REGULATION OF MUC1-SPECIFIC IMMUNITY BY $CD4^+$ T CELLS

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MUC1 is a large glycoprotein that is expressed on ductal epithelial cells and also the majority of epithelial adenocarcinomas. Dysregulated expression of aberrantly glycoslyated MUC1 in carcinomas allows tumor-specific recognition of MUC1-derived epitopes by antibodies and T lymphocytes. However, despite the ability of CTL to specifically recognize and kill MUC1$^+$ tumor cells, immune responses in cancer patients fail to prevent tumor progression. The failure of patients' immune systems to eradicate MUC1$^+$ tumors has been linked with their inability to mount MUC1-specific helper T cell responses.

Here we show that CD4$^+$ T cells play a central role in both the enhancement and suppression of MUC1-specific immune responses. Using MUC1-Tg mice as a model for tolerance to self-expressed MUC1, we show that MUC1-specific regulatory T cells (Tregs) respond to stimulation with MUC1 in the absence of a CD4$^+$ T helper response. The Treg:Th imbalance in MUC1-Tg mice causes the suppression of MUC1-specific immunity. This suppression can be overcome by providing functional Th cells from WT mice. To focus our studies on MUC1-specific CD4$^+$ T cells, we created a TCR-transgenic mouse whose CD4$^+$ T cells are specific for an MHC Class II-restricted epitope derived from unglycosylated MUC1. Using these mice, we have confirmed that adoptive transfer of MUC1-specific CD4$^+$ T replaces the MUC1-specific T cell help that is missing in MUC1-Tg mice and restores their ability to respond to MUC1 vaccines. This work shows that the generation of CD4 helper T cell responses is critical to establishing effective immunity to MUC1$^+$ cancers.

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DEDICATION

To my parents, Maureen and Peter Turner, who, through their immeasurable sacrifices, love and support over the last 29 years, have made it possible for me to pursue my dreams.

To my grandparents, Sidney Turner and Eileen Bill, who both died from cancer.

PREFACE

This is the section where one is expected to write something really profound. However, that is not my style. This dissertation is not about me, or about the countless times I worked through the night, only to obtain negative results. Neither is this intended to be seen as a self-important attempt at over-stating the importance of my work. This is simply an account of a series of experiments that were designed to shed light on one small, yet fundamental part of the incredibly complex puzzle that is tumor immunology. I hope that it makes interesting reading, or at the very least, a useful door-stop.

I will simply leave you with the following quote from the book "Masquerade" by Terry Pratchett, which describes perfectly my experience as a graduate student…

"…no matter how hard a thing is to do, once it <u>has</u> been done it'll become a whole lot easier and will therefore be done a lot. A huge mountain might be scaled by strong men only after many centuries of failed attempts, but a few decades later Grandmothers will be strolling up it for tea and then wandering back afterwards to see where they left their glasses."

STATEMENT OF PROBLEM

Cancer is the second leading cause of death after heart disease. Of all cancers, those of epithelial origin (lung, colon, pancreas etc) occupy the top five positions in terms of mortality (1). The failure of conventional therapies to combat these diseases, in particular, pancreatic cancer has fuelled the search for novel forms of treatment.

The discovery that tumor-associated antigens can be recognized by antibodies and T lymphocytes (2-4) strengthens the case that tumor-specific immune responses have the capacity to protect against various forms of cancer. One such tumor-associated antigen is the epithelial cell mucin, MUC1, a large glycoprotein that is expressed on ductal epithelia and also on a wide range of epithelial carcinomas (5). Cancer-associated changes in post-translational processing and expression of MUC1 allow MUC1-specific T cells to target $MUC1^+$ tumors without harming the surrounding normal tissues. This makes MUC1 it an attractive target for the immunological treatment of most adenocarcinomas (6).

MUC1-specific immune responses can be detected in cancer patients (7-9). However, they are unable to prevent tumor progression. It is thought that MUC1-specific immune responses are weakened in cancer patients by the absence of MUC1-specific "T cell help", which is provided by $CD4^+$ "helper" T lymphocytes (Th cells).

The following pages contain a description of a series of experiments that utilized a well-defined set of reagents, including a novel TCR transgenic mouse, to determine the role that MUC1-specific $CD4^+$ T cells play in modulating the immune response to MUC1.

1. INTRODUCTION

1.1. Cancer and the Immune System

In 1909, Paul Ehrlich first conceived the idea that the immune system might have the capacity to protect individuals from developing carcinomas (10). However, it was not until 1957 that Thomas and Burnet formally proposed the hypothesis of cancer immunosurveillance (11-13). This hypothesis implicates the immune system as a sort of watch-dog, lying in wait for cells to turn cancerous, and states that:

> "…small accumulations of tumour cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumour and no clinical hint of its existence."

Considerable evidence now exists to indicate that this is, indeed, the case. However, the immune surveillance system is not perfect; eventually a cancer cell arises that is able to evade the immune system long enough to grow into a detectable tumor mass. A battle then ensues between the cancer cells and the immune system that is trying to suppress their growth. If the cancer cells proliferate faster than they are being killed, the tumor will grow in size. Conversely, an effective anti-tumor immune response will kill the cancer cells faster than they can multiply, in which case the tumor will shrink or "regress". The outcome depends on many factors: on the immune system side, genetics, age of the individual, and previous immune history determine the ability to respond efficiently; on the tumor side, specific mutations leading to loss of antigenicity and

acquisition of the ability to produce immunosuppressive factors, determine the susceptibility to immune control.

In the half-century since Burnet's seminal paper, immunologists have worked to understand the mechanisms behind the anti-tumor immune response (14) in an attempt to harness its potential to provide effective protection against a wide array of different malignancies (2,15-18). In order to fully understand how the immune system responds to tumors, it is important to understand the interaction of the major cell types that are involved in the anti-tumor immune response.

