Immunity and tolerance to the tumor-associated antigen MUC1

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Human mucin 1 (MUC1) is a highly glycosylated transmembrane glycoprotein that is expressed on the luminal surface of ductal epithelial cells. Human adenocarcinomas overexpress MUC1 as a tumor-associated antigen (TAA) that presents to the immune system peptide epitopes and glycopeptide epitopes with tumor specific carbohydrates, such as mono- and disaccharides known as Tn, sialyl-Tn, and TF antigens. Studies in MUC1 transgenic (MUC1-Tg) mice have indicated that, compared to transgene negative wild-type (WT) mice, the MUC1-Tg immune system maintains a certain level of tolerance to the MUC1 peptide, reflected most notably in decreased CD4 T cell help. We made a novel observation that in contrast to suppressed responses to the MUC1-peptide vaccine, vaccination of MUC1-Tg mice with tumor-associated MUC1 glycopeptide resulted in effective anti-MUC1 immunity similar to that elicited in WT mice. We hypothesized that the tumor-associated glycopeptides were seen as foreign and therefore not subject to tolerance. To study CD4 T cell responses to MUC1 glycopeptides we generated glycopeptide GST(GalNAc;Tn)A specific, MHC-Class II restricted CD4 T cell hybridoma, RF6. We cloned the RF6 TCR (RFT; Vα4.1Jα16-Vβ15Jβ1.3) and generated TCR transgenic mice RFT-Tg. Using the RFT-Tg mice and the previously generated MUC1 peptide-specific TCR transgenic VFT-Tg mice, we show that peptide-specific VFT CD4 T cells transferred to MUC1-Tg mice are suppressed through mechanisms of peripheral tolerance, which are not induced against MUC1-glycopeptide specific RFT CD4 T cells. We show that peptide-specific CD4 T cells are transiently activated upon transfer into MUC1-Tg mice, suggesting that

MUC1-peptide epitope is presented in the periphery in healthy mice as well as in tumor bearing mice. In contrast, MUC1-glycopeptide epitopes are tumor specific and thus treated as foreign antigens in MUC1-Tg mice, resulting in effective activation of glycopeptide-specific CD4 T cells. Simultaneous activation of glycopeptide-specific T cells can break tolerance of peptide-specific T cells. Our findings with MUC1 apply to other TAA that contain some epitopes that are "self" and subject to self tolerance and other epitopes that are tumor specific ("foreign") and not affected by tolerance. Understanding this distinction is very important in the development of effective and safe cancer vaccines.

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PREFACE

I would like to acknowledge the guidance given by Dr. Olja Finn, my dissertation advisor and mentor. Thank you, Olja. I would also like to thank all the members of the Finn Lab, past and present, for insightful discussions and assistance.

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Contributors to this thesis work are indicated as co-authors in the publications listed at the beginning of Chapter 1 and 2. In addition, Michael S. Turner provided the data for Figure 15.

1.0 INTRODUCTION

Portions of Chapter 1 have been adapted from "Tumor antigen-based immunotherapy and immunoprevention of cancer", Ryan, S.O., K.R. Gantt, and O.J. Finn. 2007. *Int Arch Allergy Immunol* 142:179-189; with permission from S. Karger AG, Basel. Copyright permission is kept on file with Sean O. Ryan.

Important to our understanding of the immune system's response to cancer has been resurgence in patient studies and development of relevant animal models that support the immunosurveillance hypothesis. Proposed in 1970 by Burnet and Thomas, the term 'immunosurveillance' implies that many tumors occur but never progress to clinical disease because they are cleared naturally by the immune system (1). This hypothesis postulates that cancer can overcome the individual response when it evolves to evade immune defenses, or when the immune system becomes compromised.

Immunosurveillance was difficult to test experimentally when originally proposed and remained primarily a hypothesis for many years. This was partly due to the lack of an appropriate mouse model. The evidence that the immune system prevents tumor development became clear as differences between mouse strains were better understood and reagents that addressed the role of cytokines and other immune mediators became available (1). For example, IFNγ and perforin were shown to be necessary for tumor rejection in mice. When IFNγ responses were eliminated, such as in IFNγ receptor- or STAT1-deficient mice or with monoclonal blocking antibody to IFNγ; susceptibility to methylcholanthrene-induced tumors was increased.

These same IFNγ-unresponsive mice crossed with p53 tumor suppressor-deficient mice showed an increase in tumor burden when compared to mice with p53 deficiency alone, implying that IFNγ is necessary for continued protection against tumors. Perforin-deficient mice demonstrated a similar susceptibility to tumor formation compared to controls (1).

Spontaneous murine tumor models based on transgenes that carry known oncogenic mutations, such as K-ras, INK4A and PTEN, are making it possible to determine what early steps in tumor development are subject to immunosurveillance (2, 3). The ability to time and examine early neoplasia in mice will allow an evaluation of which immune effector cells, cytokines, and other immune components are induced and necessary for eradication of transformed cells.

1.1 IMMUNE RESPONSE TO TUMORS

With the acceptance of the immunosurveillance hypothesis it has become apparent that giving the immune system the appropriate stimulus will allow it to overcome evasion and eliminate tumors. Of course, by the time most patients are diagnosed with a malignancy, they have been harboring the tumor for some time and are usually immunosuppressed (4). This is likely to be the reason that early detection and treatment of disease result in the best clinical prognosis as treatment occurs before immunosuppressive effects have developed fully (5, 6). Increasingly, solutions to the cancer problem are focused on immunoprevention and early stage immunotherapies (Figure 1). In addition to recent preclinical studies (7-9), the utility of prophylactic vaccination for immunoprevention of cancer is gaining attention after the FDA

approval of a multivalent vaccine against human papilloma virus that prevents viral infection and thus cervical cancer (10).

The immune responses that are most effective against tumor growth are those generated through a well-balanced and well-timed interplay between the innate and the adaptive immune system. Antigens are presented by activated dendritic cells (DCs) to CD4 T cells, which in turn coordinate CD8 cytotoxic T lymphocytes (CTL) and antibody production by B cells (11). In some cases the contribution of activated NK and NKT cells, which like CTLs use perforin and granzymes to destroy their targets, have been shown to be necessary for tumor control (12, 13).

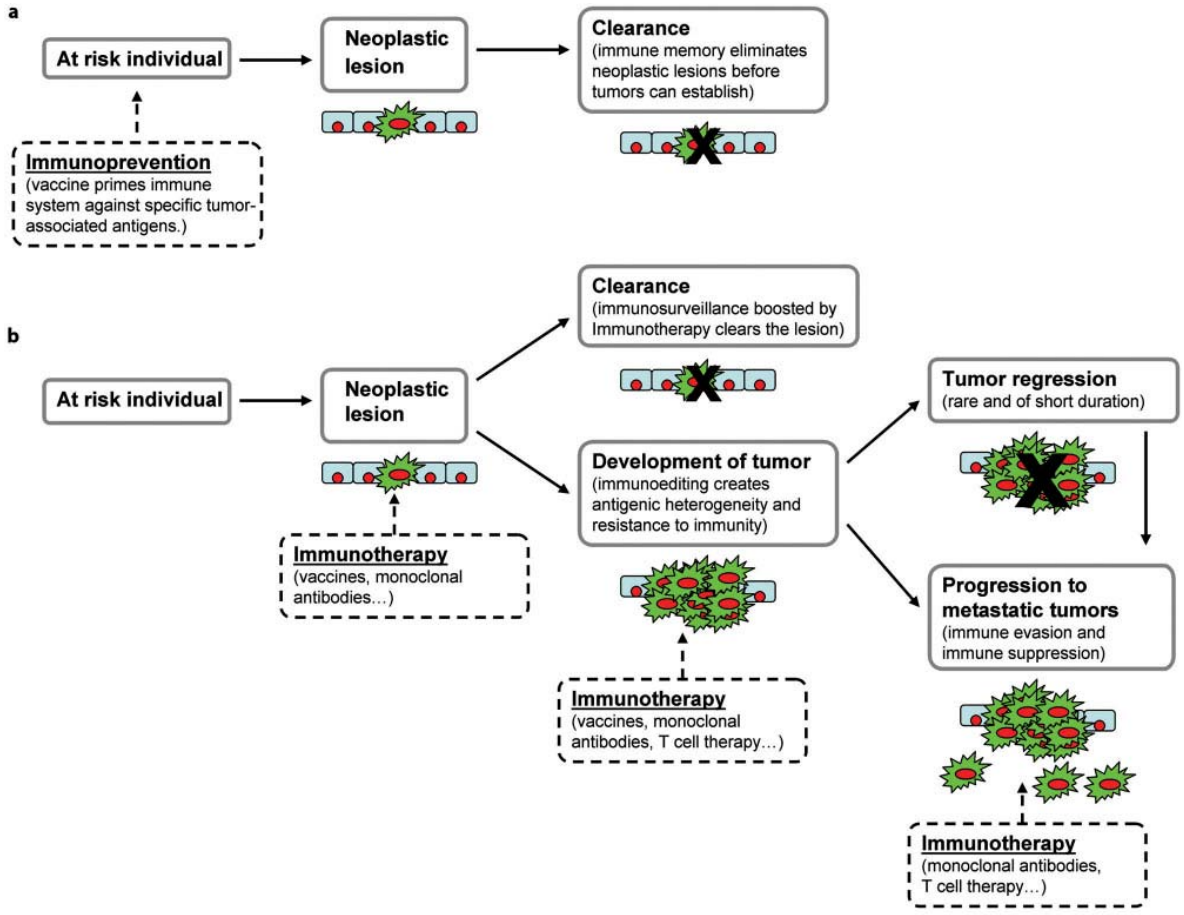

Figure 1. The difference between immunoprevention and immunotherapy is in the timing and the expected outcome.

a) Immunoprevention uses a vaccine in an individual at risk for developing cancer, to generate immunologic memory that prepares the immune system to detect and eliminate future premalignant lesions. b) Conversely, immunotherapy addresses the disease after it is already diagnosed. Immunotherapies can take the form of vaccines or monoclonal antibody therapies among others. Depending on when the disease is detected, immunotherapies can be given early (neoplastic lesions) or late (primary and metastatic tumors) in the disease process. Due to limitations in detecting neoplastic lesions most immunotherapies are given at later stages of disease and show success primarily in the setting of a minimal tumor burden.

When the balance between the innate and the adaptive immunity is skewed in favor of innate immune responses, this can also exacerbate disease and facilitate tumor growth. Some components of the innate immune response found in tumors, such as macrophages and mast cells, have been correlated with increased tumor growth and metastasis (14, 15). Upon activation, these cells release oxidants, prostaglandins, cytokines, and other components that directly and indirectly cause tissue destruction and remodeling. Peripheral granulocytes from patients with metastatic adenocarcinomas have been shown to release hydrogen peroxide which in turn impairs T cell function (4). Tumor-infiltrating macrophages can secrete matrix metalloprotease-9 (MMP9) that releases VEGF from extracellular matrix stores, increasing angiogenesis at the tumor site and suppressing DC function (16).

Chronic inflammation, characterized by sustained activation of many players in the immune system, is suspected to drive premalignant lesions into fully malignant states. Inflammation can occur in response to chronic infections with a diverse array of pathogens such as hepatitis viruses or *Helicobacter pylori* (17). Sustained inflammation can also be the result of an individual's inability to restore immune homeostasis after infection. The increased expression of COX-2, an enzyme in the prostaglandin biosynthetic pathway, has been correlated with colorectal cancer (18). Similarly, blocking the function of this enzyme with nonsteroidal anti-

inflammatory drugs has correlated with decreased cancer risk in humans, particularly in colon cancer (19). Consequently, halting chronic inflammation may restore the immunosurveillance mechanisms necessary to control transformation and neoplastic growth.

In contrast to chronic inflammation, acute inflammation has been shown to eliminate some cancers. One current therapy based on the principle of eliciting strong acute inflammation uses bacillus Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium tuberculosis*, to treat bladder cancer (20). Intravesicular instillation of the bacterium into the bladder activates resident DCs, which then increases antigen presentation and cytokine production. The first immune-based therapy that used acute inflammation to fight against cancer was the administration of 'Coley's toxins'. William Coley had observed that patients with sarcomas that also suffered from erysipelas, a severe bacterial skin infection coupled with a fever, experienced remission of their tumors. Based on this evidence, he subsequently injected patients with a combination of heat-killed *Streptococcus* and *Serratia marcescens* (21-23). Now it is understood that the bacterial products Coley injected provided the 'danger signals' that induced DCs to give efficient costimulation to both the innate and the adaptive arm of the immune system (24). Additionally, the increased inflammation and necrotic tissue at the site of the sarcoma, where the injection of bacterial products was made, would have increased the number and efficiency of antigen-presenting cells (APCs). Without antigen presentation and strong costimulation, some tumors can progress unchecked by the immune system.

The above examples describe instances where tumor progression or tumor rejections are byproducts of the immune response activated through dangers other than the tumor itself. While one can learn a great deal from studying these processes, especially regarding the immune effectors and their targets on tumor cells, only an elicited tumor-specific immune response can

be turned into a therapy with a reproducible outcome. For generating the ultimate anti-tumor effector cells, tumor-specific antigens need to be presented on professional APCs. The most potent of these are DCs, which are being targeted *in vivo* or manipulated *in vitro* for use as adjuvants in numerous current therapies (11).

1.2 TUMOR IMMUNOTHERAPY

Several approaches to target tumors are currently being pursued that exploit the specificity of the immune system. The first among these is the vaccine approach. Traditionally vaccines have been exceptionally effective at priming the immune system to protect against infectious diseases. Based on this successful protection against infectious disease, vaccines may also be effective at priming an individual's immune system to control their own malignancies. Choosing an adjuvant and target antigen has proven challenging and success is likely to result from polyvalent approaches that utilize as many aspects of the immune response as possible. Timing is also important; the most effective vaccination against pathogens occurs before infection and this is likely to be most effective for malignancies as well (25). Therapeutic vaccines that treat patients after diagnosis of malignant disease are currently being tested (26). However, the immense success of the one prophylactic cancer vaccine that immunizes against human papilloma virus and protects against cervical cancer has reinforced that prevention is better than therapy during disease (27). Additional evidence that immunologically relevant events protect against developing malignancies later in life would argue that such prophylaxis is the route to follow. One recent report from our laboratory demonstrates that bone breaks, contraceptive use, mastitis,

and pelvic surgeries are protective against ovarian cancer with the hypothesis that the anti-MUC1 responses induced during those episodes provide protection from malignancies (28).

1.3 ANTI-TUMOR VACCINES

When vaccinating an individual, the goal is to provide an effective immune memory response to antigen challenge. Whether the targeted antigen comes from a pathogen or is from a transformed cell, the same principles apply. There are three main questions to answer when developing a vaccine: what antigen(s) to target, what is the ideal time and route of vaccine delivery, and what adjuvant to use in order to elicit a desired type of immune response?

1.3.1 Tumor Antigens

When vaccinating against an infectious agent, the antigens used are foreign to the host immune system and thus are free from many of the complicating concerns of cancer antigens being self-antigens. Because malignant transformation comes from the 'host material' immune responses against many of the antigens expressed on tumors are either subject to self-tolerance or potentially could result in autoimmunity if tolerance is broken. However, there are several classes of antigens that make good potential targets for use in vaccines directed against tumors because their restricted expression or expression pattern is characteristic of tumors rather than healthy tissue, and thus not hindered by self-tolerance or prone to generate adverse autoimmune effects.

Some selected characteristics of tumor antigens that make them useful targets for immunotherapy are: (1) common expression on a variety of carcinomas thus making the vaccine more broadly applicable, (2) stable expression through different stages of tumor development so that stem cells, progenitor cells and mature tumor cells can all be targets of the elicited immune responses, and (3) indispensable for tumor survival and thus not susceptible to immunoediting (29). Many tumor antigens identified to date have these characteristics. They belong to several categories that include products of mutated genes, viral antigens, differentially expressed antigens and tissue-restricted antigens. Most tumor antigens characterized to date are differentially expressed antigens and tissue-restricted antigens.

1.3.1.1 Differentially Expressed Tumor Antigens

Early attempts at understanding tumor immunity were based on the assumption that the immune system can only recognize molecules that are expressed in tumors as a result of many oncogenic events, and not on normal cells. However, this assumption proved incorrect and the majority of molecules identified as human tumor targets were found to have the same gene and protein sequence as the normal cells that gave rise to the tumor. The differences seen by the immune system were instead quantitative, such as antigenic overexpression, and/or qualitative, such as aberrant posttranslational modifications. We describe below three well-known tumor antigens that belong to this category and also fulfill the criteria for good vaccine candidates.

Human mucin 1 (MUC1) has been studied as a tumor antigen and target for immunotherapy for over a decade. In healthy tissues, MUC1 is expressed at low levels on the apical surface of ductal epithelial cells as a heavily glycosylated transmembrane protein. Conversely, in the majority of human adenocarcinomas, MUC1 is overexpressed and hypoglycosylated (30, 31).

Many patients with MUC1+ tumors have low levels of specific CTLs (32, 33) and low antibody titer (34, 35), and both types of responses have been shown to be specific for the polypeptide core of MUC1. Even though the patients have detectable tumor MUC1-specific immune responses, most succumb to their disease. As a preclinical model, we have shown the safety and immunogenicity of three different MUC1-based vaccines in the MUC1-transgenic mouse (36). In addition, we showed an important difference in the anti-MUC1 responses when MUC1 is expressed as a self-molecule as compared to the wild-type mouse where it is not endogenously expressed. We and others have attributed this difference to the hypo-responsiveness in the MUC1-specific CD4 T helper cell compartment (36-38). A new opportunity for MUC1 tumor vaccine preclinical studies has emerged with the development of spontaneous tumor models in the MUC1-transgenic mouse (39-41). These will allow a more physiological comparison to humans concerning the effectiveness of MUC1-specific vaccines. In addition to mouse studies, we have used a MUC1 peptide with adjuvant LeIF (*Leishmania braziliensis*-derived protein) vaccine in nonhuman primates that was safe, tolerable, and capable of inducing an anti-MUC1 cellular immune response (42). More recently we completed a phase I trial using a vaccine composed of the MUC1 100-amino acid core polypeptide with the adjuvant SB-AS2 (monophosphoryl A, purified saponin and an oil-water immersion) to treat pancreatic cancer patients after tumor resection (43). This trial showed the vaccine to be safe and to have the potential of inducing MUC1-specific immune responses.

HER2/neu (also known as Erb-B2) is expressed as a 185-kDa glycoprotein surface receptor, member of the epidermal growth factor family of tyrosine kinase receptors. HER2/neu functions in cell cycle regulation and its expression on healthy tissues is low (44). Numerous adenocarcinomas show HER2/neu overexpression, including those of the breast, ovary, colon,

prostate, and lung. HER2/neu overexpressing tumors have been characterized as being more aggressive and linked to shorter patient survival (45).

A finding that indicated the potential of HER2/neu as a vaccine candidate and target for immunotherapy was the presence of preexisting anti-HER2/neu immune responses in cancer patients with HER2/neu-overexpressing tumors. As in the case of MUC1 immunity, even though the response is too low to effectively clear the established tumor, it indicates that the adaptive immune repertoire had not been depleted of HER2/neu-specific B and T cells through tolerance mechanisms to self-proteins (46).

The most notable HER2/neu-based immunotherapy is the humanized anti-HER2/neu antibody Herceptin® (trastuzumab). Originally generated and studied in mice, Herceptin is currently in phase III trials, where it has been shown to confer longer disease-free survival in HER2/neu+ breast cancer patients (47, 48). In addition to Herceptin, invoking cellular anti-HER2/neu responses has been shown to control tumor growth in spontaneous breast cancer models in HER2/neu-transgenic mice (49, 50) and breast and ovarian cancer patients (51).

Cyclin B1 was recently identified to be a tumor antigen by our group and shown to be constitutively overexpressed in the cytoplasm of tumor tissue and tumor cell lines (52). Under normal conditions cyclin B1 is transiently expressed in the nucleus as a mediator of the G2-M phase transition in the cell cycle. Our group and others have shown cyclin B1 expression to be regulated by p53, and overexpression in transformed tissue has been associated with the deregulation of this well-studied tumor suppressor gene (53, 54). Cyclin B1 deregulation has been associated with oncogenesis as well as poor prognosis (55).

Cyclin B1 overexpression and anti-cyclin B1 immune responses have been identified in patients with various adenocarcinomas, including those of the colon, pancreas, breast and lung

(52, 56-58). Current preclinical studies on human cyclin B1 have been limited to human tissue samples and cell lines. Our group has performed studies examining human cyclin B1 immune responses and mouse cyclin B1 anti-tumor vaccines (59, 60) using the $p53^{-/-}$ mouse model of spontaneous carcinoma (61).

1.3.1.2 Tissue-Restricted Antigens

In contrast to differentially expressed antigens, it was gradually recognized that some tumor antigens were not expressed on the normal tissue that tumors originated from. These tumor antigens are referred to as tissue-restricted antigens because their expression in healthy individuals is either restricted to fetal development, before the adaptive immune system completely matures, or restricted to immune privileged sites and not accessible to immune surveillance. As such, they make excellent vaccine targets, as previously arising tolerance and autoimmunity are less likely to be complicating factors.

Identified in 1965 (62), carcinoembryonic antigen (CEA) has been widely studied for its role as a tumor marker and tumor antigen. CEA is a 180-kDa glycoprotein, found both at the cell surface and in a secreted form. CEA is categorized as an oncofetal antigen, expressed at high levels in the fetal gut during development, and more recently CEA was found in some cases to be expressed at low levels in the adult colon (63, 64).

A clear function of CEA in healthy and transformed tissue has yet to be defined. Studies have indicated a role as an intercellular adhesion molecule (65, 66) and when overexpressed, an inhibitor of anoikis (67), a form of apoptosis triggered by insufficient cell-matrix contacts (68). In addition, CEA overexpression on neoplastic tissue has a role in tumor progression and metastasis (69). Tumor CEA has an aberrant glycosylation pattern (70, 71) and is overexpressed on a large number of adenocarcinomas, including 90% of gastrointestinal, colorectal, and

pancreatic cancers, 70% of non-small cell lung cancers, and 50% of breast cancers (72). The tumor expression profile of CEA on a large number of different adenocarcinomas, in combination with its possible role in tumor progression and metastasis, makes CEA a promising target for immunotherapy.

CEA-transgenic mice have been used for preclinical studies of various forms of CEA-targeted immunotherapies. Vaccines using recombinant pox viral vectors (vaccinia, ALVAC, fowlpox), DCs, or anti-idiotype antibodies have been shown to generate anti-CEA-mediated protective immunity to subsequent tumor challenge (72). The anti-CEA immune response was also demonstrated in HLA-A2:CEA double transgenic mice (73). Importantly, these studies showed that intrinsic tolerance to CEA could be overcome in the CEA transgenic mice without causing any adverse autoimmune effects (74, 75). Following the animal studies, several human studies and phase I trials have been completed showing safety as well as induction of anti-CEA immune responses and anti-tumor effects (76).

The NY-ESO-1 antigen falls into a subcategory of tissue-restricted antigens, the cancer/testis antigens. Expression of these antigens is normally restricted to germ cells and trophoblasts but is also expressed on a wide variety of cancers. Over 40 antigens have been identified as cancer/testis antigens with NY-ESO-1 having been the most studied to date due to its strong tumor-specific immunostimulatory capacity (77). Completed clinical studies using an NY-ESO-1 and ISCOMATRIX adjuvant vaccination strategy showed the vaccine to be well tolerated, safe, and capable of inducing potent immune responses (78), as well as possible clinical responses (79). Currently a number of clinical trials treating various cancer types are being conducted using differing combinations of NY-ESO-1 and adjuvants (80).

In recent years there has been a trend for anti-tumor vaccines to include multiple tumor antigens (81, 82). Considering the rapid growth rate and genetic instability of growing malignancies, combining multiple antigens into a single vaccine will help to prevent development of tumor antigenic loss variants (83, 84). In addition to addressing tumor antigenic loss variants, other tumor escape mechanisms need to be considered. These mechanisms can include activity of T regulatory cells, expression of regulatory molecules, and/or production of anti-inflammatory cytokines (85, 86).

1.3.2 Vaccine Design

Currently most therapeutic vaccines use single antigens and most are chosen for their ability to elicit cytotoxic T cell responses (87). There is evidence from animal studies, however, that for full tumor control involving establishment of a strong memory response, more may be needed than just an effector T cell response (88). A vaccine that can elicit a more comprehensive immune response involving helper and cytotoxic T cells, as well as a strong antibody response, is likely to be the vaccine that can provide effective tumor control. This type of vaccine will require either multiple tumor antigens or multiple epitopes derived from the same antigen, as well as adjuvants that stimulate good innate immune responses and production of cytokines important for supporting both arms of the adaptive immune system. In our own studies of MUC1 vaccines we have favored a long peptide (100 amino acids) as antigen because it contains epitopes recognized by CTL, helper T cells (89, 90) and B cells (35). The use of longer peptides is now advocated by other groups as well (91).

How a vaccine is administered is another important consideration in vaccine design. We discussed above how important timing relevant to disease occurrence is likely to be in

vaccination. The route of entry for antigens and associated adjuvants is equally important (92, 93). Traditionally, vaccines have been injected intramuscularly, regardless of the site of cancer. This is suboptimal in two very important ways. Intramuscular injection is likely to elicit systemic immunity. Cancers that occur in mucosal sites, particularly those involving mucosal epithelium might require a good mucosal immune response instead and thus mucosally administered vaccines would be a better strategy. The microenvironment of gastric and gynecologic mucosa is a tolerogenic one that adenocarcinomas evolve within, and to target them appropriately a mucosal route of administration of antigen and potent mucosal adjuvants are necessary (93). An example of a successful mucosal vaccine is FLUMIST^{TM}, an intranasal vaccine against influenza. This vaccine as well as other effective vaccines against pathogens can inform cancer vaccine design. Adjuvants were mentioned several times above in connection with vaccine design. The reason is that administering antigens that do not activate APCs and induce high levels of costimulatory molecules would simply induce tolerance.

One example of a promising adjuvant is heat-labile toxin of *Escherichia coli* (LT). When given topically over the site of antigen injection, LT is believed to activate APCs found in the skin (Langerhans cells) which take up antigen and become potent immunostimulatory cells (94). The topical LT adjuvant given with an influenza vaccine has shown an increase in influenza-specific immune response in elderly adults (>60 years) compared to vaccine alone (95). This finding has implications to tumor immunotherapy due to the fact that more cancers predominantly arise in the elderly. More recently we have shown in a mouse model the effectiveness of LT adjuvant in boosting effective anti-tumor immunity (59).

Because of their ability to take up antigens and process them into MHC I- and MHC II-restricted peptides that can prime or boost tumor-specific CD8 and CD4 T cells, and because

they can be activated to express costimulatory molecules and various important cytokines, DCs are known as 'nature's adjuvants'. DC-based vaccines use autologous DCs generated *in vitro* to present tumor antigens along with potent costimulation to T cells. Immature DCs grown *in vitro* are loaded with a tumor antigen of choice, most often through the process of phagocytosis that traditionally targets antigen for MHC II presentation. This is responsible for generating a tumor-specific T helper response. Sustained responses against tumors require CD4 T cell help (11). An effective immune response against tumors needs a vigorous CD8 CTL response that requires presentation of tumor antigens in MHC I. DCs have the ability to cross-prime; antigens from the same source can be presented on both MHC I and MHC II. *In vitro*, DCs have been loaded with peptides that are specific to MHC I and MHC II by using specific amino acid sequences (96). This method is rather inefficient and is limited by the number of peptides available. However, DCs can be induced to cross present antigens depending on the presence of proinflammatory cytokines and CD40 ligation (11). Targeting of the endocytic receptor DEC-205 with an antigen-antibody conjugate has also capitalized on this pathway to target a more complete immune response (97, 98).

1.4 PRECLINICAL MODELS

The most commonly used animal models in tumor immunology research are mice. Since the majority of human tumor-associated antigens are derived from self-antigens, it is important to test anti-tumor vaccines in an environment that also expresses the tumor antigen in its normal, self-antigen form. This need led to the engineering of mice expressing human tumor antigens as transgenes, for example MUC1- and CEA-transgenic mice (75, 99). Using the transgenic mice

along with comparable transgene-negative mice can answer questions about the efficacy of the therapy, endogenous immune tolerance mechanisms, as well as possible autoimmune damage to healthy tissue.

Until recently, the majority of studies assessing the effects of *in vivo* tumor immunotherapy involved post-vaccination tumor challenge by injection of a cultured tumor cell line. Although this method has demonstrated tumor-associated antigen-specific protection, these models do not fully represent the etiology of human disease. To better mimic the interaction of the innate or vaccine-induced immune response with the slow progression of carcinoma that occurs in humans, spontaneous tumor models have been developed (2, 3, 100-103). One of the newest is a model based on a conditionally expressed mutant of Kras (104, 105). The fact that Kras is an oncogene that is commonly mutated in human tumors makes the model very attractive to study neoplastic growth and the immune responses right from the start of cellular transformation. More specifically, spontaneous tumor models allow one to follow the appearance and role of specific tumor antigens in oncogenesis and disease and the immune response against it (7).

1.4.1 Human MUC1 transgenic mouse

Initial mouse studies examining the immunogenicity of MUC1 vaccines appeared promising, showing that robust effective anti-MUC1 immunity could be generated (106, 107). However, these findings were misleading since the extracellular region of MUC1 shares little homology with mouse Muc1 (108) and thus could potentially be seen as foreign in this environment. The need to evaluate MUC1 immunity in an environment that mimics the human system led to the generation of the MUC1 transgenic (MUC1-Tg) mouse (99). The initial study using the MUC1-

Tg mouse (99) and well as subsequent studies have shown that there exists a level of self tolerance against tumor-expressed MUC1 (37), and more specifically tumor-associated MUC1 peptides (109-112).

