manipulation of components of the immune response.

1.4.1 *MUC1 Transgenic Mice*

A genetically engineered transgenic mouse has been developed to express the human form of Mucin1 (MUC1) under the control of its endogenous promoter(119). Therefore, expression is biologically relevant both quantitatively in the amount of expression and qualitatively in its natural expression pattern. The MUC1 transgenic (MUC1.Tg) mouse model is well characterized as a model for the study of tumor-associated antigens in cancer(120). MUC1.Tg mice have lead to insights in immune cell functions and interactions as well as the host response to “abnormal self.” Vaccination with MUC1 has shown the ability of MUC1.Tg mice to generate MUC1-specific immunity with no indication of autoimmunity(121). Given as a prophylaxis, MUC1 vaccine induces a protective immune response against MUC1 positive tumor

formation, and can induce tumor regression as a therapeutic regimen. In addition, differences in MUC1 vaccine design lead to differences in the immune response within the MUC1.Tg mouse(121). Importantly, the effectiveness of the MUC1-specific immune response to the tumor form of MUC1 in the MUC1.Tg mouse (where it is perceived as an abnormal version of an endogenously expressed molecule) is not as robust as compared to an environment in which it is not endogenously expressed and perceived as foreign (as in wildtype mice)(122, 123). The dampened immune response has been attributed to deficiencies in the CD4 T cell response(124). There is less of a proliferative response and lower production of IFNγ exhibited by MUC1-specific CD4 T cells in response to MUC1 antigen in MUC1.Tg mice then in their wildtype counterparts(122, 123). These findings suggest an altered detection of abnormal self versus foreign self.

The MUC1.Tg mouse model system enables dissection of a productive immune response compared to a more tolerant immune reaction induced by antigen specific responses based on environment. This enables us to investigate early antigen-specific T cell responses that result in the establishment of immunity or tolerance. We use the well-established flow cytometry technique of phospho-flow to examine the activation of early MUC1-specific T cell signaling events to MUC1 antigen in MUC1.Tg and WT mice.

1.5 PHOSPHO-FLOW

Phospho-flow is a technique that allows for the multiparameter analysis of single cells, which can be adapted for high-throughput screening ideal for clinical studies(125-129). The ability of this technique to analyze single cells in heterogeneous populations can provide insight into the

signaling pathways activated, the relationship between networks of signaling, and the signaling profiles could result in correlation to therapeutic efficacy, disease progression, and clinical outcome(129). Phospho-flow has been used to identify alterations in the signaling profile of patients with acute myeloid leukemia in response to cytokine stimulation, and demonstrated a deficiency in STAT1 signaling in response to IFNγ stimulation(130). Knowledge of alterations in the ability of cancer patients to respond to certain stimuli could lead to development of new therapeutics as well as alterations in individual treatment strategies(129). To date, phospho-flow has been employed experimentally to examine alterations in cellular signaling in individuals suffering from late stage cancer, autoimmune disorders, and infection(131, 132). In addition, there has been some initiative to use phospho-flow to predict patient response to therapy(133).

We propose that identification of the activated signaling pathways in T cells after therapeutic treatment will be predictive of the immune response that ensues. We have identified several 'phospho-fingerprint' candidates based on their known central role in signaling pathways related to T cell activation, differentiation, proliferation, and survival (Table 1). The quality (targets phosphorylated), quantity (the numbers of cells expressing the phosphorylated targets), and temporality (the time and sequence in which signaling pathways are activated) may all determine if a signaling 'fingerprint' is correlated to the induction of a protective or a tolerant response.

Table 1. Candidates for T cell 'phospho-fingerprint' in response to immunization

TH 1 Associated Signaling	Marker
TH1 (transcription factor)	Tbet
TH1: IFN signaling	STAT 1 (pY701)
TH1: IL12 signaling	STAT 4 (pY693)

Cell Survival/ Proliferation	Marker
Ras/Raf pathway (TCR/BCR activated)	ERK1/2 (pT202/pY204)
PI3K pathway	AkT (pS473)
MAPK	p38 MAPK (pT180/pY182)

Treg Associated Signaling	Marker
Treg (transcription factor)	FOXP3
Treg homeostasis (tumor micro) : IL-2 signaling	STAT 5 (pY694)
Protein synthesis, Treg generation	S6 (pS244)

TCR Associated Signaling	Marker
TCR	ZAP70 (Y319)
TCR/costim signaling (CD4)	Lck (pY505)

T Helper Associated Signaling	Marker
TH2: IL-4 signaling	STAT6 (pY641)
TH17: IL-6 signaling	STAT3 (pY705)

1.6 INTRACELLULAR T CELL SIGNALING

Environmental cues, such as cytokines and TLR ligands, as well as interactions between cells activate intracellular signaling pathways in T cells inducing and shaping their response. The signaling pathways activated determine the magnitude and duration of the T cell response. The signaling controlling cellular processes is a complex intricate network with many components of regulation. The signaling molecules we chose to examine (Table 1) are involved in T cell activation after antigen recognition, differentiation of T helper cells, and cell survival and proliferation. Here, we discuss some of the predominant effects of phosphorylation of the selected targets.

The initial T cell response to antigenic stimulation consists of two main components: T cell receptor (TCR) signaling after peptide-MHC recognition and costimulatory signaling. TCR binding to the peptide-MHC complex induces the phosphorylation of ZAP70 and Lck leading to

functionally active kinases(134). Signaling through the CD28 costimulatory receptor can lead to the up-regulation of expression of other costimulatory molecules such as CD40L and OX40(135). The activation of costimulatory signaling can affect the amplitude and persistence of both ZAP70 and Lck phosphorylation, prolonging their activation. The activation of ZAP70 and Lck leads to further phosphorylation via multiple signaling cascades leading to T cell activation and proliferation.

The microenvironment of a CD4 T cell dictates the network of signaling pathways that function together to determine helper T cell differentiation and fate(136). There are multiple subsets of CD4 T helper cells including Th1, Th2, Th17, and regulatory T cells. Intracellular signaling through the JAK/STAT pathways downstream of cytokine receptors is crucial to the initiation and maintenance of genes responsible for T cell differentiation(137, 138). Binding of IFNγ and IL-12 to their respective cell surface receptors signal through STAT1 and STAT4 respectively, skewing T helper cells to mainly a Th1 phenotype responsible for the clearance of intracellular pathogens and cellular immunity(137). The transcription factor, Tbet, is also associated with the differentiation and maintenance of the Th1 phenotype. However, signaling through the IL-4 receptor activates STAT6 that in conjunction with the transcription factor GATA3 skews T cell differentiation towards Th2 phenotype(139, 140). Th2 immunity is associated with allergy, clearance of extracellular pathogens, and induction of humoral immunity. The differentiation of the Th17 subset is associated with IL-6, IL-23, and IL-21 receptor signaling through STAT3(141-143). Th17 cells play a role in autoimmunity, tissue inflammation, and clearance of extracellular pathogens. In addition, activation of STAT5 through the IL-2 receptor is associated not only with transient T cell activation but with the development of regulatory T cells(144). STAT5 directly binds and activates the transcription

factor FOXP3 that regulates Treg development(145). On the other hand, the early activation of S6 is inversely correlated to Treg development.

There are multiple avenues that lead to cellular proliferation and survival. G protein-coupled receptors (GPCRs), ion channels, growth factors, receptor tyrosine kinases (RTKs), and integrins can activate ERK1/2 signaling through the RAS/Raf pathways leading to progression of T cell growth and proliferation(146). Activation of receptors for integrins, RTKs, cytokines (such as IL-7), and the TCR can result in the downstream activation of Akt signaling through the PI3K signaling pathway(146). Activation of Akt leads to a number of cellular activities such as increased protein synthesis (such as the synthesis of the anti-apoptotic protein BCL-2), survival, and proliferation(147). In addition, GPCRs, growth factors, DNA damage, oxidative stress, and inflammatory cytokines can trigger the p38MAPK signaling pathways that result in cytokine production, T cell anergy, and apoptosis(148).

1.7 STATEMENT OF PURPOSE

As previously discussed, immunotherapy is used widely in the clinic but its beneficial effects are realized long-term. Cancer vaccines are a long-standing interest of our laboratory. Most recently we have focused on developing prophylactic vaccines based on the tumor associated antigen (TAA) MUC1 for individuals with premalignant lesions or other diagnoses that put them at high risk for developing MUC1 positive cancer. This requires development of biomarkers to be measured early after vaccination that could predict future anti-tumor efficacy. Currently no such biomarkers exist for any therapeutic or prophylactic cancer vaccine. Our specific goal is to identify biomarkers predictive of the MUC1 vaccine efficacy that could also be generalized to

other TAA based vaccines. We hypothesize that the phospho-flow technique will allow us to detect early intracellular signaling profiles that serve as a hallmark of antigen-specific T cell activation may be used as biomarkers for future anti-cancer therapeutic efficacy.

MUC1, a highly glycosylated molecule on ductal epithelia, is aberrantly hypoglycosylated, overexpressed, and immunogenic in its tumor form. Our lab has previously found that both endogenous T cells as well as adoptively transferred TCR transgenic T cells specific for the MUC1 peptide, have reduced responses (e.g. proliferation and interferon-γ production) to MUC1 peptide vaccination in MUC1.Tg mice compared to wildtype mice. We used this model system to examine early intracellular signaling events in CD4 T cells during priming for effector cell generation, in response to two different MUC1 vaccines in MUC1.Tg versus WT mice, to define potential differences that can be correlated with differences in functional outcome. The ability to alter these signaling profiles by varying vaccine design is examined as well. The work was performed within two specific aims:

Specific Aim I (Chapter 2): Optimize the phospho-flow technique for peak detection of phosphorylation in MUC1-specific T cells in vivo responding to stimulation by MUC1 vaccines.

Prior to the determination of whether T cell signaling was suitable as a parameter to evaluate therapeutic efficacy, the limitations and successes of using the technique to evaluate antigen-specific signaling initiated in vivo needed to be assessed. We demonstrated the limitations of the technique as originally published, and optimized it to detect antigen-specific signaling in in vivo stimulated T cells.

Specific Aim II (Chapter 3): Examine intracellular signaling profiles in CD4 T cells in MUC1.Tg versus WT mice during priming for effector cell generation in response to a MUC1 peptide vaccine, and investigate how changes in vaccine design can modulate the signaling response.

MUC1.Tg and WT mice were immunized with soluble adjuvant-based and DC-based MUC1 vaccines. The activation of intracellular signaling pathways (downstream of TCR, CD28/CD40L, and cytokine receptors) in CD4 TCR transgenic T cells (MUC1 specific and OVA specific as controls) was assessed by phospho-flow cytometry, in addition to flow cytometric analysis of the cell surface phenotype. We demonstrated that there were detectable differences in the T cell signaling profiles; to a large degree in response to vaccine design and to a lesser degree between MUC1.Tg and WT mice, but especially dependent on vaccine designs within the first 24 hours post immunization.

2.0 OPTIMIZATION OF THE PHOSPHO-FLOW TECHNIQUE FOR ANALYSIS OF PRIMARY CD4 T CELL SIGNALING POST IN VIVO STIMULATION

2.1 INTRODUCTION

Biomarkers of cancer are used to evaluate treatment efficacy, disease progression and reoccurrence, and to define disease stage. Many of the existent biomarkers have low diagnostic sensitivity and specificity, and few known markers have predictive power of therapeutic responses. Early elucidation of the effect an experimental vaccine strategy has on priming the immune response, whether in animal models or clinical trials, is of extreme importance. At present, long lengths of time are required to accurately predict the immunogenicity of a vaccine. Therefore, new improved cancer biomarkers are sought after. The advent of new technology or novel development of existing technology often leads to advancements in biomarkers. Phospho-flow is one such development in the field of flow cytometry that shows promise(149).

We proposed that in addition to published applications of this technique that mostly involved homogenous activation of cell lines or very strong stimuli to primary cells, phospho-flow could be used to measure very early activation of T cell signaling networks after immunization creating T cell signaling profiles that predict the outcome of the immune response, serving as a biomarker for vaccine efficacy. The sensitivity and reproducibility of the phospho-flow technique to analyze the phosphorylative response of single cells within a population to

stimulation has been well established in vitro(125-128). These responses are prompted by strong external stimuli such as cytokines, PMA/IONO, PHA, or pervanadate treatments. Furthermore, signaling differences that have been demonstrated between signaling and healthy states are not seen at basal states of phosphorylation, and can be deciphered only in the presence of activating stimuli(130, 150). Moreover, measuring signals in vitro has the distinct advantage of simultaneous stimulation of a large group of cells resulting in a known measurable and collective peak in the response(151, 152). To our knowledge, the use of this technique to measure signaling events of cells stimulated in vivo until now has been confined to situations where the stimulus is a bolus of adjuvant or cytokine(153), antibody(154), TLR ligands(155), vaccine virus infection(155), or a soluble peptide that binds directly to the MHC(156, 157).

Many hurdles exist that may require optimization of the phospho-flow technique in order for it to be an effective means of detecting phosphorylation signatures in an antigen-specific response to immunization. The antigen-specific activation we wished to measure would presumably be a much weaker response then those illustrated above for many reasons. Antigen processing time, the time until the vaccine reaches the lymphoid organ, and the time it takes for a stable TCR:MHC synapse to form between an antigen-specific T cell and mature antigen presenting DC are all variable; consequentially the peak of the response will be difficult to detect. Furthermore, at such early timepoints many activation markers are not yet upregulated so T cells that have not yet been primed may dilute the signal. In addition, the route of administration may result in signaling differences due to timing and specific conditions within the lymphoid organ in which it is primed. Moreover, differences in vaccine design, the adjuvant present, and variability between the individual mice may affect signal to background ratio. All of these factors need to be taken into consideration, and the technique optimized to create the

most sensitive detection of phosphorylation. Here, we examine the intensity and sensitivity of detection of intracellular phosphorylation by variations in the basic phospho-flow protocol and alterations in assay design in in vitro and ex vivo assays in order to optimize the phospho-flow technique to optimize the measurement of intracellular signaling induced in primary T cells after stimulation in vivo.

2.2 METHODS

2.2.1 *Mice*

C57BL/6 (WT), BALB/c, and C57BL/6-Tg (TcraTcrb)42SCbn/J (OT-II TCR-transgenic) mice purchased from The Jackson Laboratory (Bar Harbor, ME, USA), MUC1.Tg mice obtained from Dr. S. Gendler (Mayo Clinic, Scottsdale, AZ, USA), and VFT TCR-transgenic mice (158) were housed in a AALAC approved SPF environment at the Univeristy of Pittsburgh. All breeding and experimental procedures were approved by and performed in accordance with the University of Pittsburgh DLAR and IACUC guidelines.

2.2.2 *Production of Dendritic cells derived from bone marrow (BMDC)*

Dendritic cells derived from bone marrow were generated as described previously(159). Bone marrow cells were harvested from the tibia and femur of WT mice, the RBC lysed, and plated at a density of $1.5X10^6$ cells/ml in AIM-V media (Invitrogen) containing sodium pyruvate, 2-mercaptoethanol, non-essential amino acids, and supplemented with 20ng/ml of granulocyte

macrophage colony stimulating factor (GM-CSF; Miltenyi Biotec). BMDC were harvested on day 6 of culture and loaded with either MUC1 or OVA peptide overnight in the presence of either 50µg/ml poly:ICLC (Hiltonol®) (Oncovir, Inc) or LPS. The 100-mer MUC1 peptide represents 5 repeats of a 20 amino acid sequence HGVTSAPDTRPAPGSTAPPA found in the VNTR region(160).

2.2.3 *In vitro T cell stimulation assay*

CD4 T cells from VFT TCR transgenic or OT-II TCR transgenic mice were isolated via magnetic bead separation and plated either alone or in conjunction with splenocytes (1:10) in a 6 well plate and allowed to rest for 2 hours at 37°C. A stimulant was added to the culture (cytokine, PMA/IONO, H_2O_2, DC (1:5)) and incubated for various periods of time at 37°C. Cells were fixed immediately with 1.6% paraformaldehyde final concentration (Electron Microscopy Services) for 10 minutes at room temperature. Cells were then permeabilized in ice-cold methanol for 30 minutes at 4°C and stored at -80°C until staining followed analysis by flow cytometry.

2.2.4 *Adoptive T cell transfer*

Splenocytes from VFT or OT-II transgenic mice were isolated and RBC lysed. Magnetic bead isolation of CD4 T cells was performed via manufacture's instructions (Miltenyi Biotec). Only OT-II TCR.Tg CD4 T cells were labeled with 2µmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) prior to transfer, as VFT TCR.Tg mice are on the Thy1.1

congenic background for T cell identification. $5x10^6$(per strain where co-transfer applicable) were injected via the lateral tail vein or the hind footpad 1-2 days pre vaccination.

2.2.5 *In vivo stimulation*

Mice were immunized with either ~$3x10^6$ MUC1 peptide pulsed DC, OVA peptide pulsed DC, MUC1 peptide admixed with poly:ICLC or LPS, or poly:ICLC or LPS alone by lateral tail vein injection or in the footpad. At various timepoints, lymphoid organs (ether spleens or lymph nodes) were harvested and immediately fixed by processing into a single cell suspension in 1.6% paraformaldehyde(152, 153, 156, 161) at room temperature for 10 minutes. Cells are then permeabilized by the addition of ice-cold methanol for 30 minutes at 4°C. Samples are stored long-term at -80°C.

2.2.6 *Detection of intracellular phosphorylation by flow cytometry*

A panel of intracellular candidates were selected based on signaling pathways associated with either Th1 signaling (the transcription factor Tbet as well as STAT1 (Y701) and STAT4 (Y693) associated with Th1 costimulatory cytokines), Treg (the transcription factor FOXP3, STAT5 (Y694) associated with IL-2 signaling, as well as S6 (Y641) expression that inversely correlates with Treg induction), TCR activation (ZAP70 (Y319) and Lck (Y505)), cell survival and proliferation pathways (ERK1/2(T202/Y204), Akt(S473), p38MAPK(T180/Y182)) or signaling associated with differentiation of T helper cells(STAT3(Y705), STAT4(Y693), and STAT6(Y641)) (Table 1). Staining for the intracellular phospho-specific targets was done in conjunction with staining for surface markers as previously described(125). Prior to Fc block for

20 minutes at room temperature, cells were pelleted and washed in staining buffer (PBS, 0.5% BSA, and 0.02% sodium azide) to remove residual methanol. Cells were then stained for 45 minutes at room temperature in an antibody cocktail containing two intracellular markers as well as a mixture of surface antibodies including CD4 V500, B220 PeCy7, CD90.1 APC-eFluor 780 (eBioscience), and an exclusion panel of biotin – streptavidin PE-Texas Red labeled antibodies (CD49b, TER119, CD11c, CD8a, F4/80, and CD11b) for better resolution of the populations of interest.

Gating on Exclusion-B220-CD4+ T cells, we identified the transferred MUC1-specific VFT T cell response (Thy1.1+CFSE-) and/or the OVA-specific OT-II T cell response (CFSE+Thy1.1-). Fold change was calculated by normalization of the Median Fluorescent Intensity (MFI) of the individual stimulated sample to the average MFI of the unstimulated samples. In the case of co-transfer, fold change was calculated by normalization of the MFI of VFT T cells to the MFI of OT-II T cells in that same mouse.

2.3 RESULTS

2.3.1 *Detection of phosphorylation after in vitro stimulation of established and primary cell lines*

The phospho-flow technique was established using human U937 and Jurkat cell lines; consequently it is an ideal starting point for validation of the technique. Jurkat and U937 cells were subjected to PHA stimulation in order to establish the standard levels of signaling exhibited in our hands (**Figure 2-1**). Phospho-flow was carried out as instructed in the 'Basic Protocol 1'

found in Current Protocols in Immunology, the original foundation on which all further optimization was built(161, 162). Phosphorylation was induced as expected. Before proceeding with the confirmation of cytokine induced modification of molecules, we examined if there was any background signal, which would result from culture medium or the serum in the media in which cells were allowed to equilibrate. If cells are being basally stimulated by serum or media components, low levels of detectable phosphorylation in the control cells could decrease the signal to background ratio. However, if cells are in a stressed state that may also cause baseline phosphorylation to be higher, again decreasing the detected fold change. Allowing cells to recover in media rather than PBS increased the fold change in phosphorylation detected (**Figure 2-2**a), and the presence of serum did not appear to have an effect on the intensity of phosphorylation detected (**Figure 2-2**b).

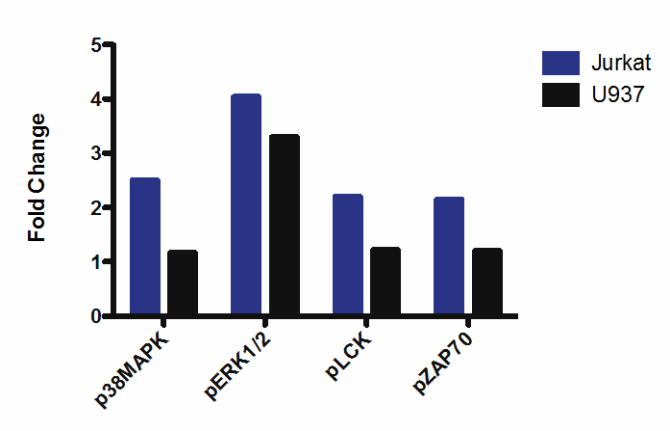


Figure 2-1. Induction of signaling in Jurkat and U937

Jurkat and U937 cell lines were stimulated with PHA for 15 minutes to detect ZAP70 (Y319), 38/MAPK (T180/Y182), ERK1/2 (T202/Y204), and Lck (Y505) phosphorylation events. Cells were fixed in 1.6% PFA for 10 minutes, permeabilized with MeOH, stained with antibody cocktail, and analyzed by flow cytometry. Increases in phosphorylation recapitulated similar increases in fold changes over unstimulated cells as reported in literature.