1.2. Induction of Tumor Immunity

1.2.1. Dendritic Cells Present Tumor Antigens to T cells

DC are the most potent antigen-presenting cells (APC) and the only ones with the ability to prime naïve T cells (i.e. T cells that never encountered their specific antigen) and are therefore the initiators of the adaptive immune response (19). Dendritic Cells act as the sentinels of the immune system. They develop in the bone marrow and then travel to other tissues, where they take up temporary residence, sampling the environment within the peripheral tissues by ingesting particulate matter, such as bacteria, viruses, or pieces of tumor cells (20). Having sampled the tissues, DC travel to the draining lymph node, where they 'present' peptides derived from "processed" antigens to antigen-specific T cells. T cells express a receptor on their surface called the "T cell antigen receptor" (TCR) (described in detail later), which allows them to recognize antigenic peptides bound to MHC Class I or Class II molecules on the DC cell surface.

Although each MHC molecule can bind to only one peptide at a time, a single DC has thousands of MHC molecules, so it can simultaneously present many different peptides on its surface.

1.2.2. DC control the decision between immunity and toleranace

Ligation of the TCR by its cognate peptide:MHC complex provides the first of two signals that are required by the T cell for its full stimulation. The second signal can come in many different guises, collectively termed "co-stimulation". Examples of co-stimulatory molecules expressed by DC are CD40, CD80, CD86, ICOS and OX40L (21-23). Originally, the presence of co-stimulatory molecules on the DC was observed to be beneficial, even essential, for optimal T cell activation (21,24-26). Recently however, it has been shown that co-stimulation also has inhibitory effects and may have a role in preventing autoimmunity by tolerizing self-reactive T cells (22,23,27,28).

The context in which the DC acquires the peripheral antigens (i.e. in the presence or absence of an inflammatory response to an insult or injury) determines its activation status and the type of costimulation that it can provide to T cells when it arrives in the lymph node. This will affect the outcome of its interaction with antigen-specific T cells.

Dendritic Cells use numerous receptors to recognize conserved ligands on potential pathogens (e.g. recognition of bacterial CpG by TLR9) (29-31). Upon detection of such pathogens, the DC become activated, upregulate co-stimulatory molecules, and begin to produce inflammatory cytokines such as IL-12, TNF-alpha (32,33). DC activated in such a fashion are able to efficiently stimulate antigen-specific T cells in the lymph nodes, which will return to the site of the infection to eliminate any infected cells. However, if the tissue is not under attack, the DC

will arrive in the lymph node in a quiescent state (low level of costimulatory molecules, no cytokine production), presenting only 'self' tissue-antigens, and reporting that everything is normal. T cells that recognize self-antigens on quiescent DC will only receive signal 1 and thus be tolerized (deleted or functionally altered) and prevented from traveling to the tissue and causing autoimmunity (34).

The terms "mature DC" and "immature DC" have been used to describe the status of the DC in these two situations and can be defined by the presence or absence of certain molecules on the surface of the DC (e.g. CD40, CD80, CD86). However, description of a DC as "mature" does not necessarily determine the outcome of its interaction with a naïve T cell; it has been shown that mature DC expressing high levels of co-stimulatory molecules can be either stimulatory or tolerogenic (23,35,36). Other molecules, such as the cytokines IL-12 and IL-10, are thought to be involved in the decision between immunity and tolerance (35), although the details of this switch are not fully understood.

The main point to be made is that DC can only instruct naïve T cells to become effector cells if they receive 'danger' signals induced by an active injury or infection (37) that result in upregulation of co-stimulatory molecules and production of inflammatory cytokines. A benign tumor, developing slowly over a long period of time, is unlikely to cause the widespread tissue destruction necessary to elicit the danger signals required for activating DC (38). Tumor immunologists wishing to elicit effective anti-tumor immunity are attempting to understand how it might be possible to dictate the context in which DC present tumor antigens, such that effective anti-tumor immunity can l be established.

1.2.3. Tumor-specific CTL and helper T cells

For many years, $CD8^+$ T cells representing primarily cytotoxic T lymphocytes (CTL) received much attention as the cells that are primarily responsible for mediating immunological rejection of tumors. Since adenocarcinomas express MHC Class I molecules and lack MHC Class II, CD8+ CTL that recognize MHC Class I peptide complexes are the cells which directly recognize tumor antigens on the surface of cancer cells (39,40) and initiate apoptotic death of tumor cells via release of lytic granules or by Fas-FasL interactions (41). CD4+ T cells, on the other hand, recognize most tumor antigens cross-presented by DC in their MHC Class II molecules. This indirect recognition of tumor antigens by $CD4^+$ cells, until recently considered to be only helper cells, has led to questioning their precise role in anti-tumor immunity.

Given a strong enough stimulus, such as highly activated DC and optimal concentrations of antigen, primary CD8 T cell responses to tumors can be generated in the absence of helper T cells (42). However, there is an apparent requirement for help from $CD4^+$ T cells in the establishment of effective CTL memory (43). Evidence from several models of acute infections suggests that $CD4^+$ T cells are required for the priming (44,45) and maintenance (46) of memory CTL, although it is unclear whether they are also required for re-stimulation of memory CTL in the recall response (44,47). These $CD4^+$ helper T cells (Th) mediate their effect through the dendritic cell. When Th cells recognize specific antigen presented on a properly activated DC, they provide signals via CD40L which 'license' the DC to provide the proper co-stimulation for antigen-specific CTL (24-26,48). There is a significant body of evidence in support of a similar role for $CD4^+$ T cells in the generation of productive tumor immunity (16,49-51). Some of the

most striking evidence is from observations that some tumors render $CD4^+$ T cells non-responsive in order to avoid rejection (52,53).

1.2.4. $CD4^+$ Regulatory T cells

A relatively recent addition to the T cell family is a sub-population of $CD4^+$ T cells called regulatory T cells (Tregs). These have the opposite function from helper T cells in that they suppress T and B cell responses. Tregs are defined by expression of CD4, CD25 and FoxP3 (54); GITR, CD62L and CD27 have been implicated as additional markers (55-57). Tregs exert their suppression either by cell-cell contact, or by secretion of the cytokines IL-10 or TGF-β(58,59). Tregs are thymically derived and have an important role in the maintenance of tolerance to self-antigens (60-64), although they also control immune responses to pathogens (65,66). The presence of Tregs in cancer patients correlates negatively with strength of the tumor-specific immune response and prognosis (67-69) and depletion of Tregs enhances the anti-tumor immune response (70,71). $CD4^+CD25^+$ Tregs were originally thought to be anergic, as they appeared to lack classical features of T cell activation such as proliferation and cytokine production (66,70). However, recent studies have shown that their proliferation can be induced by different subsets of dendritic cells (35,60,72-75).