The transgene used to generate the MUC1-Tg mouse was a 10.6-kb genomic SacII fragment containing the entire MUC1 gene sequence, as well as 1.5 kb of 5' sequence and 800 bases of 3' sequence (113). Expression of MUC1 in MUC1-Tg mice is seen in the cells and organs that normally express the molecule in humans, without expression at ectopic sites (99). In addition, adenocarcinomas induced in MUC1-Tg mice express the abnormal tumor-associated form similar to that of humans (104, 114, 115).

1.5 SELF TOLERANCE TO TUMOR-ASSOCIATED ANTIGENS

T cells specific for tumor-associated antigens (TAA) are detectable in the blood, tumors, and draining lymph nodes of individuals with cancer, yet attempts to boost those responses have yielded inconsistent results and many patients still succumb to their disease. Several new mouse models that express human tumor antigens as transgenes are beginning to reveal that in spite of the ability of many of these antigens to induce antibodies and T cells in cancer patients, they may nevertheless be subject to a certain level of self tolerance mediated by different mechanisms (37, 74, 116, 117). In the case of human melanoma, the self tolerance to TAAs is reflected in findings that immunotherapies that confer a survival benefit in patients with advanced disease can also induce autoimmune side effects (118, 119).

These relevant mouse models have shown that self tolerance (i.e. suppressed responses) exists to most of the TAAs examined to date. The suppressed responses, as compared to wild

type responses in transgene negative mice, indicate that TAA-derived peptide epitopes are seen as self and thus may not be ideal tumor-specific antigens. Recent studies have shown that both thymic (central) and peripheral tolerance can suppress T cell responses to tumor-associated peptides (74, 116, 120), yet the precise mechanisms are not clear.

1.6 MUC1 IN HEALTH AND DISEASE

1.6.1 Normal MUC1

MUC1 is expressed on the luminal surface of glandular and ductal epithelium and by some hematopoietic lineages (121) as a type 1 transmembrane glycoprotein. The MUC1 gene consists of seven exons, including exon 2 that contains a variable number of tandem repeat (VNTR) domain. This extracellular VNTR domain consists of 25-125 individual 20 amino acid sequence repeats, PDTRPAPGSTAPPAHGVTSA. The threonine and serine residues of the VNTRs carry an extensive, densely branched pattern of O-linked glycosylation that effectively masks the peptide backbone of normal MUC1 (122). The biological function of normal MUC1 has been suggested in mucociliary clearance, in addition to cell-cell adhesion and anti-adhesion mechanisms. In addition, the highly conserved cytoplasmic domain of MUC1 contains seven tyrosine phosphorylation sites indicating a role in signal transduction pathways (123).

1.6.2 Abnormal MUC1

Tumor-associated MUC1 (abnormal) expressed by transformed epithelium differs from normal MUC1. Abnormal MUC1 is characterized by its increased expression, loss of luminal polarity, and an altered glycosylation profile. This abnormal glycosylation pattern contains a predominance of shortened *O*-linked carbohydrate residues, such as the tumor-specific TF antigen (Galβ1-3GalNAc-*O*-S/T), Tn antigen (GalNAcα-*O*-S/T), and sialyl-Tn antigen (sialyl-GalNAcα-*O*-S/T), in addition previously masked peptide epitopes are now exposed (122).

The tumor-associated carbohydrate antigens are not normally exposed on healthy cells or tissues with benign disease. Conversely, ~90% of carcinoma tissues (many being adenocarcinomas) express these glycoepitopes (carbohydrates) (124). The glycoepitopes are also associated with cancer cell adhesion and aggressiveness (125-127), and have been studied as targets for immune-based elimination of carcinomas (128, 129).

Recent studies have suggested that MUC1 functions as an oncoprotien playing an active role in the malignant phenotype. This is in part based on findings that the MUC1 cytoplasmic domain interacts with ErbB receptors, c-Src, β-catenin, and p53, ERα transcription factors; suggesting a role for MUC1 in numerous signaling pathways which have links to carcinogenesis. In addition, overexpression has been shown to allow anchorage-independent cell growth and tumorigenicity [summarized here (130)].

1.7 CONCLUSION

Each cancer can be as unique as the patient that suffers from it but there are also antigenic similarities that can be exploited for immunotherapy. As the individual's immune system and neoplasm coexist for a prolonged period of tumor development, each exerts influence on the other. Two promising areas of tumor immunotherapy are vaccines and monoclonal antibody-based therapies. An important difference between these two therapies is that monoclonal antibodies are limited to the presence of disease, whereas tumor antigen-based vaccines can serve either as a tumor immunotherapy (concurrent with disease) or as a prophylactic treatment (for individuals at high risk for cancer). The best-case scenario would be to prevent disease from occurring, by using prophylactic vaccination. This has worked exceptionally well in the case of infectious disease. Development of many technologies that identify early disease and/or individuals at high risk for disease, combined with the improved understanding of the requirements for greater efficacy and safety of anti-tumor immune responses, has brought cancer immunoprophylaxis into the mainstream of cancer prevention efforts.

1.8 STATEMENT OF PURPOSE

As described above, it is now well established that the immune system plays a vital role in controlling tumor growth. A large focus of current tumor immunology involves vaccine studies attempting to harness the exquisite fine-specificity and memory capabilities of the adaptive immune system to prevent cancer occurrence. Important to successful vaccinations are

not only the use of a proper adjuvant and delivery methods, but also the choice of antigen(s) that can induce a strong, targeted cellular and humoral immune response (131). With proper immune modeling it is becoming increasingly clear that peptide epitopes derived from tumor-associated antigens are subject to pre-existing self tolerance and thus are not completely tumor-specific (74, 109, 111, 116, 117, 120). The ideal tumor-specific antigen contains epitopes that are foreign to the immune system thus allowing for tumor cell cytotoxicity while leaving healthy tissue unharmed. ***We hypothesize that immune responses to MUC1 peptide epitopes are suppressed due to tolerance mechanisms that effect functional CD4 T cell responses, but tumor-associated MUC1 glycopeptides contain tumor-specific epitopes that are seen as foreign and therefore not subject to self tolerance.*** We tested this hypothesis in two specific aims.

Specific Aim 1: Compare the relative immune responses to tumor-associated MUC1 peptide and glycopeptide antigens in WT and MUC1-Tg mice.

To address Aim 1 we have used two vaccination protocols, soluble adjuvant-based and DC-based, which are used to induce immune responses, where possible, to MUC1-peptide or MUC1-glycopeptide antigens in WT or MUC1-Tg mice. To further understand the immune responses generated to these two tumor-associated MUC1 antigens, we have also generated a panel of MUC1-responsive CD4 T cell hybridomas. The results of Aim 1 are described in Chapter 2.

Specific Aim 2: Assess possible mechanisms of tumor-associated MUC1 tolerance by examining MUC1 peptide and glycopeptide –specific CD4 T cell development and function in MUC1-Tg mice, as compared to WT mice.

To address Aim 2 we have generated a MUC1-glycopeptide –specific RFT T cell receptor (TCR) transgenic mouse, which complements a previously generated MUC1-peptide – specific VFT TCR transgenic mouse. The generation of the RFT mouse is described in detail in Chapter 3. Chapter 4 describes the results of T cell adoptive transfer experiments using peptide-specific VFT CD4 T cells and glycopeptide-specific RFT CD4 T cells in WT and MUC1-Tg recipient mice.

The appendix of this thesis contains additional observations made in the course of this study that fell outside the scope of the specific aims. These sections describe A) the possible influence of gender in antibody responses elicited by MUC1-vaccines in MUC1-Tg mice, B) the characterization of additional MUC1-peptide specific T cells found in the VFT TCR transgenic mouse, and C) phenotypic observations of mouse Thy1 expression as related to function.

2.0 TUMOR-ASSOCIATED MUC1 GLYCOPEPTIDE EPITOPES ARE NOT SUBJECT TO SELF-TOLERANCE AND IMPROVE RESPONSES TO MUC1 PEPTIDE EPITOPES IN MUC1 TRANSGENIC MICE

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2.1 INTRODUCTION

Transformed cells can aberrantly express many self-proteins that elicit immune responses. Because they are not mutated and because they are also expressed, albeit differently, on normal cells, they are generally termed tumor-associated antigens (132). Several new mouse models that express human tumor antigens as transgenes are beginning to reveal that in spite of the ability of many of these antigens to induce antibodies and T cells in cancer patients, they may nevertheless be subject to a certain level of self tolerance mediated by different mechanisms (37, 74, 116, 117). This may in part explain why patients with detectable immune responses against tumor-associated peptides still succumb to the disease, and why attempts to boost these responses using peptide vaccines have been met with only a limited success. Many of these antigens are potential sources of epitopes that are very different when derived from the tumor form of the

antigen versus the normal form found on normal cells, and it is of interest to explore if self-tolerance can be avoided by choosing epitopes that are more tumor-specific.

Most human adenocarcinomas over-express an abnormal form of the transmembrane glycoprotein mucin 1 (MUC1), characterized by exposure of the bare VNTR protein backbone in its extracellular domain due to its marked hypoglycosylation. MUC1-specific $CD8^+$ cytotoxic T cells (CTL) recognizing VNTR peptides are found in patients with $MUC1^+$ tumors indicating that an immune response can be generated against them (32, 133). However, clinical trials using MUC1 peptide based vaccines to boost this immunity have resulted in little change in CTL activity and ineffective anti-MUC1 antibody class switching beyond the IgM isotype (43, 134, 135). Studies in the MUC1-transgenic (MUC1-Tg) mouse model, which mimics the self-tolerant environment for MUC1 peptide-specific immune responses of humans, have shown that low antibody and CTL responses to MUC1 VNTR peptides are due in part to very low level of CD4 T cell responses compared to responses in transgene-negative litter mates (9, 110, 111). This is of concern since CD4 T helper cells are critical components of effective anti-tumor immunity and required for effective B cell priming and antibody isotype-switching, CD8 cytotoxic T lymphocyte (CTL) expansion, and CD8 memory responses (136).

As an alternative to tumor-derived peptides that induce low responses, tumor-associated glycoprotein antigens, such as MUC1, could be targeted via their tumor-associated glycopeptides (137). Some tumor-associated glycoproteins carry the Tn (GalNAc-O-S/T) and T (Gal-GalNAc-O- S/T) glycan antigens. These carbohydrate antigens are not normally exposed on healthy cells or tissues with benign disease. Conversely, ~90% of carcinomas (many being adenocarcinomas) express these tumor-associated carbohydrates (124). Tumor-associated glycopeptides derived from tumor MUC1 have been shown to be viable targets for T cells and anti-tumor immunity

(138-140). It has not yet been determined, however, whether tumor-derived glycopeptides would face self-tolerance similar to that of peptides. Using two different vaccination methods we show for the first time that incorporation of tumor-associated Tn antigens on MUC1 VNTR peptides can not only induce glycopeptide specific responses, but it can also boost previously suppressed MUC1 peptide-specific T cell and antibody responses in the MUC1-Tg mouse. We generated MUC1 glycopeptide-specific CD4 T cell hybridomas and could show that dendritic cells and B cells differ in their ability to present epitopes derived from the MUC1 glycopeptide, but are equally efficient in presenting the MUC1 peptide antigen.

2.2 MATERIALS AND METHODS

2.2.1 Mice and cell lines

All mice were maintained in a pathogen-free environment at the University of Pittsburgh. C57BL/6 mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and MUC1-Tg mice (99) from Dr. S. Gendler (Mayo Clinic, Scottsdale, AZ). MUC1-transgene positive and transgene negative (wild type) mice from MUC1-Tg by C57BL/6 mouse breeding were identified by PCR analysis. T cell hybridomas were generated using a similar method as previously described (141). Briefly, C57BL/6 mice were vaccinated three times using a DC-TnMUC1 vaccine (109, 141). Seven days after the final vaccination lymphocytes were collected and stimulated once *in vitro* with TnMUC1-pulsed DCs. Following *in vitro* stimulation, cells were collected and then fused with the HAT-sensitive BW5147α-β- thymoma line (142) using polyethylene glycol 1500 (Roche, Mannheim, Germany). The resulting T cell hybridomas were

screened for CD3 and CD4 expression by FACS analysis before further selection based on IL-2 production in response to TnMUC1 or MUC1p. IL-2 was measured using a BD OptEIA ELISA kit following the manufacturer's protocol (BD Biosciences, CA). The RF6 hybridoma was cloned using single cell limiting dilution.

2.2.2 MUC1 peptides and glycopeptides

The 100mer peptide (MUC1p) represents 5 repeats of the 20 amino acid sequence HGVTSAPDTRPAPGSTAPPA from the MUC1 VNTR region and was synthesized as described previously (111). The GalNAc-100mer (Tn100mer/TnMUC1) was prepared by enzymatic addition of GalNAc to the synthetic peptide substrate using a recombinant human UDP-GalNAc:polypeptide N-acetyl-galactosaminyltransferases rGalNAc-T1 as previously described elsewhere (143). The final reaction product contained a heterogeneous mixture of 9 to 15 GalNAc residues per 100mer peptide molecule that were incorporated within threonine in VTSA region and adjoining serine and threonine within GSTA region as defined previously (143). Both MUC1p and TnMUC1 were synthesized at the University of Pittsburgh Genomics and Proteomics Core Laboratories. Glycopeptides H2 [GalNAc(α1-O)], A3 [Gal(ß1-3)-GalNAc(α 1-O)] and W3 [Glc(α1-4)-Glc(ß1-4)-GalNAc(α1-O)] were synthesized as described previously (141).

2.2.3 Generation of bone marrow derived DCs (BMDC) and vaccination protocols

BMDCs were generated as previously described (141) with a few modifications. Briefly, bone marrow (C57Bl/6 mice) was collected and subjected to RBC lysis before being plated at 1 x 10^6

cells/ml serum-free AIM-V media (Invitrogen, CA) containing sodium pyruvate, nonessential amino acids, and 2-ME; supplemented with 10 ng/ml each of GM-CSF and IL-4 (a generous gift from Immunex, WA). Cells were fed on Day 3 by replacing one half of the culture volume with fresh AIM-V plus 10ng/ml GM-CSF and IL-4. DCs were purified on Day 6 of culture using a Nycoprep 1.068 (Accurate Chemical, NY) gradient.

For the DC vaccine, peptides and glycopeptides were first conjugated to BioPORTER (Sigma, MO) for 5 min at room temperature, according to manufacturer's instructions, before being added to BMDCs. BMDCs were pulsed with TnMUC1-BioPorter, MUC1p-BioPorter or PBS- BioPORTER for 4 h at 37^{o}C. The pulsed BMDCs were washed with PBS, counted, and 3 x 10^5 viable cells/100µl sterile, endotoxin-free PBS were injected s.c. in the hip area of MUC1-Tg mice. The mice were boosted twice, 3 weeks apart.

For vaccines involving the use of the adjuvant E6020 (a kind gift from Eisai, Boston, MA), 3 µg of E6020 was mixed with either TnMUC1 (2.6 µg or 104 µg) or MUC1p (2 µg or 80 µg) and injected in a final volume of 100µl (s.c.) or 200µl (i.p.) prepared in endotoxin-free PBS.

2.2.4 *In vitro* functional assays for cellular responses

One to two weeks after the third vaccination spleens and lymph nodes (inguinal, mesenteric) were collected. Single cell suspensions were prepared by mechanical disruption and RBC lysis before being cultured in 24 well plates at a density of 1-1.5 x 10^6 cells/ml. The culture medium used was cDMEM-10 (10% FBS, penicillin and streptomycin, L-glutamine, sodium pyruvate, nonessential amino acids, and 2-ME), supplemented with 20 U/ml IL-2.

Cells were tested at two time points for intracellular cytokine detection by FACS, either after an *ex vivo* stimulation or after two rounds of *in vitro* restimulation. The restimulation was

done 10 d apart, with 1 x 10^5 antigen pulsed or nonpulsed DCs for each 1 x 10^6 spleen cells in culture. The first or final stimulation, respectively, was carried out for 6 h. Brefeldin A was added during the last 4 h. Cells were stained with anti-mouse CD3 (PerCP), CD4 (APC) and IFNγ (FITC) antibodies. Isotype control antibodies were used to set up the gates. All antibodies were from BD Biosciences and staining was done according to manufacturer's protocol. Stained cells were analyzed on a LSR II Flow Cytometer using the FACSDiva data analysis software (BD Biosciences, CA). Testing for soluble cytokine production was done following a similar *in vitro* restimulation protocol as described above. A mouse anti-CD4 blocking antibody (clone H129.19; BD Biosciences, CA) was used during the final restimulation at 2.5 μg/ml. Cell culture supernatants were collected 48 h following the final restimulation and IL-5 concentrations determined by ELISA using a BD OptEIA ELISA kit following the manufacturer's protocol (BD Biosciences, CA).

For T cell hybridoma functional responses, 1 x 10^5 hybridomas were co-cultured in cDMEM-10 with 1 x 10^4 BMDCs per well (96 well plate) in the presence or absence of the indicated MUC1 antigen. In some cases BMDCs were replaced with 3 x 10^4 splenocytes (C57BL/6) per well. The final culture volume was 200μl/well and each condition was done in triplicate. After 48 h, cell culture supernatant was collected for IL-2 cytokine analysis by ELISA using a BD OptEIA ELISA kit following the manufacturer's protocol (BD Biosciences, CA). Data for all cytokine ELISA is represented as the average and standard deviation of triplicate cell culture wells.

2.2.5 Anti-MUC1 antibody ELISA

At specified time points, blood was collected and serum isolated after coagulation. The serum was stored at -80^{o}C until ready to use. Serum from individual mice was tested for the presence of MUC1-specific antibodies. 96-well Immulon 4HBX plates (Fisher Scientific) were coated overnight with 10 μg/ml of MUC1p peptide or 15 μg/ml TnMUC1 glycopeptide or 2.5% BSA to serve as negative control. Plates were then washed with PBS and blocked with 2.5% BSA. Serial dilutions of sera were done in 2.5% BSA and added to the plates in triplicate. Plates were washed three times with PBS/0.1% Tween 20, then HRP-conjugated goat anti-mouse IgG1 or pan-IgG secondary antibody (Sigma, MO) was added (diluted 1:500 in 2.5% BSA). The plates were washed three times with PBS/0.1% Tween 20 and then incubated with tetramethylbenzidine substrate (BD Biosciences, CA). The reaction was stopped with 2N sulfuric acid and the absorbance measured at 450 nm. Data was represented using the average and standard deviation of triplicate wells, after subtracting the background readings of control wells.

2.2.6 Tumor challenge

RMA-MUC1 was generated previously by transfection of the T cell thymoma line RMA with the pRc/CMV-MUC1 plasmid containing human MUC1 cDNA with 42 tandem repeats of the VNTR region (109). MUC1 expression is maintained by culturing cells with 1 mg/ml (active) G418 sulfate (Mediatech, VA). Prior to use, growing RMA-MUC1 cells were stained with fluorescent labeled anti-MUC1 antibody (HMPV) and analized by flow cytometry. MUC1 was expressed on >85% of cells. 10 d following the final vaccination mice were injected (s.c.) in the shaved hind flank with 1x10^{5} RMA-MUC1 tumor cells. Beginning on day 14 post tumor

challenge mice were observed every 2-3 days for tumor growth. Mice were sacrificed when tumor diameter measured 2cm.

2.3 RESULTS

2.3.1 MUC1 peptide (MUC1p) and MUC1 glycopeptide (TnMUC1) are handled differently by the immune systems of WT versus MUC1-Tg mice

To address the effect of tumor-associated carbohydrates on anti-MUC1 peptide immune responses in MUC1-Tg mice where the peptides may be seen as self, we compared them to responses in transgene-negative (WT) mice where the human MUC1 peptide sequence, which is not homologous to mouse Muc1, might be seen as foreign. The MUC1 vaccine was composed of a soluble adjuvant E6020, a synthetic Lipid A mimetic (144), mixed with either a 100 amino acid peptide from the MUC1 VNTR region (MUC1p) or the same MUC1p glycosylated *in vitro* to contain multiple tumor-associated Tn (GalNAc-O-S/T) moieties (TnMUC1). As we expected, E6020-MUC1p and E6020-TnMUC1 induced strong anti-MUC1 IgG antibody responses in WT mice (Figure 2A) indicating an effective T cell help for T cell dependent isotype-switch from IgM. MUC1-Tg mice were hyporesponsive to the E6020-MUC1p vaccine, producing no detectable MUC1-specific IgG antibody (Figure 2B) suggesting a lack of helper T cell activation. This result is consistent with the hypothesis that these transgenic mice may see MUC1p as a self antigen. In contrast, MUC1-specific IgG responses were observed in the majority (80%) of MUC1-Tg mice after two doses of the E6020-TnMUC1 vaccine (Figure 2B). These responses

were further boosted by giving a third vaccine dose (Figure 2D), although antibody titers were 10 fold lower than in WT mice (Figure 2C).

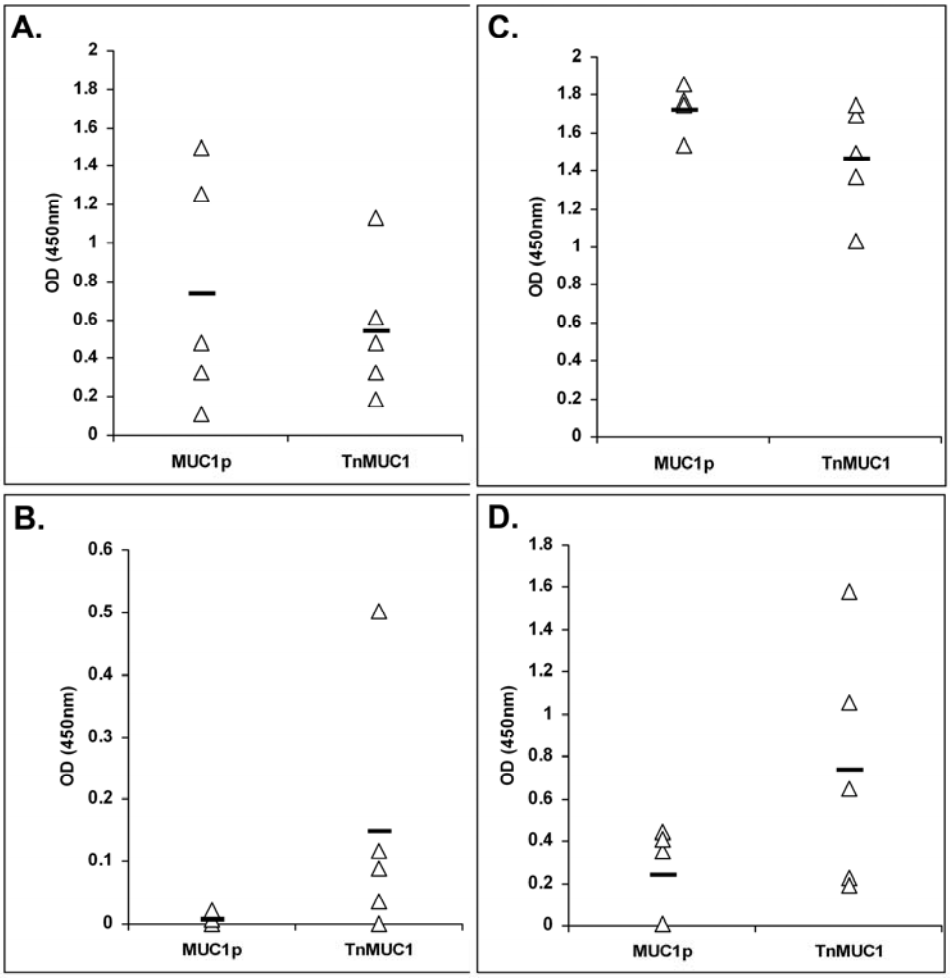


Figure 2. TnMUC1 vaccine induces anti-MUC1 IgG in MUC1-Tg mice and MUC1p vaccine does not.

WT and MUC1-Tg mice were vaccinated subcutaneously with E6020 (3 μg) adjuvant plus equimolar amounts of MUC1p (80 μg) or TnMUC1 (104 μg). Anti-MUC1 IgG was measured by ELISA at 4 d after the second vaccine dose in WT (A) and MUC1-Tg (B) mice. Antibody measurements were performed again 6 d following the final vaccine dose in WT (C) and MUC1-Tg (D) mice. Individual groups are labeled by the vaccine received. Data points represent OD values (post vaccine OD subtracted from pre-vaccine OD) at 1:1000 serum dilution (A, C) and 1:100 serum dilution (B,D). The average OD value of each individual group (n=5) is indicated by a solid bar.

Consistent with the weak MUC1-specific IgG antibody response to the E6020-MUC1p vaccine, in MUC1-Tg mice we could not detect MUC1-specific T cells. Strong MUC1p-specific antibody responses in WT mice were accompanied by MUC1-specific CD4 T cell activation (Figure 3A). The E6020-TnMUC1 vaccine induced MUC1-specific CD4 T cell responses in both MUC1-Tg mice and WT mice (Figure 3B), as measured by specific production of IL-5. We measured IL-5 instead of IFN-γ because it is a T cell specific cytokine and this way we avoid measuring IFNγ produced by other cells in culture, such as NK or NKT cells, which may have been stimulated by the adjuvant.

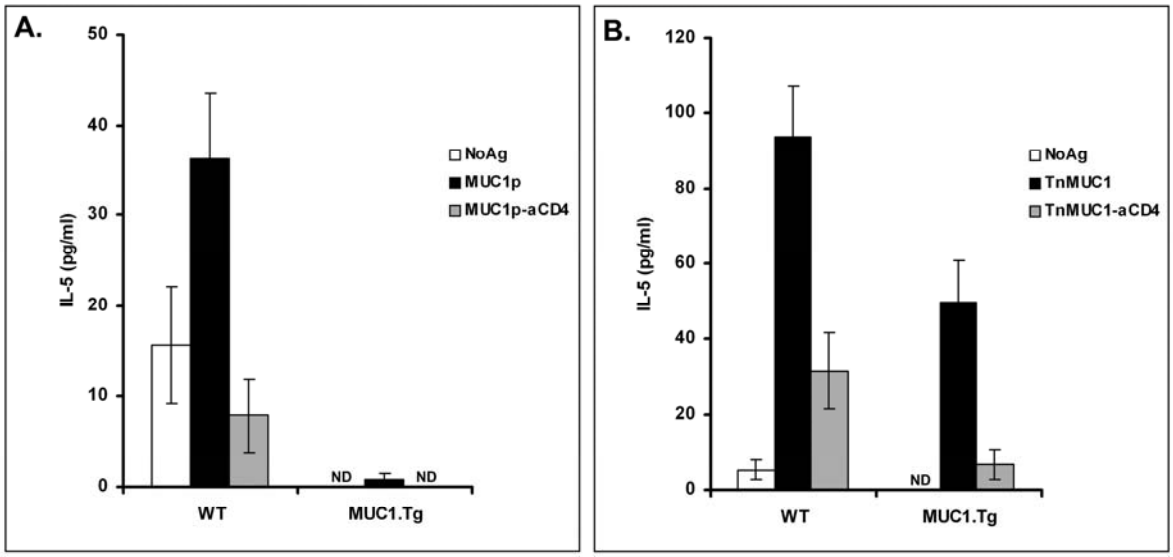


Figure 3. TnMUC1 plus adjuvant vaccine elicits CD4$^+$ T cells in MUC1-Tg mice.

WT mice and MUC1-Tg mice were vaccinated three times with E6020 (3 μg) plus equimolar amounts of (A) MUC1p (2 μg) or (B) TnMUC1 (2.6 μg). 7 d following the final vaccine mice were sacrificed and lymphocytes were collected. Cells isolated from each mouse were stimulated once *in vitro* using DCs pulsed with the same form of MUC1 used in the vaccine, followed by a second *in vitro* stimulation 7 d later. To detect MUC1-specific responses cells from each mouse were restimulated with either DC alone (NoAg, white bar), DCs pulsed with Tn (black bar), or DCs pulsed with TnMUC1 in the presence of anti-CD4 blocking antibody (gray bar). 48 h following the second stimulation cell supernatants were collected and IL-5 was detected by ELISA. Responses from representative mice per vaccine group are shown.

2.3.2 MUC1-Tg mice have improved responses to TnMUC1- versus MUC1p -pulsed dendritic cell (DC) vaccine

In addition to MUC1 plus adjuvant vaccines, we also tested anti-MUC1 peptide and glycopeptide responses when triggered by antigen-pulsed dendritic cells (DC-MUC1 vaccines). The DC-MUC1p vaccine has been used in both preclinical (109) and clinical (135) studies but it has not been compared side by side with the DC-TnMUC1 vaccine. We vaccinated MUC1-Tg mice with either DC-MUC1p or DC-TnMUC1 and following the final boost we examined the T cell response to the respective forms of MUC1 used to vaccinate. Both DC-MUC1p and DC-TnMUC1 vaccines elicited similar low percentages of MUC1-specific CD4 T cells when examined directly *ex vivo* (Figure 4A). However, expansion of MUC1-specific IFNγ-producing CD4 T cells after *in vitro* stimulation was much greater from mice vaccinated and restimulated with TnMUC1 (Figure 4B). Here we measured antigen-specific IFNγ production because unlike soluble adjuvants that can stimulate IFNγ production by other cell types, DC vaccines induce primarily IFNγ producing T cells. Paralleling the cellular response, we found that MUC1-Tg mice receiving DC-MUC1p vaccine produced much lower IgG antibody responses compared to MUC1-Tg mice vaccinated with DC-TnMUC1 (Figure 4C). Not only did the TnMUC1 vaccine induce antibody against TnMUC1 but it also induced higher IgG titers and CD4 T cell responses against MUC1p epitopes. DCs pulsed with TnMUC1 can present both peptide and glycopeptide epitopes to CD4 T cells (Figure 6) (141) and this result shows that T cells reacting to the glycopeptide epitope help the peptide-specific T cells rather than being inhibited by the mechanisms that suppress their function in the absence of the Tn antigen.