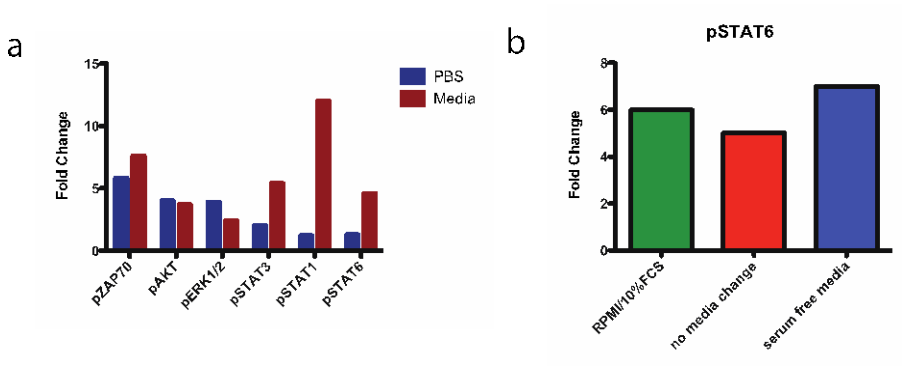


Figure 2-2. Effect of media on the strength of signal to noise ratio.

(a) Jurkat T cells that recover to resting state in medium, rather than PBS, displayed greater increases in the detection of phosphorylation signaling compared to unstimulated controls. Cells were stimulated with PMA/IONO [500ng/ml,1μM], IFNγ [1000U/ml], IL-6 [10ng/ml], IL-4 [10ng/ml]for 15 minutes to detect phosphorylation of ERK1/2 (T202/Y204), STAT1 (Y701), STAT3 (Y705), and STAT6 (Y641) respectively. Cells were stimulated with 3% H_2O_2 for 10 minutes to detect ZAP70 (Y319) and Akt (S473) phosphorylation. Cells were fixed in 1.6% PFA for 10 minutes, permeabilized with MeOH, stained with antibody cocktail, and analyzed by flow cytometry. (b) Variations in the percentage of serum in the media or changing the media to fresh media upon stimulation had no significant affect on phosphorylation levels. Jurkat cells were stimulated with IL-4 [10ng/ml] for 15 minutes either in the presence of 10% FBS, serum free media, or no addition of media with the stimulus. Cells were fixed in 1.6% PFA for 10 minutes, permeabilized with MeOH, stained with antibody cocktail, and analyzed by flow cytometry.

We next confirmed that detection of phosphorylation of specific target molecules would be induced upon upstream receptor stimulation. Homogenous cells were given a bolus of stimulus inducing rapid phosphorylation of the target until the signal peaked and began to deteriorate. Stimulation of Jurkat cells with IFNγ, IL-4, or PMA/IONO demonstrates a robust increase in the phosphorylation of STAT1, STAT4, or ERK1/2 and p38/MAPK respectively (**Figure 2-3**a). As expected, a robust increase in phosphorylation was also induced after stimulation of WT splenocytes with IFNγ, IL-4, IL-6, PMA/IONO, GMCSF, or H_2O_2 compared

to unstimulated cells (**Figure 2-3**b,c) establishing that the protocol and antibodies were effective in primary mouse cells.

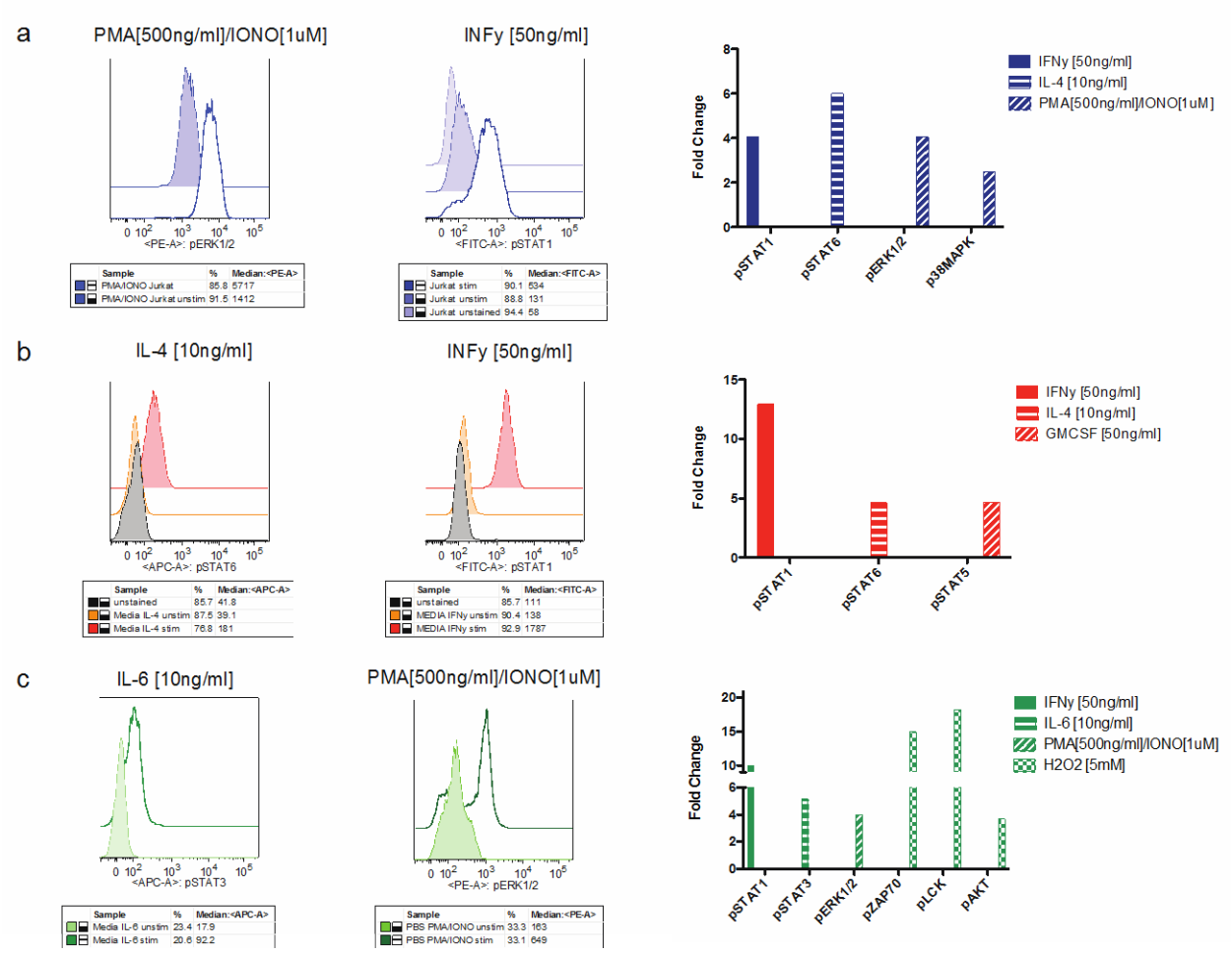
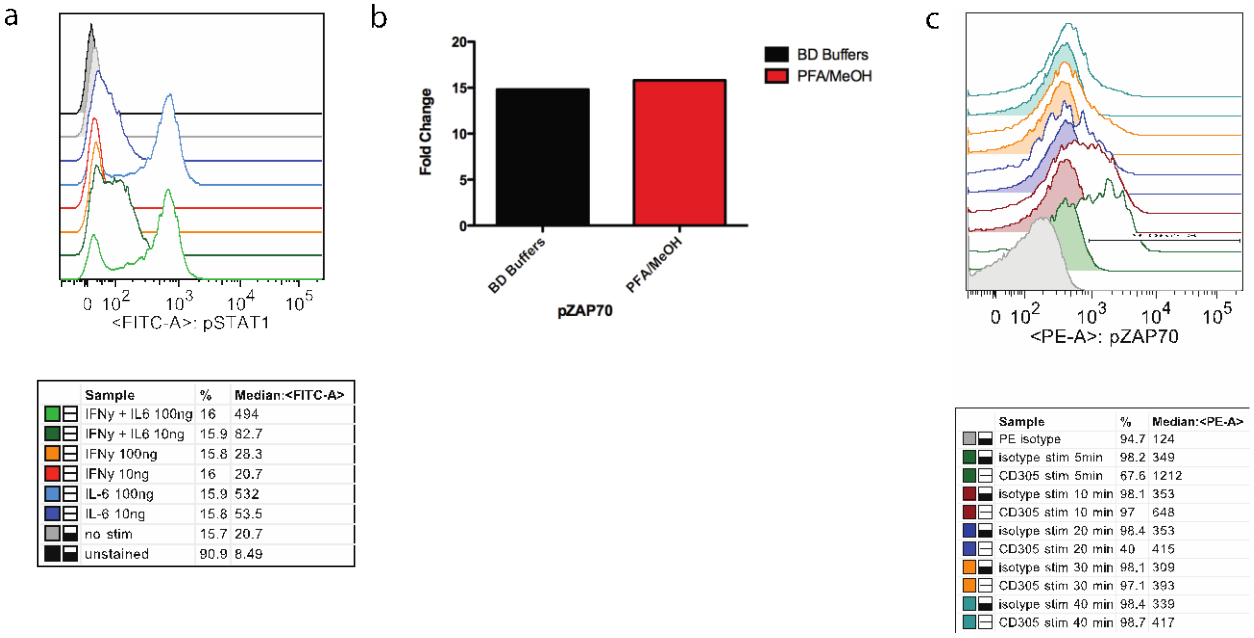


Figure 2-3. Intracellular phosphorylation of targeted molecules in human cell lines and mouse primary cell lines post stimulation

Jurkat T cells (a) and primary mouse splenocytes (b,c) were incubated with indicated stimuli for 15 minutes at 37°C, with the exception of cells examined for ZAP70 (Y319) and Lck (Y505) that were stimulated for 5 minutes. Cells were fixed in 1.6% PFA for 10 minutes, permeabilized with MeOH, stained with antibody cocktail, and analyzed by flow cytometry.

Validation of the sensitivity of the phospho-flow technique was shown by dose dependent detection of phosphorylation (**Figure 2-4**a). In addition, it was determined that the use of BD buffer systems did not increase the sensitivity of the assay to detect phosphorylation as compared to PFA/MeOH protocols (**Figure 2-4**b). We also validated that the technique was sensitive and stringent enough in our hands to detect the transient phosphorylation in cells after stimulation within a 40-minute time course. The phosphorylation of ZAP70 in Jurkat T cells was seen to peak at five minutes post stimulation with a Jurkat-specific anti-TCR antibody then progressively decrease until baseline (**Figure 2-4**c). These results were confirmed by western (data not shown).



Sample	%	Median:<FITC-A>
IFNγ + IL6 100ng	16	494
IFNγ + IL6 10ng	15.9	82.7
IFNγ 100ng	15.8	28.3
IFNγ 10ng	16	20.7
IL-6 100ng	15.9	532
IL-6 10ng	15.8	53.5
no stim	15.7	20.7
unstained	90.9	8.49

Sample	%	Median:<PE-A>
PE isotype	94.7	124
isotype stim 5min	98.2	349
CD305 stim 5min	67.6	1212
isotype stim 10 min	98.1	353
CD305 stim 10 min	97	648
isotype stim 20 min	98.4	353
CD305 stim 20 min	40	415
isotype stim 30 min	98.1	309
CD305 stim 30 min	97.1	393
isotype stim 40 min	98.4	339
CD305 stim 40 min	98.7	417

Figure 2-4. Sensitivity of detection of intracellular phosphorylation levels

(a) Splenocytes from mice were incubated for 15 minutes with either IFNγ and IL-6 or either alone at either [100ng/ml] or [10ng/ml]. Cells were fixed in 1.6% PFA for 10 minutes, permeabilized with MeOH, stained with antibody cocktail, and analyzed for STAT1 (Y701) phosphorylation by flow cytometry. (b) Mouse splenocytes were treated with H_2O_2 for 10 minutes. Half the cells were fixed in 1.6% PFA for 10 minutes and permeabilized with MeOH, while the other half were fixed and permeabilized in the BD Buffer system per manufacturer's instructions. All cells were then stained with antibody cocktail, and analyzed for phosphorylation of ZAP70 (Y319) by

flow cytometry. The BD Buffer system did not increase the sensitivity of detection. (c) Jurkat cells were incubated with a Jurkat specific anti-TCR antibody. Cells were fixed at various timepoints with 1.6% PFA, permeabilized, and analyzed as stated previously.

The antigen-specific stimulation of cells in vivo results in activation of far fewer cells than does polyclonal activation of cells in vitro. Thus, in order to analyze a significant population of antigen-specific cells the overall number of cells analyzed would need to greatly increase. To determine if staining parameters are within the thresholds of detection, we looked at a 1:5 and 1:10 dilutions of antibody based on the recommended concentration as well as varying the number of cells in the staining volume. Lower concentrations of antibody did not cause a significant decrease in the intensity of staining (**Figure 2-5**a). However, an increase in the concentration of cells revealed a threshold at the level of 10 million cells/100µl that resulted in a definitive decrease in intensity of staining (**Figure 2-5**b). Therefore, all future staining was performed at cellular concentration of no greater than 50 million cells per milliliter.

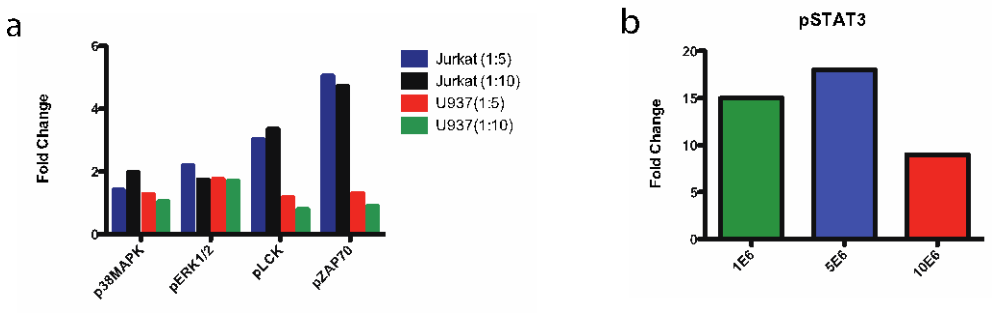


Figure 2-5. Determination of signal intensity of intracellular phosphorylation post stimulation

(a) Jurkat and U937 cells were stimulated with 3% H_2O_2 for 10 minutes and stained at the same cellular concentration but with two titrations of the anti-phospho antibodies. Cells were fixed in 1.6% PFA for 10 minutes, permeabilized with MeOH, stained with antibody cocktail, and analyzed for STAT1 (Y701) phosphorylation by flow cytometry. (b) Jurkat cells were stimulated with IL-6 [10ng/ml] for 15 minutes before fixation. Cells were stained at three different cellular concentrations and a 1:10 dilution of the recommended antibody concentration.

2.3.2 *Intracellular phosphorylation signatures of CD4 T cells after in vivo stimulation with DC loaded with antigen*

Timepoints of one and six hours were chosen to assess early signaling in a polyclonal response after DC immunization. An alloantigen was chosen since it would be expected to elicit a robust endogenous polyclonal response in comparison to the much more restricted response elicited by a foreign protein or peptide antigen. Immunization of B6 WT mice with DC loaded with BALB/c lysate produced detectable increases of phosphorylation over B6 WT mice immunized with unloaded DC (**Figure 2-6**a,b). Of note, the phosphorylation patterns were variable between mice. However, based on data obtained with an admittedly low number of mice (n=2) in the preliminary study, we proceeded to determine the signaling fingerprint of CD4 T cells responding to foreign antigen.

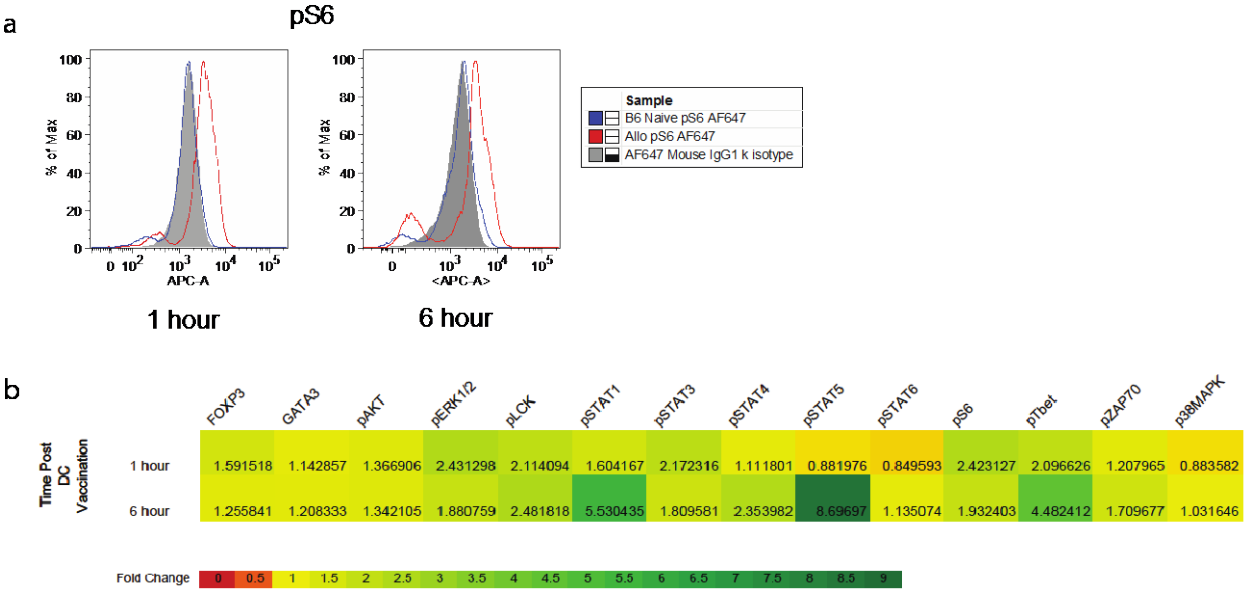


Figure 2-6. Detection of intracellular signaling molecules in wildtype mice after allo immunization

Spleens were harvested from C57BL/6 mice one and six hours after DC vaccination of BALB/c lysate or control vaccination and immediately fixed in 1.6% PFA for 15 minutes and then MeOH permeabilized. Phosphorylation

of signaling molecules was detected by flow cytometry. (a) Representative histogram of detection of intracellular phosphorylation of S6 (Y641). (b) Heatmap depicting fold changes in intracellular phosphorylation in two B6 WT mice.

The variation in signaling patterns between mice was more evident in attempts to determine the signaling signatures in MUC1-specific CD4 T cells responding to immunization with DC loaded with MUC1 peptide in MUC1.Tg mice (n=3) and WT mice (n=3) at various early timepoints (1, 6, 12, and 24 hours). Although variation from mouse to mouse within groups was sizeable, and the baseline phosphorylation levels of unstimulated mice appeared high creating a large signal to noise ratio; several differences in signaling between MUC1.Tg and WT mice were observed. MUC1-specific CD4 T cells exhibited greater expression of phosphorylated STAT1 and Tbet at 24 hours post vaccination as compared to MUC1-specific T cells in the wildtype mice, as well as an increased phosphorylation of STAT4 at 1 hour (**Figure 2-7**a). The observed initial increase in activation of Th1 associated molecules at 24 hours suggests differentiation into the Th1 lineage cell fate in MUC1.Tg mice. In addition to increasing phosphorylation of Th1 associated pathways, MUC1-specific T cells also exhibited an increase in ZAP70 phosphorylation and a decreased phosphorylation of Lck tyrosine 505 in MUC1.Tg mice (**Figure 2-7**b). When this tyrosine is phosphorylated it promotes the formation of a closed conformation, which prevents Lck activation. Initial activation of signaling molecules downstream of the TCR is greater in MUC1-specific CD4 T cells in MUC1.Tg mice versus WT. However MUC1.Tg mice appear to have a dichotomous response, T cells from MUC1.Tg mice display an impaired ability to increase phosphorylation of early transient activation markers FOXP3 and STAT5 respectively in MUC1.Tg as compared to WT (**Figure 2-7**c). In addition, MUC1-specific T cells in WT but not MUC1.Tg display an impaired ability

to increase phosphorylation of S6, which is inversely proportional to later Treg development(163). More endogenous regulatory cells are observed post vaccination in MUC1.Tg mice, and MUC1-specific CD4 T cells do not decrease regulatory signaling within the first 24 hours (**Figure 2-7**c). In addition, phosphorylation of ERK1/2 decreased in MUC1-specific cells in WT but not MUC1.Tg mice at one hour while phosphorylation levels of 38/MAPK decreased in MUC1-specific cells in MUC1.Tg but not WT at six hours post vaccination. Activation of Akt was equivalent over the first 24 hours in MUC1.Tg and WT mice. Taken together these data suggest less proliferation of MUC1-specific T cells in MUC1.Tg compared to WT mice (**Figure 2-7**d). While initial experiments appeared to indicate that differences in the quality of early signaling in response to MUC1 immunization in MUC1.Tg compared to WT mice, statistical significance was not reached perhaps due to the large variability within each group.

The number of cells that exhibited phosphorylation of the targeted molecules was then examined to see if a difference existed in the quantity of the response, i.e. the number of cells responding (**Figure 2-8**). Fewer VFT T cells activated STAT1 and Lck but more activated Tbet in MUC1.Tg mice than in WT mice. However, again no statistically significant differences were seen in the number of cells with increased phosphorylation of these targets between the two groups. We thought that perhaps the variability could be reduced and a robust accurate T cell signature produced if we could corroborate the results with in vitro findings in the same system.