The balance between immunity and tolerance is therefore an active process involving the interaction of DC and $CD4^+$ T cells (20,43). CD8 T cells seem to take more of a passive role; they are either stimulated by the DC, or they remain ignorant. Therefore, DC and $CD4^+$ T cells are the controllers of the adaptive immune response, whereas CD8 T cells simply follow their

lead. By dictating the nature of the Th-DC interaction, one might hope to influence the establishment of effective tumor-specific memory CTL responses.

1.3. Mucin Expression in Gastrointestinal Cancers

Gastrointestinal cancers originate from malignant transformation of normal epithelial cells that line the gastrointestinal tract and various other ductal structures. The same cell type gives origin to other human solid tumors, such as breast cancer, non-small cell lung cancer, kidney cancer, bladder cancer, prostate cancer, etc. Because of this, tumors that appear to be widely different, e.g. colon cancer and breast cancer, express in common numerous molecules that are characteristic of epithelial cells. Among many such molecules, those belonging to the mucin family have been very extensively studied due to their special characteristics that can help define and diagnose various gastrointestinal pathologies, including cancer.

1.3.1. The Mucin family of glycoproteins

Mucins are large secreted and/or transmembrane glycoproteins, predominantly expressed on the luminal surfaces of wet epithelia (5). The mucin family has steadily grown over the last 15 years, numbering currently fourteen different members encoded by genes MUC1-MUC14. Their pattern of expression varies depending on the histological site as well as the integrity of the site, and the gradient from normal to diseased state. The common feature that is shared by all mucins, which distinguishes them from mucin-like glycoproteins, is a large region composed of variable number of tandem repeats (VNTR). The amino acid sequence and length of the tandem repeats is different for each mucin gene.

1.3.2. Glycosylation of Mucins

More than 50% of molecular weight of mucins is derived from O-linked carbohydrates attached to numerous serine and threonine residues found in the tandem repeats. Addition of sialic acid to the terminal sugars gives these molecules their mucinous character (76-80). Early studies with monoclonal antibodies reactive with epithelial tumors revealed that some antibodies recognized only mucin epitopes uniquely expressed on malignant cells (9,81,82). It is now understood that this is due to aberrant glycosylation of tumor mucins, which creates tumor specific glycoepitopes as well as tumor specific peptide epitopes (81,83-86). Attachment of O-glycans to the mucin peptide backbone and subsequent elongation and branching of carbohydrate chains occurs in the Golgi apparatus and is initiated by polypeptidyl N-acetylgalactosaminyltransferases (GalNAc-T's), which transfer N-acetylgalactosamine (GalNAc) to the Ser/Thr residues. Further monosaccharides, mainly galactose (Gal) and/or N-acetylglucosamine (GlcNAC), are then added to form long, branched carbohydrate chains. Finally, α-anomeric monosaccharides, such as α-fucose, α-sialic acids, α-Gal or α-GalNAc, are added to the periphery in different combinations to form blood-group carbohydrate antigens such as A, B, H and the Lewis group (87). Cancer cells exhibit changes in the composition and relative levels of glycosyltransferases that perform O-glycosylation of mucins. Furthermore, an increase in sialyltransferases (88), which leads to premature addition of sialic acid, acts as a stop signal preventing further elongation and results in the truncation of the carbohydrate chains (89,90). These cancer-associated changes result in qualitatively as well as quantitatively different mucin glycosylation, producing carbohydrates that are not found on normal cells and have been used as tumor markers. The best examples are … T, …sT, …Tn and …sTn (91,92).. Decreased glycosylation of the polypeptide core also exposes peptide epitopes on tumor mucins that are not exposed on normal mucins.

These new tumor specific mucin epitopes offer a unique opportunity to design mucin-based diagnostic as well as therapeutic approaches for managing human epithelial tumors, such as gastrointestinal tumors. In the rest of the chapter we will provide examples of how cancer-associated changes in mucin expression have been used already in diagnosis and therapy of GI malignancies and foreshadow future uses of some of the most promising candidates for immunotherapy and prevention of GI cancers.

1.3.3. Mucin Expression Patterns in healthy & diseased tissues

Various members of the mucin family are expressed in different regions and on different cell-types within the GI tract. Furthermore, the expression patterns of individual mucins correlate with different stages of disease and with prognosis *(Table 1)*. Characterization of these expression patterns is now maturing to the point that it can provide methods for early detection of cancer-associated changes within the mucosa, which in turn will allow timely intervention. Below are examples of the best-studied preneoplastic and cancerous states that are characterized by specific patterns of mucin expression.

1.3.3.1. Esophageal mucins

Normal esophagus expresses low levels of MUC5B in the submucosal glands and MUC1 and MUC4 in the stratified squamous epithelium. Two types of malignancies arise from these tissues, esophageal adenocarcinomas and esophageal squamous cell carcinomas. Barrett's metaplasia (Barrett's Esophagus) is a premalignant lesion of the esophagus that usually precedes Barrett's adenocarcinoma. For a long time, it was unclear whether Barrett's Esophagus

represented an intermediate stage of transformation towards full-blown carcinoma, or whether the two states occurred independently of each other. In recent years, mucin gene expression has been used to show that the former is the case, by characterizing the steps involved in the transition from normal epithelial cells in the esophagus, through Barrett's Esophagus to esophageal adenocarcinoma (93,94). Mucin patterns were also used to further differentiate esophageal adenocarcinomas from squamous cell carcinoma. Upregulation of MUC1 and MUC4 in the absence of mucins 2, 3 and 5, is a diagnostic mucin signature of squamous cell carcinomas. Barrett's adenocarcinomas, on the other hand, were found to upregulate mucins 2-6 but not MUC1. Interestingly, of the Barrett's adenocarcinoma-associated mucins, MUC2, MUC3 and MUC4 correlated with intestinal metaplasia, whereas expression of MUC5AC and MUC6 was observed in gastric metaplasia (94). From these studies, it appears that gastric and intestinal metaplasia of the esophagus are intermediate steps in the development of Barrett's adenocarcinoma, but not esophageal squamous cell carcinoma.