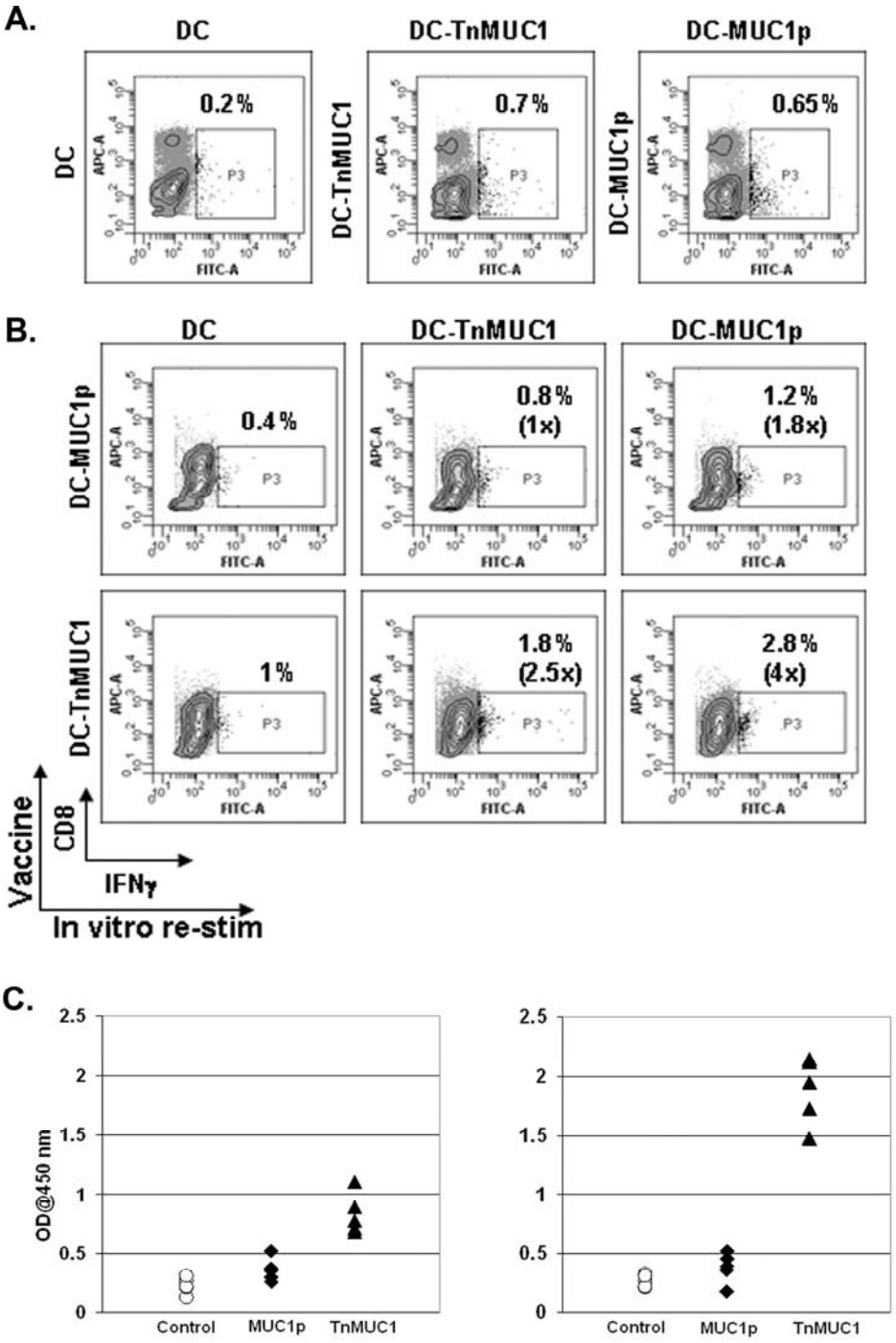


Figure 4. TnMUC1-pulsed DC vaccine induces antibody and cellular responses in MUC1-Tg mice and improves MUC1 peptide specific immunity.

(A) Ex vivo staining for intracellular IFNγ in spleen cells from mice vaccinated with either control (nonpulsed) DC, MUC1 peptide-pulsed DC (DC-MUC1p) or MUC1 glycopeptide-pulsed DC (DC-TnMUC1). Staining was performed using a pooled fraction of spleen cells from 3 mice in each group. The cells were stimulated for 4 h with

their respective vaccine in the presence of Brefeldin A. Numbers shown represent percentage of T cells secreting IFNγ. (B) Intracellular staining for IFNγ after two *in vitro* re-stimulations. Spleen cells from DC-MUC1p (upper row) or DC-TnMUC1 (lower row) vaccinated mice were re-stimulated twice with either control DC (left), DC-TnMUC1 (middle) or DC-MUC1p (right). (C) MUC1-specific serum IgG1 antibodies detected by ELISA. The plates were coated with either MUC1p (left) or TnMUC1 (right). The serum was diluted 1:150.

2.3.3 RMA-MUC1 tumor challenge

RMA-MUC1 is a mouse T cell thymoma that expresses human MUC1 from an expression vector. RMA-MUC1 has been shown to express epitopes present on both glycosylated normal MUC1 and underglycosylated tumor MUC1 and the cell line grows well as a subcutaneous (s.c.) solid tumor (109).

We used RMA-MUC1 tumor challenge to determine if the immune responses generated by the E6020-MUC1p and E6020-TnMUC1 vaccines (Figure 2) could provide tumor protection in MUC1-Tg mice, compared to WT mice. Only WT mice vaccinated with E6020-MUC1p showed a significant protection against RMA-MUC1 tumor growth over adjuvant-only control mice (Figure 5A). Reflecting the MUC1p hyporesponsiveness in MUC1-Tg mice shown previously, E6020-MUC1p vaccination did not confer RMA-MUC1 tumor protection in these mice (Figure 8B). However, results from the E6020-TnMUC1 vaccinations were inconclusive since the positive control WT mice did not elicit RMA-MUC1 tumor protection (Figure 5C), thus it was not surprising that vaccination did not provide protection in MUC1-Tg mice (Figure 5D).

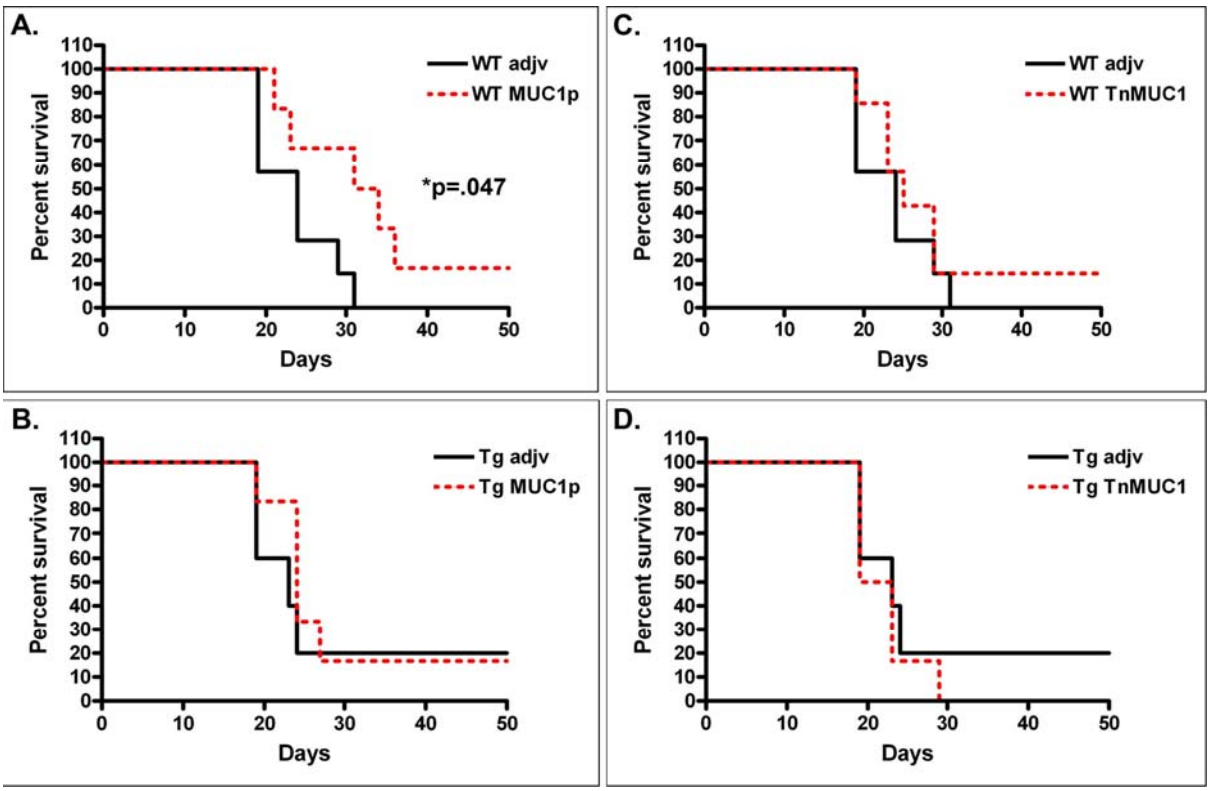


Figure 5. Vaccination of MUC1-Tg mice with soluble adjuvant plus MUC1p or TnMUC1 does not provide protection from future RMA-MUC1 tumor challenge.

WT (A,C) mice and MUC1-Tg (Tg; B,D)) mice were vaccinated three times (i.p) with E6020 (3 μg) alone (adjv) or plus equimolar amounts of (A,B) MUC1p (80 μg) or (C,D) TnMUC1 (104 μg). 10 d following the final vaccination mice were injected (s.c.) in the hind flank with $1x10^5$ RMA-MUC1 tumor cells. Beginning on day 14 post tumor challenge mice were observed every two days for tumor growth. Mice were sacrificed when tumor growth measured 2 cm in diameter. Kaplan-Meier curves show the combined results of two separate experiments with a total of 5-7 mice per group. P values were calculated using the log-rank test.

2.3.4 Dendritic cells and B cells differ in MUC1 antigen presentation

Due to the different outcomes we saw in immune responsiveness to MUC1p versus TnMUC1 with both the adjuvant-based and DC-based vaccines, we postulated that there may be inherent differences in how the immune system encounters the two forms (145). The soluble antigen

delivered with the adjuvant might be presented *in vivo* by multiple antigen-presenting cell (APC) populations, not only DC, and different APC might differ in their ability to take up and present the two different forms, MUC1p and TnMUC1. To test this *in vitro* we needed MUC1-specific T cell lines; however, these are difficult to establish and maintain long-term. As an alternative, we generated T cell hybridomas with specificity for TnMUC1-derived epitopes and used them to explore differences in antigen presentation.

Our previous work had shown that MUC1 VNTR peptides either unglycosylated (MUC1p) or carrying the tumor-associated Tn antigens (TnMUC1) are taken up by DCs where both forms are processed into peptides presented in MHC class II for peptide-specific CD4 T cell recognition (141). To better understand if TnMUC1 can also be a source of glycopeptides able to activate glycopeptide-specific CD4 T cells, we generated a panel of $CD4^+$ T cell hybridomas using T cells from WT mice vaccinated with DC-TnMUC1. We first tested polyclonal hybridoma populations for the presence of glycopeptide specificity. We found that these cell populations were specific for the immunogen TnMUC1, but responded differently when the antigen was presented on bone marrow derived DCs (BMDC) versus splenocytes. Furthermore, we found that TnMUC1-pulsed DCs can present both glycopeptide and peptide epitopes. For example, when BMDCs were used as APCs, hybridoma population RF78 responded strongly to both TnMUC1 and MUC1p, as monitored by their level of IL-2 secretion (Figure 6A). However, when splenocytes, consisting mainly of B cells (70%), were used as APCs, the response to TnMUC1 by the same RF78 population decreased, while responses to MUC1p remained high (Figure 6A). This was seen with another hybridoma population, RF141, which also responded strongly to TnMUC1 when BMDCs were used as APCs, but not when splenocytes were used to present the antigen (Figure 6B). Experiments were repeated using lipopolysaccharide (LPS)

activated B cells as APC and the same results were obtained as with total spleen cells (data not shown), suggesting that B cells can present MUC1 peptides but not glycopeptides. We confirmed that peptide epitopes are derived from TnMUC1 and that DCs are the only APC capable of presenting TnMUC1-derived epitopes by using the single-cell cloned VF5 hybridoma (Figure 6C), previously characterized for specificity to an peptide epitope derived from MUC1p (141).

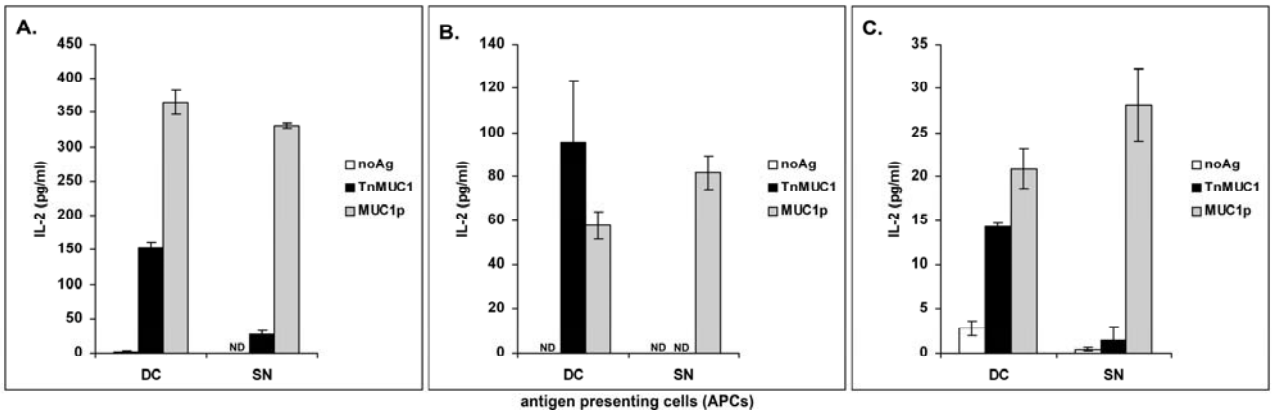


Figure 6. Dendritic cells and splenocytes differ in TnMUC1 antigen presentation.

Antigen specific responses by CD4 T cell hybridomas were measured by IL-2 detection by ELISA after 48 h stimulation. Hybridoma population RF78 (A), RF141 (B) or VF5 hybridoma clone (C) were stimulated with either DC or spleen cells (SN) loaded with nothing (white bar), TnMUC1 (black bar) or MUC1p (gray bar). Data are represented as the average and standard deviation of triplicate wells.

2.3.5 DC process and present epitopes from TnMUC1 that are recognized by T cells only if glycosylated

To identify T cell hybridoma clones responding exclusively to glycoepitopes, we subcloned the RF141 population described above and isolated the RF6 clone that responds to DCs pulsed with TnMUC1 and not MUC1p, suggesting that only glycopeptides and not peptides are recognized by its T cell receptor (TCR). We determined that RF6 responds to glycopeptides carrying the Tn

antigen on the GS(GalNAc-O-T)A motif (Figure 7A). In addition, RF6 responsiveness decreased when the number of carbohydrate residues O-linked to the threonines in the GSTA motif increased from 1 (optimal) to 3 (Figure 7A). At high antigen concentrations we observed some cross-reactivity with unglycosylated epitopes derived from MUC1p that decreased precipitously at lower antigen concentration, showing that inclusion of the carbohydrate increases the binding affinity the RF6 TCR and that its preferred epitope is the glycopeptide (Figure 7B). Further characterization of the RF6 hybridoma showed that its TCR is MHC class II (A^b) restricted (Figure 8).

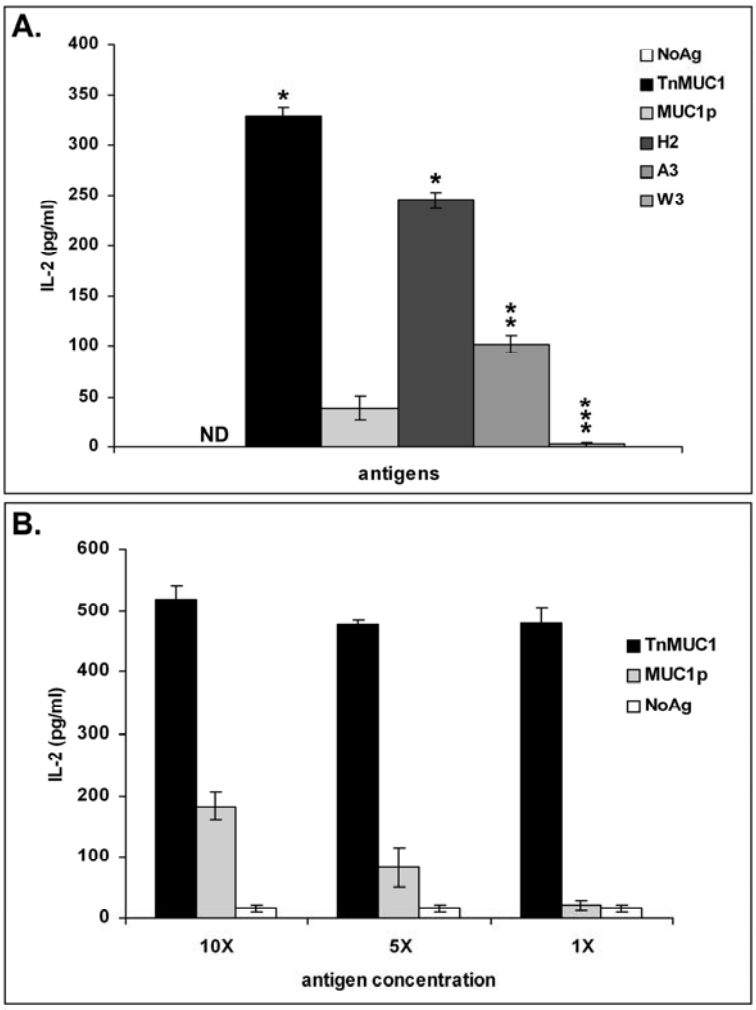


Figure 7. RF6 hybridoma response to TnMUC1-derived epitopes is glycosylation dependent.

(A) RF6 was stimulated with BMDCs alone (NoAg, white bar) or with different MUC1 peptides: TnMUC1, MUC1p, H2 [GalNAc(α1-O)], A3 [Gal(ß1-3)-GalNAc(α1-O)], and W3 [Glc(α1-4)-Glc(ß1-4)-GalNAc(α1-O)] (B) Cross reactivity with peptide epitopes at high antigen concentrations. RF6 were stimulated with the same number of DC loaded with 26, 13, 2.6 µg/ml TnMUC1 or 20, 10, 2 µg/ml MUC1p (10X, 5X, 1X, respectively). Data are represented as the average and standard deviation of triplicate cell culture wells.

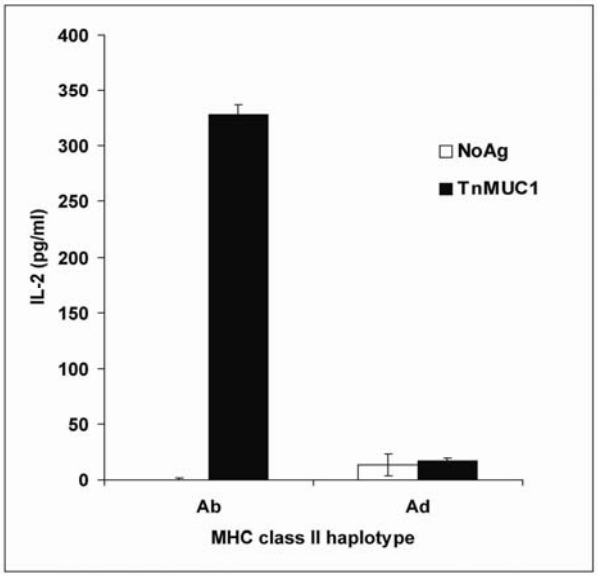


Figure 8. The RF6 hybridoma is I-A^b (MHC class II) restricted.

RF6 hybridomas were co-cultured with BMDCs derived from mice expressing different MHC class II haplotypes; C57BL6 mice (A^b) or BALBc mice (A^d). The RF6 hybridomas and BMDCs were either cultured alone (NoAg) or with 20µg/ml TnMUC1 (TnMUC1). Antigen specific responses were measured by IL-2 detection by ELISA after 48 h stimulation. Data are represented as the average and standard deviation of triplicate cell culture wells.

2.4 DISSCUSION

Past and present studies in the MUC1-Tg mouse model have indicated that in the context of a "self" environment immune responses to unglycosylated MUC1 VNTR peptides face self tolerance. (9, 37, 109-111). As a strategy to increase the potency of MUC1-peptide vaccines, we altered the peptide immunogen by incorporating tumor-associated glycans within its structure (i.e. Tn antigen), thus more closely resembling epitopes that would be displayed on $MUC1^+$ tumors and on APCs that cross-present $MUC1^+$ tumors to T cells in patients.

In the present study we show that incorporation of tumor-associated Tn antigens on MUC1 VNTR peptides (TnMUC1) can boost MUC1 peptide-specific CD4 T cells and promote effective antibody responses that are otherwise suppressed in the MUC1-Tg mouse. *In vitro* studies indicate that DCs are the main APC capable of cross-presenting TnMUC1 for CD4 T cell stimulation. Ensuring that vaccines will target TnMUC1 to DCs *in vivo* would be beneficial not only because they are the professional APCs responsible for driving strong adaptive immune responses, but also because the optimal MUC1 antigen is not presented by other APCs. Based on our observations, it could be extrapolated that intradermal delivery of the vaccines would be optimal and adjuvants that are administered via this route and able to stimulate dermal DCs would be expected to be more efficient than those administered via an intramuscular route.

An efficient humoral response has been elicited previously in the MUC1-Tg mouse with a vaccine composed of MUC1 VNTR peptides carrying Tn antigens linked to keyhole limpet hemocyanin (KLH) (146). In this formulation, KLH served as a strong inducer of helper T cell responses. However, since this help is not related to MUC1, these types of vaccines providing heterologous help would not be expected to induce effective anti-tumor memory T cells. MUC1 glycopeptide-specific T cells, on the other hand, can provide MUC1-specific help at the time of

priming and also at any later time when, as memory cells, they are challenged with the same antigen expressed on tumor cells.

By directly comparing antibody responses induced by MUC1p and TnMUC1 vaccines in WT versus MUC1-Tg mice, we noticed that even though TnMUC1 can elicit responses in MUC1-Tg mice, those responses were not as strong as the response in WT mice. We are postulating that peptide epitopes that are processed from TnMUC1 simultaneously with the glycopeptide epitopes may induce regulatory T cells that may have a suppressive effect on the glycopeptide-specific T cells as well, albeit not to the same extent. Further studies are needed to define the epitopes derived from TnMUC1 that would have only the stimulatory effect and delete those that induce regulatory T cells. The T cell hybridomas we described will be useful reagents for such studies. We note that the T cell receptor of the RF6 hybridoma that we described above as being dependent on the glycosylation of the threonine in the GSTA motif of the VNTR region, has the same specificity as antibodies generated using TnMUC1 coupled to KLH (146, 147). This epitope is thus recognized both in its native form on the whole antigen as well as a processed peptide in MHC class II.

The RMA-MUC1 challenge experiments further reflected the hyporesponsive environment to MUC1p in MUC1-Tg mice, compared to WT mice. However, the results were inconclusive concerning the TnMUC1 vaccinations. We could not interpret the results in MUC1-Tg mice due to the lack of tumor protection in TnMUC1-vaccinated WT mice (positive control). These inconclusive results could be related to the small size of the experimental groups receiving E6020-TnMUC1 vaccination (WT, n=7; MUC1-Tg, n=6), thus outliers within the groups can more easily distort the outcome. It is possible that RMA-MUC1 is not a proper model of TnMUC1 anti-tumor immunity. Although we have shown that RMA-expressed MUC1

has exposed VNTR peptide regions and hypoglycosylation (109), the presence and density of tumor-associated Tn antigens on this mouse thymoma line has not been examined. However, in a model of spontaneous colon carcinoma (115) we have found that prophylactic TnMUC1 vaccination completely protects mice from tumor occurrence (Beatty PL, et. al., manuscript in preparation), indicating that TnMUC1 can induce effective anti-tumor immunity.

Studies have shown that different forms of MUC1, depending on glycosylation, are endocytosed by DCs though different means (145, 148), yet DCs are not the only APCs that can stimulate CD4 T cell response to a vaccine containing soluble antigen. We show a difference in the ability of BMDCs and splenocytes to present TnMUC1 derived epitopes. While BMDC preparations are routinely 80-90% $CD11c^+$, the majority of APCs found in bulk splenocytes are B cells (~70%) and splenic DCs make up only a minor percentage. We postulate that the difference in TnMUC1 presentation is due to the absence on B cells of the receptor for TnMUC1, namely the macrophage galactose-type lectin (MGL). The MGL receptor is expressed on immature DCs and macrophages but not on B cells in both mouse (149) and human (150). In addition, the MGL receptor has been shown to specifically bind the Tn antigen (GalNAc) of glycosylated MUC1 and facilitate its internalization into DCs (148, 151). By nature immature DCs that express the MGL receptor are highly endocytic but are weak stimulators. Thus soluble TnMUC1 should be administered with a potent adjuvant that will properly mature endogenously antigen-loaded DCs (151).

Because of its antigenicity and escape from tolerance in MUC1-Tg mice, TnMUC1 should be evaluated in clinical trials. Like many others, we have evaluated MUC1 VNTR peptides, completing a trial of a DC-MUC1p vaccine (135) and two trials of MUC1p vaccine using two different adjuvants (43, 134). Taken together with the studies in the preclinical

MUC1-Tg mouse model, the low level responses in patients to the peptide vaccine suggest that improvements are needed. Choosing MUC1 epitopes that are more tumor-specific and less self-like should lead to an improved response. This strategy applies to other tumor-associated antigens that are uniquely modified on tumors, such as those with altered glycosylation, phosphorylation (152) and other post-translational modifications. Focusing the vaccines on altered rather than self epitopes should improve both efficacy and safety of cancer vaccines.

3.0 GENERATION OF A MUC1 GLYCOPEPTIDE-SPECIFIC T CELL RECEPTOR TRANSGENIC MOUSE

3.1 INTRODUCTION

Previous studies have suggested that weak immune responses to the MUC1 VNTR peptides (MUC1p) in MUC1-Tg mice is due to a level of self-tolerance, which is not seen in transgene negative WT mice. The weak anti-MUC1p T helper cell response appears to be central to the self-tolerance seen in MUC1-Tg mice (37, 111). However, the function and fate of those respective CD4 T cells in the MUC1-Tg environment has not been directly shown. In addition, the respective MUC1-glycopeptide (TnMUC1) -specific CD4 T cell function and fate is not known, although we have shown that humoral and CD4 T cell responses are improved using TnMUC1 vaccines compared to MUC1p vaccines (112).

In order to specifically address the fate and function of tumor-associated MUC1 peptide (MUC1p) -specific CD4 T cells in the MUC1-Tg mouse, we recently generated the MUC1p-specific VFT TCR-Tg mouse (153). To complement the study of MUC1p-specific CD4 T cells in the MUC1-Tg mouse, we describe here the generation of a MUC1 glycopeptide (TnMUC1) – specific TCR-Tg mouse. The TnMUC1-specific TCR (RFT) was identified and cloned a from the MHC class II (A^b) -restricted RF6 CD4 T cell hybridoma (Figure 7,8). We show that

expression of the RFT transgenic Vα4.1-Jα16:Vβ15-Jβ1.3 TCR in the RFT-Tg mouse results in TnMUC1-specific primary CD4 T cells.

3.2 MATERIALS AND METHODS

With exception to the PCR reaction used in the 5' RACE protocol (Invitrogen, CA), a basic PCR reaction [10′94°C/(1′94°C, 1′56°C, 1′72°C, 30× cycles)/10′72°C] including 2.5μM $MgCl_2$ and AmpliTaq Gold polymerase (Applied Biosystems, CA) was used. For RT-PCR, cDNA was prepared using a GeneAmp kit (Applied Biosystems). Many of the methods described below have been adapted from a previous study that generated the VFT-Tg mouse line (153). Molecular weight DNA ladders were purchased from Invitrogen or BioRad, where indicated.

3.2.1 Mice

All mice were maintained in a specific pathogen-free environment at the University of Pittsburgh. C57BL/6 mice and B6.SJL-PtprcaPepcb/BoyJ mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and MUC1-Tg mice (99) from Dr. S. Gendler (Mayo Clinic, Scottsdale, AZ). Transgene positive and negative (wild type) mice were identified by PCR analysis of isolated tail tissue DNA.

3.2.2 MUC1 peptides and glycopeptides

The 100mer peptide (MUC1p) represents 5 repeats of the 20 amino acid sequence HGVTSAPDTRPAPGSTAPPA from the MUC1 VNTR region and was synthesized as described previously (111). The GalNAc-100mer (Tn100mer/TnMUC1) was prepared by enzymatic addition of GalNAc to the synthetic peptide substrate using a recombinant human UDP-GalNAc:polypeptide N-acetyl-galactosaminyltransferase rGalNAc-T1 as previously described elsewhere (143). The final reaction product contained a heterogeneous mixture of 9 to 15 GalNAc residues per 100mer peptide molecule that were incorporated within threonine in the VTSA region and the adjoining serine and threonine within the GSTA region as defined previously (143). Both MUC1p and TnMUC1 were synthesized at the University of Pittsburgh Genomics and Proteomics Core Laboratories. The 20mer glycopeptide H2 [GalNAc(α1-O)] was synthesized as described previously (141).

3.2.3 T cell hybridomas

T cell hybridomas were generated using a similar method as previously described (141). Briefly, C57BL/6 mice were immunized three times using a DC-TnMUC1 vaccine (109, 141). Seven days after the final vaccination lymphocytes were collected and stimulated once *in vitro* with TnMUC1-pulsed DCs. Following *in vitro* stimulation, cells were collected and then fused with the HAT-sensitive BW5147α-β- thymoma line (142) using polyethylene glycol 1500 (Roche, Mannheim, Germany). The resulting T cell hybridoma populations were screened for CD3 and CD4 expression by FACS analysis before further selection based on IL-2 production in response to TnMUC1 or MUC1p –pulsed DCs. IL-2 was measured using a BD OptEIA ELISA kit

following the manufacturer's protocol (BD Biosciences, CA). The RF6 hybridoma was cloned using single cell limiting dilution. Three limiting dilutions were preformed to ensure cell clonality (original name for RF6 was SR141.8.1.6) before TCR molecular cloning. The 58a-b- hybridoma is a variant of the DO11.10 hybridoma that lacks expression of its functional endogenous TCR (154).