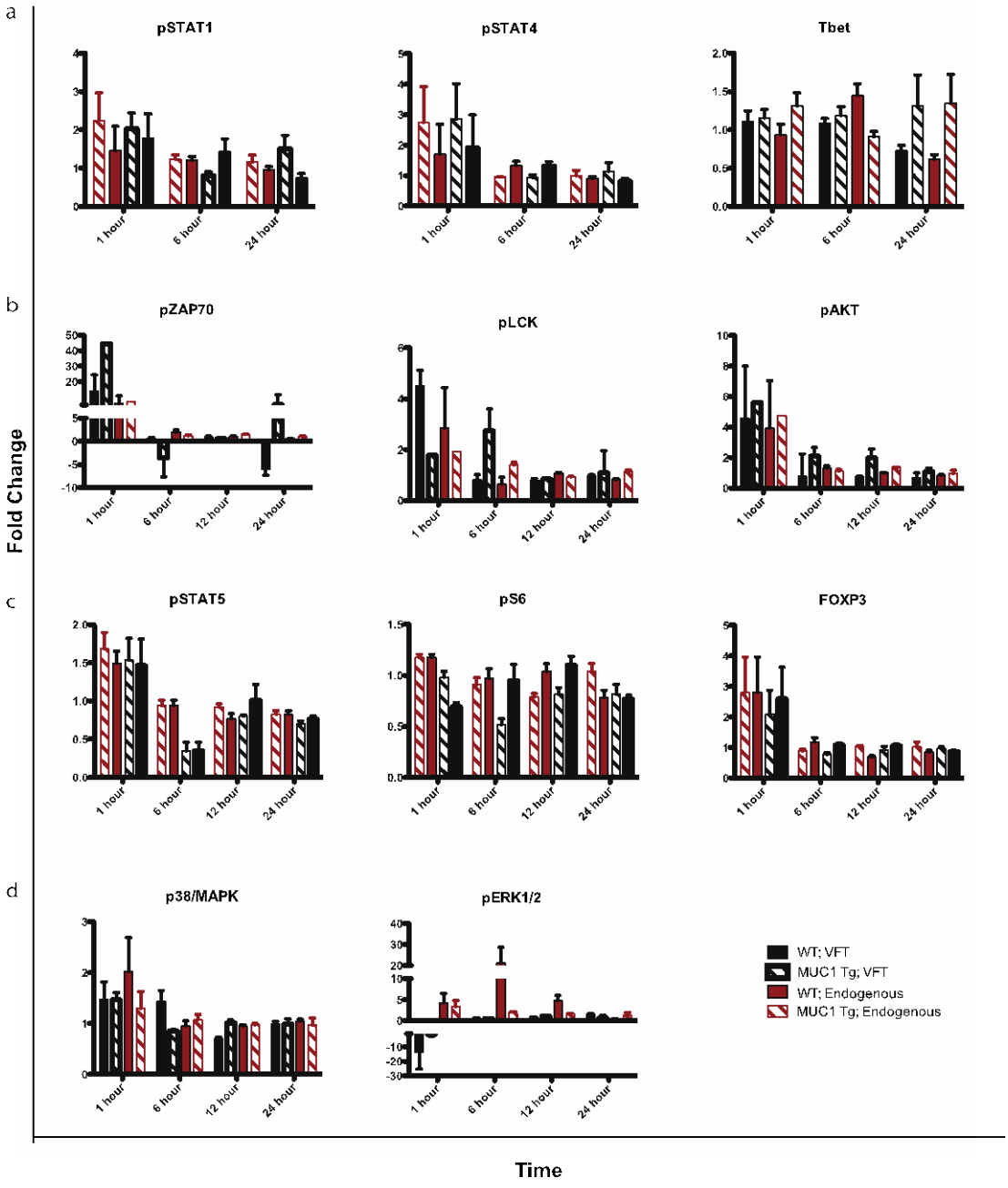


Figure 2-7. In vivo T cell 'phospho-fingerprint' in response to MUC1 pulsed DC vaccination

MUC1-specific T cells from the VFT TCR Tg mouse were adoptively transferred into MUC1.Tg and WT mice one day prior to DC vaccination with MUC1 100mer or unloaded DCs (n=3 mice/group). Spleens were fixed and methanol permeabilized immediately upon harvest 1, 6, 12, and 24 hours post vaccination. Phosphorylation of classical Th1 signaling proteins (a), proteins downstream of TCR activation (b), molecules associated with regulation (c), and proteins involved in cell cycle, survival, and signal transduction downstream of inflammatory mediators (d) were detected by flow cytometry.

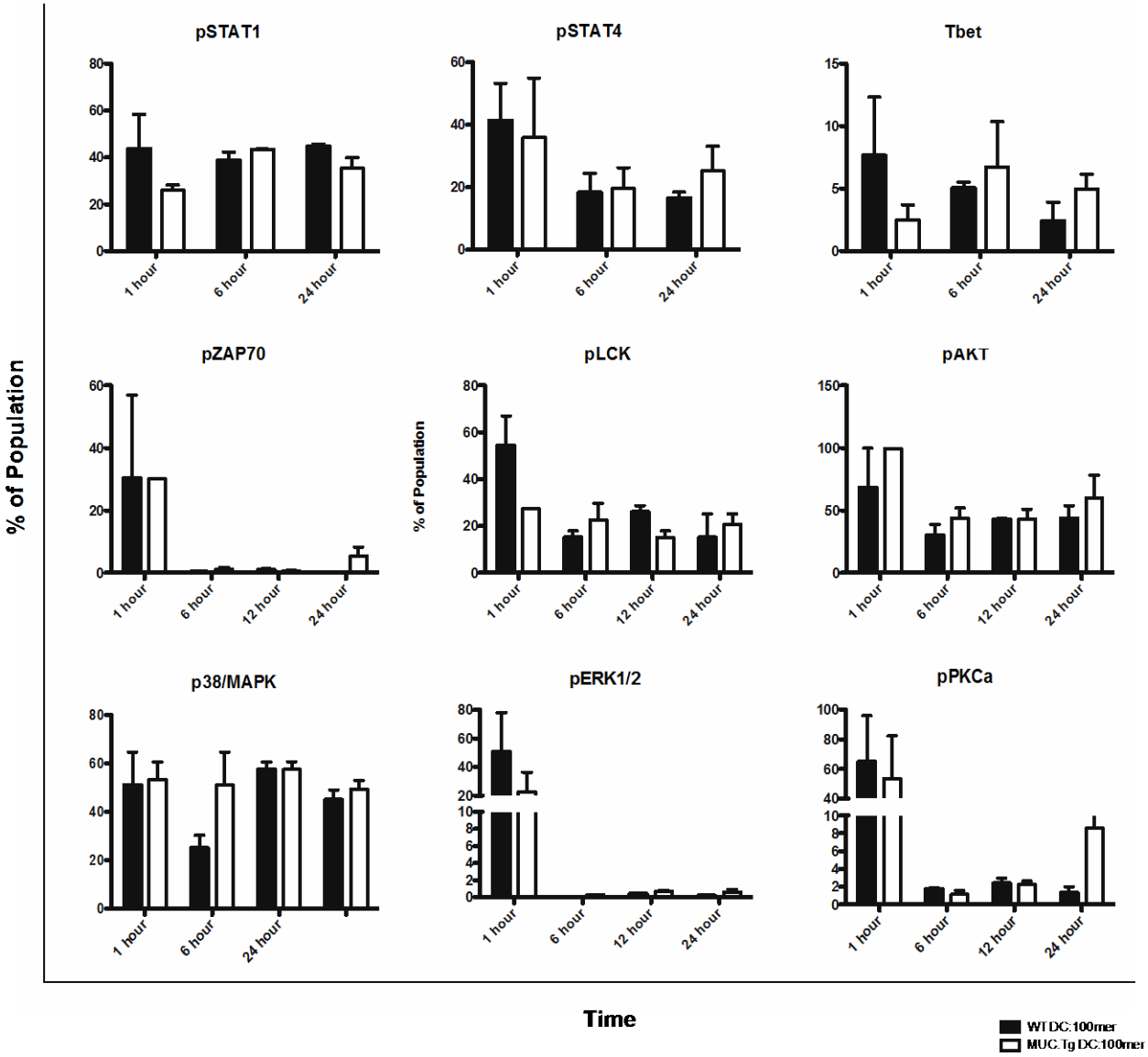


Figure 2-8. Percentages of cells expressing phosphorylated candidates

VFT T cells were adoptively transferred into MUC1.Tg and WT mice one day prior to i.v. injection of poly:ICLC MUC1 pulsed DC or unloaded DCs (n=3 mice/group). Spleens were harvested 1, 6, 12, and 24 hours post vaccination, and the percentage of T cells exhibiting phosphorylation of the indicated targets above that of unstimulated T cells was analyzed.

2.3.3 *Detection of intracellular signaling pathways in MUC1-specific CD4 T cells in vitro stimulation with DC*

Variability observed upon ex vivo examination of signaling events that occurred in vivo could be caused by a multitude of factors: the time it takes the antigen to reach the lymph node, the time elapsed between initial APC:T cell interaction and fixation of the cells, and/or dilution of the signal resulting from only a few cells in the MUC1-specific population responding to antigen at the specific timepoint. To address these facets of the response, we examined the phosphorylation status of the targeted molecules in VFT CD4 T cells after in vitro stimulation with either unloaded DC or DC pulsed with MUC1 100mer peptide. Since VFT CD4 T cells demonstrated normal proliferative and functional properties in WT mice, compared to MUC1.Tg, the deficiency in response is not inherent to the VFT T cell, but rather the microenvironment is the likely reason for the differences in functional and proliferative responses. To accurately model the in vivo signaling response in vitro, we co-cultured the VFT CD4 T cells with either MUC1.Tg splenocytes or WT splenocytes. After allowing the cells to rest for two hours at 37°C to reach equilibrium, either MUC1 pulsed DC or unloaded DC were added to stimulate the culture. Cells were harvested at 15min, 30 min, 45 min, 1 hour, 6 hour, and 24 hour after stimulation. Standard fixation, permeabilization, and staining were performed followed by analysis. Unfortunately, the groups stimulated with MUC1 pulsed DC demonstrated no significant increase in phosphorylation over the baseline signaling seen by stimulation with DC alone in either MUC1.Tg or WT mice (**Figure 2-9**).

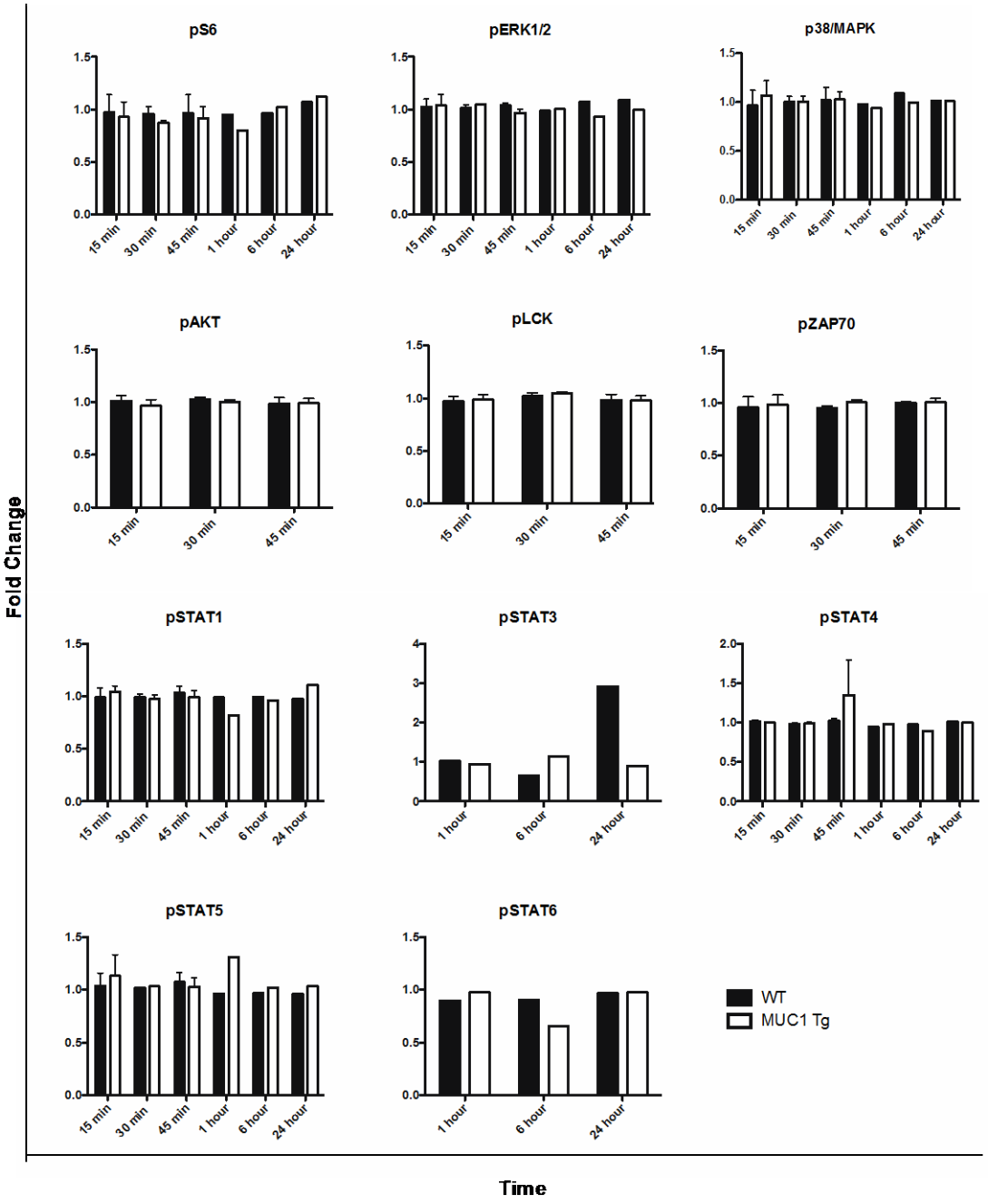


Figure 2-9. Intracellular signaling detected at various timepoints after in vitro stimulation with DC pulsed with MUC1 peptide

MACs purified CD4+ T cells from VFT TCR transgenic mice were co-cultured with either MUC1.Tg or WT splenocytes at a 1:10 ratio and allowed to rest for 2 hours at 37°C. Cells were stimulated with either poly:ICLC matured MUC1 pulsed DC or unloaded DC at a 1:5 ratio. At various timepoints, cells were fixed in 1.6% PFA, permeabilized, stained with antibody cocktail, and analyzed by flow cytometry.

Since the levels of phosphorylation were high in both groups stimulated with DC pulsed with antigen as well as the DC matured without antigen, it is possible that in vitro-matured DC were highly immunogenic themselves and caused activation of the non-cognate antigen specific cells. Therefore, we examined if the in vitro stimulation of T cells with in vivo-generated DC harvested from spleens of vaccinated mice could recapitulate intracellular signaling differences detected ex vivo after in vivo stimulation of T cells, while increasing the signal strength. Neither the use of bulk splenocytes (**Figure 2-10**a) nor MACS isolated CD11c+ DC (**Figure 2-10**b) harvested from mice immunized with MUC1 100mer admixed LPS in comparison to LPS alone enhanced the strength of the antigen-specific phosphorylation signal-to-noise ratio.

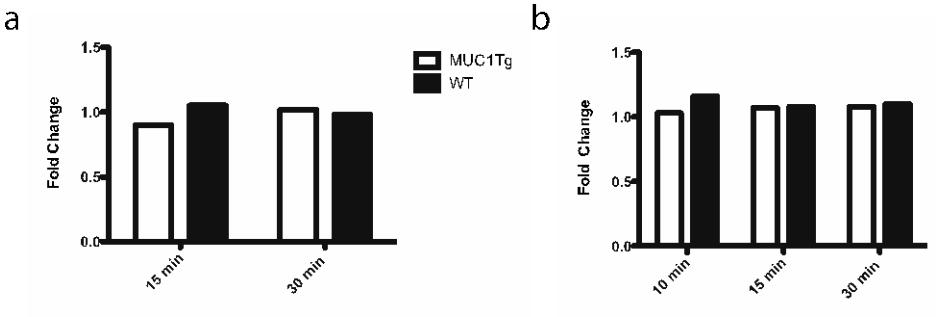


Figure 2-10. In vitro stimulation of T cells with DC matured in vivo

MUC1.Tg and WT mice were immunized i.v. with MUC1 100mer admixed LPS or LPS alone. Spleens were harvested after 48 hours, and either (a) bulk splenocytes or (b) MACS isolated CD11c+ DC were co cultured with VFT CD4 T cells for various timepoints.

At this point, in vitro DC stimulation of MUC1-specific T cells was not yielding the robust signaling we had anticipated would be present. The differences we had initially seen in T cell activation after DC immunization in vivo, appeared dependent on the microenvironment or events that transpire in vivo that were not being successfully replicated in vitro. Therefore, we examined if signaling events in CD4 T cells activated in vivo by DC immunization could be enhanced and sustained upon re-stimulation ex vivo. However, we found that MUC1-specific

CD4 T cells exhibit some differences in phosphorylation after PHA re-stimulation compared to in vivo signaling at three hours post vaccination and is not consistently a more robust signal (**Figure 2-11**).

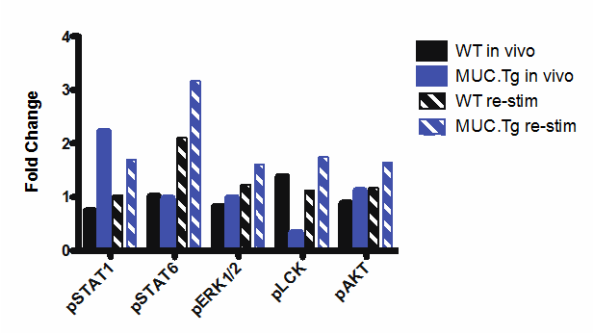


Figure 2-11. Differences in CD4 T cell signaling after re-stimulation

VFT T cells were adoptively transferred into MUC1.Tg and WT mice one day prior to immunization with poly:ICLC matured DC pulsed with MUC1 100mer or DC alone. Three hours post immunization, spleens from WT and MUC1.Tg mice were harvested. One half of the splenocytes from each mouse were fixed in 1.6% PFA and MeOH permeabilized for staining, and the other half were allowed to equilibrate to baseline for two hours at 37°C. Cells were restimulated with PHA for fifteen minutes, fixed, and permeabilized. Cells were stained and analyzed.

2.3.4 *Neither antigen nor route of administration impacted the detection of signaling, but co-transfer of cells recognizing a non-cognate antigen as a baseline improved data quality*

Although MUC1 is a potent TAA, there are foreign antigen systems that have demonstrated a much more robust response, such as OT-II TCR transgenic CD4 T cells that recognize their cognate antigen ovalbumin (OVA). We therefore tested whether the changes detected in a more robust TCR transgenic system would be more pronounced. However, intracellular phosphorylation detected after both in vitro (**Figure 2-12**a) and in vivo (**Figure**

2-12b) stimulation of OVA-specific CD4 T cells were similar in intensity as the signal strength in MUC1-specific T cells.

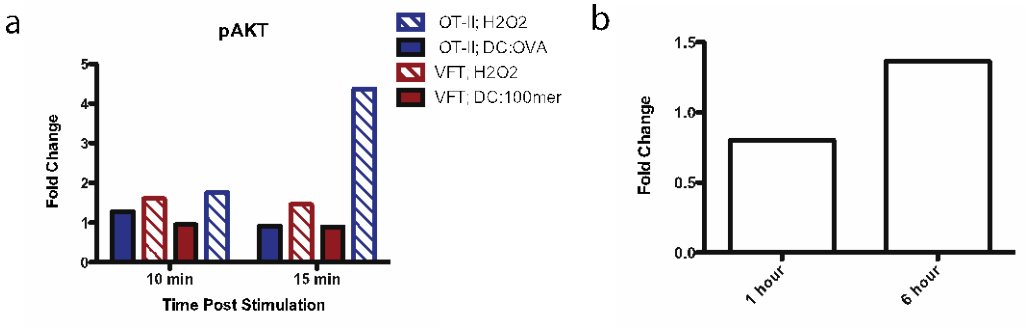


Figure 2-12. Intracellular signaling in OVA-specific cells in response to stimulation

(a) Phosphorylation of Akt in OT-II and VFT CD4 T cells in response to in vitro stimulation for fifteen minutes with H_2O_2 or DC pulsed with cognate antigen were compared by phospho-flow analysis. (b) MACS purified OT-II CD4+ T cells were adoptively transferred to B6 WT mice one day prior to immunization with poly:ICLC matured DC pulsed with OVA peptide. Spleens were harvested one and six hours post immunization. Cells were fixed, permeabilized and phosphorylation was analyzed by flow cytometry.

Decreasing the variability detected in responses, as well as accounting for any other intrinsic factors that may be contributing to such variability in individual mice could reduce the background noise, thereby increasing the window of detection. The co-transfer of TCR T cells whose cognate antigen is not endogenous to the system, i.e. OVA-specific T cells, could permit better control for inter-mouse variation by creating a baseline of phosphorylation within the same mouse in which the MUC1-specific response is being measured. Obviously, useful results from such an approach are contingent on there being a low to no bystander effect on T cell activation. Indeed, normalization of MUC1-specific CD4 T cells to co-transferred OT-II CD4 T cells post immunization with MUC1 pulsed DC (**Figure 2-13**b) yields a more robust phosphorylation signature then normalization to unloaded DC immunization in separate mice (**Figure 2-13**a).