Expression of MUC1 and MUC2 has been correlated with malignant potential of esophageal dysplasia (95,96). MUC1 is absent in dysplastic epithelium but upregulated in both Barrett's adenocarcinoma (95) and squamous cell carcinomas (96), which makes it a good diagnostic marker for cancer. MUC2 is also a good diagnostic marker because it is present in dysplastic lesions but completely absent in cancerous lesions. While mucins that are diminished in expression or completely turned off in the progression from normal to dysplastic to cancerous state can be used to properly diagnose a biopsy, the mucins that are upregulated in cancer, such as MUC1, can also be used as targets for therapy.

1.3.3.2. Gastric mucins

As with the esophageal cancers, as well as most other carcinomas, there is a series of sequential steps in the development of gastric adenocarcinomas, starting with gastritis and passing sequentially through intestinal metaplasia, dysplasia, intramucosal carcinoma and finally invasive carcinoma (97-99). In parallel with disease progression, there exists a spectrum of mucin expression, with certain mucins being associated with specific stages of disease and in some cases correlating also with clinical outcome. In normal gastric mucosa, MUC1, MUC5AC and MUC6 are expressed in a cell type-specific manner, with MUC1 and MUC5AC found in superficial epithelium and MUC6 in the deep (antral) glands (100). MUC2 is not found in normal gastric epithelium, although it is expressed by goblet cells during intestinal metaplasia. MUC1 expression increases during the gastric adenoma to carcinoma progression and correlates with poorer outcome in patients with gastric carcinomas (100). Conversely, patients with $MUC2^{+}$ tumors show better survival. The other mucin genes do not show a reproducible or characteristic pattern of expression in gastric carcinomas, even though increase in heterogeneity of mucin gene expression is frequently seen and appears to correlate with advanced stages of gastric cancers (101).

1.3.3.3. Pancreatic mucins

Three main types of ductal pancreatic tumors have been characterized with respect to mucin gene expression: (1) Invasive ductal adenocarcinoma, which is the most common, highly malignant and carries the worst prognosis; (2) Intraductal papillary mucinous neoplasia (IPMN), which are usually relatively benign; (3) Mucinous cystic neoplasia (MCN), which almost exclusively only affect women and, if noninvasive, can be treated simply by resection of the pancreatic tail.

Pancreatic tumors have different mucin expression patterns and these correlate with aggressiveness of the tumors. MCN express MUC5AC ubiquitously, and MUC2 in goblet cells. MUC1 is only expressed in areas of invasion (102). IPMN are similar, in that MUC5AC is widely expressed, MUC2 is found only in the dark cell type, and MUC1 was observed in the compact cell type and in areas of dark cell type that showed carcinomatous change and invasiveness (103). In pancreatic ductal adenocarcinoma, however, the pattern is quite different. Upregulation of MUC1 and MUC6 and de-novo expression of MUC5AC occurs early in pancreatic intra-epithelial neoplasia and is maintained in the majority of ductal adenocarcinomas. MUC2 is rarely expressed. (104,105).

The distinct patterns of mucin expression in these different forms of pancreatic cancer suggest different pathways of oncogenesis, which may require different forms of treatment. However, there are some common characteristics from which some important conclusions can be drawn. In common with other gastro-intestinal epithelia, over-expression of MUC1 and loss of MUC2 in the pancreas correlates with invasiveness and poor prognosis. Hence, pancreatic adenocarcinomas that have the combined phenotype of $MUC1^{+}$ $MUC2^{-}$, show worse patient survival rates than MCN or IPMN. Because of the high level of expression of MUC1 in pancreatic adenocarcinomas, and many cancer-associated changes in MUC1 glycosylation, this mucin has been extensively explored as a target for immune attack on tumor cells, as well as an immunogen to elicit tumor-specific immune responses.

1.3.3.4. Colonic mucins

Normal colon is characterized by the expression of MUC2, MUC3 and MUC4, but that pattern changes in a very characteristic fashion during the progression from polyps to adenomas to adenocarcinomas. While MUC1 is not expressed in normal colonic epithelium, it is found in the majority of adenomatous dysplasia and almost all colorectal adenocarcinomas (106,107). Conversely, loss of MUC2 expression is observed in over 50% of severely dysplastic adenomas and carcinomas. This correlates with the Ki-67 proliferation index, suggesting a link between decreased MUC2 expression and malignant transformation (106). A recent report showed conclusively that mice made genetically defective in the MUC2 gene, frequently develop intestinal adenocarcinomas and rectal tumors (108). Thus, MUC1 and MUC2 expression can be correlated with disease progression, with MUC2 having an apparent role in the suppression of colorectal carcinomas.

MUC4 is another mucin whose expression is lost in polyps and adenomas (109). Evidence from other epithelial organs suggests a link between MUC4 and highly invasive carcinomas, although this correlation is not clear for intestinal epithelia. MUC5AC and MUC6 are both expressed mainly in the intermediate developmental stages of intestinal adenocarcinomas. Neither one is expressed in the normal lower intestine, yet they are expressed at a high level in moderate dysplasia and at a lower level in carcinomas (110,111).