3.2.4 Identifying the RF6 hybridoma T cell receptor

The TCR α and β chains of the RF6 hybridoma (112) were identified by RNA ligase-mediated 5' rapid amplification of cDNA ends (RLM-RACE; 5' RACE) using a GeneRacer Kit (Invitrogen) following the manufacturer's protocol, followed by sequencing and BLAST searches. To begin, total cellular RNA was isolated from the RF6 hybridoma using an RNeasy kit (Qiagen, CA). In addition to the reagents supplied by the Generacer Kit, we used the constant-region specific reverse primers 5'-ACAGCAGGTTCTGGGTTCTGG-3' (CαGSP) and 5'-GAGACCTTGGGTGGAGTCAC-3' (CβnestIII)(153) in the α and β chain PCR amplification and sequencing reactions, respectively. The amplified TCR fragments were sequenced at the University of Pittsburgh sequencing facility. Identification of the RF6 TCR (Vα4.1-Jα16 and Vβ15-Jβ1.3) was done by comparative analysis using online BLAST searches (NCBI) and other published references (155-158). The identified TCR gene segment rearrangements were confirmed by PCR analysis using internal V and J region-specific primers; CAGCAGAGGTTTTGAAGCTAC (Va4.1f) and GGTGCCAGATCCGAAGTAAA (Ja16r), or GCACTTTCTACTGTGAACTCAGC (Vb15f) and CGGCTTCCTTCTCCAAAATA (Jb1.3r). An additional primer specific for the Vα2 gene segment was used as a control; GATTCACAATCTTCTTCAATAAAAGGG (Va2m).

3.2.5 Cloning the RF6 TCR

RF6 hybridoma genomic DNA was isolated using a DNeasy kit (Qiagen, CA). The rearranged RF6α and RF6β TCR V-J gene segments, including ~100bp of intron sequence downstream of the J gene segments, were amplified by PCR and cloned using the TOPO TA Cloning Kit (Invitrogen). The cloning primers used to amplify RF6α were 5'-TCTCCCGGGAGGCTTCATGACTGTGATGCT-3' (Va4.1c; XmaI site added) and 5'-ATTCGCGGCCGCGGACAAGGATCCAAGCTAAAG-3' (Ja16c; NotI site added). The cloning primers used to amplify RF6β were 5'-TCTCTCGAGCACTGAAGATGTTACTGCTTCTA-3' (Vb15c; XhoI site added) and 5'-ATTCCCGCGGCATGCATGAGCAGAGGAGTT-3' (Vb1.3c; SacII site added). The presence and integrity of cloned RF6α and RF6β TCR V-J gene segments in TOPO plasmids were confirmed by PCR and DNA sequencing using the Va4.1f-Ja16r and Vb15f-Jb1.3r internal primers (described above). Using restriction endonuclease digestion, RF6α and RF6β were removed from the TOPO plasmid and ligated (T4 ligase) into the TCR cassette vectors pTα and pTβ respectively (159). pTα- RF6α and pTβ- RF6β vectors were sequenced to confirm intact cloned fragments. Restriction endonucleases and the T4 ligase were purchased from New England Biolabs (Ipswich, MA) and used according to the manufacturer's protocol.

3.2.6 Ectopic RF6 TCR expression and functional analysis

$1x10^7$ D10 cells (160) or 58a-b- hybridomas (154) were co-transfected with 10μg of pTα-RF6α, 10μg pTβ-RF6β, and 1μg pCD4 DNA by electroporation in .4cm cuvettes at 260mV and 960μF (Bio-Rad, CA). The mouse CD4 expression plasmid (pCD4) was a kind gift from Dr. Carrie

Miceli (UCLA, CA). D10 cells were analyzed 48 hrs post transfection by RT-PCR using RF6 TCR V-region forward (f) primers and C-region reverse primers (described above). After resting transfected 58a-b- cells overnight in cDMEM-10 the cultures were supplemented with 500μg/ml (active) G418 sulfate (Mediatech). The resulting growth-selected hybridomas were analyzed by flow cytometry for CD3 and CD4 expression. The identified CD3 and CD4 expressing 58a-b- hybridoma population (58pRFTpCD4) was stimulated for 48 hrs with BMDCs pulsed with 20ug/ml TnMUC1, H2, or MUC1p antigen. Supernatants were analyzed for presence of IL-2 using a BD OptEIA ELISA kit following the manufacturer's protocol (BD Biosciences, CA).

3.2.7 Generating the RFT-Tg mouse line

Before supplying the transgenic mouse facility (University of Pittsburgh) with the pTα-RF6α and pTβ-RF6β constructs, the vectors were linearized and the prokaryotic DNA was removed using SalI (pTα) or KpnI (pTβ) restriction endonuclease digestion followed by electrophoresis using a 0.8% agarose gel. Vector fragments were purified from gel slices using a QIAquick Gel Extraction Kit (Qiagen). The University of Pittsburgh Transgenic Mouse Facility carried out the microinjection of B6.SJL-PtprcaPepcb/BoyJ (B6-CD45.1^{+}) embryos with the linear pTα-RF6α and pTβ-RF6β constructs separately or together. Founder RF6α, RF6β, and RF6αβ mice were identified by PCR of tail tissue DNA using internal primers specific for the Vα4.1-Jα16 and Vβ15-Jβ1.3 rearrangements (described above). Identified founders were bred with B6.SJL-PtprcaPepcb/BoyJ mice to ensure germ-line transmission of the transgenes. The single transgenic RF6α and RF6β mouse lines were interbred to generate double transgenic RFT-Tg mice.

3.3 RESULTS

3.3.1 Identifying the RF6 TCR by 5' RACE

The TCR α and β subunits contain both 5' variable (V-segment, J-segment) and 3' constant (C-segment) regions of sequence based on DNA alignments (155). In the TCR α and β loci there are numerous different V and J exons that could combine during T cell development by somatic recombination events resulting in inter- T cell V-region heterogeneity. Due to the high level of heterogeneity, it is not possible to predict the TCR V-region sequences of an α/β T cell. On the other hand, the TCR α and β loci have only limited C-region exons, thus the sequence of this region can be predicted in all α/β T cells. 5'RACE is a useful method for identifying complete gene transcripts when only the 3' sequence is known. Using this method we identified the TCR Vα4.1-Jα16 and Vβ15-Jβ1.3 variable regions of the RF6 hybridoma (Figure 9).

>RF6 alpha VJC cDNA
CGACTGGAGCACGAGGACACTGACATGGACTGAAGGAGTAGAAAACTGTCGAGATGGGTCTAAAGATGAACTCTTCTCCAGGC
TTCATGACTGTGATGCTCCTCATATTCACAAGGGCCCATGGAGACTCAGTGACTCAGACGGAAGGTCAAGTGGCCCTCTCAGA
AGAGGACTTTCTTACGATACACTGCAACTACTCAGCCTCAGGGTACCCAGCTCTGTTCTGGTATGTGCAGTATCCCGGAGAAGG
TCCACAGTTCCTCTTTAGAGCCTCAAGGGACAAAGAGAAAGGAAGCAGCAGAGGTTTTGAAGCTACATATGATAAAGGGACCAC
CTCCTTCCACTTGCGGAAAGCCTCAGTGCAAGAGTCAGACTCGGCTGTGTACTACTGTGCTCTGGGTGATCTGA**CTAATTACA
ACGTGCTTTACTTCGGATCTGGCACCAAACTCACTGTAGAG**CCAAACATCCAGAACCCAGAACCTGCTGT

>RF6 beta VJC cDNA
CGACTGGAGCACGAGGACACTGACATGGACTGAAGGAGTAGAAAAGACCTCTCTTAAGCGAAGGTGGTGTGAAGTCAACACTG
AAGATGTTACTGCTTCTATTACTTCTGGGGCCTGGCTGTGGGCTTGGAGCACTCGTCTATCAATATCCCAGAAGAACCATCTGT
AAGAGTGGAACTTCCATGAGGATGGAGTGTCAAGCTGTGGGTTTTCAGGCAACTTCTGTAGCTTGGTATCGTCAATCGCCTCAA
AAGACATTTGAACTGATAGCACTTTCTACTGTGAACTCAGCAATCAAATATGAACAAAATTTTACCCAGGAAAAATTTCCCATCAG
TCATCCCAACTTATCCTTTTCATCTATGACAGTTTTAAATGCATATCTTGAAGACAGAGGCTTATATCTCTGTGGTGCTAGGGTGG
GACAGAA**TTCTGGAAATACGCTCTATTTTGGAGAAGGAAGCCGGCTCATTGTTGTAG**AGGATCTGAGAAATGTGACTCCACC
CAAGGTCTC

Figure 9. The RF6 TCR variable region contains the Vα4.1-Jα16 and Vβ15-Jβ1.3 gene segments.

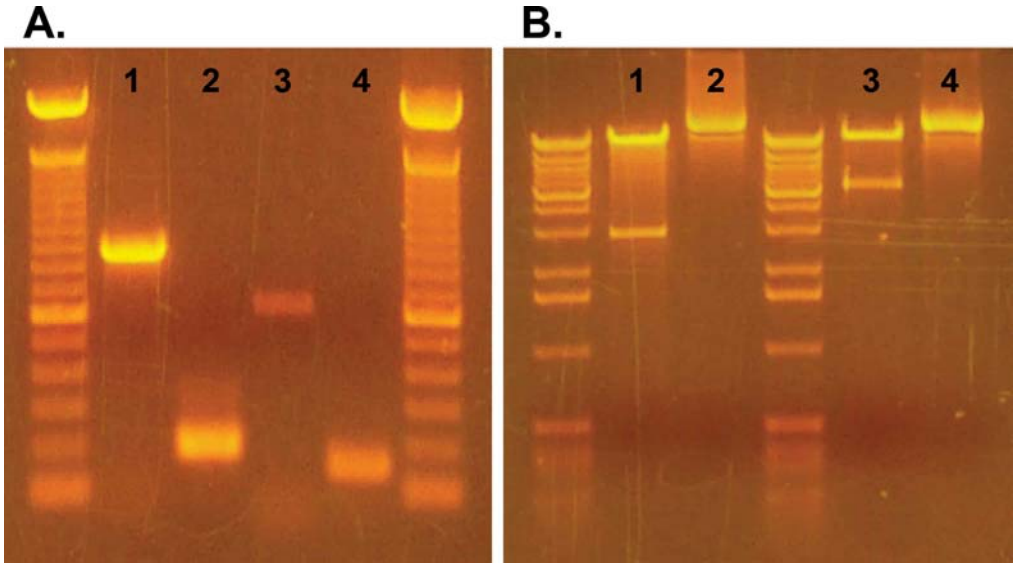
The final cDNA amplified products from 5'RACE were inserted into TOPO-TA cloning plasmids and propagated in TOP-10 *E. Coli* following the manufacture's protocol. The plasmids were sequenced and the DNA sequence of the alpha (top) and beta (bottom) inserts is shown. By comparison with known mouse TCR chain sequences, the RF6 TCR variable regions were identified as Vα4.1-Jα16 and Vβ15-Jβ1.3. The V-J-C coding regions of the cDNA

inserts are separated as indicated (**). The 5' GeneRacer oligos are shaded in green and the DNA primer binding sites have been underlined.

3.3.2 Cloning the RF6 TCR in pTcass expression vectors

The pTcass vectors were developed to aid the cloning and expression of recombinant TCR molecules for use in generating TCR-Tg mice. The advantages of the pTcass vectors are 1) short segments of rearranged α and β variable regions can easily be inserted, 2) transcription of these constructs is driven by the natural TCR promoter and regulatory elements, and 3) the constant regions are maintained within the vectors and allow for natural RNA splicing of the V-J to C-regions (159).

The recombined V-J gene segments of the RF6 TCR were amplified from genomic DNA and cloned in the TOPO-TA vector. We included 92bp and 106bp of additional 3' sequence downstream of the J alpha and J beta gene segments, respectively, to allow for correct splicing of the cloned V-J segments to the constant regions encoded by the vectors (161). The V-J segment inserts were then sub-cloned into their respective pTcass vectors. Recombinant pTα-RF6α and pTβ-RF6β vector constructs were identified by PCR (Figure 10A). In addition, we used restriction endonuclease digestion to confirm that the prokaryotic vector DNA could be separated from the pTα-RF6α and pTβ-RF6β vector (Figure 10B). The restriction endonuclease digestion and gel analysis also confirmed that recombination events had not altered the vector backbone composition, which we had observed in other cloned pT-RF6 vector preparations. After sequencing the pTα-RF6α and pTβ-RF6β vectors we found that the genomic Vα4.1 and Vβ15 gene segments contain intron sequences (Figure 10C) that appear to be spliced out in mature mRNA (Figure 9).



C.

>RF6 alpha VJ gDNA

TCTCCCGGGAGGCTTC**ATG**ACTGTGATGCTCCTCATATTCAGTAAGTAGAGTGACCTTAAACTTCTCACGTTTTTATGGCAGAAT
ATTTTTGAGCCCTAAATTCATCTAAGAGCTTGGTCTTTAATGACATTCATATCAGTTTTTGTTTTGTTTTGTTTTTCTTCCCCAGCA
AGGGCCCATGGAGACTCAGTGACTCAGACGGAAGGTCAAGTGGCCCTCTCAGAAGAGGACTTTCTTACGATACACTGCAACTA
CTCAGCCTCAGGGTACCCAGCTCTGTTCTGGTATGTGCAGTATCCCGGAGAAGGTCCACAGTTCCTCTTTAGAGCCTCAAGGG
ACAAAGAGAAAGGAAGCAGCAGAGGTTTTGAAGCTACATATGATAAAGGGACCACCTCCTTCCACTTGCGGAAAGCCTCAGTG
CAAGAGTCAGACTCGGCTGTGTACTACTGTGCTCTGGGTGATCTGACTAATTACAACGTGCTTTACTTCGGATCTGGCACCAAA
CTCACTGTAGAGCCAAGTAAGTTATTGTTACCCACCCATTATGCTAAATTCCTCCTACTCGCTCTATATTTGCTTTGAGTTCTCTT
TAGCTTGGATCCTTGTCCGCGGCCGCGAAT

>RF6 beta VJ gDNA

TCTCTCGAGCACTGAAG**ATG**TTACTGCTTCTATTACTTCTGGGGCCTGGTATGAGCCATGCTATACCTTGAAGTTTGTCTGTGTG
AGTGTGCATGTGTATGAGACTGAATGTCTGTATAAGTGTATGCGTTATCCTGAGTGAAATAGTGAAGATTCTTTGTCAATCATTTG
TGCTGATCAGATGAGATGGCACATCTGGGGCCACCTAAGGTGTAATTATCAACACCATCCACGGAAAGTGAAGGACTGTGAATT
CTTGAGTATCTCAGAAGATACTTGCATAAGTGCAAGGAGGATTTTCCAGGAACTTAAAGAAAAATGGAGTGAACCCAAATTTTCT
GGGATTATCTTTGACTCAGGCTCATTCTACTTCTGGGGCTTCTGAAGTCTTTAATAATCCTGCCCTCTGTTTTGGCAGGCTGTGG
GCTTGGAGCACTCGTCTATCAATATCCCAGAAGAACCATCTGTAAGAGTGGAACTTCCATGAGGATGGAGTGTCAAGCTGTGG
GTTTTCAGGCAACTTCTGTAGCTTGGTATCGTCAATCGCCTCAAAAGACATTTGAACTGATAGCACTTTCTACTGTGAACTCAGC
AATCAAATATGAACAAAATTTTACCCAGGAAAAATTTCCCATCAGTCATCCCAACTTATCCTTTTCATCTATGACAGTTTTAAATGC
ATATCTTGAAGACAGAGGCTTATATCTCTGTGGTGCTAGGGTGGGACAGAATTCTGGAAATACGCTCTATTTTGGAGAAGGAAG
CCGGCTCATTGTTGTAGGTAAGTTAGGGCCAAATGGCTGGGTACTGGGTGAAGTTTCTCTCTGAGGTGCCCAGGTTGCAGTGT
ACTTCTATCAGAAAGAACTAAACTCCTCTGCTCATGCATGCCGCGGGAAT

Figure 10. pTα-RF6α and pTβ-RF6β insert and vector integrity confirmation.

Amplification products from PCR reactions that used A) pTα-RF6α vector with (1) cloning Va4.1c:Ja16c and (2) internal Va4.1f:Ja16r primers and pTβ-RF6β vector with (3) cloning Vb15c:Ja1.3c and (4) internal Va15f:Ja1.3r primers were run on a 1.2% agarose gel with 100bp DNA ladder (Invitrogen). B) Restriction endonuclease digestions of pTα-RF6α with (1) SalI or (2) vector alone and pTβ-RF6β with (3) KpnI or (4) vector alone were run on a 1% agarose gel with a 1kb DNA extension ladder (Invitrogen). These identified pTα-RF6α and pTβ-RF6β vector clones were sequenced and the C) DNA sequence of the alpha (top) and beta (bottom) inserts is shown. The ATG start codons are emboldened. Internal non-coding intron sequence is shaded in blue, downstream J region non-

coding intron sequence is shaded green, and the cloning and internal DNA primer binding sites have been underlined.

3.3.3 Confirming ectopic RF6 TCR vector expression and function

Before using the pTα-RF6α and pTβ-RF6β constructs to generate a RF6 TCR transgenic (RFT-Tg) mouse we wanted to confirm that the recombinant TCR could be expressed from the pTcass expression vectors and functionally respond to its cognate epitope. We first transfected a CD4 T cell line (D10) with the pTα-RF6α and pTβ-RF6β constructs. Since Vα4 or Vβ15 –specific antibodies are unavailable we could not delineate RF6 TCR cell surface expression on D10, which expresses its own endogenous TCR. RNA was collected from transfectants (D10pRFT) to detect transcription of the RF6 TCR by RT-PCR analysis. The PCR reaction of D10pRFT -derived cDNA used a V-region -specific forward primer with a constant-region -specific reverse primer. Within the pTcass expression vectors, the V-J segments of both the alpha and beta chain are separated by greater than 2000bp of noncoding DNA sequence (159). As indicated by the size of the amplified products, less than 300bp (Figure 11A), RNA splicing had successfully joined the cloned variable region with the downstream constant region.

To determine whether vector-expressed RF6 TCR transcripts can be translated to form functional TCR molecules we co-transfected a TCR-deficient mouse T cell hybridoma (58a-b-) with the pTα-RF6α and pTβ-RF6β constructs, along with a mouse CD4 expression plasmid (pCD4). Cells were transfected at a 1:10 pCD4: pT-RF6 ratio to increase the probability that cells receiving the pCD4 selection plasmid would also receive the pT-RF6 constructs. In addition to providing a neomycin resistance gene, allowing for G418 growth selection, pCD4 was used to ensure CD4 co-receptor expression on transfected hybridomas. We found

expression of the endogenous CD4 molecule on 58a-b- cells to be unstable. The G418-resistant transfectants were further screened for CD3 surface expression by flow cytometry to identify RF6 TCR expressing 58a-b- cells (58pRFTpCD4) (Figure 11B). Using an *in vitro* functional assay, we show that the 58pRFTpCD4 cells respond to the minimal H2 glycopeptide epitope but not to the 100mer peptide (MUC1p) (Figure 12A), similar to the responsiveness of the RF6 hybridoma (Figure 12B). Surprisingly, 58pRFTpCD4 cells did not appear to respond to TnMUC1-derived glycopeptide epitopes. The strong responsiveness of RF6 to TnMUC1 -pulsed DCs confirms that the DCs were presenting the epitopes to 58pRFTpCD4.

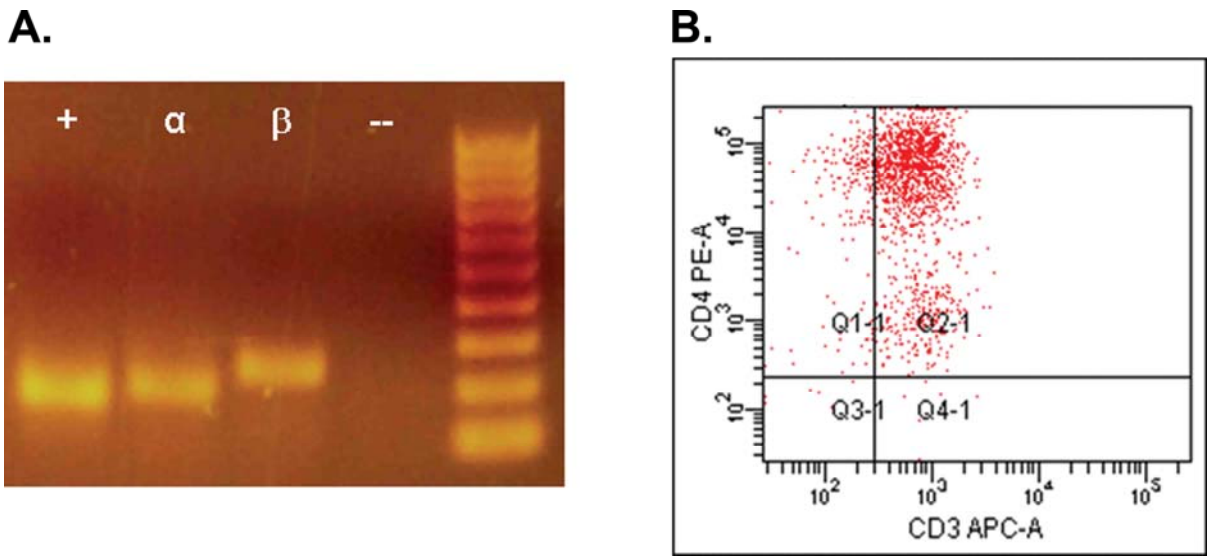


Figure 11. The pTα-RF6α and pTβ-RF6β expression vector constructs express RF6 TCR mRNA transcripts and productive TCR protein complexes.

The pTα-RF6α and pTβ-RF6β constructs were co-transfected along with a mouse CD4 expression plasmid (pCD4). Following transfection of the A) D10 cell line, mRNA was isolated from cell lysates for RT-PCR analysis. The PCR reactions contained primers specific for the D10 α chain (+, Va2m:CαGSP); RF6 α chain (**α**, Va4.1f:CαGSP); RF6 β chain (**β**, Vb15f:CβnestIII); or without primers (**--**). A 100bp DNA ladder (BioRad) was used to confirm the size the PCR products. To identify RF6 TCR cell surface expression from the pTα-RF6α and pTβ-RF6β vectors we

performed a similar co-transfection into the B) 58a-b- hybridoma (endogenous TCRα-β-) followed by G418 growth selection. Growing cells were further analyzed for CD4 and CD3 cell surface expression by flow cytometry.

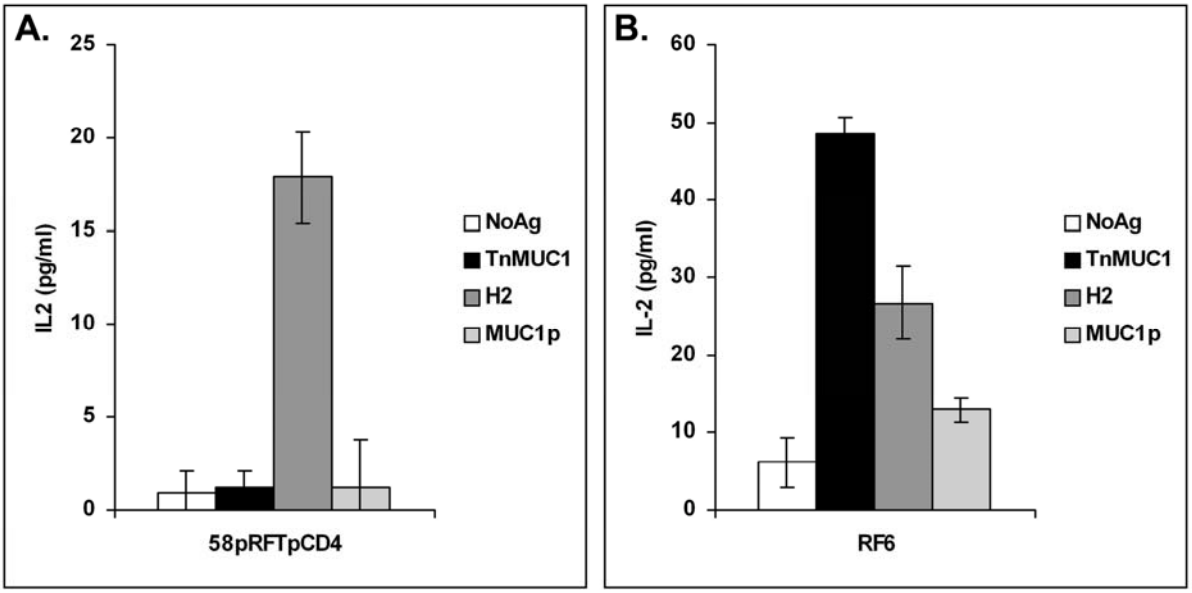


Figure 12. The RFT TCR expressed by the 58a-b- hybridoma functionally responds to the cognate glycopeptide epitope.

The 58pRFTpCD4 hybridoma was tested for TnMUC1 responsiveness in a functional co-culture assay, similar to the assay used in Chapter 2 to assess hybridoma specificity. DCs pulsed with TnMUC1, H2, or MUC1p were co-cultured with A) 58pRFTpCD4 hybridomas or B) RF6 hybridomas. After 48 hrs culture supernatants were collected and analyzed by ELISA for IL-2 levels. Data are representative of three independent experiments.

3.3.4 Generation and functional confirmation of the RFT-Tg mouse line

B6-CD45.1^{+} embryos were microinjectioned with linear pTα-RF6α and pTβ-RF6β constructs (lacking prokaryotic DNA) together or separately, followed by implantation into pseudopregnant B6-CD45.1^{+} female mice. The resulting 65 live births yielded founder RFTαβ-Tg (1 of 30), RFTα-Tg (1 of 21), and RFTβ-Tg (1 of 14) mice. These founders were bred with wild type mice

to confirm germ-line transmission of the transgenes. The RFT alpha and beta transgenes from the double transgenic founder were always transmitted together to offspring, indicating that the gene insertions are on the same chromosome. However, all offspring (F2 and F3 generations) of the double transgenic founder (RFTab) tested did not have detectable TnMUC1-responsive T cells (Figure 13B). Analysis of splenic T cell Vα2 and Vβ8.1/8.2 and Vβ6 chain usage by flow cytometry indicated that there was no skewing of the TCR repertoire compared to transgene negative littermates (WT) (Figure 13A). On the other hand, the cross-breeding of offspring from the single transgenic RFTα-Tg and RFTβ-Tg founders, resulting in double transgenic RFT-Tg mice (RFTbXa), did show skewing of the TCR repertoire (Figure 13A) and the CD4 T cells were TnMUC1-responsive (Figure 13B,C). This indicates that either one or both of the TCR transgenes in RFTab mice are not properly expressed and suggests a defect with the transgene chromosomal insertions. Thus, the RFT-Tg mouse line that is used in all subsequent experiments is the result of interbreeding the single transgenic RFTα-Tg and RFTβ-Tg lineages.

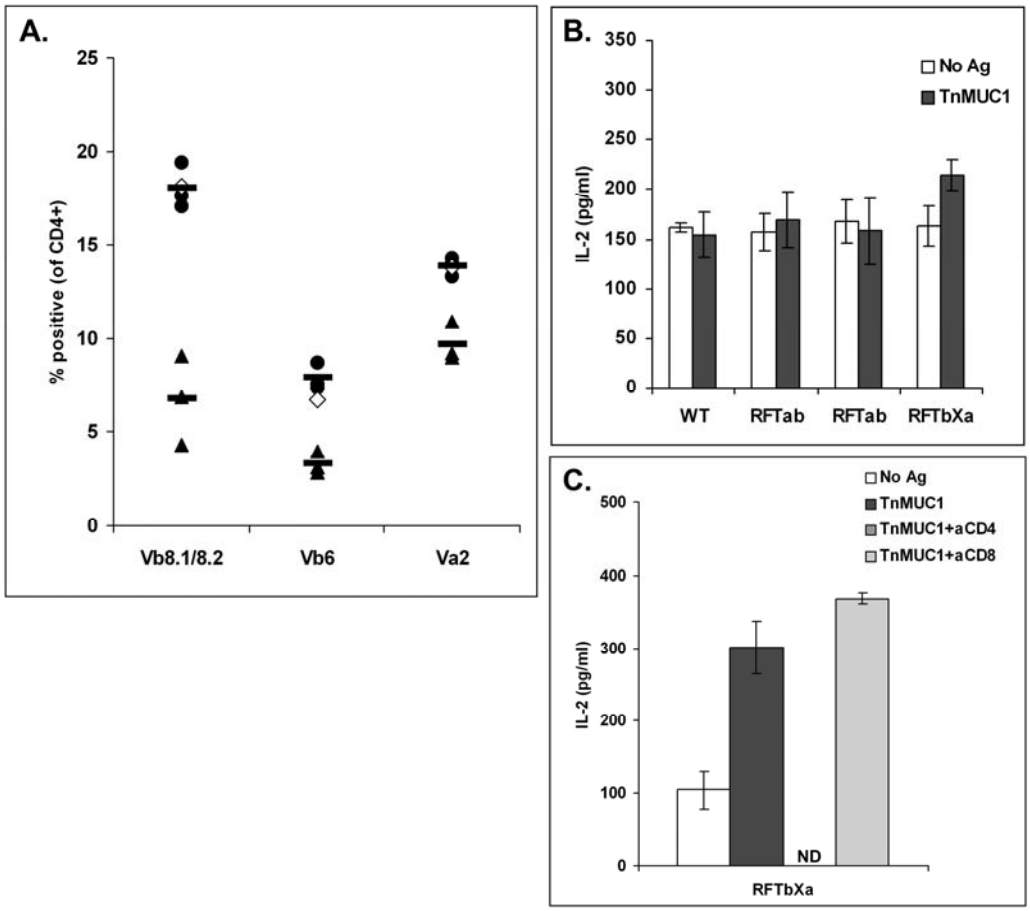


Figure 13. CD4 T cells from only one of two RFT double transgenic (RFT-Tg) mouse lineages have a non-wild type (WT) TCR repertoire and respond to TnMUC1.