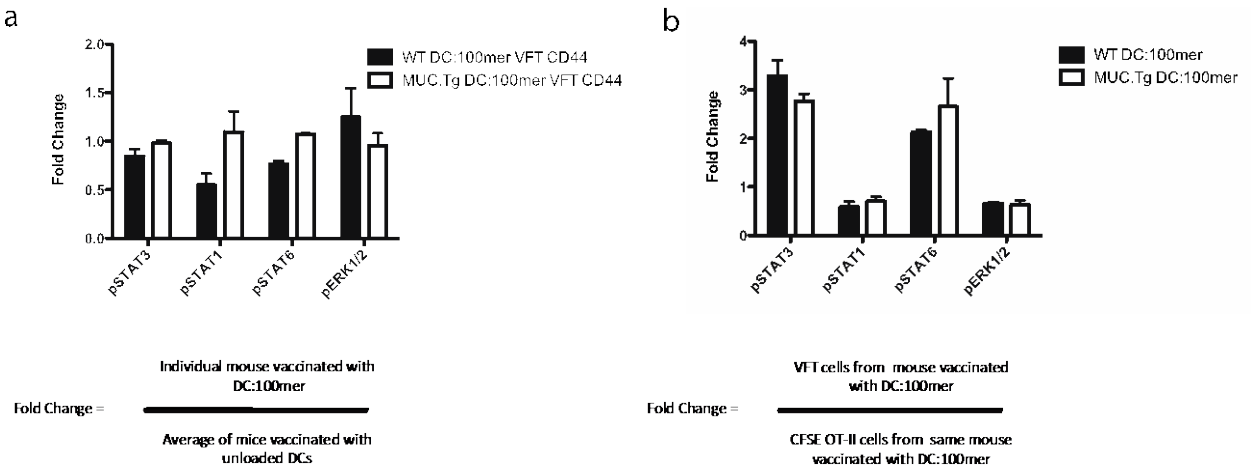


Figure 2-13. Improved detection of inducible signaling when non-cognate antigen-specific T cells are co-transferred to determine basal levels of phosphorylation

Either MACS isolated VFT CD4+ T cells (a) or a combination of VFT and CFSE-labeled OT-II T cells (b) were transferred into MUC1.Tg and WT mice two days prior to immunization. Spleens were harvested three hours post immunization with poly:ICLC matured DC pulsed with MUC1 100mer peptide or unloaded DC. Cells were fixed, permeabilized, and phosphorylation assessed by flow cytometry.

Another factor that may affect the quality of signaling or detection thereof is the route of administration of the antigen. Antigen delivered intravenously will travel through the lung to the spleen, while antigen delivered to the footpad, a combination of intradermal and subcutaneous injection goes directly to the draining popliteal lymph node. However, neither the concentration of the T ells present in the lymph node nor the route the DC travel (a shorter more direct path bypassing the lung) had an impact on the intensity of the phosphorylation signal detected (**Figure 2-14**).

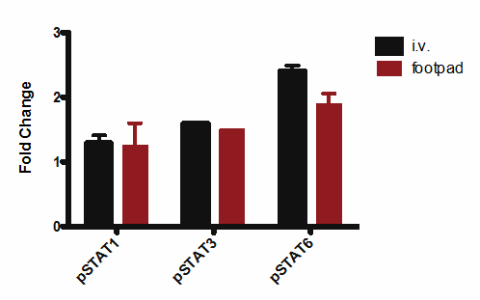


Figure 2-14. Strength of the signaling events detected were similar across different routes of administration and lymphoid organs.

Equivalent numbers of MACS purified CD4+ VFT and OT-II T cells were adoptively co-transferred to MUC1.Tg mice. One day later mice received either an i.v. or footpad injection of poly:ICLC matured DC pulsed with MUC1 100mer peptide. Three hours post injection spleens (i.v.) or popliteal lymph nodes (footpad), respectively, were harvested. Cells were, fixed, permeabilized, and phosphorylation analyzed by flow cytometry.

2.4 DISCUSSION

There are several phospho-flow protocols in the literature that vary slightly in incubation times, incubation temperatures, and the concentration of fixative used. Many permutations of small technical details such as antibody concentrations, the duration of incubation with antibodies, fixative concentration, methanol permeabilization time, removal of fixative prior to permeabilization, procedural temperatures, as well as rehydration of the sample prior to staining were all examined. Many factors had no effect, but any parameter permutations that did increase the signal to noise ratio such as rehydration and the removal of fixative prior to permeabilization were reflected in the modified phospho-flow protocol used for all troubleshooting to boost signaling detection presented here.

The robust signaling seen after cells were stimulated in vitro with cytokines, prompted us to try to optimize the detection of antigen receptor signaling and boost the phosphorylation detected. In vitro experiments, including co-culture with MUC1.Tg or WT splenocytes and stimulation with DC activated in vivo, demonstrated no differences in signaling in CD4 T cells upon external stimulation indicating the importance of cues from the microenvironment which we were not able to recapitulate in vitro. Therefore, we tried to optimize signaling detection by the development of an ex vivo re-stimulation assay that would capture more robust differences in phosphorylation. Ex vivo experiments demonstrated that the ex vivo signaling fingerprint did not always reflect the same phosphorylation patterns before and after re-stimulation. Furthermore, re-stimulation did not consistently result in the detection of greater levels of phosphorylation. In addition, the use of a different adjuvant or antigen system did not result in greater detection of phosphorylation. However, co-transfer of TCR transgenic T cells that recognized a cognate antigen in conjunction with non-cognate TCR transgenic T cells as a baseline of phosphorylation, resulted in a greater window of phosphorylation detection and reduced the variability of the calculated fold change between mice. This optimization in experimental design allows for the use of the phospho-technique to assess phospho-signatures after in vivo stimulation of cells. These phospho-signatures can be predictive of the efficacy of vaccines in preclinical models, correlated to experimental outcomes, and eventually used as a biomarker of immunogenicity and disease outcome in clinical trials.

3.0 EARLY IN VIVO SIGNALING PROFILES IN MUC1-SPECIFIC CD4 T CELLS RESPONDING TO TWO DIFFERENT MUC1 VACCINES IN TWO DIFFERENT MICROENVIRONMENTS

Chapter 3 has been adapted from Reichenbach, D.K., and O.J. Finn. Early invivo signaling profiles in MUC1-specific CD4T cells responding to two different MUC1 vaccines in two different microenvironments. 2013. *OncoImmunology*. 2(3); with permission from Landes Biosciences.

3.1 INTRODUCTION

Cancer immunotherapy is gaining recognition not only as an important addition to standard radiation and chemotherapeutic approaches but also as an effective monotherapy(38). Cancer vaccines are one form of immunotherapy that could benefit advanced cancer patients by boosting their anti-cancer immune response, as well as by aiding in cancer prevention for individuals who are at high risk for cancer. Over the last three decades, there have been great advances in the characterization of immune responses in cancer patients and types of immunity required to control various tumors. This research has also identified numerous tumor antigens recognized by tumor specific T cells that have been used to develop and test cancer vaccines. Preclinical animal models, in particular genetically engineered mice, have been very useful in testing

immunogenicity of cancer vaccines and anti-tumor efficacy. These studies have shown that to be effective a vaccine needs to elicit a vigorous effector T cell response, including a robust memory response. The ability of a vaccine to do this depends on the choice of tumor antigen(s), choice of adjuvant(s), and the patient's immune competence. The majority of well-characterized tumor antigens (66) are tumor-associated antigens that are closely related to self-antigens and may be subjected to various degrees of self-tolerance. The choices of adjuvant and antigen delivery systems (e.g. loaded on DC, in viral vectors, conjugated to antibodies targeting DC) are important determinant of both the strength and the type of immune response elicited. These variables, in addition to others, determine the efficacy of a single vaccine across patients. Cancer vaccine efficacy can be evaluated by two outcomes: 1) immunogenicity, measured as production of new antibodies and T cells several weeks after vaccination, and 2) tumor control, measured weeks after vaccination in mouse models or months and years after vaccination in patients. In the case of vaccines for cancer prevention, evaluation of efficacy would be even more delayed. According to many animal models and some clinical trials, these two outcomes are tightly correlated – the more robust the antibody and T cell responses induced by the vaccine, the better tumor control in the future. The goal of the work we report here was to evaluate in vivo a technique that has been used successfully to measure activation of T cells in vitro, in order to determine if an early T cell activation signature can be obtained in primary T cells that might be developed as a predictive biomarker of a vaccine efficacy.

CD4 T cells play a central role in the generation and quality of CD8 cytotoxic T cells (CTL), antibodies, and the induction of memory. In addition, they participate in the activation and recruitment of innate effector cells to the tumor site(164-166). Therefore, the ability of a vaccine to activate CD4 T cells could be an important biomarker of its efficacy.

Mucin 1 (MUC1) is an O-linked glycosylated transmembrane protein normally expressed on the apical surface of ductal epithelial cells, but it is also found aberrantly expressed in a broad spectrum of adenocarcinomas. Upon malignant transformation of cells, MUC1 loses polarity and becomes overexpressed and hypoglycosylated revealing a region of tandem repeats of a 20 amino acids sequence rendering itself immunogenic. Low T cell and antibody responses to MUC1 have been seen in patients with adenocarcinomas prompting development of vaccines to boost that response.

Previously, we have shown that a MUC1 peptide loaded DC (DC-MUC1) vaccine elicits more effective anti-tumor responses than a MUC1 peptide in conjunction with poly:ICLC (MUC1-poly:ICLC) vaccine(121). Furthermore we showed that MUC1-specific T cells proliferate less and produce less IFN-γ in response to both vaccines in MUC1.Tg compared to WT mice(158, 160). These findings yielded a relevant model system for investigating potential early biomarkers that are associated with these different outcomes.

Development of flow cytometry based assays, a technique known as phospho-flow, has allowed for complex signaling networks to be identified within single cells(125-128). Phospho-flow has already been used to investigate STAT activation in T cells from late stage cancer patients(132), shifts in signaling potentials in leukocytes of acute myeloid leukemia patients(130), deficiencies of STAT signaling in monocytes of HIV infected individuals(131), and even attempts to predict clinical responsiveness to therapies for Rheumatoid Arthritis(133). Here we use this technique for the first time on tumor antigen specific primary T cells to evaluate differences in the quality (what pathways are activated and their kinetics) and the quantity (strength of phosphorylation) of immune responses elicited by two different MUC1 vaccines in two different mouse strains. We hypothesized that differences we previously reported in the

immunogenicity and efficacy of these vaccines in different hosts, measured weeks and months after vaccination, might be predicted by the state of CD4 T cell activation immediately after vaccination. We show that this is indeed the case and that the phosphorylation profile of MUC1-specific TCR transgenic CD4 T cells at 3, 6, 12 and 24 hours after vaccination is different in response to soluble peptide plus adjuvant versus peptide loaded on DC.

We propose that other vaccines can be similarly compared to identify the most immunogenic ones, with the most robust T cell activation signature to select best candidates for further development.

3.2 METHODS

3.2.1 *Mice*

C57BL/6 (WT) and C57BL/6-Tg (TcraTcrb)42SCbn/J (OT-II TCR-transgenic) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), MUC1.Tg mice were originally obtained from Dr. S. Gendler (Mayo Clinic, Scottsdale, AZ, USA), and VFT TCR-transgenic mice were generated at the University of Pittsburgh transgenic mouse facility as previously described(158). All mice were housed in a specific pathogen free environment. All breeding and experimental procedures were approved by and performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Pittsburgh.

3.2.2 *Generation of Bone Marrow Dendritic Cells (BMDC)*

BMDC were generated as previously described(159). In brief, bone marrow cells were harvested from the tibia and femur of WT mice. Following RBC lysis, BM cells were plated at $1.5X10^6$ cells/ml in AIM-V media (Invitrogen) containing sodium pyruvate, 2-mercaptoethanol, non-essential amino acids, and supplemented with 20ng/ml of granulocyte macrophage colony stimulating factor (GM-CSF; Miltenyi Biotec). On day 3 of culture, half the medium was replaced with fresh AIM-V and 20ng/ml of GMCSF. On day 6 of culture, BMDC were harvested, counted, and loaded with MUC1 peptide overnight in the presence of 50µg/ml poly:ICLC (Hiltonol) (Oncovir, Inc). The 100-mer MUC1 peptide represents 5 repeats of a 20 amino acid sequence HGVTSAPDTRPAPGSTAPPA found in the VNTR region of Mucin 1. Its synthesis and quality assurance was carried out at the University of Pittsburgh Genomics and Proteomics Core Laboratories(160).

3.2.3 *Adoptive T cell transfers*

Spleens were harvested from VFT and OT-II TCR transgenic mice, mechanically processed into single cell suspensions, and subjected to RBC lysis. CD4 T cells were negatively isolated by magnetic bead separation per manufacturer's instructions (Miltenyi Biotec). VFT TCR-Tg CD4 T cells were distinguishable from endogenous cells via Thy1.1 congenic background, therefore only OT-II TCR-Tg CD4 T cells were labeled with 2µmol/L carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) prior to transfer. Equivalent numbers of CD4 VFT and OT-II T cells ($3x10^6$ -$5x10^6$/strain) were injected via the lateral tail vein 1-2 days pre vaccination.

3.2.4 *In vivo Stimulation*

For immunization, each mouse received either a DC-based vaccination of $3x10^6$ MUC1 peptide loaded BMDC or a MUC1- poly:ICLC vaccination comprising of 100µg MUC1 peptide and 50µg of poly:ICLC by lateral tail vein injection. Splenocytes were harvested and processed for phospho-flow analysis as described previously(152, 153, 156, 161). In brief, spleens were harvested at various timepoints post immunization and fixed immediately by processing into a single cell suspension in 1.6% paraformaldehyde (Electron Microscopy Services). After fixation for 10 minutes at room temperature, cells were permeabilized with the addition of ice-cold methanol to a final concentration of 80%. Samples were incubated for 30 minutes at 4°C before being stored at -80°C.

3.2.5 *Optimization of the phospho-flow technique for analysis of primary T cells*

Intracellular phospho-specific staining was done in conjunction with staining for surface markers as previously described. (125) Briefly, cells were washed twice in staining buffer (PBS, 0.5% BSA, and 0.02% sodium azide) before blocking with an anti-CD16/32 antibody for 20 minutes at room temperature. Cells were stained for 45 minutes at room temperature with a mixture of surface antibodies including CD4 V500, B220 PeCy7, CD90.1 APC-eFluor 780 (eBioscience), and an exclusion panel of biotin – streptavidin PE-Texas Red labeled antibodies (CD49b, TER119, CD11c, CD8a, F4/80, and CD11b) for better resolution of the populations of interest. Two intracellular or phospho-specific antibodies (Tbet Alexa Fluor 647, Akt Alexa Fluor 647 (pS473), ERK1/2 PE (pT202/pY204), Lck Alexa Fluor 647(pY505), p38 MAPK Alexa Fluor 647 (pT180/pY182), Stat1 PE (pY701), Stat3 PE (pY705), Stat4 PE (pY693), Stat5 PE (pY694),

Stat6 Alexa Fluor 647 (pY641), S6 Alexa Fluor 647 (pS244), Zap70 PE (Y319)) were used per sample. After staining, all samples were washed and resuspended in staining buffer. All antibodies were products of BD Biosciences unless noted otherwise.

All samples were run on the LSR Fortessa and analyzed with Flowjo software (Treestar). The transferred MUC1 stimulated VFT CD4 T cell population (exclusion$^-$CD4$^+$B220$^-$ CD90.1$^+$CFSE$^-$) as well as the co-transferred control OT-II population that does not respond to MUC1 peptide (exclusion$^-$CD4$^+$B220$^-$CD90.1$^-$CFSE$^+$) were identified. Median Fluorescent Intensity (MFI) was calculated for each population within the sample and used to calculate fold change upon stimulation after normalization to the corresponding fluorescence minus one (FMO) control (fold change = absolute value ([MFI $_{\text{stimulated}}$ /MFI $_{\text{stimulated FMO}}$]/ [MFI $_{\text{unstimulated}}$ /MFI $_{\text{unstimulated FMO}}$]))

3.3 RESULTS

3.3.1 *Kinetics of TCR signaling in MUC1.Tg and WT mice in response to two different MUC1 vaccines*

We adoptively transferred MUC1-specific VFT T cells and control OT-II T cells into WT or MUC1 transgenic mice and immunized the mice with either DCs loaded with MUC1 peptide (DC-MUC1) vaccine or MUC1 peptide admixed with the adjuvant poly:ICLC (MUC1-poly:ICLC) vaccine. We collected splenic T cells from 3 mice per group at each time point. After vaccination with the DC-MUC1 vaccine, MUC1-specific T cells displayed high levels of Zap70 phosphorylation at 3hours in MUC1.Tg mice that was transient and not present in WT mice. At 6

hours the levels of Zap70 phosphorylation in MUC1.Tg mice was still present but at a much lower level, closer to that seen in WT mice (**Figure 3-1**a). The percentage of MUC1-specific T cells expressing phosphorylated Zap70 was low (~2%) in both strains. In contrast, phosphorylation of Lck, which results in stabilization of the inactive conformation, was low but measurable at all time points and present in a large percentage of T cells (**Figure 3-1**b). In the case of the MUC1-poly:ICLC vaccine, MUC1-specific T cells had equivalent levels of phosphorylated Zap70 at 3 hours in WT and MUC1.Tg mice and a higher number of activated T cells at early time points. As with the DC-MUC1 vaccine, Lck phosphorylation was measurable at 6 hours (**Figure 3-1**a) and in a larger number of T cells in both MUC1.Tg and WT mice (**Figure 3-1**b). Similar activation through their TCR and similar signaling of MUC1-specific T cells in response to both types of vaccines and in both MUC1.Tg and WT mice suggests that it is not this first step that is responsible for the ultimate difference in the vaccine outcome.

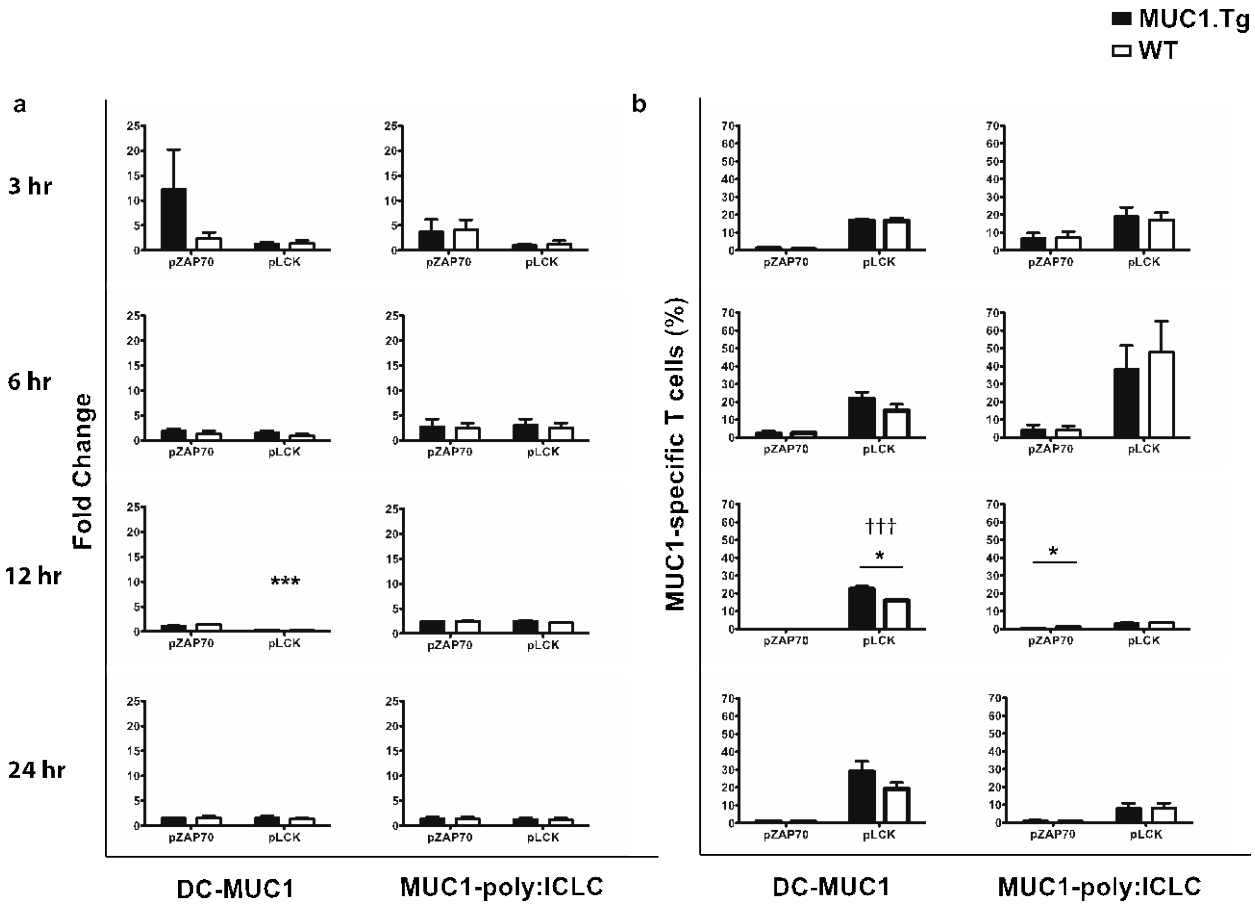


Figure 3-1. Signaling through the TCR

(a) Levels of phosphorylated ZAP and Lck were examined at 3, 6, 12, and 24 hours post vaccination with either DC-MUC1 (n=6/strain/timepoint except 12 hour (n=3)) or MUC1-poly:ICLC (n=6/strain/timepoint except 6 hour (n=5)) in MUC1.Tg or WT mice. The levels of phosphorylation in MUC1-specific T cells responding to DC-MUC1 vaccine was significantly different then seen in response to MUC1-poly:ICLC vaccine at (*$p<0.05$) 12 hours by unpaired t-test. (b) The corresponding number of THY1.1+ cells expressing the phosphorylated targets at each timepoint in response to vaccination was determined. The percent of cells responding to DC-MUC1 vaccine was significantly different by unpaired t-test (†††$p<0.001$) at 12 hours in comparison to responses to MUC1-poly:ICLC vaccine.