Table 1. Expression Patterns of gastro-intestinal mucins in normal and tumor tissues

Site	MUC1	MUC2	MUC3	MUC4	MUC5AC	MUC5B	MUC6
Normal Esophagus	+[a]	-	-	+	-	+	-
Esophageal Squamous Carcinoma	++	+/-	-	++	-	-	-
Barrett's Metaplasia	-	+ Int [f]	+ Int	+ Int	+ Gastric	-	+ Gastric
Barrett's Adenocarcinoma	+/-	+/-	+	+	+	-	+
Normal Stomach	+	-	-	-	+	ND[b]	+
Gastric IM[c]	+/-	+			+/-	ND	+/-
Gastric Cancer	++/-	+	+	+	-	ND	+/-
Normal Pancreas	+	-	+/-	-	-	+	+
IPMN[d]	-	+/-	ND	ND	+	ND	ND
MCN[e]	-	+ Goblet cells	ND	ND	+	ND	-
Pancreatic Adenocarcinoma	++	-	ND	+	+	ND	++
Normal Intestine	-	++	ND	ND	-	ND	-
Intestinal Adenoma	+	+	ND	ND	++	ND	++
Intestinal Carcinoma	++	+/-	ND	ND	+	ND	+

[a] Increase in the number of +'s indicates an increase in the level of expression.
[b] ND, Not Determined
[c] IM, Intestinal Metaplasia
[d] IPMN, Intraductal Papillary Mucinous Neoplasia
[e] MCN, Mucinous Cystic Neoplasia
[f] Int, Intestinal

1.4. Mucin-based Therapy

The cancer-associated changes in mucin expression described above suggest that certain mucins may be used as targets for treatment and prevention of specific GI cancers. For most of the mucins, however, there is not always a clear-cut difference of expression between normal and neoplastic tissues and thus targeting those molecules may not under most circumstances be tumor specific. For example, even though MUC5AC is overexpressed in many gastrointestinal cancers, it is also expressed on the normal epithelium in many parts of the GI tract. MUC2 is the most abundantly secreted intestinal mucin and is over-expressed in all *mucinous* tumors of colon, breast, ovary and pancreas (112,113). However, in adenocarcinomas, MUC2 appears to act as a tumor suppressor and loss of its expression is associated with a more aggressive phenotype. Therapy-induced selection pressure against the expression of MUC2 could lead to the outgrowth of $MUC2^-$ tumors, which evidence suggests, would be even more aggressive than the original tumor. Another factor to consider when designing mucin-based therapies is that most of these molecules are shed or secreted by tumor cells and may not be suitable targets for some therapies. Of the 14 mucins described so far, only MUC1, MUC4 and MUC5AC are transmembrane, cell-associated molecules that could be used to target tumor cells with various forms of therapy. MUC1 is by far the best characterized for its tumor specific expression and antigenicity.

1.4.1. Mucin-based immunotherapy

There are compelling reasons to consider mucins as potential immunotherapy targets. The major ones are the difference in gene expression between normal and abnormal tissues, described above, and the cancer-associated pattern of glycosylation that distinguishes cancer mucins from normal mucins. By directing immune responses against the cancer-associated forms, one can

expect to elicit protective or therapeutic immunity without causing collateral autoimmune damage. Cancer associated mucin forms can be targeted through passive immunotherapy with preformed immune effector mechanisms, such as antibodies or T cells that can be administered to the patient in hope of destroying tumor cells remaining after surgery or chemotherapy. The other alternative is active specific immunotherapy where mucin molecules are used as immunogens in vaccines designed to elicit anti-mucin and therefore anti-tumor immunity.

Antibody mediated therapy for cancer (114) has gained in popularity in recent years due to successes obtained with such therapy in breast cancer (115) and B cell lymphomas (116). Even though one of the more successful antibody trials has been in colon cancer (117) using an antibody against a tumor glycoprotein similar to a transmembrane mucin, therapy with anti-mucin antibodies has not been sufficiently explored in GI cancers. As mentioned above most mucins are secreted molecules and thus have not been considered good targets for such therapy. This concern is justifiable in cases where the antibody is used to deliver drugs, toxins or radioisotopes to cancer cells. However, antibodies may serve additional functions, one of which is the formation of immune complexes with soluble mucins that could be taken up by Fc receptors on dendritic cells. This route of endocytosis favors processing and presentation of antigens in the class I pathway (118,119) and may help generate additional mucin specific CTL. At the very least, the cell-associated mucins should be considered as targets for immunotherapy of GI cancers. An antibody against a cell membrane associated mucin MUC1 did show positive result when used to treat ovarian cancer (120) and there are reasons to believe that other tumors that overexpress tumor forms of MUC1 should be candidates for therapy with that antibody. It is

hoped that newly aroused interest in antibody therapy in general will provoke greater efforts in testing anti-mucin antibodies in therapy of GI cancers.

1.4.2. Immune responses to MUC1

Of all the gastrointestinal mucins, MUC1 has the strongest association with aggressiveness/malignancy and prognosis (121). MUC1 is normally highly glycosylated and its normal expression is at very low levels and restricted to the apical surface of ductal epithelia. Thus, the potentially antigenic peptide core of native MUC1 is anatomically and sterically inaccessible to the immune system, especially the antibodies. During the process of carcinogenesis, normal epithelial cell polarity is lost, such that on tumor cells there is no clear distinction between the basolateral and apical surfaces. This results in expression of MUC1 over the entire tumor cell surface (121) where it is for the first time accessible to the cells of the immune system and specific antibodies. Furthermore, cancer-associated truncation of O-linked carbohydrate chains creates tumor-specific and immunogenic T and Tn antigens (87,122,123) on the protein core of the tandem repeats, which can also be sialylated (sT and sTN), thereby creating at least four tumor antigens that can be recognized by antibodies (91,123). Tumor-specific T cells can also distinguish cancer-associated peptide and glycopeptide epitopes from normally glycosylated MUC1 epitopes (8,9,124).

1.4.3. Anti-MUC1 immunity induced by tumor growth

Many studies have shown that patients suffering from tumors that overexpress MUC1 possess antibodies and T cells that recognize tumor forms of MUC1. However, these humoral and cellular immune responses that are induced during tumor development are very weak, with

antibody titers and T cell frequency being considerably below what would be expected to be a therapeutic level. Nevertheless, in some instances the presence of antibodies against MUC1 can be correlated with favorable disease outcome (i.e. patients with MUC1-specific antibodies did not experience recurrent tumors) (125). Although the presence of MUC1-specific antibodies is indicative of an immune response, antibodies alone have little effect, if any, on the growth of solid tumors (126). It is also striking that MUC1-specific antibodies that are induced in response to the tumor, are predominantly of the IgM type (127). This indicates that MUC1-specific B cells do not receive the help from antigen-specific $CD4^{\pm}$ T cells necessary to allow them to switch to the production of other immunoglobulin isotypes.