We received three founder mice containing genomic RFT TCR transgenes; RFTab (double transgenic), RFTa (RFTα, single transgenic), and RFTb (RFTβ, single transgenic). The RFTα-Tg and RFTβ-Tg mice were interbred to generate the RFTbXa double transgenic lineage. A) Splenocytes were collected from either WT mice (●), RFTab mice (◊), or RFTbXa mice (▲) and labeled with antibodies specific for CD3, CD4 and the Vb8.1/8.2, Vb6, and Va2 TCR chains. The average percentage of RFTab mice or RFTbXa mice T cells ($CD3^+CD4^+$) expressing the indicated TCR chains is shown (—). To assess functional antigenic responsiveness T cells were isolated from either the A) blood or C) spleen and co-cultured with DCs in the absence (No Ag) or presence of 26 µg/ml TnMUC1 in triplicate. Where indicated 2.5 µg/ml anti-CD4 (aCD4) or anti-CD8 (aCD8) blocking antibody was added. After 48 hrs culture supernatants were collected and analyzed by ELISA for IL-2 levels (ND=not detectable).

3.4 DISCUSSION

Using a similar method as that used to generate the MUC1-peptide specific TCR transgenic (VFT-Tg) mouse (153), we have generated a novel MUC1-glycopeptide specific TCR transgenic (RFT-Tg) mouse. We successfully identified and cloned the Vα4.1-Jα16:Vβ15-Jβ1.3 glycopeptide-specific TCR from the RF6 CD4 T cell hybridoma. Interestingly, a past study has shown that the Vα4 and Vβ15 chains that comprise the RFT TCR also predominate in other glycopeptide-specific TCRs (162).

The RFT-Tg mouse T cells responded to TnMUC1-derived epitopes (Figure 13B,C) and the minimal H2 glycopeptide epitope (data not shown) similar to the RF6 hybridoma (Figure 7,12B). This indicates that the inability of transfected 58pRFTpCD4 cells to respond to TnMUC1-derived epitopes (Figure 12A) is not due to an inherent defect in the recombinant TCR. Rather, the discrepancy may indicate a TCR-independent difference between the RF6 hybridoma/primary T cells and the 58a-b- hybridoma's ability to recognize TnMUC1-derived epitopes on DCs. It has been shown that the context in which DCs encounter antigen can affect the outward presentation and responsiveness of T cells (163). It is known that TnMUC1, as opposed to unglycosylated peptide antigens, is endocytosed by DCs by a MGL-receptor mediated mechanism (148), which in turn may lead to the co-expression of additional cell surface molecules that aid in the recognition of processed TnMUC1. The H2 antigen, possibly due to its small size and limited glycosylation, may participate in a divergent antigen processing and presentation pathway than TnMUC1.

Although there are multiple advantages to using the pTcass TCR expression vectors (161) we have also found disadvantages, which we believe are due to the large size of each vector (>20 kb). We have found that the vectors are stable when stored at -80^{o}C in buffer, but in multiple

cases the vectors appeared to alter their conformation after propagation in bacteria, which we have seen previously (153). The result was an apparent decreased size of the vectors (by agarose gel electrophoresis) along with resistance to endonuclease digestion at known cut sites, which indicates that recombination events may be occurring during replication in bacteria (153). In addition, the large size of the pT-RF6 constructs made the D10 cell and 58a-b- hybridoma co-transfection experiments difficult because the molar ratio of pT-RF6 copies per cell must be kept low due to the cytotoxicity of high DNA concentrations, thus transfection efficiency was consistently low.

4.0 CD4 T CELL TOLERANCE VERSUS IMMUNITY TO PEPTIDE AND GLYCOPEPTIDE EPITOPES OF THE TUMOR ASSOCIATED ANTIGEN MUC1 IN MUC1-TG MICE

4.1 INTRODUCTION

Transformed cells can aberrantly express many self-derived molecules that can elicit measurable immune responses. If their protein sequences are not mutated and they are also expressed, albeit differently, on normal cells, they are generally termed tumor-associated antigens (132). Although many human tumor antigens can induce antibody and T cell responses in cancer patients, studies in transgenic or knockout mouse models indicate that a level of central and/or peripheral self-tolerance may hinder effective anti-tumor immunity (37, 116, 117, 120, 164). This may in part explain why cancer patients with detectable immune responses against tumor-associated antigenic peptides still succumb to their disease, and why attempts to boost these responses have been met with only a limited success.

Tumor-associated antigens are sources of many epitopes; those that are derived from the tumor form of the antigen can be very different from those derived from the normal form that is found on healthy tissue. A safer and potentially much more effective alternative to breaking self-tolerance in order to elicit tumor immunity (131), which brings the risk of autoimmunity, is to elicit immunity to epitopes that are less self and more tumor-specific (foreign). Abnormal

expression of many self molecules by transformed cells, such as over-expression, unscheduled or constitutive expression, mislocalization and abnormal posttranslational modifications provide a spectrum of tumor-specific epitopes. An example we studied here is the tumor-associated antigen Mucin 1 (MUC1). Most human adenocarcinomas over-express and aberrantly glycosylate this transmembrane glycoprotein, characterized by exposure of short *O*-linked sugar chains and areas of non-glycosylated protein backbone in the extracellular variable number of tandem repeats (VNTR) region. Each tandem repeat is a 20 amino acid sequence, HGVTSAPDTRPAPGSTAPPA, which can vary from 25-125 repeats per allele, effectively dominating the extracellular domain of MUC1 (122). MUC1-specific $CD8^+$ cytotoxic T lymphocytes (CTL) that recognize VNTR peptides are found in patients with $MUC1^+$ tumors indicating that an immune response can be generated against them (32, 133). However, clinical trials using MUC1 VNTR peptide-based vaccines to boost this immunity have resulted in little change in CTL activity and ineffective anti-MUC1 antibody class switching beyond the IgM isotype (43, 134, 135). Studies in the MUC1-transgenic (MUC1-Tg) mouse model have shown that low antibody and CTL responses to MUC1 VNTR peptides are due in part to MUC1 peptide-specific CD4 T cell hypo-responsiveness compared to responses in transgene-negative (wild type; WT) littermates (9, 110, 111). This is of concern since CD4 T helper (Th) cells are a critical component of effective anti-tumor immunity and required for effective B cell priming and antibody isotype-switching, CTL expansion, and CD8 T memory cell responses (136).

As an alternative to tumor-derived peptides, tumor-associated glycoprotein antigens, such as MUC1, could be targeted via their tumor-associated glycopeptides (137). Some tumor-associated glycoproteins carry the Tn (GalNAc-O-S/T) and T (Gal-GalNAc-O- S/T) glycan antigens. These core carbohydrates are not normally exposed on healthy cells or tissues with

benign disease, yet they are found in ~90% of carcinomas (many being adenocarcinomas) (124). Tumor-associated glycopeptides derived from the mucin MUC1 have been shown to be viable targets for T cells and anti-tumor immunity (138-140), and we have shown that, in the MUC1-Tg mouse model, responses to MUC1 glycopeptide are improved over those obtained against the MUC1 peptide alone (112). To dissect the nature of self-tolerance mechanisms arising *in vivo* from antigens derived from a MUC1 peptide harboring Tn antigens (TnMUC1) versus its unglycosylated MUC1 peptide (MUC1p), we generated two new MUC1-specific T cell receptor (TCR) transgenic mice; one that specifically recognizes TnMUC1 epitopes and the other selective for MUC1p determinants. We show that MUC1p-specific CD4 T cells are not deleted through central tolerance mechanism(s) during thymic development or later in the periphery of MUC1-Tg mice. Suppression of these T cells occurs upon antigenic stimulation, indicated by their attenuated antigen-specific proliferation in MUC1-Tg recipients compared to WT recipients. Conversely, MUC1 glycopeptide-specific CD4 T cells respond similarly in both WT and MUC1-Tg mice. The similar responsiveness of MUC1 glycopeptide-specific CD4 T cells in both WT and MUC1-Tg mice mimicked that of ovalbumin (ova) -specific (OTII) CD4 T cells, indicating that the MUC1 glycopeptide antigen is not seen as a self molecule in MUC1-Tg mice. In addition, we show that co-activation of MUC1 glycopeptide-specific T cells confers “help” to MUC1 peptide-specific T cells, bringing their level of activation up to that seen in WT mice.

4.2 METHODS

4.2.1 Mice and cell lines

All mice were bred and maintained in a specific pathogen-free environment at the University of Pittsburgh and were treated under IACUC-approved guidelines in accordance with approved protocols. C57BL/6, B6.PL-Thy1a/Cy, and B6.SJL-PtprcaPepcb/BoyJ mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and MUC1-Tg mice (99) from Dr. S. Gendler (Mayo Clinic, Scottsdale, AZ). MUC1-transgene positive and transgene negative (wild type; WT) mice from heterozygous breeding were identified by PCR analysis. VFT and RFT TCR transgenic mice were generated at the University of Pittsburgh transgenic mouse facility.

4.2.2 Generation of VFT mice

The TCR alpha chain of the VF5 hybridoma (141) was amplified by 5' RACE using a Generacer Kit (Invitrogen, Calsbad, CA) in combination with reverse primers specific for TCR-Cα. The TCR beta chain was amplified using degenerate Vβ primers (165) and reverse primers specific for TCR-Cβ. The identity of the amplified TCR chains was determined by sequencing, performed at the University of Pittsburgh Sequencing Core Facility. The rearranged Vα2.5-Jα49 and Vβ6-Jβ2.5 TCR gene segments, including ~150bp of intron downstream of the J gene segments, were amplified from genomic DNA of the VF5 hybridoma and cloned using the pcDNA3.1/V5-His vector (Invitrogen). Intact cloned TCRα or TCRβ fragments were identified by sequencing, then sub-cloned into the TCR cassette vectors pTα or pTβ respectively (161), re-

sequenced and tested for functional expression by transfection into DO11.10 hybridomas. Founder VF5α and VF5β mice were generated by microinjection of B6.PL-Thy1a/Cy (CD90.1^{+}) embryos with linear pTα-VF5α and pTβ-VF5β constructs, respectively. VF5α and VF5β founder mice were identified by PCR of tail tissue DNA using primers specific for the Vα2-Jα49 and Vβ6-Jβ2.5 rearrangements, then crossbred to produce double-transgenic VFT-Tg mice.

4.2.3 Generation of RFT mice

Generation of RFT-Tg mice is described in detail in Chapter 3. Briefly, the TCR alpha and beta chains of the RF6 hybridoma (112) were amplified by 5' RACE using a Generacer Kit (Invitrogen), and identified to have Vα4.1-Jα16 and Vβ15-Jβ1.3 arrangements. Founder RF6α and RF6β mice were generated by microinjection of B6.SJL-PtprcaPepcb/BoyJ (CD45.1^{+}) embryos with linear pTα-RF6α and pTβ-RF6β constructs, respectively. Founder mice were identified by PCR of tail tissue DNA using primers specific for the Vα4.1-Jα16 and Vβ15-Jβ1.3 rearrangements, then cross-bred to produce double-transgenic RFT-Tg mice.

4.2.4 Flow cytometry

Cells were labeled with indicated antibodies at a dilution of 1:50 in FACs buffer (PBS, 5% FBS, .01% sodium azide) for 30 minutes on ice. Further intracellular labeling was carried out using the BD Cytofix/Cytoperm Solution Kit following the manufacture's protocol (BD Biosciences, San Jose, CA).

4.2.5 Bone marrow transplantation

Lineage negative (Lin^{neg}) bone marrow precursors were purified from VFT-Tg mice using a Lineage Cell Depletion Kit (Miltenyi, CA). WT or MUC1-Tg recipient mice were irradiated (900Rad) 4h hours prior to injection via tail vein with 10^5 Lin^{neg} cells, plus $2x10^5$ host-type BM cells to ensure survival (166). Spleens were harvested from recipient mice 5-6 weeks post transfer and single cell suspensions were analyzed by flow cytometry. Briefly, cells were labeled with indicated antibodies to determine the presence of VFT T cells and their respective intracellular FOXP3 expression.

4.2.6 T cell adoptive transfer

T cells were purified from the spleens of VFT-Tg, RFT-Tg, or OTII-Tg donor mice by CD3 negative selection using magnetic antibody cell sorting (MACS) microbeads following the manufacturer's protocol (Miltenyi Biotech, Auburn, CA). In some experiments, purified donor T cells were labeled with 5μM CFSE (Molecular Probes, Carlsbad, CA) prior to transfer. 3-5 x 10^6 donor T cells were transferred to recipient mice by lateral tail vein injection. For experiments involving *in vivo* stimulation of donor T cells, recipient mice were vaccinated with antigen-loaded or non-loaded DCs, administered by lateral tail vein (i.v.) injection.

4.2.7 MUC1 peptides and glycopeptides

The 100mer peptide (MUC1p) represents 5 repeats of the 20 amino acid sequence HGVTSAPDTRPAPGSTAPPA from the MUC1 VNTR region and was synthesized as described

previously (111). The GalNAc-100mer (Tn100mer/TnMUC1) was prepared by enzymatic addition of GalNAc to the synthetic peptide substrate using a recombinant human UDP-GalNAc:polypeptide N-acetyl-galactosaminyltransferase rGalNAc-T1 as previously described (143). The final reaction product contained a heterogeneous mixture of 9 to 15 GalNAc residues per 100mer peptide molecule that were incorporated within the threonine of the VTSA region and adjoining serine and threonine within the GSTA region as defined previously (143). Both MUC1p and TnMUC1 antigens were synthesized at the University of Pittsburgh Genomics and Proteomics Core Laboratories.

4.2.8 Generation of bone marrow derived dendritic cells (DC) and vaccination protocol

DCs were generated as previously described (141) with a few modifications. Briefly, bone marrow cells from C57Bl/6 mice were subjected to RBC lysis, then plated at 1 x 10^6 cells/ml in serum-free AIM-V media (Invitrogen) containing sodium pyruvate, nonessential amino acids, and 2-ME; supplemented with 10ng/ml of GM-CSF (R&D Systems, Minneapolis, MN). Cells were fed on Day 3 by replacing one-half of the culture volume with fresh AIM-V plus 10ng/ml GM-CSF. DCs were purified on Day 6 of culture using a Nycoprep 1.068 (Accurate Chemical, Westbury, NY) gradient. For *in vivo* vaccinations, DCs were loaded with peptides and/or glycopeptides overnight at 37^{o}C in the presence of 10ng/ml GM-CSF. The loaded DCs were washed with PBS and administered to recipient mice by i.v. injection (3-5 x 10^5 viable cells in 200µl PBS/mouse).

4.3 RESULTS

4.3.1 MUC1 peptide-specific CD4 T cells are not deleted in the thymus of MUC1-Tg mice but their function is suppressed in the periphery

Previous work has indicated that MUC1-specific tolerance in the MUC1-Tg mouse is responsible for the hypo-responsiveness of MUC1-specific CD4 Th cells *in vivo* (99, 109, 111). Recent studies in MUC1-Tg mice have detected MUC1p-specific CD4 T cells *ex vivo* using alternative vaccination strategies (9, 110) and *in vitro* using T cell cloning techniques (our unpublished data) suggesting that anti-MUC1p CD4 T cells have not simply been deleted. Similarly, although MUC1p-specific CD4 T cells have been difficult to detect directly *ex vivo* from cancer patients, they have been detected *in vitro* using T cell cloning techniques (89). To better address the function of MUC1p-specific T cells in an environment where MUC1 is present as a self molecule, we generated MHC class II (I-A^b) –restricted, MUC1 peptide (MUC1p)-specific VFT TCR (Vα2.5-Vβ6) transgenic mice, which produce high numbers of functionally responsive CD4 T cells (Figure 14). The VFT-Tg mice ($CD90.1^+$) allow us to specifically explore for the first time whether MUC1p-specific CD4 T cells (VFT) that express a TCR selected in the WT environment where MUC1 is a foreign antigen, would be subject to central or peripheral tolerance in MUC1-Tg mice ($CD90.2^+$), where MUC1 is a self-antigen.

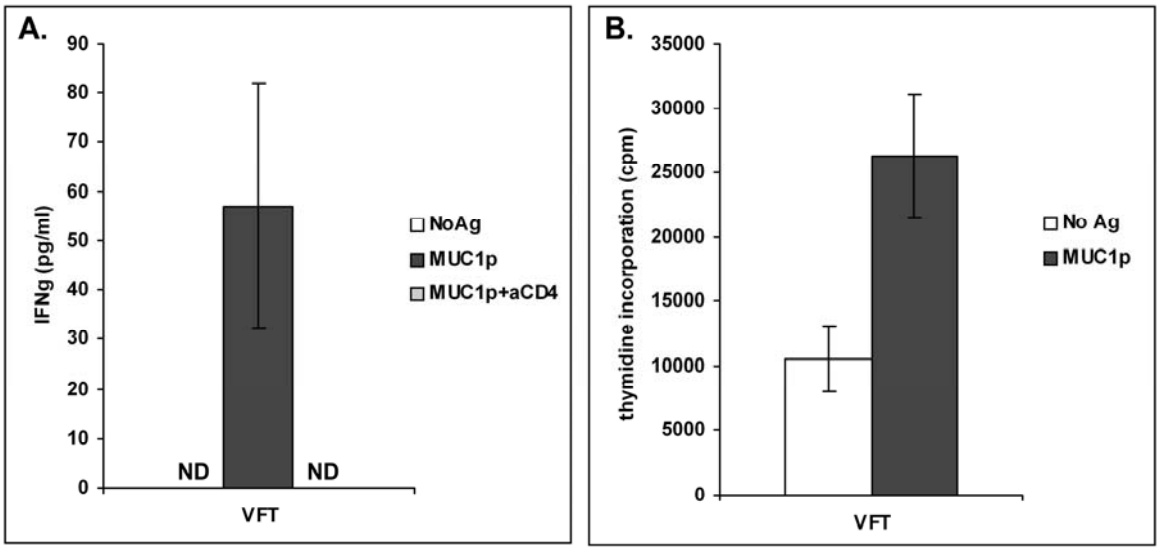


Figure 14. VFT-Tg CD4 T cells are functionally responsive to their cognate MUC1p epitope.

T cells were isolated from VFT-Tg mouse spleens and cultured in 96 well plates in the absence (NoAg) or presence of 20μg/ml MUC1p. DCs were used as antigen presenting cells in all conditions. Blocking anti-CD4 (aCD4) antibody was used at 2.5μg/ml were indicated. After three days, supernatant from A) VFT T cell cultures was collected for IFNγ (IFNg) analysis by ELISA and the cells were cultured for an additional 18-20 hours in the presence of tritiated thymidine. B) thymidine incorporation is shown as counts per minute (cpm). Each condition is shown as the average and standard deviation of three separate wells per condition. Results are representative of greater than three independent experiments.

We transferred lineage negative VFT-Tg bone marrow precursors along with bulk WT bone marrow to lethally irradiated WT and MUC1-Tg mice. The WT bone marrow served as a source of erythrocytes to prevent anemia in lethally irradiated recipients. Five weeks later we found no significant difference between WT and MUC1-Tg recipient mice in the percentage of VFT CD4 T cells that were able to develop and migrate to the periphery (Figure 15A). In addition, the percentage of those VFT CD4 T cell emigrants expressing a $V\alpha2^+$ TCR was not significantly different (data not shown). These findings indicate that the MUC1p-specific CD4 T

cells were not subjected to either thymic negative selection or peripheral clonal deletion in MUC1-Tg mice.

To address the possibility that the MUC1p-specific CD4 T cell precursors in MUC1-Tg mice might have developed into natural T regulatory (Treg) cells due to potential encounters with MUC1p in the thymus, we examined FOXP3 expression in the VFT CD4 T cell thymic emigrants. While we did find $FOXP3^+$ VFT CD4 T cells, there was no significant difference in their percentage in WT versus MUC1-Tg recipient mice (Figure 15B). Thus, VFT CD4 T cells developing in the MUC1-Tg recipients were neither preferentially programmed in the thymus to be natural Treg cells, nor converted to inducible $FOXP3^+$ Treg cells in the periphery.

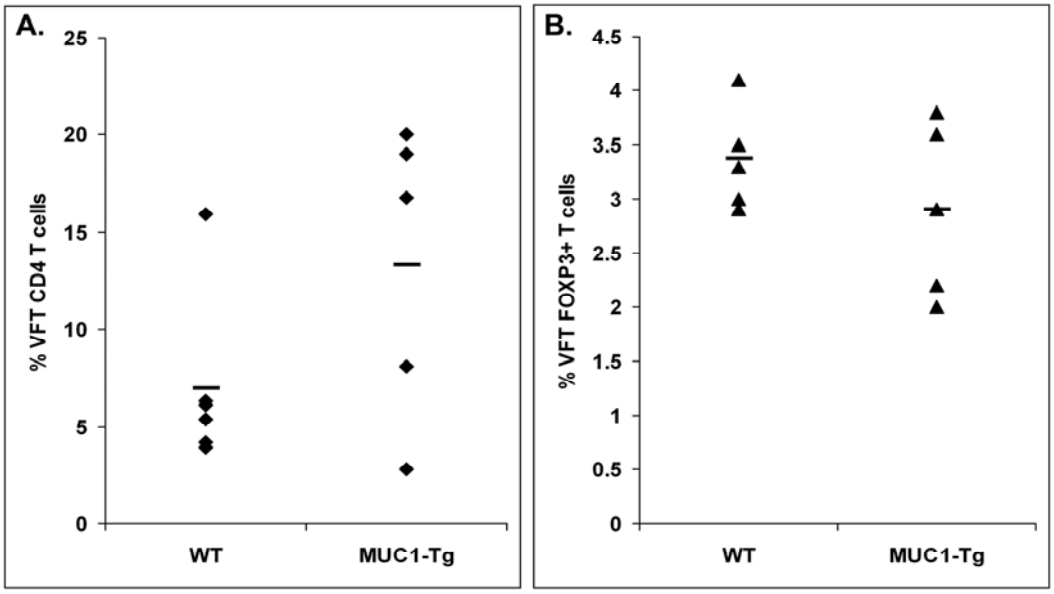


Figure 15. VFT precursors develop through the thymus and enter the periphery at equal levels in WT and MUC1-Tg mice.

Recipient mice (WT, n=6 and MUC1-Tg, n=5) were lethally irradiated prior to bone marrow transfer. Five weeks post VFT bone marrow transfer, the presence of mature VFT CD4 T cells in the spleens of recipient mice was assessed by flow cytometry. A) Percent of donor cells ($CD90.1^+$) in the $CD3^+CD4^+$ gated population of each

recipient mouse. B) Intracellular FOXP3 expression in donor cells. These data are representative of two independent experiments.

Mature T cells were then isolated from VFT-Tg mouse spleens and transferred into recipient WT and MUC1-Tg mice to examine the function of these cells in the periphery. Five to 7 days later, some of the recipient mice from both groups were sacrificed to examine donor cell responses to possible endogenous antigenic stimuli. VFT cells were identified by their expression of the congenic molecule CD90.1 and by the expression of Vα2 TCR chain. At 5-7 days post transfer, VFT CD4 T cells could easily be detected. A significantly higher percentage of VFT CD4 T cells had also proliferated in MUC1-Tg recipients as compared to WT recipients, as monitored using the fluorescent label CFSE [serving as a marker for cell proliferation] (Figure 16A). In addition, those proliferating VFT CD4 T cells in MUC1-Tg mice had a lower CFSE mean fluorescence intensities (MFI) than those in WT mice, indicating that the former had gone though more cell divisions (Figure 16B). The proliferating cells in the MUC1-Tg mice did not appear to be converted into $FOXP3^+$ Treg cells as percentage of these cells at 5-7 days post transfer was comparable in WT and MUC1-Tg mice (Figure 16C). There was also no significant expansion of VFT cells in the MUC1-Tg mice as shown by the same overall percentage of transferred cells in WT and MUC1-Tg recipients (Figure 16D).

The remaining recipient mice were vaccinated twice with MUC1p-loaded dendritic cells with the addition at the time of injection of soluble MUC1p (DC-MUC1p) (109) to stimulate the VFT CD4 T cells *in vivo*. Following the final DC-MUC1p vaccination, a significant expansion of the VFT CD4 T cell population was observed in WT recipients, indicating an effective T cell stimulation (Figure 16E). Conversely, the VFT CD4 T cells failed to expand in MUC1-Tg recipients that received the same DC-MUC1p vaccine (Figure 16E). Some recipient mice were

sacrificed 5 days following the primary DC-MUC1p vaccination and their splenocytes stimulated *ex vivo*. T cells from WT recipients previously vaccinated with DC-MUC1p produced significant levels of IFNγ in response to MUC1p (Figure 16F), whereas those from MUC1-Tg recipients responded very weakly (Figure 15G).

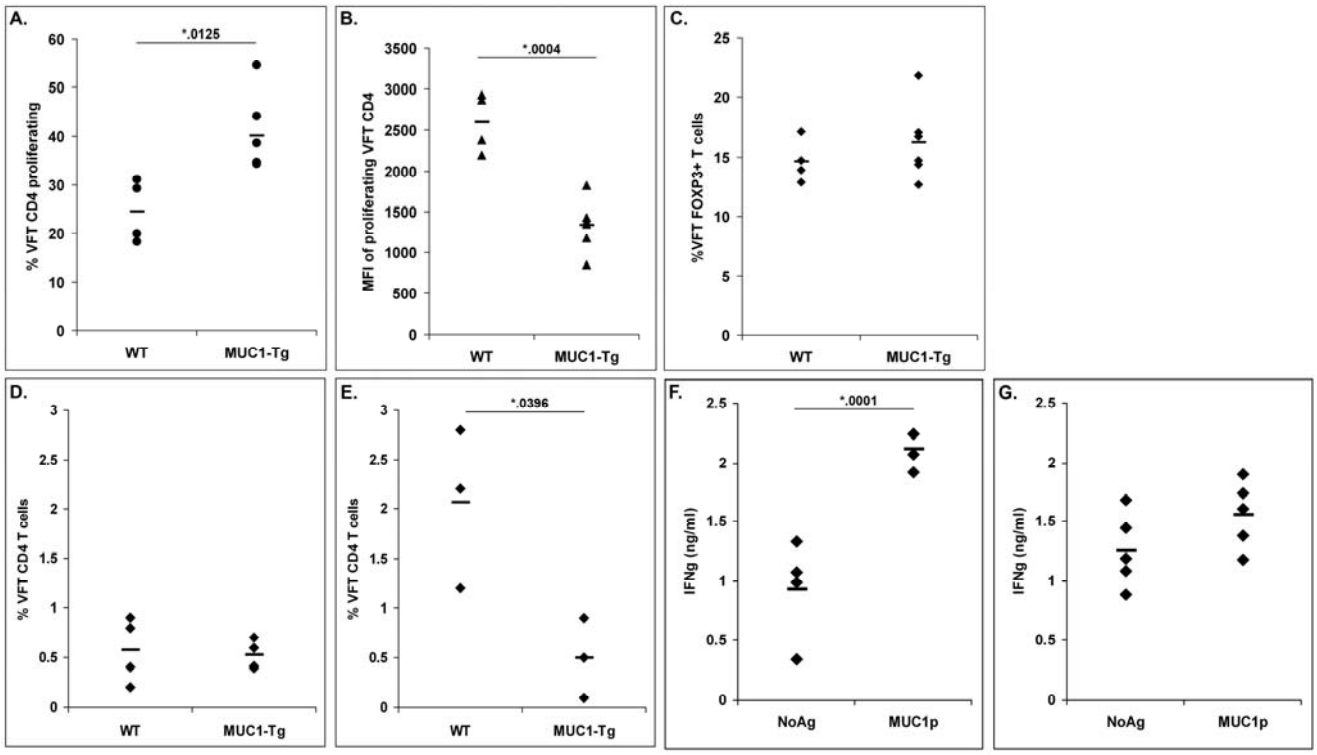


Figure 16. MUC1p-specific VFT CD4 T cells proliferate in the periphery to endogenous stimulation but are suppressed to MUC1p vaccination in MUC1-Tg mice.

3-5x10^6 CFSE-labeled VFT T cells were transferred (i.v.) to recipient mice. Five to 7 days following adoptive transfer, some recipient mice (WT, n=6; MUC1-Tg, n=6) were sacrificed and the presence of donor VFT CD4 T cells ($V\alpha2^+CD4^+$ $CD90.1^+$) in the spleen was determined by flow cytometry. VFT CD4 T cell proliferation is shown as A) percentage of cells with decreased CFSE florescence and B) CFSE mean fluorescent intensity (MFI) of dividing cells. C) Percent VFT CD4 T cells that are $FOXP3^+$. D) Percent of VFT CD4 T cells with respect to the recipient CD4 T cell population prior to vaccination (WT, n=4; MUC1-Tg, n=4) and E) following two doses of the DC-MUC1p vaccine (i.v.) (WT, n=3; MUC1-Tg, n=3). F) WT (n=4) and G) MUC1-Tg (n=5) mice were sacrificed

following the primary DC-MUC1p dose and IFNγ production from recipient mouse splenocytes was measured by ELISA after a 72 hr of *in vitro* MUC1p stimulation (NoAg = No antigen control). Each data point is the average of triplicate wells per condition for individual mice. The p values were calculated using an unpaired t test.

4.3.2 MUC1 glycopeptide-specific CD4 T cells are not suppressed in MUC1-Tg mice

To study MUC1 tumor-associated glycopeptide specific CD4 T cell responses, we generated MHC class II (I-A^b) –restricted, MUC1 glycopeptide (TnMUC1) -specific RFT TCR (Vα4.1-Vβ15) transgenic mouse. The specificity of the RFT-Tg T cells for the MUC1 glycopeptide is shown in Figure 17.