3.3.2 *Activation of cell cycle and survival pathways post MUC1 vaccination in WT and MUC1.Tg mice*

Phosphorylation of ERK1/2 has been shown to be critical for T cell activation and proliferation in response to alloantigen in vivo(167) and was of interest for MUC1-specific responses. We investigated MUC1-specific T cells responding to the two MUC1 vaccines in WT or MUC1.Tg mice for differences in phosphorylation of ERK1/2 downstream of TCR signaling in the Ras/Raf pathway and in two other tyrosine kinases, 38/MAPK and Akt, involved in cell survival and proliferation pathways. Both Akt and 38/MAPK have also been shown to have roles in T cell differentiation and function(168, 169), and their activation is dependent on upstream co-stimulatory signaling(170, 171).

We found differences in the kinetics of ERK1/2 phosphorylation in MUC1.Tg compared to WT mice after DC-MUC1 vaccine. At six hours, in MUC1.Tg mice we saw a small percentage of MUC1-specfic T cells experiencing a peak in ERK1/2 phosphorylation that quickly decreased by 12 hours after vaccination (**Figure 3-2**). The signal peaked in WT mice at 12 hours post vaccination. Activation of 38/MAPK and Akt in response to DC-MUC1 was similar. MUC1.Tg and WT mice exhibited low levels of 38/MAPK and Akt phosphorylation in a small percentage of MUC1-specific T cells. Phosphorylation of ERK1/2, 38/MAPK, and Akt in response to the MUC1-poly:ICLC vaccine was minimal but equivalent in MUC1.Tg and WT mice. Although activation of cell cycle and survival pathways in MUC1.Tg and WT mice following MUC1-poly:ICLC immunization trended the same in both the intensity and the percentage of cells, there was an initial increase in the subpopulation of MUC1-specific T cells expressing phosphorylated 38/MAPK and ERK1/2 not seen after DC-MUC1 immunization

(**Figure 3-2**b). In summary, differences can be seen in ERK1/2 phosphorylation but not in 38MAPK and Akt phosphorylation.

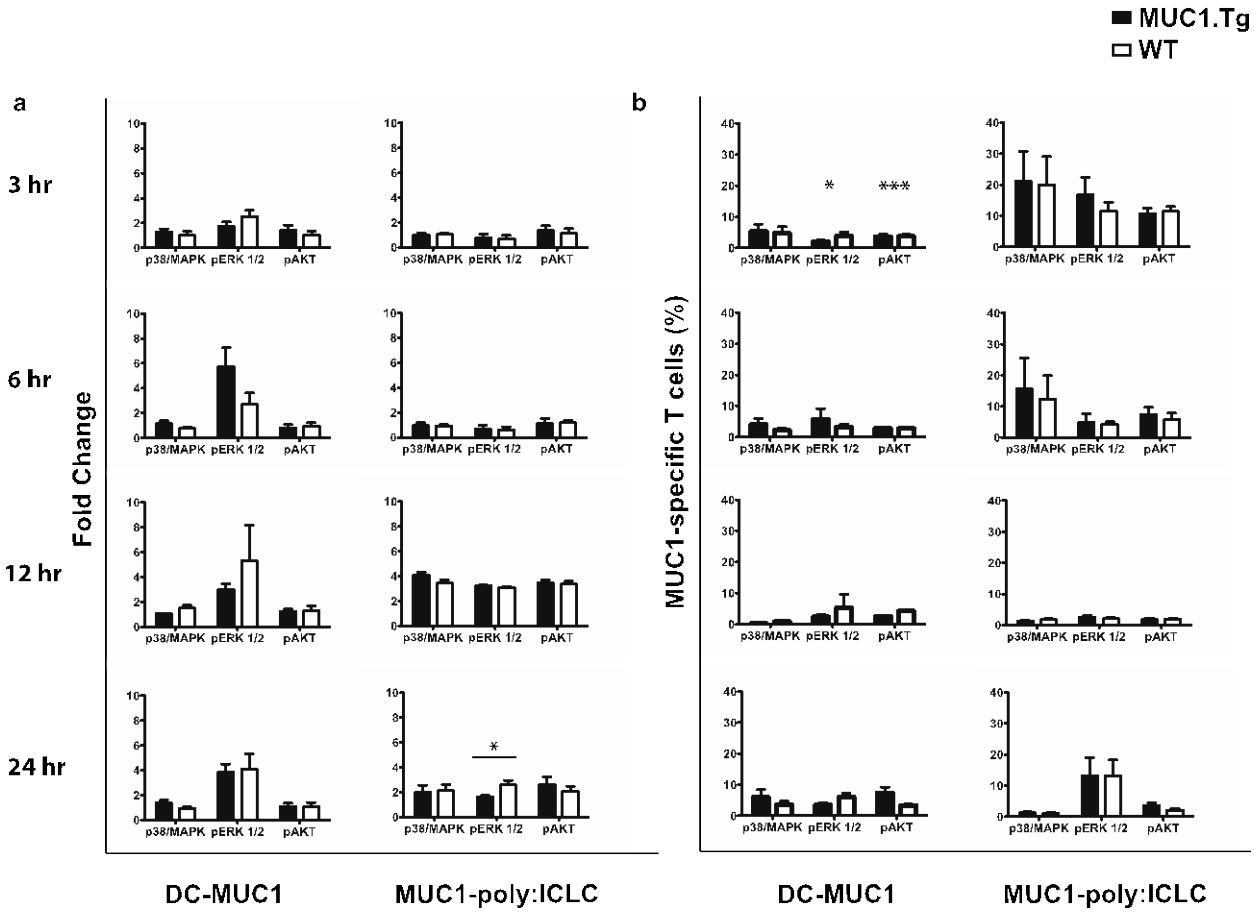


Figure 3-2. Activation of cell cycle and survival proteins

(a) Key signaling molecules in the MAPK, Ras/Raf, and PI3K pathways (38/MAPK, ERK1/2, and Akt respectively) were analyzed for changes in phosphorylation in MUC1-specific T cells at 3, 6, 12, and 24 hours post immunization with either a DC-MUC1 (n=6/strain/timepoint except 12 hour (n=3)) or MUC1-poly:ICLC (n=6/strain/timepoint except 6 hour (n=5)) vaccines. The levels of phosphorylation in MUC1-specific T cells responding to DC-MUC1 vaccine was significantly different then seen in response to MUC1-poly:ICLC vaccine at (*$p<0.05$) 24 hours by unpaired t-test. (b) The percentage of MUC1-specific cells that elicited phosphorylation of p38/MAPK, ERK1/2, and Akt in response to the vaccines were determined. The percent of cells responding to DC-MUC1 vaccine was significantly different by unpaired t-test (pERK1/2; *$p<0.05$ and pAKT; ***$p<0.001$) at 3 hours in comparison to responses to MUC1-poly:ICLC vaccine.

3.3.3 *Activation of Th1 associated signaling molecules in response to MUC1 vaccines in WT and MUC1.Tg mice*

We have previously published elicitation of MUC1-specific Th1 responses in both MUC1.Tg and WT mice following immunization with DC-MUC1 vaccine, but at the same time a decrease in IFNγ production by MUC1-specific cells in MUC1.Tg mice compared to WT mice. (158) Expecting this to be indicative of differences in the percentage of cells or the degree of phosphorylation of Th1 associated signaling molecules(160), we examined potential differences in phosphorylation of the Th1 associated signaling molecules downstream of IFNγ receptor signaling and IL-12 receptor signaling, STAT1 and STAT4 respectively, as well as expression of the transcription factor Tbet. MUC1-specific T cells displayed phosphorylation of STAT1 to a much higher degree than STAT4 in both MUC1.Tg and WT mice at 3 and 6 hours post vaccination (**Figure 3-3**a). Although activation of STATs occurs equivalently in both groups of mice after soluble vaccination, MUC1-specific T cells in MUC1.Tg mice exhibit greater activation of STAT1 in a higher percentage of the population at 3 hours after DC immunization than in WT mice (**Figure 3-3**). In addition, from 6 through 24 hours post immunization there were consistently a greater percentage of MUC1-specfic T cells exhibiting STAT1 signaling in response to DC-MUC1 than MUC1-poly:ICLC vaccine (**Figure 3-3**b). Thus DC-MUC1 vaccine activates more robust Th1 signaling than MUC1-poly:ICLC vaccine in both strains of mice.

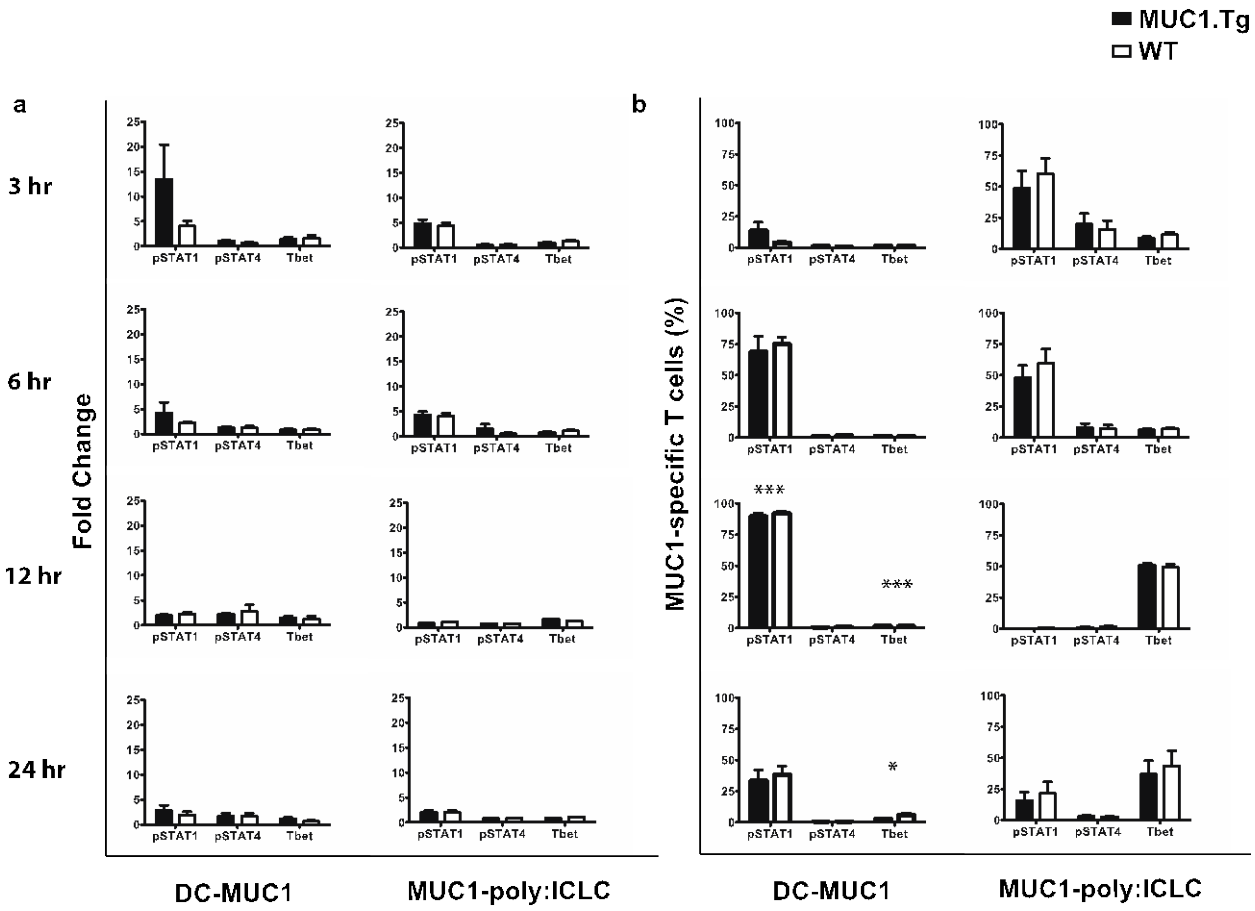


Figure 3-3. Th1 associated signaling

(a) Phosphorylation of signaling molecules downstream of cytokine receptors activated in a Th1 response, STAT1 and STAT4, as well as the expression transcription factor Tbet were examined at 3, 6, 12, and 24 hours post immunization with DC-MUC1 (n=6/strain/timepoint except 12 hour (n=3)) or MUC1-poly:ICLC (n=6/strain/timepoint except 6 hour (n=5)) vaccines. (b) Percent of MUC1-specific T cells that exhibited phosphorylation of STAT1 or STAT4 or expressed Tbet above baseline was identified. The percent of cells responding to DC-MUC1 vaccine was significantly different by unpaired t-test (***$p<0.001$) at 12 and (*$p<0.05$) 24 hours in comparison to responses to MUC1-poly:ICLC vaccine.

3.3.4 *Th2 and Th17 associated signaling responses to MUC1 vaccines in MUC1-specific T cells*

We also examined the phosphorylation status of STAT6 and STAT3 in order to potentially be able to predict if a vaccine might be activating Th2 or Th17 cells that are not routinely measured in response to cancer vaccines. STAT3 activation promotes Th17 differentiation(142, 172), while Th2 development requires STAT6 activation in addition to STAT3(173). Following both DC-MUC1 and MUC1-poly:ICLC immunization, MUC1-specific T cells in MUC1.Tg and WT had equivalent percentages of cells exhibiting similar levels and kinetics of STAT6 and STAT3 phosphorylation. In general, DC-MUC1 vaccine elicited robust STAT3 signaling in over 50% of MUC1-specific T cells in the first 24 hours (**Figure 3-4**b), with little to no increases in STAT6 activation. Furthermore, at 24 hours post immunization, there is greater phosphorylation of STAT3 in MUC1.Tg mice than in WT mice. Conversely, MUC1-poly:ICLC immunization demonstrated greater levels of STAT6 activation than STAT3 at all timepoints (**Figure 3-4**a). Although there is initially a larger percentage of cells exhibiting STAT6 activation, as time progresses the population of MUC1-specific cells with activated STAT6 decreases to barely detectable levels while the population exhibiting STAT3 phosphorylation remains constant and high (**Figure 3-4**b).

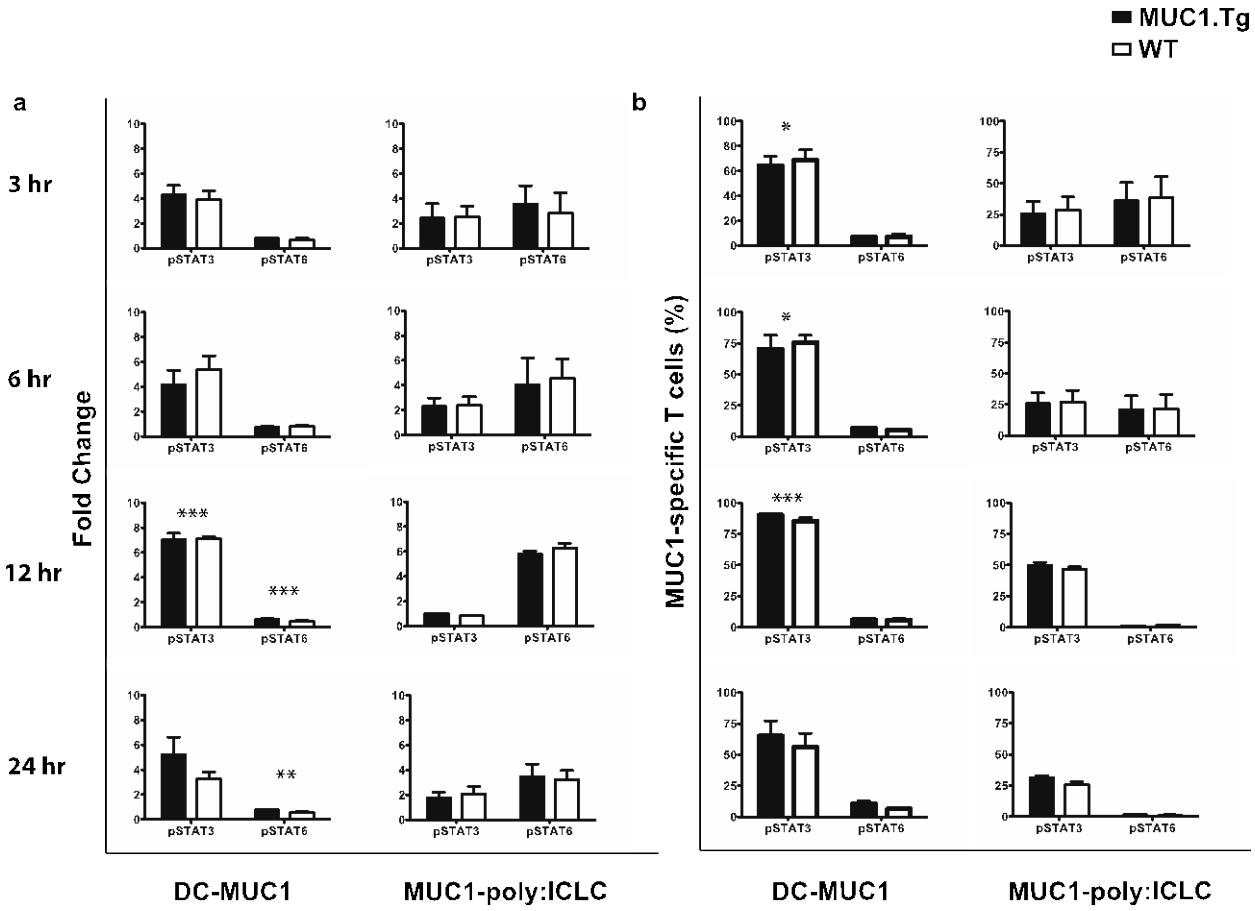


Figure 3-4. Th2 and Th17 signaling

(a) Phosphorylation of STAT3 and STAT6 signaling molecules in response to DC-MUC1 (n=6/strain/timepoint except 12 hour (n=3)) or MUC1-poly:ICLC (n=6/strain/timepoint except 6 hour (n=5)) vaccines in MUC1.Tg or WT mice was analyzed 3, 6, 12, and 24 hours post immunization. The levels of phosphorylation in MUC1-specific T cells responding to DC-MUC1 vaccine was significantly different then seen in response to MUC1-poly:ICLC vaccine at (***$p<0.001$) 12 and (**$p<0.01$) 24 hours by unpaired t-test. (b) The percent of MUC1-specific cells that phosphorylated STAT3 or STAT6 was determined. The percent of cells responding with STAT3 phosphorylation to DC-MUC1 vaccine was significantly different by unpaired t-test (*$p<0.05$) at 3, 6, and (***$p<0.001$) 12 hours post immunization in comparison to responses to MUC1-poly:ICLC vaccine.

3.3.5 *Fewer MUC1-specific T cells show S6 activation after DC-MUC1 vaccine compared to MUC1-poly:ICLC vaccine*

We have previously published that regulatory T cells (Treg) affect MUC1-specific CD4 T cell responses in the MUC1.Tg mouse(160). To assess if activation of Treg can be predicted for different vaccines in different hosts, we examined phosphorylation of S6 kinase downstream of the Akt/mTOR pathway that inversely correlates with subsequent Treg development(163) as well as STAT5 (the IL-2 receptor signaling molecule) and expression of the Treg transcription factor FOXP3. We found no differences in these molecules between T cells in vaccinated MUC1.Tg versus WT mice, with the exception of FOXP3 expression being higher in MUC1.Tg mice at the 6-hour time point post DC-MUC1 vaccination (**Figure 3-5**a). There was a difference however between the vaccines. MUC1-poly:ICLC vaccine elicited a much greater percentage of cells displaying S6 phosphorylation and significantly fewer cells exhibiting STAT5 phosphorylation then their counterparts receiving the DC-MUC1 vaccine (**Figure 3-5**b).

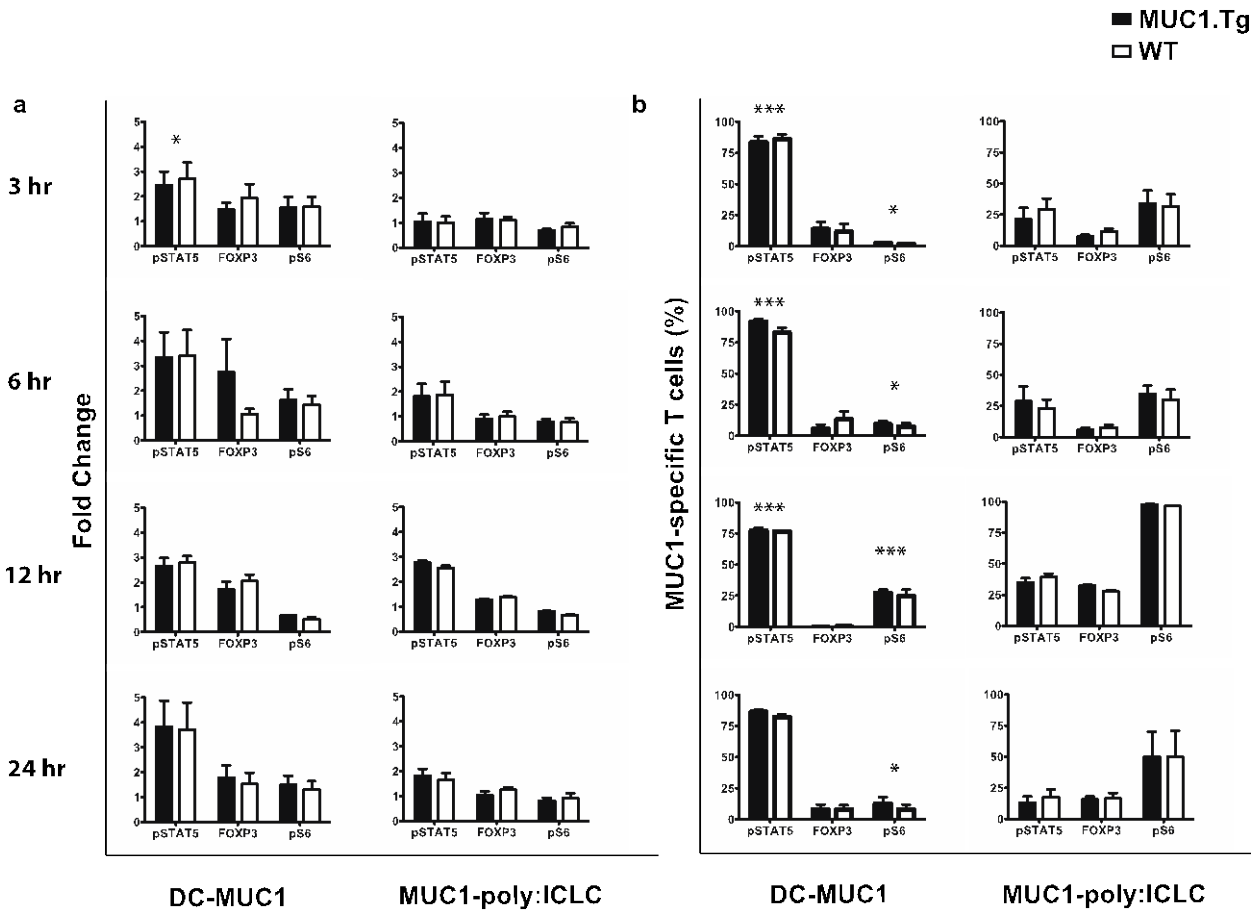


Figure 3-5. Pathways associated with signaling through T regulatory cells

(a) Expression levels of FOXP3 and phosphorylation levels of STAT5 and S6 were examined at 3, 6, 12, and 24 hours post DC-MUC1 (n=6/strain/timepoint except 12 hour (n=3)) or MUC1-poly:ICLC (n=6/strain/timepoint except 6 hour (n=5)) vaccination in MUC1.Tg and WT mice. The levels of phosphorylation in MUC1-specific T cells responding to DC-MUC1 vaccine was significantly different then seen in response to MUC1-poly:ICLC vaccine at (*$p<0.05$) 3 hours by unpaired t-test (b) The percent of MUC1-specific cells altering expression of FOXP3 or phosphorylation of STAT5 or S6 was determined. The percent of cells responding with STAT5 phosphorylation to DC-MUC1 vaccine was significantly different by unpaired t-test (***$p<0.001$) at 3, 6, and 12 hours post immunization in comparison to responses to MUC1-poly:ICLC vaccine. While the percentage of cells phosphorylating S6 in response to DC-MUC1 compared to MUC1-poly:ICLC differed at all timepoints ((*$p<0.05$) 3, 6, and 24 hours; (***$p<0.001$) 12 hours).