MUC1-specific T cells are also induced in patients during the growth of MUC1 expressing tumors. The first to be described were derived from lymph nodes of pancreatic, breast and colon cancer patients and shown to recognize underglycosylated MUC1 on tumors in an MHC-unrestricted manner (8). These MHC-unrestricted CTL proliferate in response to tumor-derived MUC1 in the absence of antigen presenting cells, and they can lyse $MUC1^{+}$ target tumor cells, regardless of the HLA alleles that they express. Structural analyses of un-glycosylated MUC1 offered an explanation for its distinctive antigenic properties related to the ability to activate specific T cells. Every 20 amino acid-long tandem repeat on the MUC1 polypeptide core contains five proline residues, which give a rigid rod-like structure to the protein. Protruding from each tandem repeat is a beta-turn helical knob, which corresponds to the PDTRP sequence (128). It is this sequence that is recognized by antibodies and T cells from cancer patients (9,129,130). This particular epitope, as well as potentially others, are located on the tandem repeat structure that imparts an antigenic multivalency, such that each mucin molecule may be

bound by many T cell receptors on a single T cell. It has been shown that more than two repeats are required to activate T cells in an MHC un-restricted fashion. MUC1 engineered to have only two repeats must be expressed at very high levels on the target cell surface to elicit a T cell response (131). Binding of many T cell receptors along the length of one MUC1 molecule allows them to be cross-linked sufficiently to induce the intracellular signals required for T cell activation (132).

CTL can also recognize MUC1 in the context of MHC class I molecules. Peptide binding studies were performed with 8, 9 and 10 amino acid long peptides derived from the MUC1 tandem repeat sequence to investigate their ability to bind to various human HLA-class I molecules and be presented to T cells. Peptide STAPPAHGV was found to bind to HLA-A1, -A2.1, -A3, and -A11 (133). T cells from healthy A-11^{+} donors were stimulated when this peptide was presented by HLA-A11 and a secondary response to this peptide was observed in LN cells from an HLA-A11+ cancer patient. A subsequent study showed that a longer MUC1 peptide containing five tandem repeats primed a broader CTL repertoire that was restricted by HLA-A1 (134). Studies in mice expressing a transgene for HLA-A2 have shown that immunization with MUC1-derived peptides produces CTL that lyse human $A2^{+}$ PBMC's loaded with the same peptide (135). This evidence that tumor growth can elicit antibodies and MHC-restricted and un-restricted CTL responses, albeit to very low, non-therapeutic levels, proves the immunogenicity of tumor MUC1 and supports current efforts to design vaccines that will specifically amplify those immune responses.

Given the central role that T cell help plays in coordinating and enhancing immune responses, the absence of a MUC1-specific $CD4^+$ T cell response in cancer patients may be the major obstacle in the generation of effective, long-term immunity to MUC1. If we can understand the reasons for inefficient helper T cell stimulation, it will be possible to correct this problem through therapy directed towards providing MUC1-specific helper T cells. They could be generated in vitro by priming on DC that have processed MUC1 antigen, expanded to large numbers and adoptively transferred into the patient. The presence of helper T cells may boost proliferation of existing CTL as well as help B cells switch from making only MUC1-specific IgM, to other Ig isotypes. Alternatively, patients who have not been extensively treated and are not immunosuppressed might be vaccinated with MUC1 forms that can be processed in vivo and presented to helper T cells. The preclinical studies in mice described here aim to test these approaches.

1.4.4. Preclinical models of MUC1 immunotherapy in MUC1-Tg mice

Studies in mice have shown that both $CD4^+$ and $CD8^+$ T lymphocytes can respond specifically to tumors that express MUC1 (6). However, the amino acid sequence of murine Muc1 shares very little similarity with that of human MUC1 (convention is that murine Muc1 is written in lower case, whereas human MUC1 is written in upper case). Therefore, the robust cellular and humoral responses to human MUC1 seen in WT mice are immune responses to a 'foreign' antigen and may not accurately represent responses that could be expected in patients where MUC1 for the most part is a self-molecule. The need for a model which better mimics the situation in humans prompted the creation of MUC1-transgenic (MUC1-Tg) mice (136,137), in which human MUC1 is expressed as a self-antigen and is regulated by its endogenous promoter.

These mice express and glycosylate MUC1 with the same cell-type and tissue-type specificity as humans (138). They have been studied for their ability to mount anti-MUC1 immune responses following vaccination, as well as bred with mice engineered to develop various tumors, in order to study MUC1 responses during tumor growth and tumor rejection. For example, a mouse model of MUC1 pancreatic cancer was developed by crossing MUC1 transgenic mice with transgenic mice that express the first 127 aa of the SV40 large T antigen under the control of elastase promoter (ET). 50% of the mice develop tumors at 12 weeks of age and clinical signs of disease at 21 weeks of age (139). The double transgenic mice (MET) develop pancreatic tumors that express human MUC1 (138). The mice exhibit dysplasia at birth that progress to microadenomas and then to full-blown adenocarcinomas. The tumors express high levels of underglycosylated MUC1, like human tumors, and that is accompanied by circulating MUC1 in the serum and presence of low titers of anti-MUC1 IgM (as in cancer patients).

Unlike wild-type (WT) mice, MUC1-Tg mice fail to initiate IgG class switching or mount $CD4^+$ T cell responses to $MUC1^+$ tumors (50,51) in response to many standard immunization protocols. In that way also, they mimic the human situation and have been used by several groups as a pre-clinical model to evaluate different vaccine strategies as methods of inducing a protective immunity to $MUC1^+$ tumors. Immunization with dendritic cells fused to $MUC1^+$ tumor cells induces MUC1-specific humoral and cellular immunity capable of rejecting experimental pulmonary metastases of the same cell line (140). Effector CTL isolated from these mice lysed the $MUC1^+$ tumor cells *in vitro*. In addition LN cells from immunized mice responded *in vitro* to exogenous MUC1, and the response was dependent on CD4, MHC-class II and costimulatory molecules B7.1, B7.2 and CD40L (141). These results suggested that fusion

of DC with tumor cells provided the MHC class II presentation of MUC1 as well as costimulation required to activate MUC1-specific $CD4^+$ T cells, which in turn enhanced the CTL response to mediate rejection of $MUC1^+$ metastatic foci.