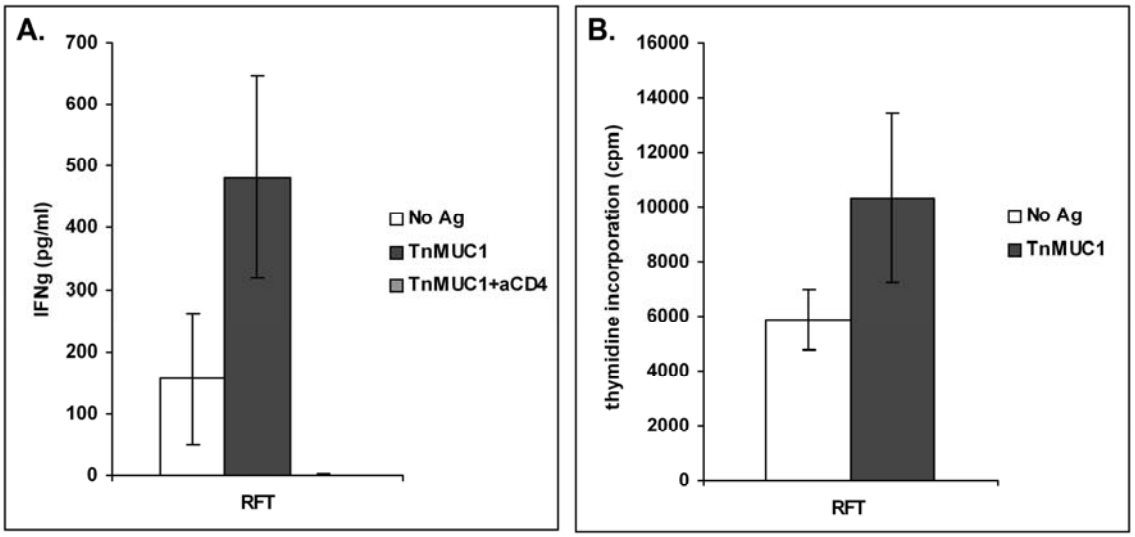


Figure 17. RFT-Tg CD4 T cells are functionally responsive to their cognate TnMUC1 epitope.

T cells were isolated from RFT-Tg mouse spleens and cultured in 96 well plates in the absence (NoAg) or presence of 26μg/ml TnMUC1. DCs were used as antigen presenting cells in all conditions. Blocking anti-CD4 (aCD4) antibody was used at 2.5μg/ml were indicated. After three days, supernatant from A) RFT T cell cultures was collected for IFNγ (IFNg) analysis by ELISA and the cells were cultured for an additional 18-20 hours in the presence of tritiated thymidine. B) thymidine incorporation is shown as counts per minute (cpm). Each condition is shown as the average and standard deviation of three separate wells per condition. Results are representative of greater than three independent experiments.

Similar to the above experiments with the peptide-specific VFT T cells, RFT CD4 T cells ($CD45.1^+$) were labeled with CFSE and transferred into WT and MUC1-Tg recipients ($CD45.2^+$). At 5-7 days post transfer we could detect a slight increase in RFT CD4 T cell proliferation in MUC1-Tg recipients compared to WT recipients (Figure 18A,B), yet there was no significant difference in the overall percentage of RFT CD4 T cells surviving in WT and MUC1-Tg recipients (Figure 18C).

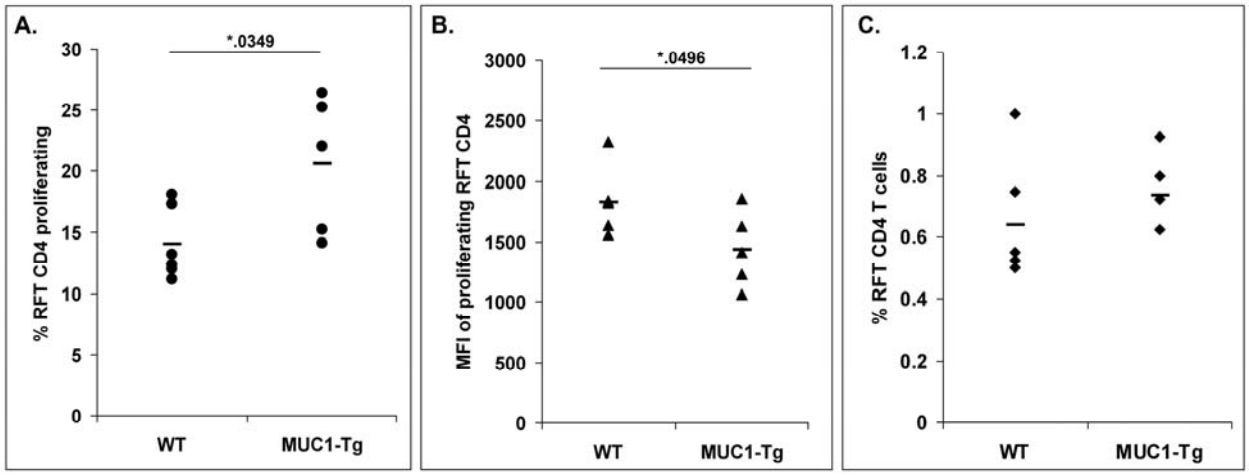


Figure 18. TnMUC1-specific RFT CD4 T cells proliferate to endogenous stimulation in the periphery of MUC1-Tg mice.

3-5x106 CFSE-labeled T cells were transferred (i.v.) to recipient mice (WT, n=6; MUC1-Tg, n=5). 5-7 days following adoptive transfer, recipient mice splenocytes were analyzed by flow cytometry for the presence of RFT CD4 T cells ($CD3^+CD4^+CD45.1^+$). RFT CD4 T cell proliferation is shown as A) percent of cells with decreased CFSE florescence and B) CFSE mean fluorescent intensity (MFI) of dividing cells. C) Percent of RFT CD4 T cells with respect to the recipient mouse CD4 T cell population. Data is representative of two independent experiments. The p values were calculated using an unpaired t test.

We next addressed the functional difference between RFT and VFT CD4 T cells, especially the possibility that glycopeptide specific RFT cells might not be suppressed in MUC1-Tg mice because, unlike VFT cells that recognize a normal (self) peptide, RFT recognize an abnormal glycopeptide that would not be considered a self epitope. We transferred CFSE-labeled T cells into WT and MUC1-Tg recipients one day prior to a single vaccination with DC loaded with either MUC1-p or TnMUC1. We confirmed that indeed RFT CD4 T cells proliferated to the same extent in response to TnMUC1 stimulation in both the WT and the MUC1-Tg environment (Figure 19A). In contrast, there was again a significant inhibition of VFT CD4 T cells in response to MUC1p (Figure 19B). Furthermore, VFT CD4 T cells that did proliferate in the MUC1-Tg mice underwent fewer cell divisions than VFT T cells in WT recipients (data not shown). Similar results were obtained using CD4 T cells from OTII TCR-Tg mice, specific for the foreign antigen chicken ovalbumin (167). CFSE-labeled OTII T cells were transferred to WT or MUC1-Tg recipients and stimulated *in vivo* with DC loaded with ovalbumin peptide. As with RFT CD4 T cells, transferred OTII CD4 T cells responded to antigenic stimulation similarly in WT versus MUC1-Tg mice (Figure 19C). To ensure that our DC-based vaccinations were generating antigen-specific CD4 T cell responses, all T cell adoptive transfer experiments contained groups of WT recipient mice that received DCs alone and showed no T cell proliferation (unpublished data).

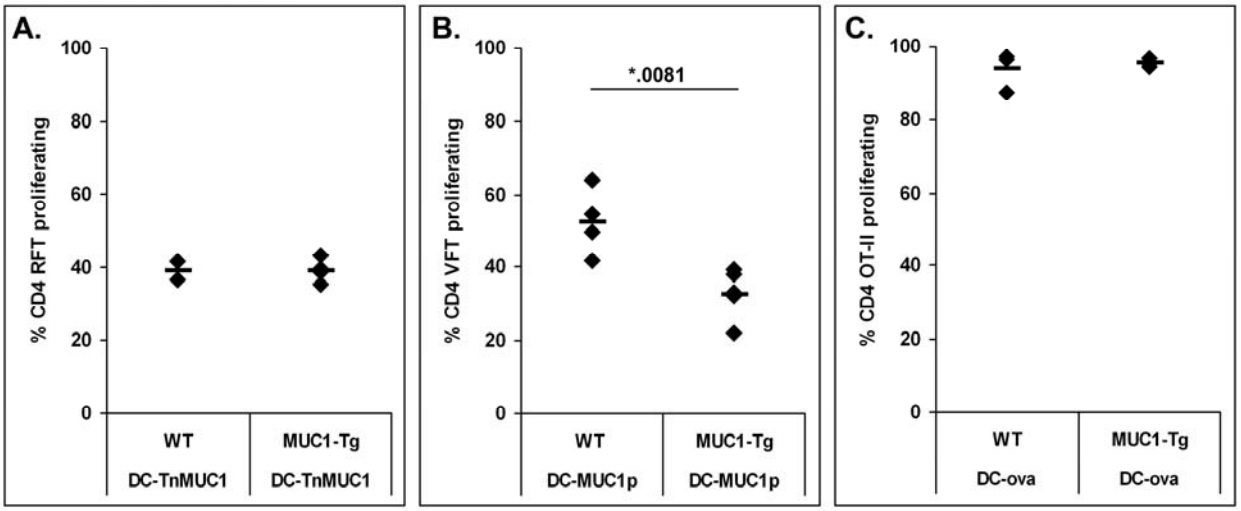


Figure 19. MUC1p-specifc suppression of VFT CD4 T cells, but not RFT or OTII CD4 T cells.

3-5x106 CFSE-labeled RFT, VFT and OTII T cells were transferred (i.v.) to WT and MUC1-Tg recipient mice. One day later, mice were vaccinated (i.v.) using DC-TnMUC1, DC-MUC1p, or DC-ova, respectively. 4-5 days following the vaccination, the spleens of recipient mice were analyzed by flow cytometry for the presence of donor A) RFT CD4 T cells ($CD45.1^+$) (WT, n=2; MUC1-Tg, n=4) or B) VFT CD4 T cells ($V\alpha2^+CD90.1^+$) (WT, n=4; MUC1-Tg, n=5) or C) OTII CD4 T cells ($CFSE^+$) (WT, n=3; MUC1-Tg, n=3) as a percentage of total $CD3^+CD4^+$ cells. Proliferation of donor cells was determined by a decrease of CFSE fluorescent intensity. The p value was calculated using an unpaired t test.

4.3.3 Simultaneous activation of glycopeptide and peptide-specific T cells overcomes suppression of peptide-specific T cells

Peptide-specific VFT and glycopeptide-specific RFT CD4 T cells were mixed at a 1:1 ratio, labeled with CFSE, and transferred to WT and MUC1-Tg recipients. One day later, recipient mice were vaccinated with DCs loaded with both MUC1p and TnMUC1 to ensure that both glycopeptide and peptide epitopes would be presented by the same DC. When RFT and VFT T cells were concurrently activated *in vivo*, we saw no suppression of VFT CD4 T cells in MUC1-Tg recipients compared to WT recipients (Figure 20A). Importantly, RFT CD4 T cells were

themselves not suppressed in the presence of VFT cells that were expected to be suppressed with concurrent MUC1p presentation (Figure 20B). When a co-transfer experiment was performed of VFT T cells and the foreign antigen (ovalbumin)-specific OTII CD4 T cells, the suppression of the VFT CD4 T cells in MUC1-Tg recipients was also overcome by co-stimulation with MUC1p and ovalbumin (Figure 20C). Like RFT cells, OTII CD4 T cells responded at similar levels in both environments (Figure 20D).

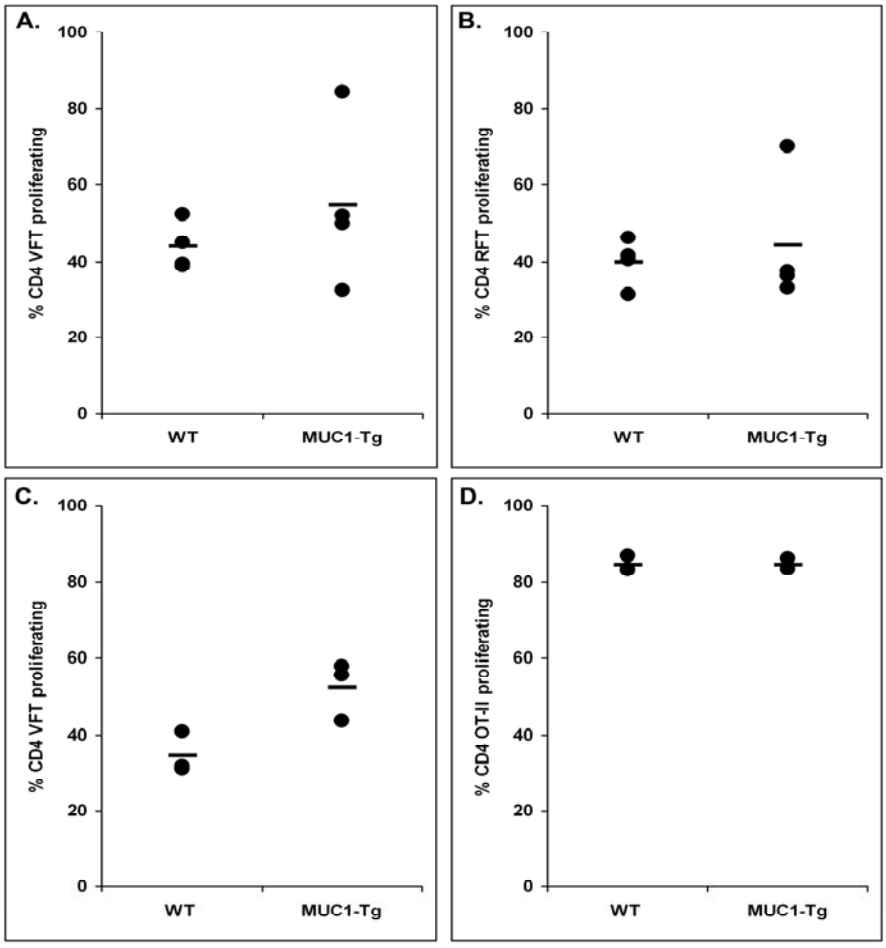


Figure 20. Stimulation of foreign antigen-specific CD4 T cells can help break endogenous MUC1p-specific CD4 T cell tolerance.

3-5x106 CFSE-labeled A,B) VFT and RFT T cells (WT, n=4; MUC1-Tg, n=4) or C,D) VFT and OTII T cells (WT, n=3; MUC1-Tg, n=3) were co-transferred (i.v.) to recipient mice (WT and MUC1-Tg). One day later, mice were

vaccinated (i.v.) using DC-(MUC1p+TnMUC1) or DC-(MUC1p+ova), respectively. Four to 5 days following the vaccination, the spleen of each recipient mouse was analyzed by flow cytometry for the presence of either A) VFT CD4 T cells ($V\alpha2^+CD90.1^+$) and B) RFT CD4 T cells ($CD45.1^+$) or C) VFT CD4 T cells and D) OTII CD4 T cells ($CFSE^+$) as a percentage of total $CD3^+CD4^+$ cells. The proliferation of donor cells was determined by a decrease of CFSE fluorescent intensity.

4.4 DISCUSSION

Past and present studies in the MUC1-Tg mouse model of human responses to the MUC1 tumor antigen have indicated that in the context of the "self" environment, immune responses to unglycosylated MUC1 VNTR peptides are greatly reduced thus compromising anti-MUC1 tumor immunity (9, 37, 109-111). As a strategy to increase the potency of MUC1 vaccines, we altered the peptide immunogen by adding tumor-associated Tn glycans to more closely resemble epitopes that would be displayed on $MUC1^+$ tumors and on APCs that cross-present tumor MUC1 to T cells in patients.

To directly study the fate and function of tumor-associated MUC1-specific CD4 T cells in MUC1-Tg mice, we generated two new TCR-Tg mice, one bearing a peptide-specific TCR and the other a glycopeptide-specific TCR. We show that MUC1 peptide-specific CD4 T cells are not deleted through central tolerance when developing in MUC1-Tg mice, but rather are under the control of peripheral tolerance. MUC1 glycopeptide-specific CD4 T cells, on the other hand, are unrestrained by peripheral suppression, resembling CD4 T cells specific for the foreign antigen ovalbumin. When co-activated, MUC1 glycopeptide-specific CD4 T cells provide "help" to the otherwise suppressed MUC1 peptide-specific CD4 T cells in the MUC1-Tg mouse.

The best known mechanism of central tolerance occurs by negative selection, whereby self-reactive T cells are deleted from the repertoire (168, 169). More recently, it has been shown that developing thymocytes can differentiate into CD4 T regulatory (Treg) cells due to positive signals received by self antigen recognition in the thymus (170-172). Normal, fully glycosylated MUC1 is expressed by human medullary thymic epithelial cells (mTEC) (173) and could theoretically influence the MUC1-specific T cell repertoire. Ectopic expression of other peripheral-tissue antigens by mTECs has been reported to result in central tolerance [reviewed in (174, 175)], including that to tumor-associated carcinoembryonic antigen (CEA) (120, 173). However, we have published that complete glycosylation of the MUC1 VNTR region prevents efficient processing and presentation of peptide epitopes by DC (145, 176), thus, the extent to which the MUC1p-specific T cell repertoire might be affected by self-tolerance was not clear. By comparing MUC1p-specific T cell development in WT versus MUC1-Tg mice, we did not detect any difference in efficiency of emigration from the thymus, suggesting that the MUC1-GVTSAPDTRPAP peptide is either not presented to developing thymocytes, or not at sufficient levels to induce deletion or conversion to Treg cells.

Although peripheral tolerance is not as well understood as central tolerance, studies have shown that auto-reactive T cells specific for model self-antigens (177) and tumor-associated antigens (74, 116) that are expressed in peripheral tissues can be tolerized at peripheral sites. Proliferation of transferred VFT CD4 T cells in unvaccinated MUC1-Tg mice and not in WT mice suggests that peptide epitopes are being presented in the periphery. The similar levels of $FOXP3^+$ VFT CD4 Treg cells in the periphery of WT and MUC1-Tg mice indicate that the weak responses induced by MUC1-peptide vaccines in MUC1-Tg mice might be related to mechanisms other than Treg cell induction. We believe the proliferation of RFT CD4 T cells in

unvaccinated MUC1-Tg mice is also in response to peptide epitopes. We previously reported that the RF6 hybridoma, source of the RFT TCR, weakly cross-reacts with MUC1p while responding with high affinity to TnMUC1 (112). This finding would in part explain the observed proliferation of RFT CD4 T cells in MUC1-Tg mice (weak MUC1p recognition), yet the lack of suppression observed following DC-TnMUC1 vaccination (strong TnMUC1 recognition).

It should be noted that there have been no reports of MUC1p vaccine-induced autoimmunity in MUC1-Tg mice (9, 37, 109), in primates (42, 178, 179), or in human clinical trials (43, 135). Thus, it is likely that MUC1p epitopes are presented at low levels and in the absence of co-stimulation, which does not allow peptide-specific T cells to become effector cells and pose threat to normal tissues. This postulated *in vivo* response may lead to T cell anergy (180) or generation of MUC1p-specific Treg cells (111) that in turn suppress responses to exogenous MUC1p vaccinations. The end result is that when this epitope is presented in a highly immunogenic context via DC-MUC1p vaccination, expansion of fully functional peptide-specific effector T cells is prevented in the periphery. This inability of VFT CD4 T cells to expand *in vivo* helps to explain why MUC1p-specific CD4 T cells are difficult to detect after $MUC1^{+}$ tumor challenge or MUC1p vaccinations in humans and MUC1-Tg mice. Recently a population of extrathymic autoimmune regulator (AIRE)-expressing cells (eTACs) were characterized in secondary lymphoid organs (181). These cells could be the source of MUC1 peptides being presented to CD4 T cells in the periphery, rather than the non-lymphoid ductal epithelial tissue (non-MHC class II expressing) where MUC1 is normally expressed.

Gerloni et. al. (110) have previously shown that by providing a non-self determinant (heterologous help) together with MUC1p they could activate previously hypo-responsive

MUC1p-specific CD4 T cells. We show that TnMUC1 provides a similar help to MUC1p-specific T cells but with one important difference. While both the foreign epitope and the glycopeptide epitope can serve the helper function and improve responses at the time of priming, only the glycopeptide epitope will be present and available to perform that function again if a tumor recurs or a new $MUC1^+$ tumor arises.

Beyond traditional peptide-specific T cell responses, recent studies have shown that T cells (via the TCR) can respond to peptides that contain post-translational modifications, such as phosphorylation and glycosylation, in the context of MHC class I and II molecules (182). Dysregulated cellular glycosylation has been related to autoimmune diseases, where the abnormal protein glycosylation activates effector T cell responses resulting in autoimmune cytotoxicity (183, 184). However, in the case of tumor immunity, the ability to direct a similar cytotoxic response against malignant tissue would be beneficial. The fact that the majority of cell proteins are glycosylated and that protein glycosylation is known to be dysregulated in cancer cells (185-187), makes targeting of tumor-specific glycopeptides, such as those derived from tumor-associated MUC1 (112, 140, 141) a viable alternative to peptide targets, which have been shown to face self tolerance mechanisms.

5.0 THESIS CONCLUSIONS AND SIGNIFICANCE

In this study we show that immune responses in a MUC1 "self" environment (MUC1-Tg mouse) are suppressed to MUC1 peptide epitopes, but can be improved if the MUC1 antigen incorporates a tumor-specific carbohydrate (Tn) that creates a MUC1 glycopeptide (TnMUC1) that is perceived as foreign. In addition, it appears that the TnMUC1 form of the antigen can only be taken up by DCs for antigen processing and presentation, while splenic B cells do not appear capable of the same function. We have shown directly that MUC1p-specific CD4 T cells (VFT) are suppressed in the periphery of MUC1-Tg mice, rather than subject to central tolerance. These data are the first to directly define the development and functional fate of MUC1p-specific CD4 T cells in the MUC1-Tg mouse. The peptide epitopes, while thought to be processed and presented primarily from tumor-associated MUC1, appear to be presented to some degree also from normal tissues in the periphery by MHC class II. The presence of MUC1p epitopes in the non-diseased state may serve as a method used by MUC1-Tg mice, and possibly humans, to educate the CD4 T cell repertoire to be self tolerant of MUC1p. In contrast, aberrant glycosylation of MUC1 by tumor cells would yield glycopeptides that would not have been encountered previously and should for all practical purposes be treated as foreign antigens. To assess T cell responses to these glycopeptides we generated a MUC1-glycopeptide (TnMUC1) – specific TCR transgenic (RFT-Tg) mouse line. We show that these TnMUC1-specific CD4 T cells (RFT) are not suppressed in the MUC1-Tg mouse, indicating that tumor-associated

TnMUC1 is seen as a foreign molecule in the "self" environment and thus capable of generating more effective immune responses. In addition, we show that TnMUC1-specific CD4 T cells provide help to otherwise suppressed MUC1p-specific CD4 T cells in the MUC1-Tg mouse.

Since the infancy of anti-tumor vaccines and immunotherapies, studies have employed the immune system in attempts to specifically target malignancies without harming healthy tissue. Human clinical trials and studies in relevant pre-clinical models have shown that self-tolerance can be a significant obstacle in generating effective anti-tumor immunity. Unlike viral peptides and subunit anti-viral vaccines, many tumor-associated antigen (TAA) peptides appear to not be foreign to the immune repertoire and thus anti-tumor vaccines utilizing these antigens face a barrier of immune tolerance and possible autoimmune induction (74, 188, 189). We show in this study that rather than using a self tolerant TAA MUC1 peptide with a potent adjuvant to break endogenous tolerance, we can induce more effective immune responses by focusing on glycopeptide epitopes from TAA MUC1 that are tumor-specific (i.e. foreign). In addition to tumor-associated glycosylation, differential phosphorylation and the resulting phosphopeptides are being studied for their ability to elicit anti-tumor immune responses (152). We propose that for more successful anti-tumor vaccines we will need to go beyond the peptide sequence and incorporate additional properties of TAAs (i.e. post translational tumor-associated glycosylation) in order to reach the goal of effective and safe tumor-specific immunity. These larger studies are now possible in part due to advances in glycobiosynthesis techniques that have made it more practicable to use high grade glycopeptide-based vaccines in controlled pre-clinical studies and in human clinical trials.

This study also serves as a proof of concept. Hypothetically there are many tumor antigens expressed by malignant cells, in addition to MUC1, that have a tumor-associated

glycosylation pattern. The defects in normal glycosylation that can result in tumor-associated glycosylation patterns are global in the cell (186, 187) and would affect all glycoproteins using the same glycosylation machinery. This could be significant since it has been estimated that more than half of all proteins are glycoproteins (185). Although studies are limited, tumor-associated glycosylation has been reported on other TAAs (70, 71).

Continued studies are needed to fully understand the specific MUC1-glycopeptide epitopes that can generate tumor-specific responses. The tumor-associated glycosylation pattern of MUC1 is more diverse than Tn antigens and this potentially diverse repertoire of glycopeptide epitopes should be evaluated for immunity in MUC1-Tg mice. We have previously shown that MUC1 VNTR peptides glycosylated with tumor-associated T antigens (Gal-GalNAc-O-T/S) are recognized by CD4 T cells in the WT mice (141), but a similar study in MUC1-Tg mice has not been performed. In addition, studies are needed to understand inherent differences in immune responses to peptides and glycopeptides, beyond self or non-self antigen discrimination. For example, as we have shown in Chapter 2, the repertoire of APCs responsible for processing and presenting peptide and glycopeptide antigens can differ, which could further affect the outward immune response. Furthermore, DCs endocytose peptide and glycopeptide antigens using a variety of different non-receptor and receptor-mediated mechanisms (145, 148, 190, 191) which in turn can influence the context of epitope presentation and type of T cell stimulation (163, 191, 192).

The RFT-Tg and VFT-Tg mice are valuable resources that can be used to continue to further our understanding of tumor-associated MUC1 immunity in health and disease. In our MUC1-Tg/IL10$^{-/-}$ mouse model (115) we can use RFT and VFT CD4 T cells to define the role of adaptive MUC1-specific immunity in controlling inflammation and inflammatory bowl disease

(IBD) related to a preventative effect on the development of colitis-associated colon cancer (Beatty, PL, et al., manuscript submitted). Furthermore, in spontaneous oncogenic carcinoma models (104, 115) the RFT T cells could be used to study tumor-associated MUC1 immunity during early tumorigenesis. In addition, the efficacy of MUC1 glycopeptide-specific T cells to provide anti-tumor therapeutic and prophylactic immunity can be evaluated using adoptive transfer of effector/memory RFT CD4 T cells. The self tolerance of MUC1p-specific VFT T cells in the MUC1-Tg mouse provides a novel model of peripheral CD4 T cell tolerance to a peripheral tissue antigen that is endogenously expressed under its own promoter, which can be analyzed against other models of peripheral T cell tolerance to tumor antigens (74, 116, 193) and model self antigens (177, 194, 195). Interestingly, most models of peripheral tolerance are focused on CD8 T cells. VFT T cells can be used to specifically identify the source of peripheral MHC class II presentation of peptide epitopes (116). Hypothetically these same antigen presenting cells in the mouse peripheral environment would be present in humans, which could further the understanding of TAA peripheral self tolerance in humans.

In addition, these peptide- and glycopeptide- specific CD4 T cells can be used to more fully understand how antigen presenting cells differ in antigen endocytosis and presentation based on antigen glycosylation. Furthermore, *in vivo*, we can more closely examine the effect of directed DC uptake and presentation of TnMUC1 on the resulting cellular and humoral immune response, compared to uptake and presentation of MUC1p by DCs and B cells.

5.1 RELEVANCE TO CLINICAL TRIALS OF MUC1 CANCER VACCINES

FDA approved use of prophylactic cancer vaccines (immunoprevention) is currently limited to prevention of tumor-associated viral infections caused by HPV and HBV. Although these vaccines are safe and appear effective, most cancers arise from oncogenic cellular changes not caused by infection. However, there have yet to be clinical trials of prophylactic cancer vaccines using autologous tumor-associated antigens, due in part to an assumed high risk of autoimmune side effects. An increasing number of studies in genetically engineered mice that express tumor-associated antigens as self molecules have shown that prophylactic cancer vaccines using various autologous tumor-associated antigens are safe, although, these pre-clinical studies show that the endogenous immune system maintains a level of self tolerance to epitopes derived from these tumor-associated antigens [reviewed in ref. (196)].

In disease-free individuals (absent pre-neoplastic lesions), our data suggests that a prophylactic MUC1 vaccine consisting of peptide and glycopeptide epitopes would be more effective in priming anti-tumor MUC1 immunity, as compared to MUC1p epitopes alone (Chapters 2 and 4). In addition to prophylactic MUC1 vaccines, individuals with identifiable neoplastic lesions, thus at higher risk of developing malignancies, should have an improved clinical outcome after receiving cancer vaccine immunotherapy (boosting immunity) at this early stage, as compared to receiving the therapy at a later stage of disease (Section 1.1, Figure 1B). In a recent study we found that immune surveillance in patients with premalignant and malignant changes that are associated with abnormal MUC1 expression (non-advanced adenomas, advanced adenomas, or colorectal cancer) had significantly higher anti-MUC1p IgG antibody levels compared to normal controls (197). This suggests that abnormal MUC1 epitopes are

eliciting immunity at early stages and that boosting immunity with a cancer vaccine may resolve the disease before it progresses.