3.3.6 *Differences in signaling profiles hours post vaccination correlate with elicited T cell populations*

We analyzed cell surface activation markers on VFT T cells retrieved from WT or MUC1.Tg mice vaccinated with DC-MUC1 or MUC1-poly:ICLC vaccine whose signaling events we determined above. We found that a large percentage of these cells expressed CD25 regardless of their origin (MUC1.Tg vs WT mice) for the first 12 hours post DC-MUC1 immunization. By 24 hours, however, the percentage of cells expressing CD25 had dropped significantly in WT mice. Conversely, CD69 expression was low for the first 6 hours post vaccination peaking at 12 hours with greater expression on cells from WT mice (**Figure 3-6**a).

Since S6 inversely correlates with the development of Treg, we determined the percentage of FOXP3+CD4+ T cells in MUC1.Tg and WT mice post secondary boost with either DC-MUC1 or MUC1-poly:ICLC peptide vaccine. Approximately 3% of CD4 T cells were FOXP3 positive in MUC1.Tg and WT mice vaccinated with MUC1-poly:ICLC. In contrast, DC-MUC1 vaccine was more effective at stimulating Treg development, yielding twice as many FOXP3+ T cells (**Figure 3-6**b).

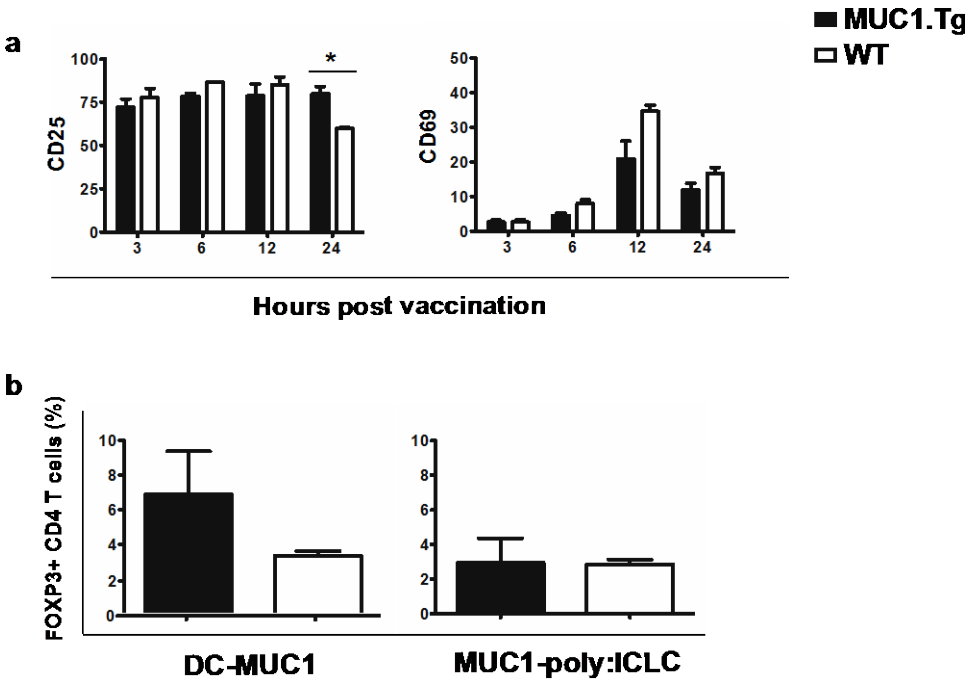


Figure 3-6. Small differences in signaling yield differences in T cell outcome.

(a) The expression of cell surface activation markers, CD25 and CD69, was examined on MUC1-specific T cells 3, 6, 12, and 24 hours (n=3/strain/timepoint) post immunization with DC-MUC1 in MUC1.Tg and WT mice. * Indicates a p value = 0.012 by unpaired t-test. (b) MUC1.Tg and WT mice (n=3/strain) were vaccinated and boosted twice at four-week intervals. The endogenous CD4 T cell populations were examined at 24 hours post secondary boost for FOXP3 expression.

Having a quantitative data set representing early activation of all the pathways that we chose because of their known importance in the development of an immune response to different vaccines in different hosts, allowed us to compile them together into one activation fingerprint. **Figure 3-7** represents signaling signatures of MUC1-specific CD4 T cells responding to MUC1 vaccines in MUC1.Tg mice, where MUC1 is a self-antigen, and in WT mice where it is a foreign antigen. It is an example of the potential of this approach that will need to be validated further by comparing other vaccines and relating it to their known outcomes.

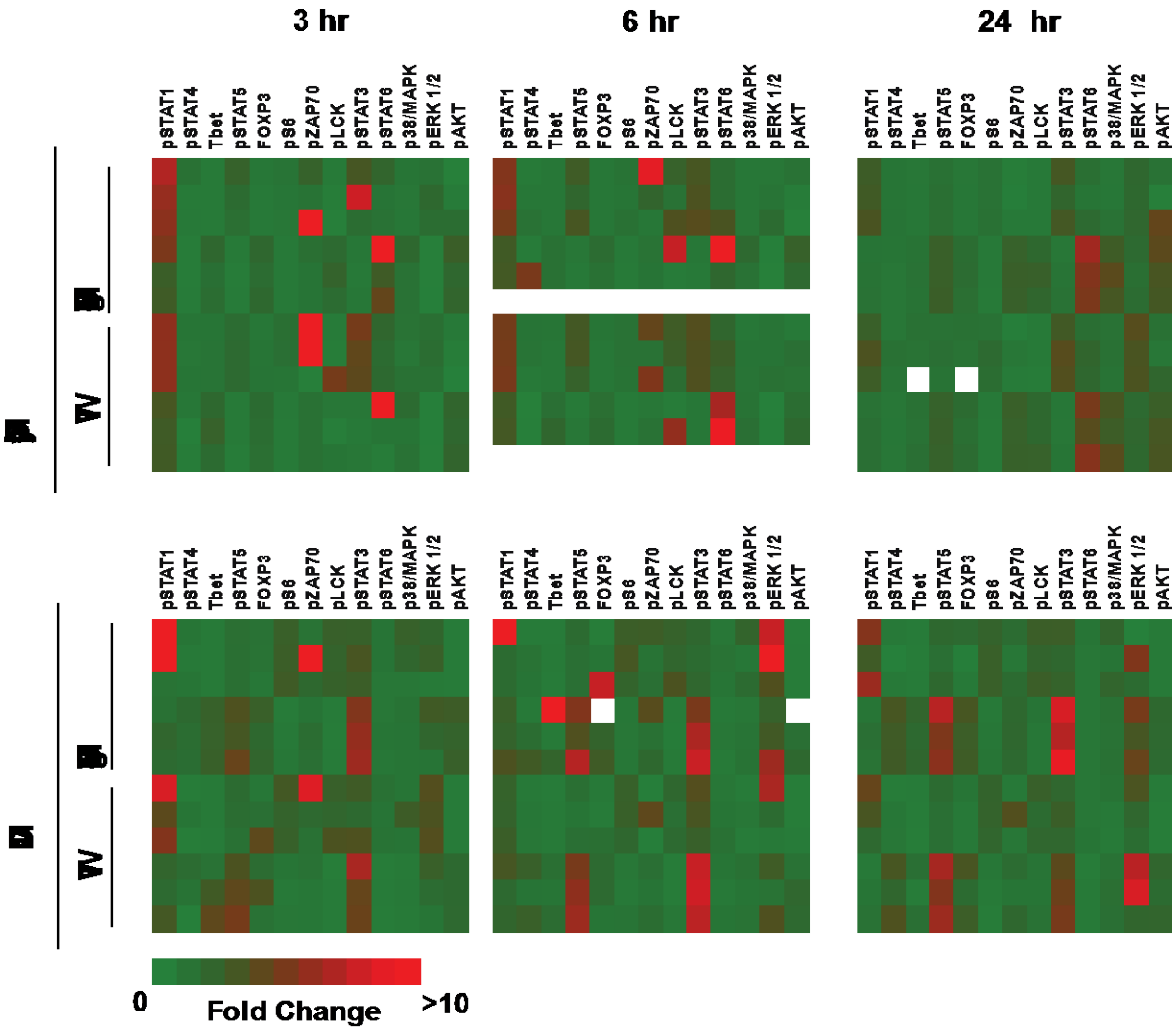


Figure 3-7. Proposed model for a MUC1-specific CD4 T cells in vivo activation "fingerprint"

The profile of the CD4 T cell population early post-vaccination composed of TCR, cell cycle and survival, and molecules associated with T helper and Treg activation in MUC1-specific T cells in response to DC-MUC1 and MUC1-poly:ICLC vaccines at 3, 6, 12, and 24 hours.

3.4 DISCUSSION

The phospho-flow technique has proven dependable and reproducible in vitro(130). There has been success in transferring this technique to in vivo models of stimulation in which a bolus of adjuvant(153) or a soluble antigen that can bind directly to the MHC cleft are administrated(156) but many hurdles still preclude its use for antigen-specific events. The time it takes for an antigen to be processed and the TCR:MHC synapse to form between DC and T cells within the spleen or lymph node can be variable between vaccines and between hosts. Furthermore, only a small number of cells within the population are being activated at a given moment with transient phosphorylation, therefore the signal is easily diluted, especially at very early time points. Nevertheless, we demonstrate that phosphorylation signatures of MUC1-specific T cells reacting to MUC1-poly:ICLC vaccination can be compared to DC-MUC1 vaccination and differences can be identified in the degree of signaling associated with Th1, Th2, Th17 and Treg responses, as well as activation of molecules that are key to cell survival and proliferation.

In future studies it may be necessary to expand the panel of signaling molecules for a more precise T cell activation signature in response to vaccination. Many of the signaling molecules we included have numerous downstream targets in different signaling pathways that they activate, and can also have multiple upstream modifiers. This could hinder interpretation of the activation and differentiation of the T cell response and in turn affect prediction of vaccine efficacy. For instance, an increase in phosphorylation of Akt could be indicative of cell growth, proliferation, or autophagy inhibition. A decrease in Akt activation suggests suppression of cell cycle progression but it may also be indicative of immune function, as it has been shown necessary for Treg suppressive function(174). Signaling molecules within the cell may have various upstream activators; IL-6 receptor, IL-21 receptor, and CD40L all signal through

STAT3. The role of STAT3 signaling has been well defined in the differentiation of T helper subsets, however it has also been shown to play a role in the maintenance and development in memory(175). In addition, the synthesis of IL-17 is dependent on downstream mediators of MAPK cascades that require the activation of 38/MAPK in CD4 T cells therefore placing 38MAPK in a critical role in IL-17 production(176). Increases in phosphorylation in MAPK following immunization may be indicative of increased cell survival or perhaps a skewing towards a Th17 response. These instances of crosstalk just demonstrate the importance of understanding the context and cell type in which signaling pathways are activated in order to extrapolate later effects on function or immune response.

All caveats acknowledged, we were still able to demonstrate differences between the phosphorylation of signaling pathways, the intensity of phosphorylation of a protein, and the kinetics of activation dependent on whether it was a foreign (MUC1 in WT mice) or abnormal self-antigen (MUC1 in MUC1.Tg mice) being recognized by the immune response. Modulation of these responses was possible by changing the design of the vaccine (peptide loaded DC or peptide in conjunction with adjuvant) creating unique signatures. This suggests that with additional optimization and proper validation, just the signaling fingerprint obtainable within a few days post vaccination can serve as a biomarker of a vaccine efficacy. Moreover, it can be a good way to assess if changes in design, modulation of its components, adjuvants and vehicles of delivery, produce a better vaccine.

4.0 DISCUSSION

Evaluation of the effectiveness of a vaccine within an earlier time frame post administration is desirable to determine if the desired immune response is occurring. The earlier the detection, the more quickly changes to therapeutic regimens can be implemented resulting in better establishment of the desired immune response. To our knowledge, our work discussed here is the first to demonstrate that early T cell signaling signatures detected within 24 hours post vaccination were different dependent on vaccine design and host environment. This suggests that T cell signaling signatures could be used to decipher the immune response to a vaccine in the context of the microenvironment, therefore serving as a biomarker of vaccine efficacy. Extrapolating from differences in the outcomes of tumor challenge that we already know exist after vaccination with the two MUC1 vaccines in the MUC1.Tg versus WT models, differences in these 'fingerprints' of activation may be correlated to differences in the outcome of disease. Furthermore, it can be an effective way to evaluate if changes in design, modulation of its components, adjuvants and vehicles of delivery, produce a better vaccine. Because signaling is universal, the detection of a T cell activation 'fingerprint' representative of induction of protective immunity versus that of a tolerant response could be universally applied to any antigen, tumor model, or even disease.

As previously discussed, the use of phospho-flow to examine differences in signaling in in vitro assays has been well characterized and reproducible. The ability of this technique to

identify differences in the signaling potential of lymphocytes and monocytes from the PBMCs of patients suffering from diseases such as leukemia has been demonstrated. We demonstrated that with additional optimization and proper validation, the signaling fingerprint obtainable within just a few days post vaccination can serve as a biomarker of a vaccine efficacy. We acknowledge that there are still hurdles in the development of this technique before it is applicable for preclinical and clinical usage as a biomarker of therapeutic efficacy. Biologically, more precise signatures to identify entire pathways being activated as well as the activation of apoptosis mediators will need to ensue. The expansion of the signaling panel as well as validation of responses at several timepoints will help to achieve a more decipherable, reproducible signature of the requirements to induce protective immune responses. In addition, although there has already been some standardization and development of a small array to minimize variability and increase ease of use, further development and standardization is necessary as well as ensuring that it is not cost prohibitive. We believe that the uses of a signaling fingerprint as a biomarker of immune function has great potential and if realized could lead to advances in the evaluation of immunogenicity. Deciphering signaling fingerprints would yield an immediate appraisal of the effectiveness of a therapy, could be a very early predictor of outcome of disease, and may prove to be a useful tool to predict the treatment strategy that would be most useful for an individual based on the signaling mediators in the environment to which the cells are nonresponsive. Additionally, examination of signaling signatures can be extended to various cell types to gain a comprehensive awareness of the microenvironment, and perhaps where immune mediators are failing due to immune suppression.

The recently developed technique of cytometric mass spectrometry, also know as CyTof™ (‘cytometry by time of flight’), would address many of the non-biologic technical

concerns of the phospho-flow technique. The limits of fluorescence detection have the majority of cytometers able to differentiate up to 13 channels simultaneously, and state of art technology allows up to 17 markers of detection(177). This limits the phospho-flow technique in the detail that it can extrapolate from samples due to limits in reagents. Moreover, the compensation between channels becomes increasingly complex as the number of markers increases and can complicate the interpretation of the data. In addition, another major limitation is cellular auto-fluorescence, which is not an issue with CyTof™. CyTof™ uses time of flight mass spectrometry coupled with heavy metal elemental tags to deliver a sensitive, high-resolution ability to analyze cellular markers(178). Although early studies have used approximately 30 parameters simultaneously, up to 100 different markers could be used theoretically(178). In preliminary studies, intracellular and surface CyTof™ analysis has revealed detailed signaling patterns of cellular subsets that were not possible to detect via phospho-flow(179).

An evident trend seen in a diverse array of immunotherapies in clinical trials, is the need for new additional parameters and biomarkers to evaluate the efficacy of treatments and better correlates of long-term disease outcome. The Response Evaluation Criteria in Solid Tumors or World Health Organization standard of initial regression of tumor size used for chemotherapeutic agents may not be a true evaluation of the effectiveness of an immunotherapeutic agent due to the length of time it may take to mount an immune response as well as the immunosuppressive environment that may need to be overcome(1, 180). Increasing clinical data has demonstrated that some patients that initially demonstrated tumor growth went on to develop long-term survival with tumor control or regression. Several initiatives to revise the standards of immunotherapeutic evaluation have resulted in new criteria to develop more meaningful endpoints: detection of reproducible biomarkers of early cellular activation with minimal

variability in the assay and correlation to clinical outcome, identification of clinically measurable patterns of anti-tumor immunity by new immune related criteria, and different statistical approaches for survival analysis(181).

We hypothesized that the identification of early intracellular signaling of T cells induced within a day after vaccination may serve as a biomarker of the effect of therapy on the immune response and correlate with disease outcome. T cell signatures could be obtained prior to treatment in order to assess the host environment, any detectable deficiencies in the immune response, and perhaps some insight into the cellular composition of the population. The response patterns may be able to direct clinicians to what therapeutic strategies could best benefit an individual patient. Importantly, fingerprints of T cell activation could be assessed early post-therapy to identify if patients are responding to treatment. If the signaling signature of a patient does not progress post therapy to that of a protective immunity inducing 'fingerprint', therapeutic options could be readdressed. This would bypass the need to wait for months or years to determine disease outcome providing a better opportunity to successfully create long lasting immunity. Although the phospho-flow technique used to decipher T cell signatures may be too variable between samples and challenging to optimize to decipher signaling signatures of in vivo events, we were able to distinguish differences and similarities to responses across different vaccine designs and host environments. Evidence of differences in T cell signaling signatures in response to different therapeutic regimens as well as differences in response to microenvironments is promising as a potential parameter by which to evaluate efficacy of immunotherapies, in particular prophylactic vaccines in which efficacy and outcome of potential disease are exceedingly long-term investigations.

Currently most immunotherapies are targeting a single antigen(182) but creation of vaccines against multiple tumor antigens may provide a more robust immune response against a tumor and prevent evasion of the immune response(183-185). Intricate model systems would need to be developed to determine differences in the efficacy of multiple tumor antigens including varying concentrations of antigens, variety of antigens, and combinations of antigens. The combination of multiple immune therapies targeting different stages or aspects of tumor progression has shown promise in the clinical setting. Treatment of melanoma patients with IL-2 and gp100 induced control of tumor growth(186). Countless therapeutic amalgamations could exist, such as combined CTL-4 and PD1 antibody therapies to prevent negative regulation of T cell activation and effector mechanisms (1). The detection of a 'fingerprint' of a signaling signature of protective immune responses versus that of tolerant immune responses could simplify and hasten preclinical modeling of different immunotherapeutic regimens by streamlining the process. As the current trend in cancer immunotherapy is the combination of multiple immune therapies, simplification, standardization, and high throughput parameters of evaluation are going to become significantly more important. New parameters such as 'synergistic toxicity' will need to be assessed(1). Identification of the best combination of candidates, consideration of the stage of the immune response to target, and the dose and schedules of the immunotherapeutic regimens in order to evaluate efficacy and choose those that are most effective will become increasingly complex.

Adding to the complexity, an important and interesting finding is that many of these tumor-associated antigens are not only found in malignancies but after disease or other severe inflammatory events. T cells specific for the abnormal form of MUC1 have been found and expanded in vitro from not only cancer patients but healthy individuals as well (87, 92).

Antibodies to abnormal MUC1 have been detected in women during lactation or with mastitis(187). Infection of cells by the herpesviridae family members, VZV(188) and CMV(189), leads to the overexpression of cyclin B1 and its detection in the cytoplasm. In these instances the immune system is building a memory response to multiple initiating events that may cause a cell to react in a certain way. Therefore, earlier events in life may cause memory that is protective for an individual from cancer or encounters with a newly encountered infection later in life. Recent studies have shown that women having experienced two or more severe inflammatory events that most likely lead to abnormal MUC1 expression (viral infection such as mumps, pelvic surgery, mastitis, etc.) showed a significant reduction in their risk of ovarian cancer(190). These data suggest that universal vaccines may be developed that would encompass boosting the immune response against cancer and infectious agents simultaneously. If a universal vaccine is to come to fruition, the ability to quickly analyze the immune response to different antigens and combinations there of will be very important. Again, the use of phospho-flow or a similar technique to distinguish the signaling 'fingerprint' of targeted immune cells after therapeutic intervention could serve as a biomarker of vaccine efficacy and an early indicator of whether or not the therapeutic regimen is inducing the desired productive/protective response. Defining a protective signaling signature of immune cells could serve as a biomarker of vaccine efficacy in addition to standard immunogenicity parameters that would be representative of a universal robustness and protective response against many disease initiating events.