Inasmuch as the goal is to induce MUC1-specific immunity against MUC1+ tumors, dendritic cell-based vaccines that utilize defined MUC1 protein or peptides instead of whole tumor cells or tumor cell lysates, offer a safer and better controlled alternative. However, MUC1-Tg mice immunized sub-cutaneously with MUC1 peptide-loaded DC mount mediocre CTL responses and virtually undetectable $CD4^+$ T cell responses (50). The goal of the studies described here is to determine why these $CD4^+$ T cell responses are so weak, and whether improving them will result in improved immunity to $MUC1^+$ tumors.

1.5. The T Cell antigen Receptor

Chapter 3 describes the generation of a novel transgenic mouse, VFT, and its use in the elucidatation of the response of MUC1-specific $CD4^+$ T cells to MUC1. In order to understand the procedure used in the identification and cloning of the MUC1-specific TCR expressed in this mouse, one must be familiar with the basic components of the TCR and how they combine differently in each T cell to form a unique receptor that confers antigen specificity upon that T cell.

1.5.1. Structure of the TCR

Each T cell carries a unique TCR on its surface and each TCR is specific for a different antigen. Despite this massive variation in the TCR repertoire, there are certain structural features that are common to every TCR. All TCRs are transmembrane heterodimers, consisting of a TCRα chain and a TCRβ chain. Each TCR chain is sub-divided into smaller regions, V (variable), J (joining), D (diversity; TCRβ only) and C (constant) (142). Each of these regions is encoded by a different gene segment, correspondingly called V, D, J and C. So the TCR expressed at the T cell surface is formed by the association of a TCRα chain (Vα-Jα-Cα) with a TCRβ chain (Vβ-Dβ-Jβ-Cβ).

The genes that encode the TCR alpha and beta chains respectively are located in two distinct chromosomal locations (143). Within the TCR loci, multiple copies of the V, (D) and J gene segments are arranged in series. In the TCRα locus, there are over 80 consecutive Vα gene segments, (Vα1, Vα2, Vα3…etc), followed by multiple Jα gene segments (Jα1, Jα2…etc) and a single Cα gene segment. The TCRβ locus contains a series of Vβ gene segments, (Vβ1, Vβ2,

Vβ3…etc), followed by two clusters of Dβ-Jβ-Cβ gene segments. Each D-J-Cβ cluster contains multiple Jβ genes flanked by a single 5' Dβ and a single 3' Cβ gene. (i.e. Dβ1, Jβ1.1, Jβ1.2…Jβ1.6, Cβ1 and Dβ2, Jβ2.1, Jβ2.2, Jβ2.3…Jβ2.7, Cβ2).

These multiple gene copies probably arose over generations of evolution by gene duplication, and much of their sequence is very similar, often being referred to as the "framework" of the TCR. However, considerable sequence variability exists in the parts of the V and J regions which interact with the cognate antigen-MHC complex. These regions of so-called hypervariability are termed the complementarity determining regions (CDR)s. CDR1 and CDR2 are located in the variable gene segments; CDR3 is located at the junction of the V (D) and J gene segments (142).

1.5.2. V-D-J DNA Recombination

Each TCR is constructed from several pre-formed genetic units during T cell development in the thymus. This occurs via a series of DNA recombination events which result in the random association of different V, D and J gene segments to form a full-length TCR (144-148). For example, the MUC1-specific TCR discussed later in this chapter contains the gene segments Vα2.7-Jα49-Cα and Vβ6-Dβ2-Jβ2.5-Cβ2.

Although the association of the VDJ gene segments is random, it is temporally highly regulated; the TCRβ chain is constructed first, followed by the TCRα chain. The first DNA rearrangement aligns a Jβ gene segment next to the Dβ gene segment from that Jβ cluster (e.g. Dβ2 with Jβ2.5).

Then a second rearrangement in the TCRβ locus introduces a Vβ gene segment into the Dβ-Jβ exon. Any intervening Vβ, Dβ or Jβ gene segments are permanently excised from the chromosome of that particular T cell and can no longer be included in the T cell receptor expressed by that T cell. The deleted portions of the TCR loci are retained in mature T cells as circularized DNA fragments known as TCR excision circles (149,150). Multiple VDJ recombination events may be necessary to produce a complete TCRβ chain, expressed as one open reading frame. The integrity of the TCRβ chain is tested by its ability to associate with a pre-TCRα chain in the pre-TCR complex (151). If this association is successful, the pre-TCR will be expressed at the cell surface, which signals to the TCRα locus to begin V-J recombination. Productive re-arrangement of the Vα-Jα gene segments results in expression of a full-length TCRα chain, which replaces the pre-TCRα chain in the final TCR complex.

1.5.3. Allelic Exclusion

Every T cell carries TCR loci on maternal and paternal chromosomes. Therefore, developing T cells have the potential to execute V(D)J recombination in two TCRα loci and two TCRβ loci. If this were to happen, every T cell would carry two TCRα chains and two TCRβ chains. Random association of the TCRα and -β chains would result in the expression of four TCRs on the T cell surface, each with a different specificity. To ensure that each T cell has only a single antigen-specificity, VDJ recombination is limited to only one TCRα and one TCRβ locus by a process termed allelic exclusion (152,153). Once VDJ recombination has produced a full-length TCRβ chain, further VDJβ recombination is suppressed at that locus and at the TCRβ locus on the other chromosome. Subsequent recombination of the TCRα genes is not affected by allelic

exclusion of the TCRβ locus. However, when a productive VJα re-arrangement is achieved, further VJα recombination at the TCRα locus is similarly suppressed.

It is possible to take advantage of the allelic exclusion mechanism in order to express a TCR of known specificity in all T cells of a TCR-transgenic mouse. The presence of pre-arranged TCR alpha and beta chains (even on a different chromosome from those which carry the endogenous TCR loci), and their expression in developing T cells, is sufficient to suppress VDJ recombination at the endogenous TCR loci (154). Thus, the vast majority of mature T cells in a TCR-Tg mouse will express the cloned TCR of interest.