We recently initiated the first clinical trial of a cancer vaccine (MUC1p plus poly-ICLC) for colon cancer prevention, designed to boost MUC1-specific immunity in high risk individuals with advanced adenomatous polyps. Early results indicate that the vaccine elicits MUC1p-specific IgG antibody isotype-switching. Based on the data of our present pre-clinical study, the clinical results suggest that tumor-associated MUC1, expressed by these pre-neoplastic polyps (198-201), may have elicited effective MUC1p priming by simultaneously stimulating tumor-associated MUC1 glycopeptide-specific CD4 Th cells. Although the early findings in this clinical trial show that vaccination with MUC1p alone is sufficient to boost immunity in multiple patients, our data suggests that changing the vaccine to include TnMUC1 may further increase the patient response rate and boost an expanded repertoire of MUC1-specific anti-tumor T cells, further improving effective anti-tumor immunity.

Our study on tumor-associated MUC1 immunity suggests that other TAAs, many of which contain "self" epitopes, subject to self tolerance (74, 99, 116, 117, 120, 164, 188, 189), should be re-evaluated in the appropriate self environment. Presumably these TAAs also contain "abnormal-self" epitopes that are tumor-specific ("foreign") and not affected by tolerance, thus priming the level of anti-tumor immunity detectable in cancer patients. In the absence of self tolerance, immune responses to "abnormal-self" epitope vaccines may be stronger and more tumor-specific, and thus less likely to elicit autoimmunity. Understanding this distinction is very important in the development of effective and safe prophylactic cancer vaccines.

APPENDIX A

MUC1-TG MOUSE GENDER INFLUENCES ANTIBODY RESPONSES ELICITED BY DC-MUC1 VACCINES

Related to their prowess as potent antigen presenting cells, dendritic cells (DCs) have been used in numerous vaccination studies, serving as both an antigen delivery vehicle and adjuvant (11). We have previously used DC-based MUC1 vaccination strategies in both non-human pre-clinical models (109, 179) and human clinical trials (135).

Similar to our past studies that addressed the effectiveness of a DC-MUC1 peptide (DC-MUC1p) vaccine in MUC1-Tg and WT mice (109, 111), we wanted to use a similar system to more extensively examine the immune responses to MUC1p as compared to TnMUC1 antigen. We have shown that DC-TnMUC1 vaccinations generate improved effector responses (cellular and humoral) in MUC1-Tg mice as compared to DC-MUC1p vaccinations [see Section 2.3.2, (112)], but it was unclear whether these responses had reached wild type (WT) levels. In addition, we wanted to determine the effectiveness of the DC-TnMUC1 vaccine in eliciting protection against future $MUC1^{+}$ tumor challenge in MUC1-Tg mice. However, the results from our more recent DC-based vaccination protocols (described here) using WT and MUC1-Tg mice appeared different from the past studies.

We show that the DC-MUC1 vaccination of WT mice (positive control), irrespective of the form of MUC1 (MUC1p or TnMUC1), do not consistently induce effective anti-MUC1

immune responses. On average only about 50-60% of vaccinated WT mice had detectable titers of anti-MUC1 IgG (Figure 21D), although most WT mice did have vaccine induced IgM titers (Figure 21B). In addition, we saw a similar response rate in the MUC1-Tg mouse groups, although the MUC1-Tg mice had detectable pre-immune titers of anti-MUC1p IgM. Interestingly, upon further analysis of the results we found that within the MUC1-Tg groups it was the female mice that elicited the effective anti-MUC1 IgG isotype-switching (4 of 4) but not the male mice (0 of 8) (Figure 21D). Those female MUC1-Tg mice also had vaccine-induced anti-MUC1 IgM titers. The results shown in Figure 20 are cumulative from two independent vaccination protocols, indicating that DC-MUC1 induced effective IgG isotype-switching in female MUC1-Tg mice but was limited in male MUC1-Tg mice (Figure 22).

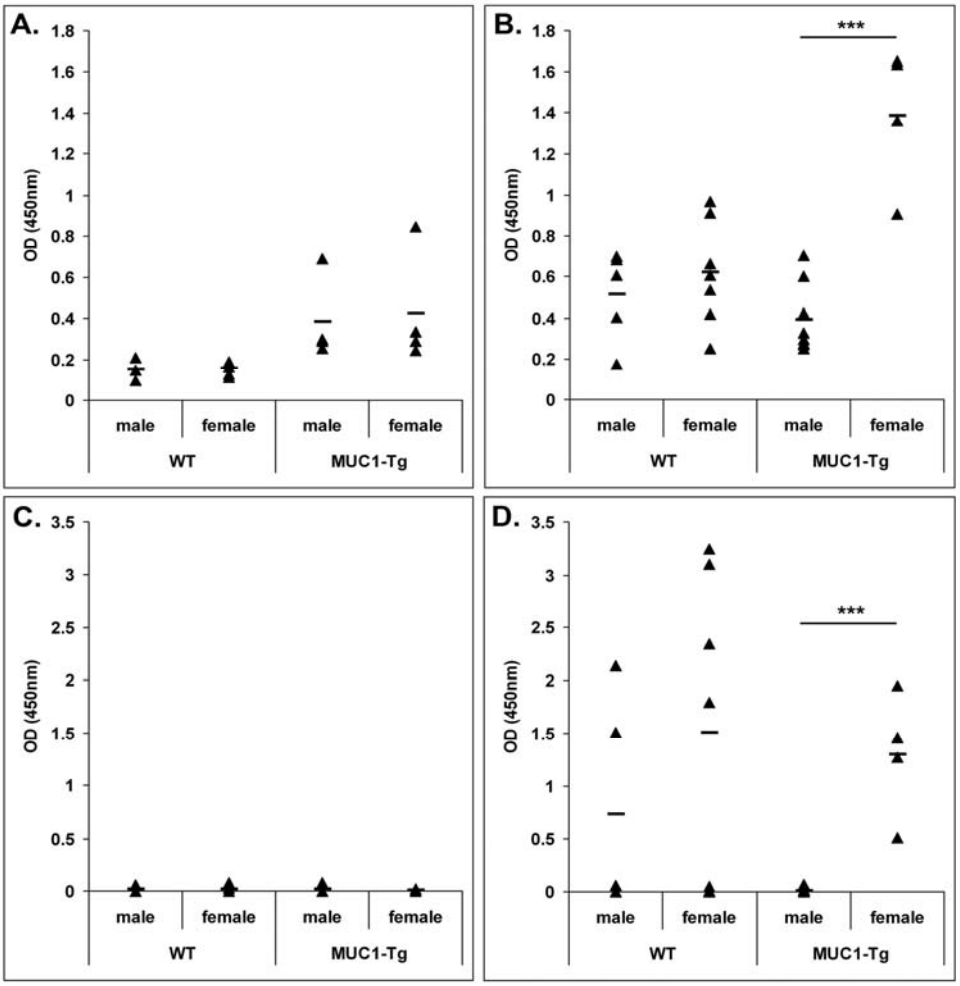


Figure 21. Female MUC1-Tg mice have improved anti-MUC1p antibody responses compared to male MUC1-Tg mice.

Serum samples were collected from representative WT (male=3, female=5) and MUC1-Tg (male=4, female=4) mice before being given three doses of DC-MUC1 vaccine (s.c.) at two week intervals. The DC-MUC1 vaccine consisted of MUC1p or TnMUC1 –pulsed DCs (2-5x10^5) plus 30µg soluble antigen. Day 7 following the final vaccine dose serum samples were collected from all vaccinated WT (male=5, female=7) and MUC1-Tg (male=8, female=4) mice. The presence of anti-MUC1p IgM antibody A) pre- and B) post- and anti-MUC1p IgG antibody C) pre- and D) post-DC-MUC1 vaccination was determined by ELISA. The data shown is the OD (optical density, 450nm) value at a 1:225 serum dilution. ***p value is less than 0.0001.

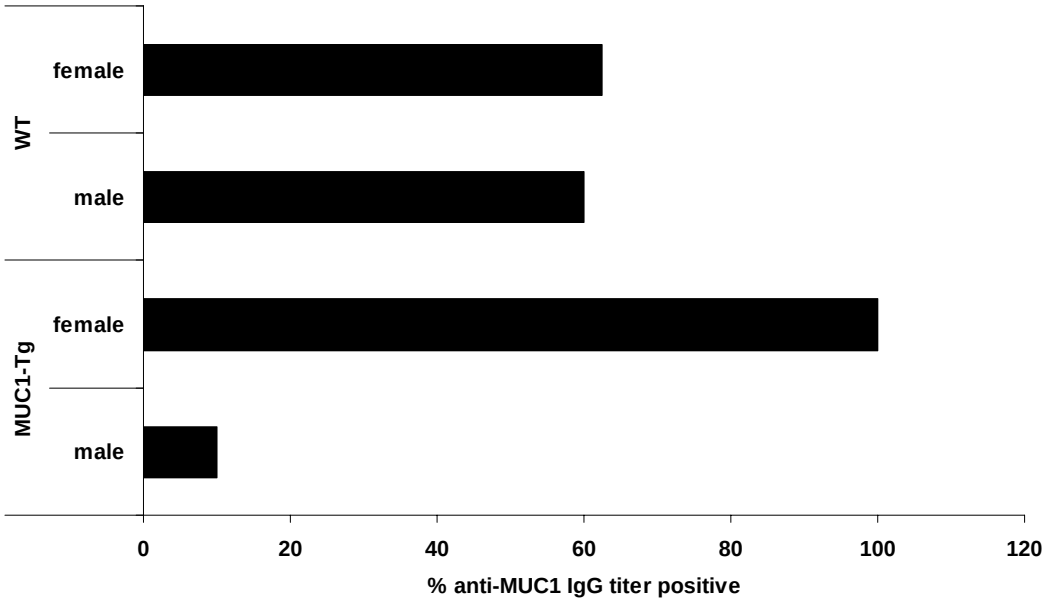


Figure 22. DC-MUC1 vaccinations induce anti-MUC1 antibody isotype-switching predominantly in female MUC1-Tg mice compared to male MUC1-Tg mice, and with increased frequency compared to WT mice. Serum samples were collected from WT and MUC1-Tg mice before being given three doses of a DC-MUC1 vaccine (s.c.) at two week intervals. The DC-MUC1 vaccine consisted of MUC1p or TnMUC1 –pulsed DCs (2-5x10^5) plus 30 μg soluble MUC1 antigen. Following the final vaccine dose serum samples were again collected from all mice. Pre- and post- vaccine serum samples were analyzed for anti-MUC1p IgG antibody titers by ELISA. An anti-MUC1 IgG titer positive mouse was identified based on an OD (optical density, 450nm) value from the post-vaccine serum that was greater than the OD value from the pre-vaccine serum or pooled nonimmunized mice serum at a 1:225 serum dilution. Results are cumulative (WT, male=10 female=8; MUC1-Tg, male=10 female=8) from two independent vaccination protocols.

A.1 DISCUSSION

We have reported previously that based on pre-existing anti-MUC1 antibodies to tumor-associated MUC1 peptides, women may be sensitized to MUC1 over time by different life

events. Life events predicting the presence of antibodies included oral contraceptive use, breast mastitis, bone fracture or osteoporosis, pelvic surgeries, and nonuse of talc in genital hygiene. The likelihood of having anti-MUC1 antibodies increased with the increased number of life events that women encountered. By the same index of events, the risk for ovarian cancer was inversely associated with number of conditions predisposing to anti-MUC1 antibodies (28). As shown in Figure 21A and in unpublished observations, MUC1-Tg mice, independent of gender, have detectable anti-MUC1 IgM antibody titers as compared to age matched WT mice. Interestingly, we show here that only the female, but not the male, MUC1-Tg mice mount effective IgG antibody responses to our DC-MUC1 vaccine.

The incomplete response rate seen in DC-MUC1 vaccinated WT mice indicates that the vaccine (given s.c.) is suboptimal for purposes of generating consistently strong anti-MUC1 responses as indicated by IgG isotype-switching. Similar MUC1 vaccinations using a soluble adjuvant produced strong anti-MUC1 IgG responses in 100% of vaccinated WT mice (see Section 2.3.1). The data also indicates that endogenous expression of human MUC1 in the MUC1-Tg mouse environment is playing an active role in suppressing humoral responses as compared to WT mice. This is supported by the MUC1-specific T cell data shown in Chapters 2 and 4. Presently, it is unclear whether the lack of isotype-switched IgG in male MUC1-Tg mice is a direct result of B cell suppression (202) or an indirect result of the suppressed CD4 T helper cell response.

It is possible that the results from these "suboptimal" DC-MUC1 vaccinations are delineating a prior priming event to MUC1 in female MUC1-Tg mice, effectively sensitizing the immune system to MUC1. In this case the DC-MUC1 vaccine acted to boost pre-existing immunity. The male MUC1-Tg mice may not have experienced those similar life events, which

resulted in no pre-existing anti-MUC1 immunity. In fact, the attempts to generate effective immunity in male MUC1-Tg mice may face the full brunt of MUC1p peripheral tolerance (see Chapter 4). On the other hand, the presence of vaccine induced anti-MUC1p IgG in female MUC1-Tg mice indicates that this priming event(s) was sufficient to partially overcome peripheral tolerance.

The priming event(s) that may have occurred in the female MUC1-Tg mice and not male MUC1-Tg mice remain unclear. All mice used in these studies were around 3-5 months of age by the end of the respective vaccination protocols and have not been previously used in colony breeding. It appears that the event that induced detectable pre-immune serum levels of anti-MUC1p IgM antibodies in female and male MUC1-Tg mice may be similar. It is clear, when excluding the WT non-responders, that the anti-MUC1 IgG titers are lower in female MUC1-Tg mice compared to WT mice, similar to other MUC1 vaccinations [see Section 2.3.1 and (109, 111)], which supports our data that show that MUC1p-specific CD4 T cells face a level of self tolerance in MUC1-Tg mice. It is interesting to note that female MUC1-Tg mice with lower IgG titers actually had higher titers of anti-MUC1 IgM, further supporting an imbalance in the effective immune response in MUC1-Tg mice compared to WT mice (111). Although not addressed here, a more specific analysis of vaccine-induced anti-MUC1 antibodies in WT and MUC1-Tg female mice may show a skewing in IgG antibody sub-isotype selection, thus reflecting a difference in the quality of the IgG response.

Possible anti-MUC1 priming event(s) unique to nonparous female MUC1-Tg mice, compared to male mice, could be related to the estrous cycle (equivalent to human menstrual cycle), in addition to female hormone control of MUC1 expression (203, 204). The female mouse estrous cycles begin around day 35 of age (205). Cycles are 4-5 days in which time the

endometrium is completely reabsorbed by the animal (covert menstruation) at the end of its reproductive cycle. By immunohistochemistry and genetic analysis of tissue samples, MUC1 is expressed throughout the genital tract and on ovarian surface epithelium in MUC1-Tg mice (206), in addition to the human female reproductive tract (207, 208), which suggests that the molecule is present for immune priming during an inflammatory event. The possible role of the estrous cycle on MUC1 immunity could be addressed by controlling the onset of puberty in juvenile female mice using pheromones derived from male (accelerating) and female (delaying) urine (209, 210). In addition, mice can be treated to induce ovulation cessation (211) or given female hormones (PMSG and hCG) to induce superovulation (212) prior to DC-MUC1 vaccinations. Similar to the suggested role of multiple pregnancies and breast feeding on MUC1 immunity in humans (28), possible differences may exist in the anti-MUC1 immune response between age-matched multiparous and nonparous female MUC1-Tg mice, as compared to similar groups of WT mice. To complement the antibody responses, the regional draining lymph nodes (para-aortic) should be examined for MUC1-responsive T cells.

The results from the DC-MUC1 vaccinations show that future studies looking at the effectiveness of MUC1 vaccines in MUC1-Tg mice, and humans, should consider gender as a variable. A significantly higher level of self tolerance appears to exist in male MUC1-Tg mice and the s.c. DC-TnMUC1 and DC-MUC1p vaccines are not sufficient to break MUC1p tolerance. On the other hand, as shown in Chapter 4, (i.v.) DC-TnMUC1 vaccination was sufficient to stimulate TnMUC1-specific CD4 T cells and break the peripheral tolerance acting on MUC1p-speicific CD4 T cells. The mice used in the T cell adoptive transfer studies were male. Although we did not address the humoral response in those experiments, it appears that the route of vaccine administration may play a role in the different responses.

APPENDIX B

CHARACTERIZATION MUC1-PEPTIDE SPECIFIC CD8 T CELLS IN THE VFT TCR TRANSGENIC MOUSE

This section of the appendix describes a novel population of MUC1p-responsive CD8 T cells found in the VFT-Tg mouse, in addition to the VFT CD4 T cells used in Chapter 4. Where noted we have used the VNTR MUC1-19mer (HGVTSAPDTRPAPGSTAPP) and -12mer (GVTSAPDTRPAP) peptides instead of the 100mer peptide that was used in previous Chapters.

B.1 VFT CD8 T CELLS ARE MUC1-PEPTIDE SPECIFIC AND MHC CLASS II RESTRICTED

The classical paradigm is that alpha/beta CD8 and CD4 T cells are restricted to antigen presented in MHC class I or II molecules, respectively. This selective recognition is related to thymic positive selection and the binding of CD4 and CD8 co-receptors to regions of the MHC molecules, which act in part to stabilize the TCR to MHC: antigen interaction. Hypothetically, since the VFT TCR originated from a CD4 T cell it should have been previously selected during thymic development for MHC class II -restricted antigen recognition. Indeed, VFT splenocytes

proliferate and secrete IL-2 and IFNγ in response to the MUC1-12mer minimal epitope and by flow cytometry the ratio of CD4:CD8 T cells (4-5:1) is skewed (data not shown) indicating preferential CD4 T cell thymic selection (213). Because the RFT-Tg mouse is not on a RAG knockout genetic background we had attributed the presence of CD8 T cells to incomplete alpha chain allelic exclusion. Although, as compared to the $CD4^+$ T cells (~55-60% $V\alpha2^+$), we consistently saw that the majority of $CD8^+$ T cells expressed a $V\alpha2^+$ TCR (>60%), suggesting that the transgene receptor was predominantly expressed on both T cells.

In subsequent *in vitro* experiments we noticed, to our surprise, that in addition to VFT $CD4^+$ T cells (Figure 23A,B), VFT $CD8^+$ T cells proliferated (CFSE dilution) in response to MUC1p (Figure 23C,D). The increased level of proliferation by VFT CD8 T cells over that of VFT CD4 T cells is seen consistently.

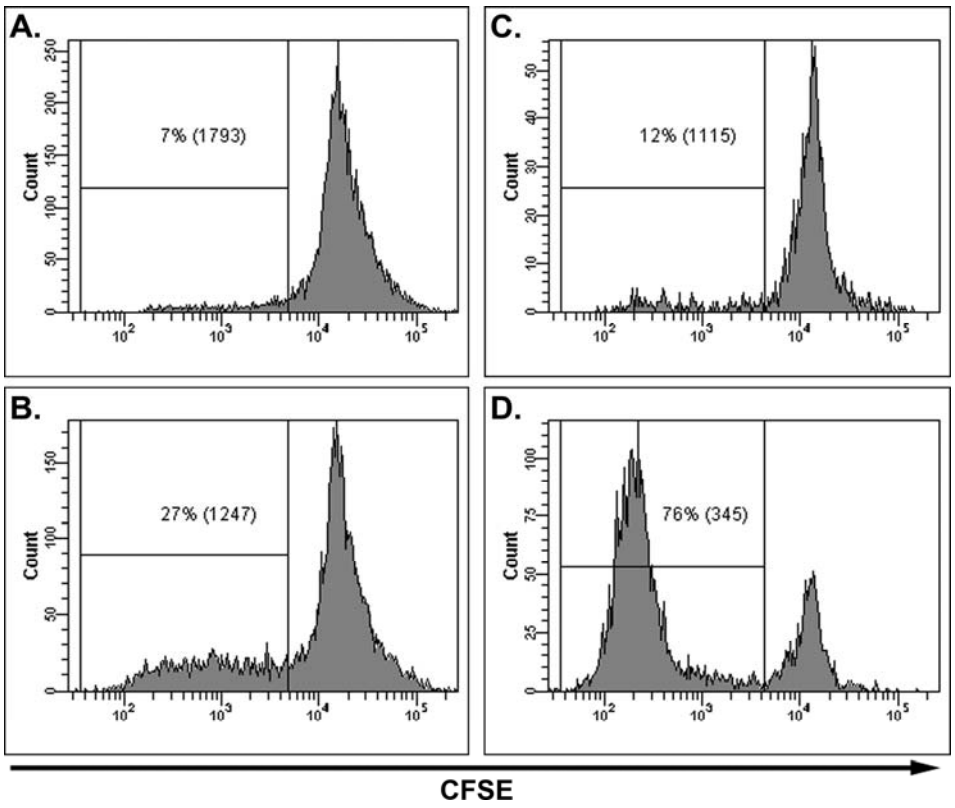


Figure 23. VFT CD4 and CD8 T cells proliferate in response of MUC1p antigen.

CFSE-labeled VFT splenocytes were co-cultured with DCs A,C) alone or with B,D) 20 µg/ml MUC1-19mer peptide. Cells were collected after 6 days and labeled with anti-Vα2, CD4, and CD8 antibodies for flow cytometry analysis. Histogram gates show the percent A,B) $V\alpha2^{+}CD4^{+}CD8^{neg}$ and C,D) $V\alpha2^{+}CD8^{+}CD4^{neg}$ VFT T cells that have decreased CFSE fluorescence. Values in parenthesis are CFSE mean fluorescent intensity (MFI) of gated cells. Results are representative of greater than three experiments.

We have shown previously that the VF5 hybridoma (source of VFT TCR) is unresponsive to MUC1p-pulsed DCs derived from B6.NOD mice ($H\text{-}2^{g7}$), mismatched in MHC class II with C57BL/6 mice ($I\text{-}A^{b}$), indicating $I\text{-}A^{b}$ MHC class II haplotype restriction. (141). To confirm the MHC-class restriction of the VFT T cell population we set up an *in vitro* stimulation using MUC1p pulsed DCs generated from WT mice, MHC class II knockout ($I\text{-}A/E^{-/-}$) mice, and

β-2 microglobulin knockout mice ($b2m^{-/-}$). We show that MUC1p-specific IFNγ production (Figure 24A) and VFT CD8 T cell proliferation (Figure 24B) require MHC class II, indicating that the VFT CD8 T cell responses are also MHC class II restricted. The minimal responses seen in I-A/$E^{-/-}$ DC cultures is likely due to contamination with WT APCs from the VFT splenocytes.

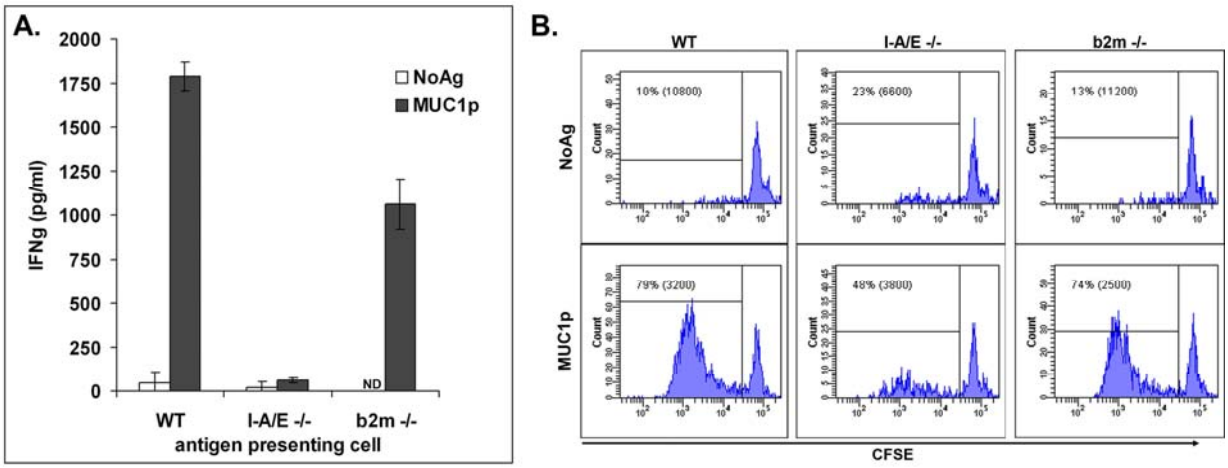


Figure 24. VFT CD8 T cells are MHC class II -restricted.

Bone marrow derived DCs were generated from C57BL/6 WT, I-A/$E^{-/-}$, or $b2m^{-/-}$ mice. DCs were pulsed overnight either with media alone (NoAg) or with 20μg/ml MUC1-19mer (MUC1p) followed by 2 washes to remove residual antigen. $1x10^5$ CFSE-labeled VFT T cells plus $1x10^4$ DCs were co-cultured per well of a 96 well plate, in triplicate. On Day 4 A) supernatants were collected for IFNγ analysis by ELISA (ND = not detectable) and B) cells were labeled with anti- Vα2, CD8, and CD4 antibodies before analysis by flow cytometry to assess proliferation (CFSE dilution). Histograms show the CFSE fluorescence within the $Va2^+CD8^+CD4^{neg}$ cell population. The percent gated cells and respective MFIs are shown. Data is representative of two independent experiments.

In addition, we found that VFT CD8 T cells responded *in vivo* to DC-MUC1p vaccination, indicated by population expansion in WT recipient mice (Figure 25A). Interestingly, this population is suppressed in MUC1-Tg mice (Figure 25A) similar to VFT CD4 T cells (see Chapter 4). Furthermore, it appeared that the VFT CD8 T cells expressing the MUC1p-

responsive transgene TCR ($V\alpha2^+$) were specifically suppressed in vaccinated MUC1-Tg mice (Figure 25B).

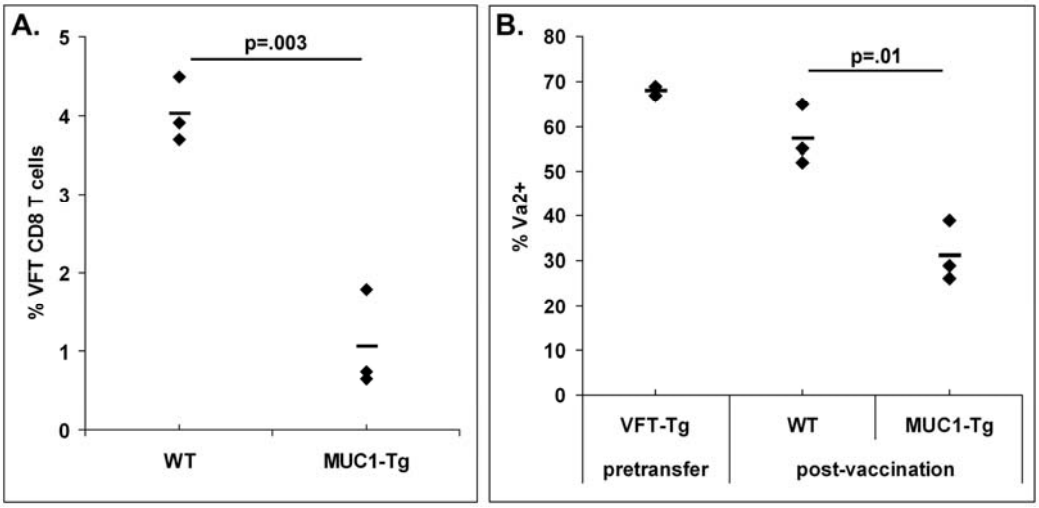


Figure 25. VFT CD8 T cell responses are suppressed in MUC1-Tg mice.

T cells were isolated by magnetic bead CD3 negative selection from VFT-Tg mouse spleens. 3-5x10^6 T cells were transferred (i.v.) to recipient mice (WT and MUC1-Tg). Day 3 after T cell transfer mice were vaccinated (i.v.) twice at a two week interval using ~7x10^5 DC-MUC1p plus 30μg soluble MUC1p per mouse. Three days following the final vaccination, recipient mice were sacrificed and their splenocytes analyzed by flow cytometry for the presence of donor A) VFT CD8 T cells ($V\alpha2^+CD90.1^+$) (WT, n=3; MUC1-Tg, n=3) as a percentage of total $CD3^+CD8^+CD4^{neg}$ T cells. In addition, B) the percentage of $V\alpha2^+$ cells within the total donor cell ($CD8^+CD90.1^+$) population before adoptive transfer and after transfer and vaccination is shown. P values were calculated using an unpaired t test.

B.2 VFT CD8 T CELLS RESPOND TO MUC1-PEPTIDE INDEPENDENT OF VFT CD4 T CELLS

Since the MUC1p-specific VFT CD8 T cell responses observed in previous experiments were in the presence of concurrent VFT CD4 T cell activation, the possibility still existed that the activation of VFT CD4 T cells might be driving bystander activation of VFT CD8 T cells. This would explain why VFT CD8 T cell function was only observed under conditions that simulated the $CD4^+$ T cells (12mer minimal epitope, MHC class II restriction). However, the *in vivo* suppression of VFT CD8 T cells expressing the MUC1p-specific TCR ($V\alpha2^+$) indicated that the cells were specifically responding to the cognate MUC1p epitope.

To determine if the VFT CD4 and CD8 T cell populations could function independently of one another we isolated the two separate populations by cytometry-based cell sorting. Using purified T cell populations and co-receptor blocking antibodies we definitively show that VFT CD4 T cells (Figure 26A) and VFT CD8 T cells (Figure 26B) are both (independently) MUC1p-specific, MHC class II -restricted T cells. Additionally, the CD4 co-receptor is required for MUC1p-specific responses by $CD4^+$ cells (Figure 26C), similar to the VF5 hybridoma (data not shown), however it appears that on $CD8^+$ cells the CD8 co-receptor may be dispensable (Figure 26D), which was not surprising because CD8 does not bind to the MHC class II molecule. This finding also explains why previous experiments using anti-CD4 blocking antibody treatment of VFT splenocytes only partially diminished MUC1p-specific soluble cytokine production and thymidine incorporation (data not shown).