A T cell signaling 'fingerprint' would also serve as a useful tool in other fields such as transplantation. T cell signatures of tolerance induction could aid in the determination of immune suppression in patients before a transplanted organ was lost or damaged. These

signaling signatures could, of course, aid in the development of new therapeutics as well as provide individual patient feedback on the suppressive therapies that would work best for the individual, similar to that discussed above. Immune signaling fingerprints have the capacity to be strong universal biomarkers that are prognostic in that deficiencies in the immune response that make an individual more susceptible may be identified, and predictive of whether a therapy, prophylactic or therapeutic, will induce the desired immune response.

APPENDIX A

Appendix A has been adapted from Reichenbach, D.K., Li, Q, Hoffman, R.A, Williams, A.L., Shlomchik, W.D., Rothstein, D.M., Demetris, A.J., and Fadi G. Lakkis. Allograft Outcomes in Outbred Mice. 2013. *American Journal of Transplantation*; with permission from John Wiley and Sons. Copyright permission is kept on file with Dawn K. Reichenbach.

ALLOGRAFT OUTCOMES IN OUTBRED MICE

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Running Title: Allograft outcomes in outbred mice

Key words: outbred, transplantation, inbreeding depression, innate immunity, complement, neutrophils

Abbreviations: CVF, cobra venom factor; O, outbred; I, inbred.

A.1 ABSTRACT

Inbreeding depression and lack of genetic diversity in inbred mice could mask unappreciated causes of graft failure or remove barriers to tolerance induction. To test these possibilities, we performed heart transplantation between outbred or inbred mice. Unlike untreated inbred mice in which all allografts were rejected acutely (6-16 days post-transplantation), untreated outbred mice had heterogeneous outcomes, with grafts failing early (<4 days post-transplantation), acutely (6–24 days), or undergoing chronic rejection (>75 days). Blocking T cell costimulation induced long-term graft acceptance in both inbred and outbred mice, but did not prevent the early graft failure observed in the latter. Further investigation of this early phenotype established that it is dependent on the donor, and not the recipient, being outbred and that it is characterized by hemorrhagic necrosis and neutrophilic vasculitis in the graft without pre-formed, high titer anti-donor antibodies in the recipient. Complement or neutrophil depletion prevented early failure of outbred grafts, whereas transplanting CD73-deficient inbred hearts, which are highly susceptible to ischemia-reperfusion injury, recapitulated the early phenotype. Therefore, outbred mice could provide broader insight into donor and recipient determinants of allograft outcomes but their hybrid vigor and genetic diversity do not constitute a uniform barrier to tolerance induction.

A.2 INTRODUCTION

Inbred mouse strains have been instrumental in uncovering fundamental immunological mechanisms that underlie transplant rejection. The utility of the inbred mouse as a pre-clinical model for testing transplantation tolerance strategies, however, has been more limited (191, 192). Administration of biological agents that readily induce tolerance in inbred mice, for example, often fail in large animals and humans. Failure has been attributed to the evolutionary separation (~ 65 million years) between mice and humans, reduced exposure of laboratory mice housed in barrier facilities to microbial pathogens, and/or the biological consequences of inbreeding (191, 192). The latter include inbreeding depression and restricted genetic diversity within and between inbred mouse strains (193-195).

Known discrepancies between mice and humans that can be attributed to evolutionary separation span both innate and adaptive immunity (196). Humanized mice in which the human immune system has replaced that of the mouse are beginning to elucidate the importance of these differences (197), but it remains unclear whether they are superior to inbred mice as pre-clinical models of organ transplantation. The impact of specific-pathogen free (SPF) housing on the ease by which transplantation tolerance can be induced in laboratory mice, on the other hand, is supported by heterologous immunity studies. Mice previously exposed to microbial pathogens generate memory T cells that cross-react with allogeneic MHC molecules and become resistant to tolerance-inducing agents (198-200). In contrast, naïve laboratory mice harbor less memory T cells and can be readily made to accept MHC-mismatched allografts. As cross-reactive memory constitutes a substantial component of the human alloreactive T cell repertoire (201, 202), heterologous immunity could provide one explanation why transplantation tolerance strategies that succeed in mice often fail in humans.

In addition to their rich repertoire of alloreactive memory T cells, humans are an outbred species characterized by considerable heterozygosity and genetic diversity. Both traits contribute to the vigorous yet diverse immune responses of humans; raising the possibility that outbreeding among humans is an important reason why transplantation tolerance or allograft acceptance strategies do not translate uniformly from inbred mice to patients. Except for one study in which outbred mice were found to require more stringent immunosuppression than their inbred counterparts to achieve islet allograft survival (203), the effect of outbreeding on allograft outcomes, particularly clinically-relevant vascularized grafts, has not been tested yet. Here we investigated the effects of outbreeding in the donor, recipient or both on the survival of vascularized heart allografts in mice.

A.3 METHODS

A.3.1 *Mice*

B6 male mice (6-8 weeks old) (Jackson Laboratory), non-sibling, CD-1 and CF-1 outbred male and female mice (4 weeks old) (Charles River Laboratory; CRL), and BALB/c, FVB, and SJL inbred male mice (6-8 weeks old) (also from CRL) were housed under SPF conditions. B6 $CD73^{-/-}$ mice were a generous gift from Linda F. Thompson (Oklahoma Medical Research Foundation). Serum and tail snips were obtained prior to transplantation. PCR analysis to type MHC II loci was performed at CRL (204). All procedures were IACUC approved.

A.3.2 *Transplantation and Tissue Processing*

Vascularized, heterotopic cardiac transplantation was performed as described (205). Vascular anatomy was similar between inbred and outbred mice and surgical mortality was equivalent in the two groups (3.8% and 4.3%, respectively). Grafts were monitored daily and harvested upon cessation of palpable heartbeat or after 75 days. Serum was collected at harvest and graft tissue was snap frozen and embedded in OCT (Sakura Finetek), or paraffin and stained with hematoxylin and eosin (H&E). Histological analysis was performed by AJD in a blinded fashion. Donor splenocytes and thymocytes were harvested at time of surgery.

A.3.3 *Mouse Treatment*

15 mg/kg MR1 (anti-CD40L mAb) and 15 mg/kg CTLA-4-Ig (BioExpess and BioXCell) were given i.p. on days 0, 2, 4, and 6 to induce long-term graft survival. C3 was depleted by injecting 10 units cobra venom factor (CVF) (Quidel Corporation) in 3 divided doses i.p. one day before transplantation. Neutrophils were depleted by injecting 250ug anti-Ly6G (1A8) antibody (BioXCell) i.v. one day prior to transplantation (206). Adenosine analog 5′-N-ethylcarboxamidoadenosine (NECA) (Sigma) was administered to CD-1 donors (0.1 mg/kg) and CD-1 recipient mice (0.01 mg/kg) i.p. 4 hours prior to transplantation.

A.3.4 *Agglutination Assay*

A panel of RBCs from 10 outbred mice were incubated with pre-transplant serum (diluted 1:2) from 7 outbred mice that had accelerated graft loss for 30 minutes at 37°C, centrifuged at 400g,

resuspended, and examined for agglutination. If agglutination was absent, the plate was washed and 10 μg anti-mouse-Ig (Chemicon) added to each well before re-assessing for agglutination.

A.3.5 *Flow Cytometry*

To measure pre-formed antibodies to donor MHC, serum was diluted 1:2 and incubated with thymocytes from their respective donors for 20 minutes at 4°C, stained with anti-IgM and anti-IgG antibodies (BD Biosciences) and analyzed on an LSRII cytometer.

A.3.6 *Immunofluorescence*

Antibody deposits were examined in allografts transplanted between outbred mice. As a positive control, we transplanted BALB/c hearts into C57Bl/6 mice pre-sensitized by transplanting BALB/c skin grafts10 days earlier. Allografts were harvested at 6 or 12 hours post transplantation and frozen in OCT. 6 μm sections were fixed, blocked (Vectabond ABC Blocking Kit, Vector Laboratories), and stained with FITC-conjugated anti-IgG or anti-IgM (Zymed Laboratories) at room temperature for 1 hour. To identify neutrophils, sections were incubated with unconjugated anti-Ly6G and biotin conjugated CD31 (eBioscience) overnight at 4°C. After incubating with secondary antibodies, the tissue was counterstained with DAPI (Molecular Probes).

A.3.7 *Enzyme-linked Immunosorbent Assay (ELISA)*

C3 serum levels were measured pre-transplantation and on days 1, 5, 7, and 11 after transplantation using mouse C3 ELISA kit (Kamiya Biomedical) per manufacturer's instructions.

A.3.8 *Statistical Analysis*

Flow cytometry data is shown as mean fluorescent intensity (MFI) and standard error of mean (SEM), and analyzed by unpaired Student's T-test. Presence or absence of early graft failure was analyzed using a two-sided Fischer's exact test. Median survival time (MST) comparisons were performed by Gehan-Breslow-Wilcoxon test.

A.4 RESULTS

A.4.1 *Genetic diversity of outbred mouse stocks*

CD-1 and CF-1 outbred mouse stocks, defined as closed populations of genetically variable animals that are bred to maintain maximum heterozygosity (207), were used as either donors or recipients of heart grafts. Mice from either stock displayed outbred vigor judged by greater body weight and larger litter size than inbred strains (http://www.criver.com). To determine their diversity, we genotyped class II H-2 loci by PCR. Of 12 alleles that were genotyped, four (*p*, *b*, *q*, and *u*) were represented in the CD-1 (n = 364) and five (*p*, *b*, *q*, *u*, and *k*) in the CF-1 stock (n = 79), resembling the diversity of an isolated 'island' population (208). The extent of

heterozygosity at these loci was 0.60 and 0.74 in the CD-1 and CF-1 stocks, respectively, which is similar to the average heterozygosity of feral mice and humans (208). Therefore, the outbred mouse stocks used in this study are somewhat limited in their diversity but maintain a significant degree of heterozygosity typical of outbred populations.

A.4.2 *Blocking T cell costimulation induces long-term allograft acceptance in outbred mice*

We hypothesized that a standard tolerogenic regimen that induces allograft acceptance in inbred mice would be less effective in outbred mice. This was tested by comparing the survival of heart allografts transplanted between disparate inbred mouse strains (BALB/c to B6, n = 41) to that of allografts transplanted between non-sibling outbred mice (CD-1 to CD-1, n = 33, or CF-1 to CD-1, n = 28). Recipients were either left untreated or received a combination of CTLA4-Ig and anti-CD154 (MR1) to block T cell costimulation at the time of transplantation. As expected, T cell costimulation blockade induced 100% allograft acceptance (graft survival > 75 days) in the inbred group while all untreated recipients rejected their allografts acutely (MST = 8 days) (Fig. 1a). In contrast, graft outcomes were not uniform in the outbred group (Fig 1b). In untreated mice, grafts failed either very early (1 - 4 days after transplantation, 10/35 or 29%), acutely (6 - 24 days, 19/35 or 54%), or underwent chronic rejection (> 75 days, 6/35 or 17%) with graft MST remaining similar to that of the inbred group (10 *vs* 8 days, $p = 0.52$). In treated, outbred mice, the response was dichotomous with 7/26 (27%) losing their grafts very early and 19/26 (73%) achieving long-term graft survival. The rate of graft acceptance in the treated, outbred group was significantly greater than that in the untreated outbred group (73% vs 17%, $p < 0.0001$). Treated, outbred recipients that did not manifest the unusual early graft failure went on to have 100% (19/19) long-term allograft acceptance similar to the treated inbred group. Allograft

acceptance after costimulation blockade occurred independent of outbred stock combination used (69% in CD-1 to CD-1 and 80% in CF-1 to CD-1, n = 16 and 10, respectively, p = 0.67). Histopathological analysis revealed mild cellular infiltration, minimal fibrosis, and focal chronic vasculopathy (intimal thickening) in long-term accepted allografts in the treated inbred and outbred groups, while long-term surviving allografts in the untreated outbred group exhibited chronic rejection, ranging from severe to focal (Fig. 1c). These data indicate that genetic diversity and heterozygosity (hybrid vigor) lead to more heterogeneous graft outcomes in untreated mice but do not constitute a uniform barrier to graft acceptance after induction therapy.

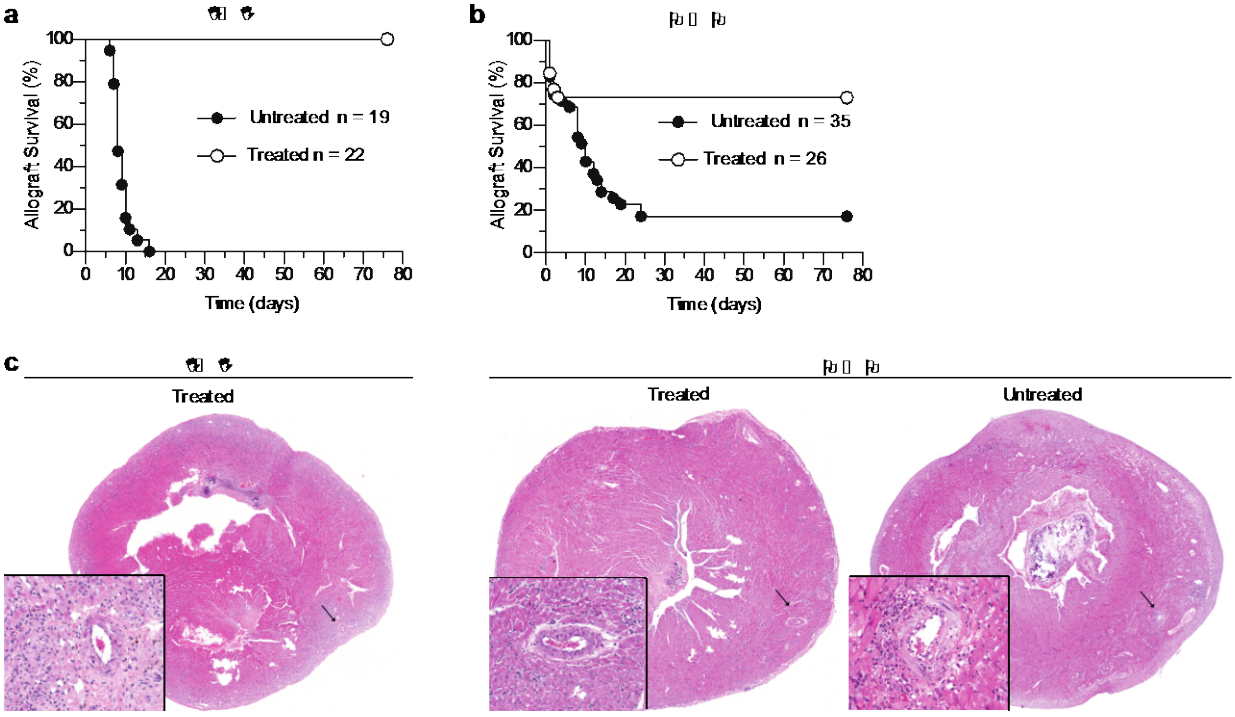

Figure 4-1. Effect of costimulation blockade on allograft survival in inbred and outbred mouse groups.

Survival of **(a)** inbred Balb/c allografts transplanted to inbred B6 recipients (I to I) and **(b)** outbred (CD-1 or CF-1) allografts transplanted to outbred CD-1 recipients (O to O) was assessed in the presence or absence of recipient treatment with costimulation blockade (MR-1 + CTLA4-Ig). **(c)** Histopathology (H&E) of allografts that survived > 75 days in treated I to I group (left panel) and either treated or untreated O to O groups (right panels). Magnification = 2x. Insets show evidence of chronic allograft vasculopathy in all groups (magnification = 30x).

A.4.3 *Increased early graft failure in outbred mice*

An unexpected finding that emerged from the above experiments is that all treated, outbred mice that lost their allografts (27%) did so between 1 and 4 days after transplantation (Fig. 1b). This accelerated graft loss was also observed in a similar proportion (29%) of untreated mice in the outbred group (Fig. 1b), but in none (0/41) of the untreated or treated mice in the inbred group ($p < 0.0001$; Fig. 1a). Early graft failure was not influenced by the particular outbred stock combination used (33% in CD-1 to CD-1 and 21% in CF-1 to CD-1, $p = 0.40$). The gross morphology and histopathology of early graft failure were distinct from classical acute cellular rejection (Fig. 2). Allografts that failed very early were significantly enlarged and were dark in color. Histopathology revealed extensive hemorrhagic necrosis with neutrophilic margination and focal neutrophilic arteritis without mononuclear cell infiltration (Fig. 2, left panels). In contrast, acutely rejected hearts in the untreated outbred group exhibited typical severe acute lymphocytic infiltration with arteritis (Fig 2, right panels). These findings suggest that the unusually high early graft failure observed exclusively in the outbred group is caused by an inflammatory or innate immune process.

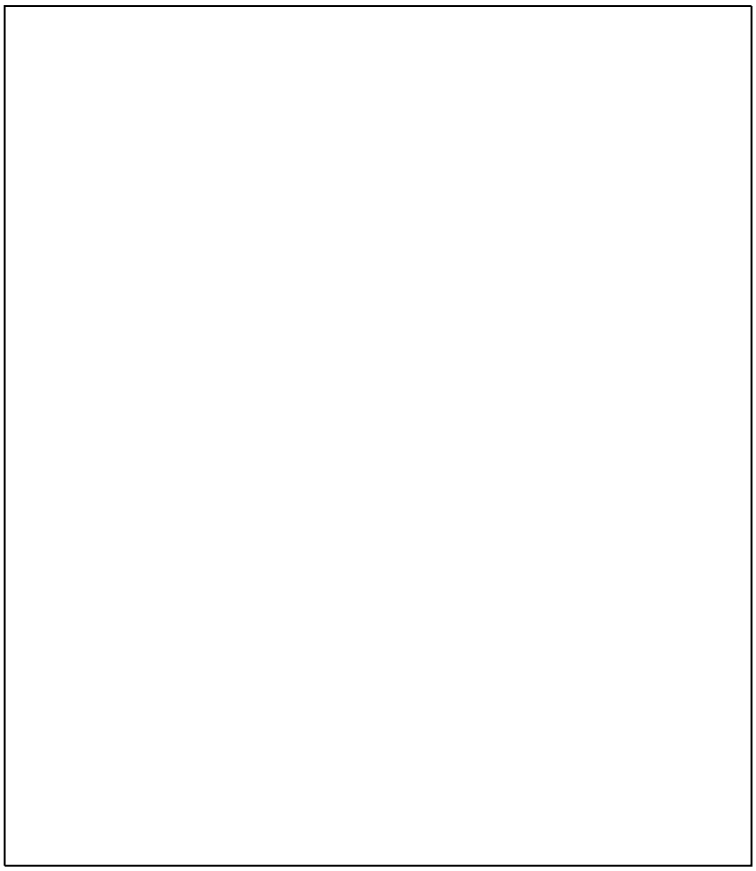

Figure 4-2. Histopathology of early graft failure

Representative pathology of cardiac allografts transplanted between untreated outbred mice that failed < 4 days (left panels) or between 6 and 24 days (right panels) after transplantation is shown. Gross appearance of allografts is shown in the top panels, H&E stained tissue sections in the middle panels (magnification = 4x), and immunofluorescent stained tissue section in the bottom panel. Insets in left middle panel highlight areas of neutrophil clot and neutrophilic vasculitis, while inset in right middle panel demonstrates lymphocytic arteritis typical of untreated acute cellular rejection. Note large area of hemorrhagic necrosis in the early graft failure but not acute cellular rejection phenotype. Ly6G, CD31 and DAPI identify neutrophils, endothelial cells, and nuclei, respectively (bottom panel; magnification = 20x).

A.4.4 *Early graft failure is dependent on donor's outbred status*

Data from mouse and human studies suggest that allograft outcomes are not only determined by the recipient's genetic background but also by that of the donor. To investigate whether recipient or donor outbred status is responsible for early graft failure, we transplanted inbred (BALB/c) hearts into outbred CD-1 mice and *vice versa*. Donors and recipients were weight-matched to avoid unanticipated surgical complications that could arise from transplanting grafts into size-mismatched recipients. In untreated recipients, early graft failure was observed in 7/26 (27%) of inbred recipients of outbred hearts (outbred to inbred, O to I, group) but in none of the outbred recipients of inbred grafts (inbred to outbred, I to O, group) (Fig. 3a). The donor outbred status effect was also observed in immunosuppressed recipients. Costimulation blockade induced 100% allograft acceptance in the inbred to outbred group, while 5/10 (50%) of grafts failed in the first 4 days in a similarly treated outbred to inbred cohort (Fig. 3b). Analysis of all outbred to outbred or outbred to inbred transplantation experiments showed that graft outcome (early failure, acute rejection, and chronic rejection) did not correlate with the degree of MHC class II mismatch between donor and recipient (Fig. 3c).

Since CD-1 animals were originally derived from the Swiss mouse stock (207), we then asked whether early graft failure is determined by genetic determinants specific to the Swiss background. To answer this question, we performed heart transplants using inbred Swiss strains as either donors (SJL to C57Bl/6, n = 23) or as both donors and recipients (SJL to FVB, n = 9). Of these, only 1/23 (3%) ($p = 1.0$ compared to I to I group) in the former and none in the latter group exhibited the early graft failure phenotype, indicating that the Swiss background of CD-1 mice does not account for the high incidence of early graft failure observed in the outbred to inbred (Fig. 2a & 2b) or in the outbred to outbred (Fig. 1b) transplantation experiments.

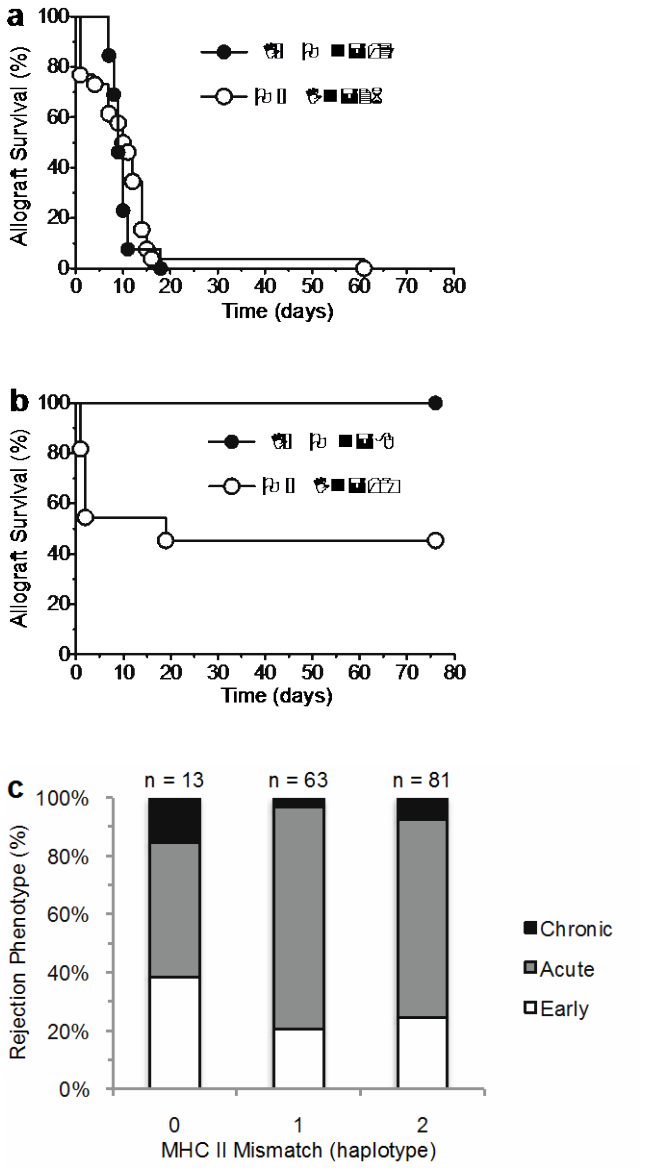

Figure 4-3. Early graft failure is dependent on donor outbred status

Survival of cardiac allografts transplanted from inbred to outbred (BALB/c to CD-1; I to O) or outbred to inbred (CD-1 to BALB/c; O to I) mice in the absence **(a)** or presence of costimulatory blockade **(b)**. Note complete absence of early graft failure phenotype in recipients of allografts from inbred donors. **(c)** Degree of MHC II mismatch between all donor-recipient pairs used in this study as well as contemporaneous experiments involving outbred mice. Note that the degree of MHC II haplotype disparity between donor and recipient does not correlate with graft loss or rejection phenotype.

A.4.5 *Role of antibodies in pathogenesis of early graft failure*

The histopathology of early graft failure described above bears striking resemblance to that of hyperacute rejection observed in sensitized transplant recipients who harbor pre-formed antibodies against donor ABO or HLA antigens (209, 210). We therefore tested for such antibodies in the pre-transplantation serum of mice that went on to develop early graft failure. Neither pre-transplantation serum from 7 such recipients (all outbred) nor serum from additional 10 untransplanted outbred mice caused agglutination of blood from a panel of 10 unrelated outbred mice (data not shown), indicating absence of significant pre-formed hemagglutinins. These results are consistent with reported lack of demonstrable expression or function of blood group antigens in mice (211, 212). We then tested whether recipients that went on to develop early graft failure had pre-formed IgG or IgM antibodies against donor MHC by incubating pre-transplantation serum with donor thymocytes. As shown in Fig. 4a, no anti-donor IgG antibodies were detected while a small increase in IgM antibodies over that found in pre-transplantation serum of one cohort of mice (O to I) that developed acute rejection could be identified, with the caveat that all groups had very low IgM levels to start with. These data indicate the absence of significant pre-formed IgG or IgM anti-MHC antibodies in recipients that develop early graft failure but do not rule out the presence of antibodies directed at other tissue antigens. To explore the latter possibility, we assessed antibody binding to graft tissue 6 and 12 hrs after transplanting outbred hearts into inbred recipients. IgM and IgG could not be detected at 6 & 12 hrs in grafts that already displayed early histopathologic evidence of hemorrhagic necrosis (Fig. 4b). Grafts harvested from prior experiments that had already developed conspicuous hemorrhagic necrosis at 24 hrs after transplantation stained strongly with fluorescein-conjugated anti-IgM or anti-IgG antibodies (micrographs not shown). The presence of significant tissue damage and hemorrhage

at the 24 hr time point, however, makes it likely that fluorescein conjugated antibodies bound to complement and Ig in the extravasated blood rather than in the heart tissue. These data therefore rule out the presence of significant, pre-formed, anti-donor antibodies in recipients that develop early graft failure but do not exclude a contribution of low titer antibodies to the observed pathology.

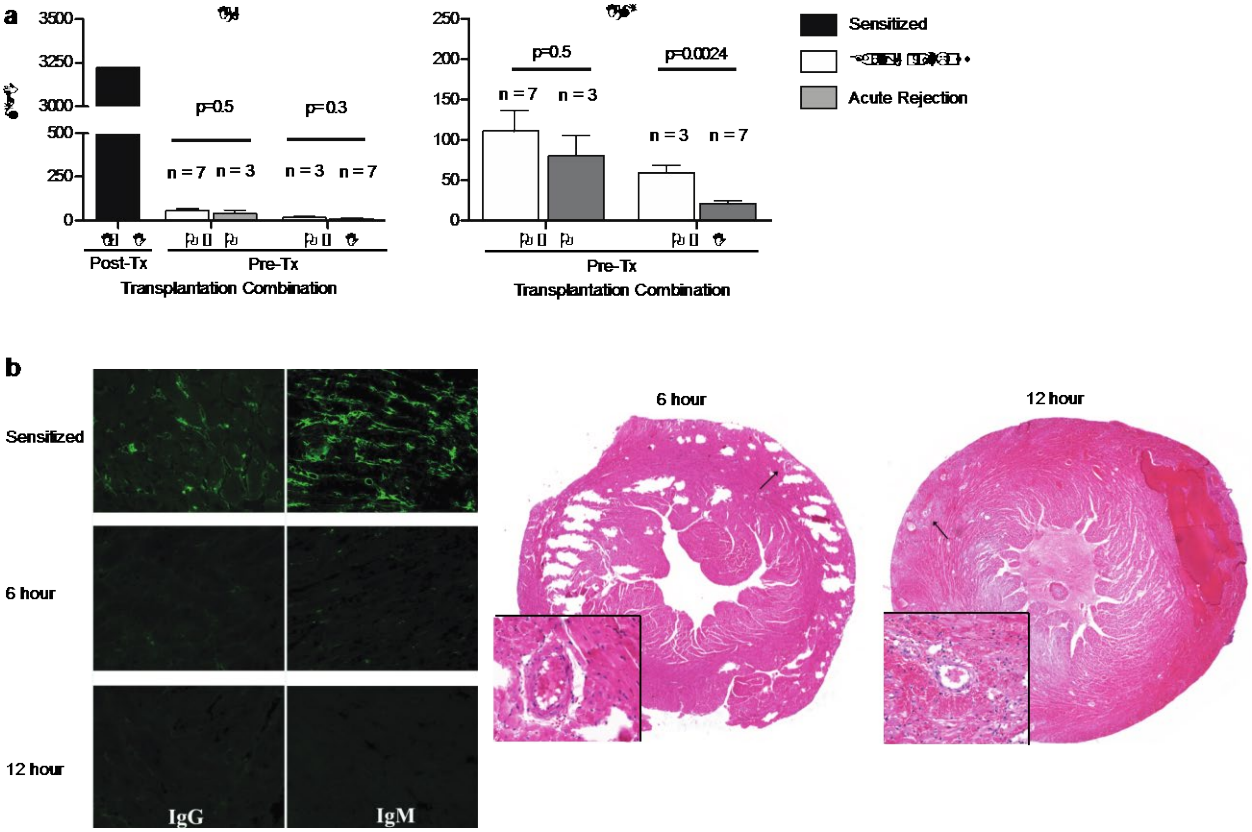

Figure 4-4. Lack of significant, preformed anti-donor antibodies in recipients that exhibited early graft failure

(a) Pre-formed, anti-donor thymocyte IgG (left panel) and IgM (right panel) antibodies in serum of recipients of outbred allografts that went on to develop early graft failure. Serum was obtained prior to transplantation in all mice except the sensitized group. MFI of isotype control antibody was subtracted in each case to determine net binding of IgG or IgM antibodies to donor thymocytes. (b) IgG and IgM deposits in cardiac allografts harvested at indicated time points after transplantation. (Top panels (positive control) show IgG and IgM deposits in

BALB/c cardiac allografts 12 hrs after transplantation to sensitized C57Bl/6 mice. Middle and bottom panels show lack of IgG and IgM deposits in outbred allografts (CD-1 to CD-1) harvested at 6 and 12 hrs after transplantation. These grafts had evidence of interstitial congestion, focal neutrophilic margination, and platelet fibrin thrombi at 6 hrs, and hemorrhagic necrosis with moderate neutrophilic margination at 12 hrs (H&E micrographs), consistent with early graft failure phenotype (magnification = 2x, inset magnification = 30x).

A.4.6 *Early graft failure is dependent on complement activation and neutrophils*

Because of the characteristics of the early graft failure phenotype observed so far, we sought to investigate innate mechanisms that could contribute to this process. We first depleted circulating C3 in outbred donor and recipient mice by administering cobra venom factor (CVF) around the time of transplantation. We found that C3 depletion completely abrogated early graft failure (n = 0/20, p = 0.02 compared to untreated O to O group) (Fig. 5a), but as C3 levels returned to baseline, 5 heart grafts failed between days 8 and 18 after transplantation with histopathologic manifestations of hemorrhagic necrosis (Fig. 5a). These results establish a cause-effect relationship between the complement cascade and early graft failure. They also confirm that the early hemorrhagic necrosis phenotype is not a reflection of an unanticipated high surgical failure rate but is a consequence of a biological process dependent on complement activation.

Since neutrophil infiltration and neutrophilic vasculitis were prominent features of grafts that failed very early (Fig. 2), we then investigated the effect of neutrophil depletion on the incidence of early graft failure. As shown in Fig. 5b, only 2/25 (8%) of neutrophil-depleted inbred recipients of outbred allografts developed hemorrhagic necrosis, which is significantly less than the incidence observed in control, neutrophil-replete mice (7/26 or 27%, p = 0.03). Of the two grafts that had hemorrhagic necrosis in the neutrophil-depleted group, one failed on day

2 with significant neutrophil infiltration, suggesting incomplete neutrophil depletion of the recipient. The other failed on day 7, again with conspicuous neutrophil but no mononuclear cell infiltration, suggesting that early graft failure phenotype could have been precipitated by return of neutrophils to the circulation. These data indicate that neutrophils are important contributors to the pathogenesis of early graft failure.

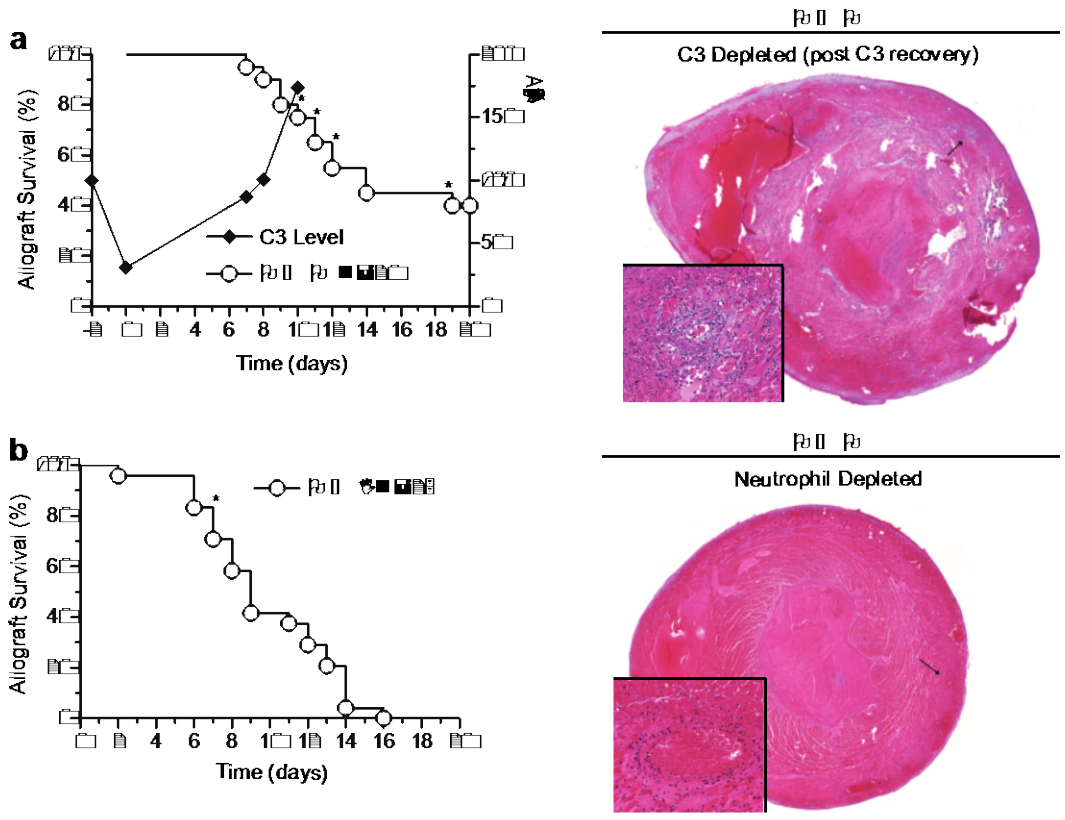

Figure 4-5. Early graft failure is dependent on complement and neutrophils.

Survival of cardiac allografts after complement depletion in both donors and recipients (CD-1 to CD-1; O to O) **(a)** or after neutrophil depletion in the recipients (CD-1 to BALB/c; O to I) **(b)**. Transplantation was performed on day 0. Average C3 levels in recipient blood are depicted as % of baseline level (right-hand y-axis in (a)). *Grafts that exhibited early graft failure phenotype by gross morphology and/or histopathology. H&E micrographs confirmed hemorrhagic necrosis and neutrophilic vasculitis in these grafts (magnification = 2x, inset magnification = 30x).

A.4.7 *Increasing the transplanted tissue's susceptibility to ischemia-reperfusion injury recapitulates early graft failure*

The pathology and mechanisms of early graft failure described so far suggest that donor factors, possibly related to susceptibility of donor tissues to ischemia-reperfusion (I/R) injury, contribute to the early graft failure phenotype. To test this hypothesis, we investigated whether transplanting heart allografts from inbred $CD73^{-/-}$ donors, known to have exaggerated tissue I/R responses (213), recapitulates early graft loss otherwise typical of outbred grafts. CD73 is an ectonucleotidase expressed on endothelial cells that downregulates I/R injury by catalyzing the hydrolyis of extracellular AMP to the anti-inflammatory metabolite adenosine (214). We found that $CD73^{-/-}$ B6 hearts transplanted to BALB/c mice exhibit high incidence of early graft failure (4/18 or 22%) similar to that of outbred allografts transplanted to inbred recipients (7/26 or 27%, $p = 1.0$). Histopathology confirmed that early graft failure was due to hemorrhagic necrosis and neutrophilic vasculitis. Conversely, treating donors and recipients with NECA, a broad adenosine receptor agonist, abrogated early graft loss in the outbred to outbred model ($n = 0/14$, $p = 0.02$). These results suggest that donor factors that underly susceptibility to I/R injury also play a role in the pathogenesis of early graft failure after transplantation.

A.5 DISCUSSION

It has been postulated that hybrid vigor and genetic diversity of the outbred human population could explain why immune therapies that are successful in inbred mouse models sometimes fail in humans (191). In this study, we found that costimulation blockade induces long-term allograft acceptance in outbred mouse stocks implying that hybrid vigor cannot account for the discrepancy between humans and inbred mice but does not completely rule out the contribution of genetic diversity. The outbred mouse stocks used in this study have been bred to maintain maximum heterozygosity (thus, hybrid vigor) but are still somewhat limited in their genetic diversity compared to the human population (208). It is possible then that costimulation blockade may fail to induce allograft acceptance if a more diverse outbred mouse population was studied. Such a population, known as Diversity Outbred (DO) mice, has been recently generated but remains limited in its availability to investigators (http://jaxmice.jax.org/strain/009376.html) (215). Investigating allograft outcomes in these mice in the future should resolve whether simultaneous presence of maximal heterozygosity (hybrid vigor) and allelic variation (diversity) impedes allograft acceptance. Moreover, it is possible that the contribution of these genetic variables is only apparent when outbred mice are housed in non-SPF facilities to increase the repertoire of alloreactive memory T cells through heterologous immunity.

A potentially interesting observation in the outbred to outbred transplantation group (Fig. 1b) is the occurrence of spontaneous long-term allograft survival (>75 days), albeit with significant chronic rejection, in approximately 17% of untreated recipients. The reason for this phenomenon was not addressed in this study but could be related to matching between donors and recipients at non-H-2 loci. By performing a large number of heart transplants between inbred mouse strains matched at either H-2 or non-H-2 loci, Peugh et al found spontaneous long-

term graft survival in 25% of recipients matched at H-2 but mismatched at non-H-2 loci (and vice versa) (216), suggesting that non-H-2 loci modulate the rejection response. Identifying such loci may be a difficult undertaking at present as it is likely that many loci with additive or opposite effects (quantitative trait loci) exist. Moreover, the spontaneous long-term allograft survival phenotype observed in our study all but disappeared when *only* donors or recipients were outbred (Fig. 3a), implying a complex interplay between donor and recipient determinants. This warrants careful analysis in the future.

A principal finding in our study is the identification of a dramatic, early graft failure phenotype characterized by neutrophilic vasculitis and hemorrhagic necrosis in 29 of a total of 97 outbred grafts (30%), but in only 1 of a total of 94 inbred grafts (1%), transplanted to either inbred or outbred mice. All transplants reported in this study were contemporaneous and were performed by the same microsurgeon (Q.L.). Moreover, surgical mortality, which occurred in the first day after transplantation due to failure of the vascular anastomosis, was equivalent in the outbred and inbred graft groups (3.8% and 4.3%, respectively). These facts make it less likely that the early graft failure phenotype was a consequence of high technical failure rate in mice that received outbred grafts. The most compelling evidence that early graft failure is a *bona fide* biological phenotype is its abrogation when specific biological mediators were eliminated (for example, after complement or neutrophil depletion) and its reappearance at later time points when the mediators had rebounded.

The timing and histopathologic hallmarks of the early graft failure phenotype and its dependence on complement but not T cell costimulation strongly indicate that it is caused by innate or inflammatory mechanisms. Although we did not identify the initial trigger of the inflammatory cascade that led to acute hemorrhagic necrosis, one possibility is the binding of

complement-fixing, low titer antibodies that evaded detection by either serology or immunofluorescence. Carroll and colleagues have shown that natural IgM antibodies present in low titers initiate ischemia-reperfusion injury in the heart, intestine, and skeletal muscles (217). These antibodies bind to self-antigens exposed after tissue ischemia and are potent activators of the complement cascade (218). Alternatively, IgM antibodies with anti-donor allospecificities may have been present in recipients that exhibited the early graft failure phenotype. Another possibility is that hemorrhagic necrosis was triggered by the alternative pathway of complement activation, which is independent of antibody binding to the affected tissue. The role of the alternative pathway in the pathogenesis of ischemia reperfusion injury has been established in a variety of experimental models (219).

The predominance of the neutrophil among inflammatory cells infiltrating grafts undergoing hemorrhagic necrosis is not surprising. Neutrophils are attracted to sites of ischemia-reperfusion injury via many mediators, including products of complement activation. The early graft failure phenotype observed in our experiments bears striking resemblance to accelerated rejection of cardiac allografts by IFNγ-deficient mice (220), and to accelerated rejection of IFNγ receptor-deficient renal allografts by wildtype recipients (221). In both cases, microscopic examination of the grafts revealed intense neutrophilic infiltration and parenchymal necrosis. As in our model, neutrophil depletion prevented accelerated rejection, further underscoring the importance of the neutrophil as a mediator of early inflammatory events after organ transplantation.

Another principal finding in our study is that the early graft failure phenotype was dependent on the breeding status of the donor and not the recipient. That donor factors, independent of MHC matching, influence graft outcomes has been documented in both mice and

humans (222). Several studies have pointed to the role of the donor complement system in influencing graft outcomes. For example, donor C3 deficiency leads to prolonged survival of kidney allografts while donor deficiency of decay-accelerating factor (DAF) accelerates cardiac allograft rejection in mice (223, 224). What donor factors influenced graft outcomes in our study is unclear. We tested whether differences in expression of the complement regulatory molecules DAF and Crry among donors correlate with presence or absence of early graft failure but did not find a significant correlation (Reichenbach, Lakkis & Heeger, unpublished). Our observation that inbred $CD73^{-/-}$ donors recapitulate early graft failure suggests that determinants of tissue susceptibility to I/R in the donor influence graft outcomes after transplantation. This warrants future studies to test whether CD39, CD73, and adenosine receptor expression in outbred mice correlates with graft outcomes.

The model of hemorrhagic necrosis described in this manuscript underscores the importance of the donor in shaping graft outcomes and provides an opportunity to gain better understanding of the inflammatory events that lead to graft injury. Interrupting these events could prevent the adverse consequences of ischemia-reperfusion injury on early graft function and possibly temper innate responses that adversely affect long-term allograft outcomes (225).

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