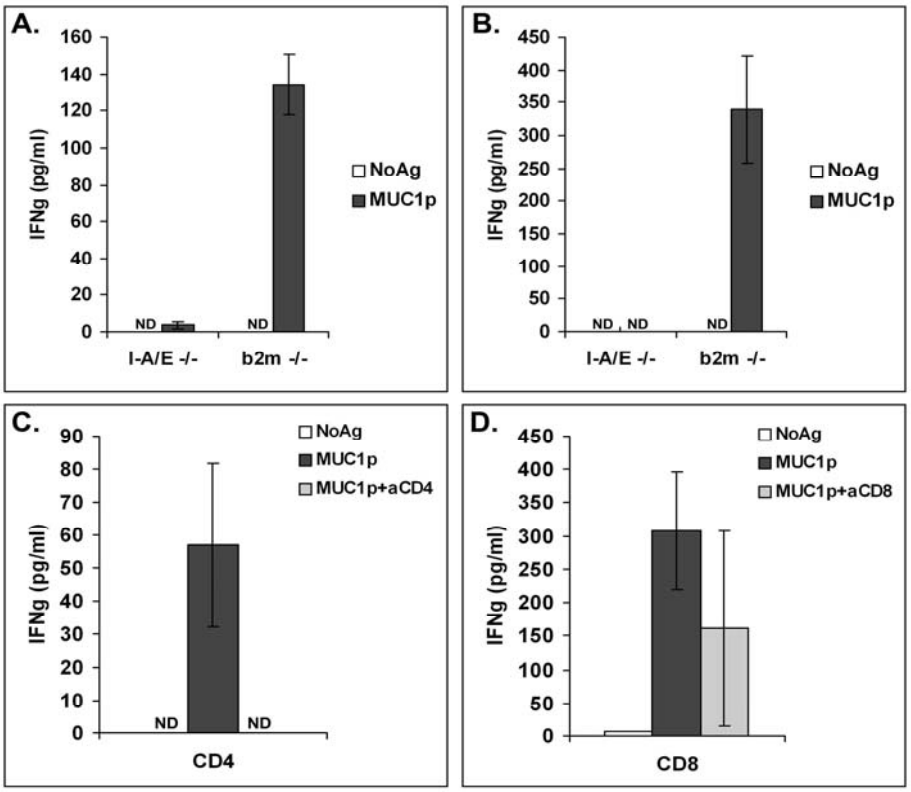


Figure 26. MHC class II -restricted VFT CD8 T cells respond to MUC1p independently of VFT CD4 T cells and CD8 co-receptor.

T cells were isolated from VFT-Tg spleens by magnetic bead CD3 negative selection and labeled with anti- CD4, CD8, CD90.1, CD25, and FR4 antibodies. Naïve VFT CD4 T cells ($CD4^{+}CD8^{neg}CD90.1^{+}CD25^{neg}FR4^{low/-}$) and VFT CD8 T cells ($CD8^{+}CD4^{neg}$) were isolated using a FACSAria cell sorter with the FACSDiva analysis software (BD Biosciences). $3x10^{4}$ A,C) CD4 T cells (plus aCD8) or B,D) CD8 T cells (plus aCD4) were stimulated in the absence (NoAg) or presence of 20µg/ml MUC1-19mer (MUC1p) with A,B) $3x10^{3}$ $I\text{-}A/E^{-/-}$ or $b2m^{-/-}$ DCs or C,D) $5x10^{4}$ irradiated (1000 RADs) WT splenocytes per well in a 96 well plate, in triplicate. 2.5µg/ml anti-CD8 (aCD8; clone 53-6.7) or anti-CD4 (aCD4; clone H129.19) blocking antibody was used were indicated. After 72 hrs culture supernatants were analyzed for IFNγ by ELISA (ND = not detectable).

B.3 DISSCUSSION

Thymic T cell development is a complex biological process based on the editing and selection of T cell receptors (TCR). By cloning and introducing the rearranged VFT TCR alpha and beta chain transgenes into a mouse germ-line the majority of developing T cells should be positively selected based on expression of the transgenic VFT TCR. Although the VFT TCR was derived from the CD4 T cell lineage (141), we have identified a population of VFT CD8 T cells in the VFT-Tg mouse that are MHC class II restricted and specific for the minimal MUC1-12mer epitope. Interesting, CD8 VFT T cells are suppressed in the self-tolerant MUC1-Tg mice, possibly by the same mechanism that suppresses VFT CD4 T cells function. We have also found that function of VFT CD8 T cells is not dependent of concurrent activation of VFT CD4 T cells, nor is it dependent on the ligation of the CD8 co-receptor.

In addition to studies of MUC1p-specific CD4 T cell immunity, the VFT-Tg mice provide an additional model that could be used to help understand CD4 versus CD8 alpha/beta T cell lineage choice. Using model systems, most of which manipulate CD4 and CD8 co-receptor expression and signaling, numerous hypotheses have been proposed to describe lineage choice, yet a clear understanding of the process still remains elusive [reviewed in ref. (214)].

There are multiple reasons that could explain the presence of the above-described VFT CD8 T cells. For example, the VFT TCR could recognize thymic positive selection signals from both MHC class I and II, which would allow for either CD8 or CD4 lineage commitment. In addition, VFT CD8 T cells could express an additional alpha chain resulting in multiple TCRs on a single cell that in turn might provide positive selection on MHC class I. We are addressing the role for incomplete allelic exclusion and VFT CD8 T cell thymic selection by crossing the VFT-Tg mouse line to the $RAG^{-/-}$ background (215). In the VFT-Tg thymus, we see a similar

percentage of $V\alpha2^+$ thymocytes in the CD4 and CD8 single positive populations (data not shown), indicating VFT thymic selection is promoting both the CD4 and CD8 lineages. The final outcome is that VFT CD8 T cells immigrate to the periphery and continue to circulate as naïve T cells until encountering their (foreign) activating ligand, MUC1p, presented in MHC class II. What is still unclear in this system, and in other systems (216), is how the VFT CD8 T cells facilitate an antigen specific, MHC class II -restricted response, independent of CD4. Because the VFT CD4 T cells are dependent on CD4 expression for function, the VFT CD8 T cells must use a yet unidentified compensatory mechanism that allows antigen-specific activation.

Previous studies have reported the presence of MHC class II –restricted CD8 T cells. Although these cells have not been clearly identified in WT mice, CD4 knockout ($CD4^{-/-}$) mice have been shown to produce large numbers of MHC class II –restricted CD8 T cells (216-218). In contrast to the low levels of IL-2 produced by traditional CD8 T cells and MHC class II -restricted CD8 T cells from $CD4^{-/-}$ mice (218), it appears that VFT CD8 T cells are the predominant IL-2 producers compared to the VFT CD4 T cells (data not shown).

APPENDIX C

THY1 EXPRESSION ON T CELLS AND THYMOMAS

Thymus antigen 1 or Thy1 (CD90) was first described over 40 years ago due to expression on thymocytes (219). As this first study alluded to, there are two allelic forms of Thy1 in mice, which are referred to currently as Thy1.1 and Thy1.2. In the majority of immunological studies Thy1 is used predominantly as a marker to differentiate donor versus recipient T cells in adoptive transfer experiments, similar to our use of Thy1.1 to identify VFT T cells in WT and MUC1-Tg mice in Chapter 4. Although studies have suggested that Thy1 functions in TCR-complex signaling, the exact physiological role of Thy1 in the immune system is still unclear [reviewed here (220, 221)]. In this section of the appendix we describe the phenotype and function of a population of Thy1-negative VFT CD4 T cells found in VFT-Tg mice. Although this population was more distinct in VFT-Tg mice, we could identify a similar, yet smaller population in WT mice. Interestingly, we found that mouse T cell hybridomas and lymphomas, in addition to the human Jurkat lymphoma line, constitutively express Thy1 alleles on viable cells, suggesting a role for Thy1 in the survival of lymphoma survival.

C.1 IDENTIFICATION OF $CD3^+CD4^+THY1^{NEG}$ T CELL POPULATION IN VFT-TG MICE

While characterizing the phenotype of VFT T cells prior to adoptive transfer, we continuously found a noticeably distinct Thy1 negative ($Thy1^{neg}$) CD4 T cell population, which was more pronounced in the spleen versus the inguinal lymph nodes (Figure 27A). This was surprising since Thy1 is considered a pan T cell marker for all thymic emigrants. There is a similar $Thy1^{neg}$ CD4 T cell population in WT ($Thy1.1^+$) mice, although it was a significantly smaller percent of the CD4 T cell population than that seen in VFT-Tg mice (Figure 27B).

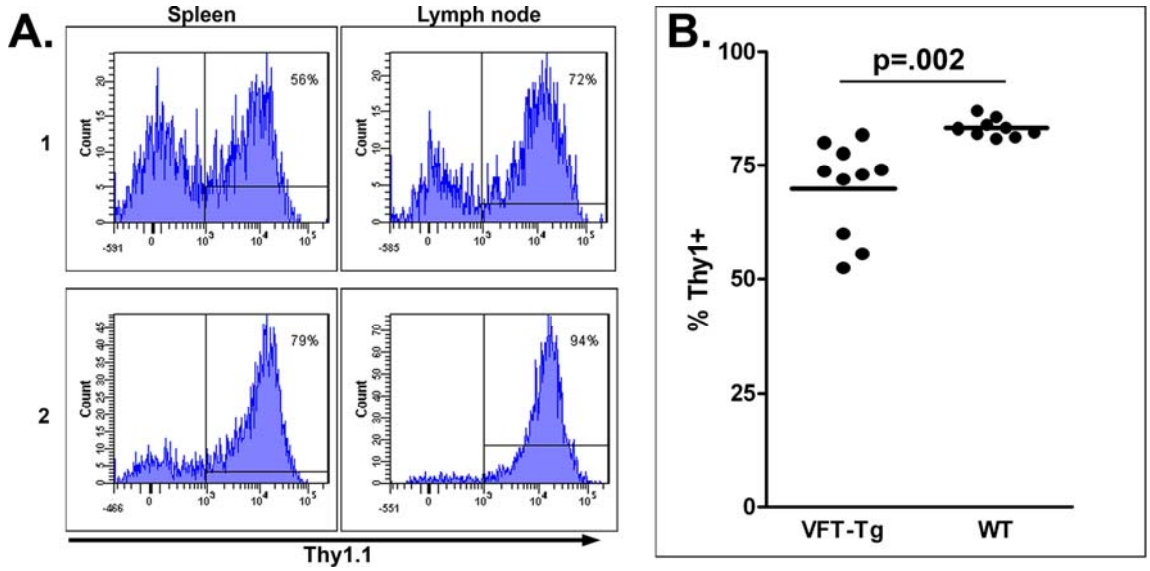


Figure 27. Populations of CD4 T cells that lack Thy1 expression exist in VFT-Tg mice.

A) Flow cytometry analysis of Thy1.1 expression on $CD3^+CD4^+Va2^+$ cells found in the spleen and lymph nodes (inguinal) of two representative VFT-Tg mice (1,2). B) Percent $Thy1.1^+$ splenocytes in the $CD3^+CD4^+$ gate from 6-12 week old VFT-Tg (n=10) and WT (n=9) mice.

Furthermore, we found that the Thy1^{+} and Thy1^{neg} CD4 T cell populations were phenotypically different. While the Thy1^{+} population contained a larger percentage of cells expressing CD62L and CD45RB (Figure 28A), cell surface molecules associated with a naïve state, the Thy1^{neg} population appeared to lack the CD62L^{+} and CD45RB medium-expressing cells (Figure 28A), suggesting a phenotype of previous activation. However, an expanded phenotypic analysis of VFT CD4 T cells showed that activation associated molecules [CD25, CD43 (1B11), CD127] were not up-regulated on Thy1^{neg} cells, as compared to the Thy1^{+} cells (Figure 28B).

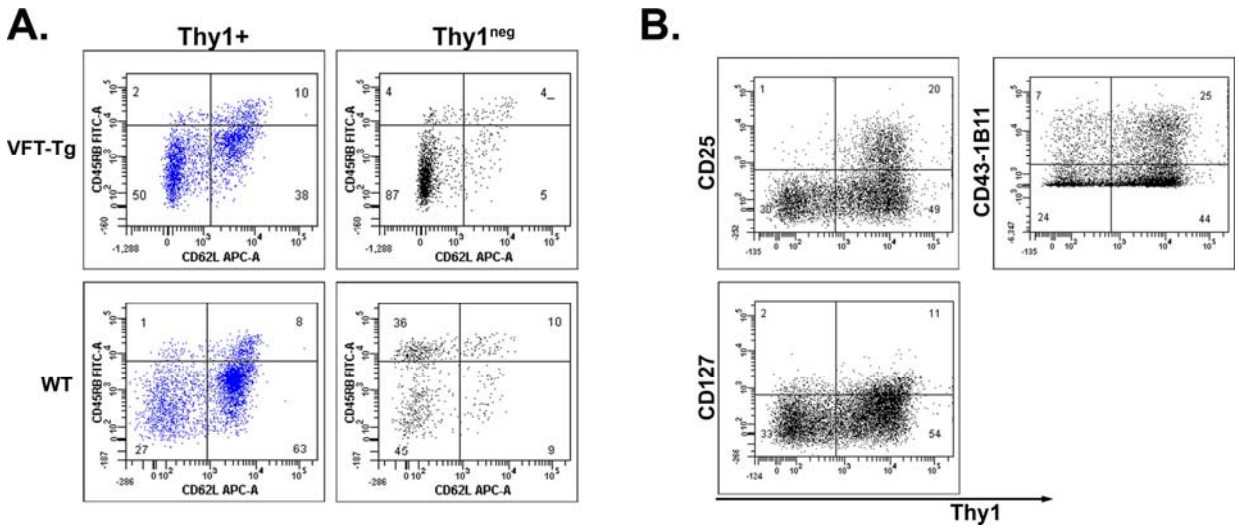


Figure 28. The Thy1 expression of CD4 T cells differentiates phenotypically distinct subpopulations.

Flow cytometry analysis of splenic CD4 T cells from A) WT (CD3^{+}CD4^{+}) and VFT-Tg (CD3^{+}CD4^{+}Vα2^{+}) mice. CD4 T cells were sub-gated on Thy1.1^{+} (left) and Thy1.1^{neg} (right) populations and the respective CD62L by CD45RB expression is shown. B) VFT CD4 T cell (CD3^{+}CD4^{+}) phenotype analysis of Thy1.1 by CD25, CD43 (1B11), or CD127. Dot plot gates in panel A have been drawn arbitrarily to separate CD62L$^{+/-}$ and CD45RB high/medium-low populations, where as gates in panel B are drawn based on negative controls.

C.2 FUNCTION OF THY1^{NEG} T CELL POPULATION

Since Thy1 expression appeared to delineate two phenotypically different CD4 T cell populations, we wanted to know whether Thy1 expression also translated to a functional difference in antigen-specific VFT CD4 T cell responses.

Interestingly, we found that MUC1p-specific proliferation was restricted to Thy1^{+} VFT CD4 cells (Figure 29A). Furthermore, the main population of proliferating cells are consistently those expressing higher levels of Thy1 (Figure 29A). The non-proliferative Thy1^{neg} T cells are not FOXP3^{+} Treg cells (Figure 29B), which could have explained the lack of proliferation *in vitro* (222). However, we did find that Thy1^{+} and Thy1^{neg} VFT CD4 T cells stimulated together produce significantly more MUC1p-specific IL-10 (Figure 29C) than Thy1^{+} T cells stimulated alone (Figure 29D), suggesting that Thy1^{neg} T cells are producing additional MUC1p-specific IL-10. In addition, we show that the Thy1^{+} population, but not Thy1^{neg}, contains the IFNγ producing VFT CD4 T cells (Figure 29D) detected in previous experiments (Figure 26). It does not appear that the IL-10 -producing Thy1^{neg} T cells suppress IFNγ production by those Thy1^{+} T cells (Figure 29D).

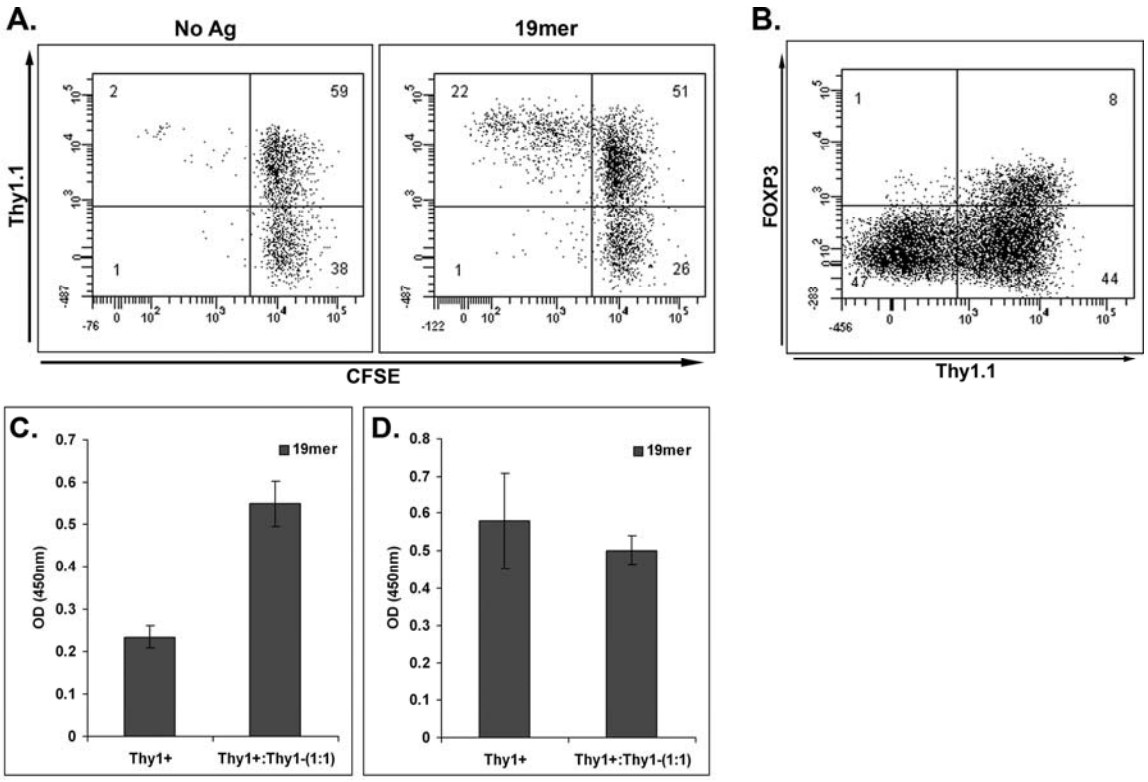


Figure 29. Thy1^{neg} VFT CD4 T cells are non-proliferative, FOXP3^{neg} cells that induce MUC1p-specific IL-10 production.

CFSE-labeled VFT splenocytes were culture in the absence (NoAg) or presence of 20µg/ml MUC1-19mer. Day 4 of stimulation cells were collected and analyzed by flow cytometry for A) Thy1.1 expression by CFSE fluorescence of the CD3^{+}CD4^{+}Vα2^{+} gated population. B) Dot plot showing Thy1.1 by intracellular FOXP3 expression in the CD3^{+}CD4^{+}Vα2^{+} gated population. Isolated VFT T cells (MACS CD3 negative selection) were further sorted (FACSAria) into CD4^{+}CD8^{neg}CD90.1^{+}CD25^{neg}FR4$^{low/-}$ (Thy1+) and CD4^{+}CD8^{neg}CD90.1^{neg} (Thy1-) populations. 3x10^{4} Thy1^{+} cells were plated per 96-well alone, or with an additional 3x10^{4} Thy1^{neg} cells (1:1). T cells were co-cultured with 5x10^{4} irradiated (1000 RADs) WT splenocytes per well in the absence or presence of 20µg/ml MUC1-19mer, in triplicate. After 72 hrs culture supernatants were analyzed for C) IL-10 and D) IFNγ by ELISA (OD = optical density). Negative control wells were below the detection of this assay and are not shown.

Furthermore, we show that Thy1^{neg} VFT CD4 T cells produce MUC1p-specific IL-10 independently of the IL-10 -producing Thy1^{+} cells (Figure 30A). The IL-10 production was also

seen when soluble anti-CD3/CD8 antibodies were substituted for MUC1p (Figure 30B), but surprisingly, we did not detect IL-10 production by the Thy1^{neg} CD4 T cell population to plate bound anti-CD3/CD28 stimulation (Figure 30C). This indicates that the APCs are providing additional cell-cell interactions needed to stimulate Thy1^{neg} CD4 T cell IL-10 production, which is not required to stimulate the IL-10 -producing Thy1^{+} CD4 T cell. Taken together, these data suggest that the lack of Thy1 expression on CD4 T cells can delineate a distinct, non-proliferative, antigen-specific IL-10 producing T cell population.

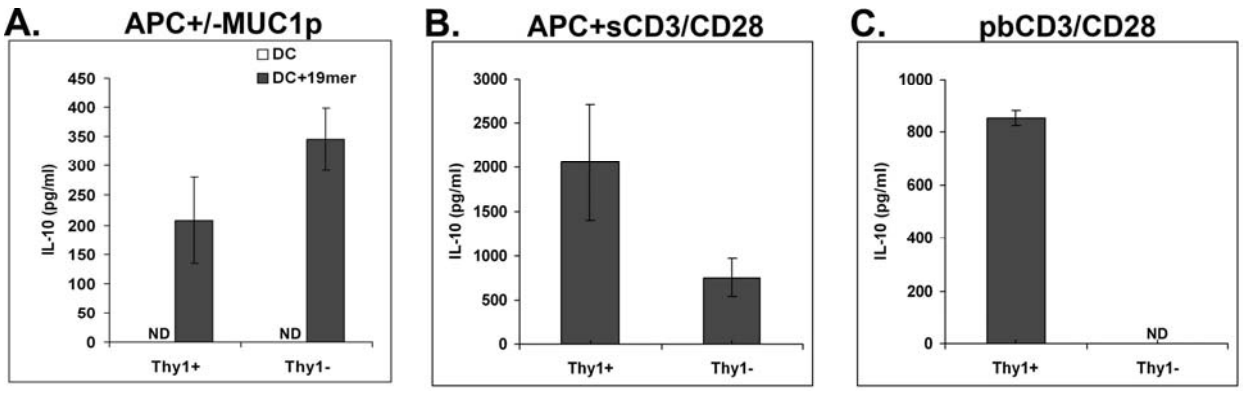


Figure 30. Thy1^{neg} VFT CD4 T cells require additional APC interactions to stimulate IL-10 secretion.

Isolated VFT T cells (MACS CD3 negative selection) were further sorted (FACSAria) into CD4^{+}CD8^{neg}CD90.1^{+}CD25^{neg}FR4$^{low/-}$ (Thy1+) and CD4^{+}CD8^{neg}CD90.1^{neg} (Thy1-) populations. The CD25 and FR4 labeling was used to exclude FOXP3^{+} Treg cells. 3x10^{4} Thy1+ or Thy1- cells were plated per 96-well with A) 5x10^{4} irradiated (1000 RADs) WT splenocytes in the absence (NoAg) or presence of 20µg/ml MUC1-19mer, B) 5x10^{4} irradiated splenocytes plus soluble anti-CD3 (.25ug/ml) and anti-CD28 (.5ug/ml) or C) alone in wells pre-coated for 2hrs at 37^{o}C with 2.5µg/ml anti-CD3 and anti-CD28. All conditions were done in triplicate. After 72 hrs culture supernatants were analyzed for IL-10 by ELISA. (ND = not detectable)

C.3 CONSERVED EXPRESSION OF THY1 ON HYBRIDOMAS AND LYMPHOMAS

In addition to the above description of Thy1 expression related to phenotype and function on primary CD4 T cells, we made an interesting observation concerning Thy1 expression on hybridoma and lymphoma cell lines. Surprisingly, all of the hybridomas we have tested express both the Thy1.1 and Thy1.2 alleles on 100% of viable cells (Figure 31A). Additionally, both the mouse EL4 (223) and BW5147a-b- (142) lymphoma lines were close to 100% Thy1.2 and Thy1.1 positive, respectively (Figure 31B), reflecting the mouse strain from which the lymphoma was derived. The RMA lymphoma line (224) also had similar Thy1 expression (data not shown). We attribute the dual expression of Thy1.1 and Thy1.2 on all the hybridomas to the source of the fusion partners. In all cases primary Thy1.2^{+} T cells were fused to either BW5147 (225) or the BW5147a-b- variant. In addition to mouse lymphoma lines, there is a similar Thy1 expression pattern on the human Jurkat lymphoma line (Figure 31C). We have found that Jurkat cells, like mouse hybridomas, down regulate CD3 expression when grown at a high cell density. Those Jurkat cells that decrease CD3 expression, or a Jurkat variant lacking cell surface CD3 expression (226), did not reflect a loss of Thy1 expression, indicating the two molecules are expressed independently.

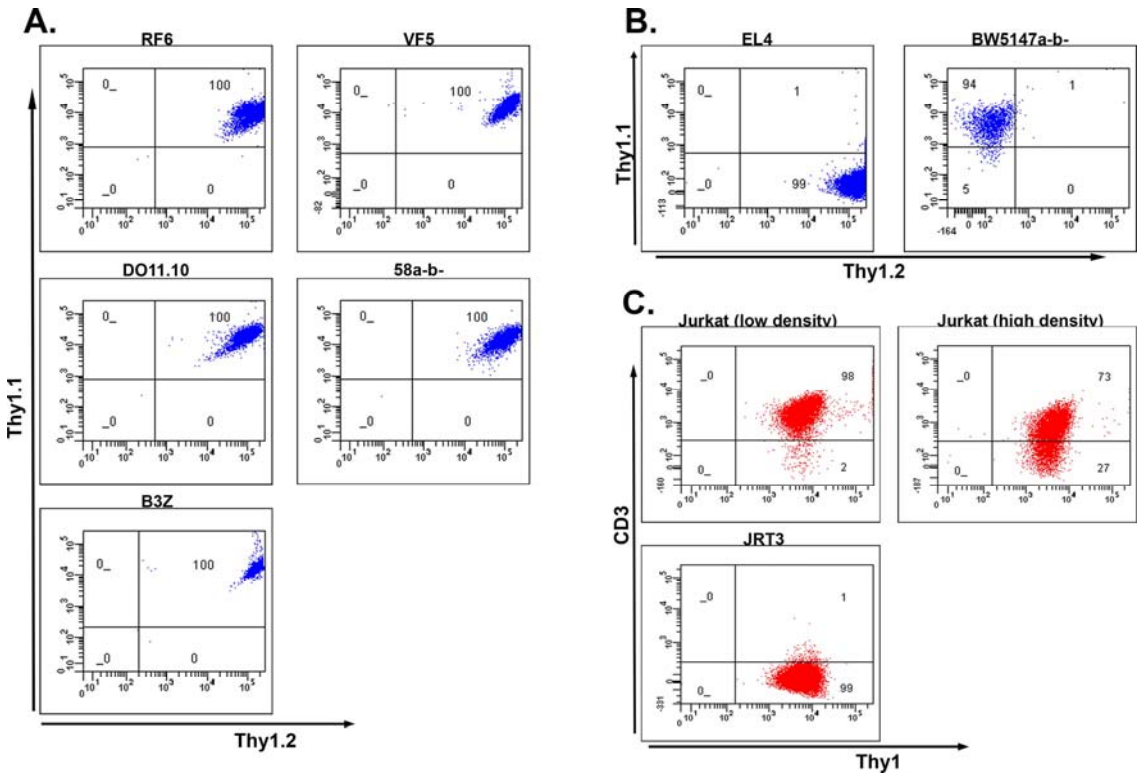


Figure 31. Thy1 expression is conserved on hybridomas and lymphomas.

A) CD4 T hybridomas RF6, VF5, DO11.10, 58a-b-, and CD8 T hybridoma B3Z and B) lymphomas EL4 and BW5147a-b- were labeled with anti-mouse Thy1.1 and Thy1.2 antibodies. C) Jurkat cells grown either at a low density (1-5x10^5cells/ml) or at a high density (>1x10^6cells/ml) and a TCR-deficient Jurkat variant (JRT3) were labeled with anti-human CD3 and Thy1 antibodies. Cells were analyzed by flow cytometry and representative histograms are shown. The percent population in each quadrant is indicated.

C.4 DISSCUSSION

Similar to previous reports (227-229), we show here that the lack of Thy1 expression segregates phenotypically different CD4 T cell populations in VFT-Tg and WT mice. The Thy1^{neg} CD4 T cell population is more prominent in VFT-Tg mice, although the reason for the expansion is

unclear. We do not believe that the specificity of the VFT TCR is resulting in an increase in the Thy1^{neg} population because we have seen a similar population in VFTβ single transgenic mice.

Similar to the presence of Thy1 in neurological synapses, Thy1 is also thought to be present in "immunological synapses" and thus could be influencing T cell to APC cell-cell interactions (221). Furthermore, Thy1 is suggested to be involved in TCR signaling based on antibody cross-linking studies (230, 231), yet studies addressing the physiological role of Thy1 in antigen-specific T cell responses are lacking. We suggest that the lack of Thy1 expression on CD4 T cells identifies a non-proliferative, IL-10 -producing population. Since the Thy1^{neg} population is producing antigen-specific IL-10, but not IFNγ, it is possible that the cells may serve a regulatory function *in vivo* (232). In addition, it still remains unclear whether the lack of Thy1 expression on CD4 T cells is playing a direct role in the antigen-specific responses, or rather serves as a marker for a sub-type CD4 T cell population.

Although Thy1 is known to be expressed on various mouse lymphomas (233, 234) and human Jurkat lymphoma (235), we suggest here that Thy1 expression is highly conserved on lymphoid tumor cell lines and required for their survival. For example, the RF6 and DO11.10 hybridomas were derived over 20 years apart, in different labs, using different BW5147 variants (112, 236). Interestingly, after the many years of cell passage and multiple transfers between different labs, all while never being intentionally selected for Thy1 expression, these hybridomas (and all others tested) have maintained constitutive Thy1 expression on all viable cells. This should be taken in the context that the T hybridomas routinely need to be re-cloned to ensure CD3 and/or CD4 expression is maintained, suggesting that those molecules are dispensable for cell survival. Continuing studies are needed to determine if Thy1 expression is constitutive on newly transformed mouse and human lymphomas.

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