2. CONTROL OF MUC1-SPECIFIC IMMUNITY BY $CD4^+$ T LYMPHOCYTES

2.1. Introduction

MUC1-specific immune responses in cancer patients are, at best, weak and insufficient to halt the progression of the developing tumor. Evidence suggests that $CD4^+$ T cells become "tolerized" to MUC1, which is a self-molecule (50,51,137,155,156). It is not yet clear whether all MUC1-specific T cells are centrally deleted or whether additional mechanisms of peripheral tolerance are at play. As $CD8^+$ T cells require help from $CD4^+$ T cells in order to establish effective CTL memory (16,42), the lack of highly effective MUC1-specific Th cells in cancer patients is thought to be a major obstacle in generating effective anti-tumor immunity.

This requirement for T cell help is exemplified by the quantitative and qualitative differences in the MUC1-specific immune responses that can be induced by vaccination of WT and MUC1-Tg mice. MUC1-Tg mice express the human MUC1 gene under the control of its endogenous promoter, such that MUC1 is expressed with similar tissue distribution in MUC1-Tg mice as in humans (136). Expression of MUC1 as a self-molecule in MUC1-Tg mice renders them tolerant to immunization with MUC1-100mer. Vaccination protocols which elicit effective MUC1-specific tumor immunity in WT mice yield only weak responses in MUC1-Tg mice and, compared with WT mice, and MUC1-Tg mice are more susceptible to challenge with $MUC1^+$ tumors (50,51,137).

When WT mice are immunized sub-cutaneously with DC that have been pre-loaded with MUC1 peptide, they generate a robust MUC1-specific T helper (Th) response which provides assistance

for all arms of the adaptive immune system (50). In the presence of MUC1-specific T cell help, CTL from DC-immunized WT mice efficiently lyse $MUC1^+$ tumors and B cells undergo efficient antibody isotype switching to produce high titers of MUC1-specific serum IgG that can be detected in sera. The same DC vaccine administered to MUC1-Tg mice induces only weak MUC1-specific CTL responses and low-titer serum IgM. MUC1-specific tolerance is particularly evident in the $CD4^+$ T cell compartment; all but the most potent vaccination protocols fail to elicit MUC1-specific $CD4^+$ T cell responses in MUC1-Tg mice (50,140,157). The lack of MUC1-specific Th responses in MUC1-Tg mice is thought to be responsible for the poor responses that are observed in the other arms of the adaptive immune system.

This is evidence to suggest that the MUC1-specific Th compartment that is compromised in MUC1-Tg mice can be restored to full functionality by supplying $CD4^+$ T cells from WT mice (51). This goes some way to proving that $CD4^+$ T cells are the key to eliciting effective immunity to MUC1. However, in those particular studies, it was not determined whether the transferred Th cells were actually MUC1-specific. Therefore, the observed improvement in the MUC1-specific CTL response may have been due to heterologous help (i.e. Th cells specific for another antigen besides MUC1).

Our goal was to determine the effect that MUC1-specific $CD4^+$ T cells have on MUC1-specific immune responses. The data presented here show that provision of WT MUC1-specific Th cells does indeed result in improved immunity to MUC1 in MUC1Tg mice. In addition, we show that, while immunization of MUC1-Tg mice with MUC1 fails to stimulate MUC1-specific Th, potent MUC1-specific Tregs are induced which are capable of suppressing MUC1-specific

immune responses both *in vitro* and *in vivo*. These results indicate that one mechanism of "tolerance" to MUC1 in Muc1 Tg mice is the imbalance between MUC1-specific Th and Treg cells, in favor of Tregs. We show that this skewing toward a regulatory response can be overcome by supplying Th cells from WT mice.

2.2. Methods

2.2.1. Mice and cell cultures

C57BL/6 MUC1-Tg mice were obtained from Dr S. Gendler (Mayo Clinic, Scottsdale, AZ) and bred in the animal facility of the Hillman Cancer Center at University of Pittsburgh School of Medicine. B6.PL-Thy1a/Cy mice were purchased from the Jackson Laboratory (Bar Harbour, MA). All mice were maintained in a specific pathogen free environment and treated in accordance with the guidelines of the IACUC of the University of Pittsburgh.

With the exception of the 44-22-1 hybridoma, all mouse cells were cultured in complete DMEM (cDMEM) containing 10%FBS, Penicillin & Streptomycin, L-Glutamine, Na Pyruvate, Non-Essential Amino Acids, Hepes Buffer, and β-2 Mercaptoethanol (amounts), plus other additives, as indicated below.

2.2.1.1. Generation of Bone Marrow Dendritic Cells and Vaccination of mice

Femurs and tibiae were removed from female C57BL/6 mice. The epiphyseal plates were cut off and bone marrow flushed out with cDMEM using a syringe. Cells were centrifuged at 300g for

5 minutes and the pellet resuspended and triturated in 1ml RBC lysis buffer (Sigma, MO) for 1min, after which 9mL cDMEM was added to dilute the lysis buffer. Cells were centrifuged and washed another three times, then resuspended at 1.5-2 million cells/mL in cDMEM containing 10ng/ml each of GM-CSF and IL-4 (a generous gift from Immunex, WA) and transferred to T75 tissue culture flasks (BD Biosciences, CA) at 15-20 million cells in 10mL. Cells were fed every two days by adding 5ml cDMEM containing 10ng/ml GM-CSF and IL-4. On day 7 of culture, cells were harvested using 2mM EDTA and separated by density centrifugation using Nycoprep 1.068 (Accurate Chemical, NY). Purified DC were loaded for 4-6 hours in polypropylene tubes with 20-50ug/mL synthetic MUC1 100mer in cDMEM at no more than 3 million cells per mL. Loaded DC were washed twice with PBS and resuspended in PBS at $5x10^5$ cells/mL. Soluble 100mer was added at 500µg/ml. Mice were injected subcutaneously in the right flank with 200µl PBS containing 10^5 loaded DC and 100µg soluble 100mer. Boosts were administered two weeks apart.

2.2.1.2. Preparation of LN and spleens

Spleens and inguinal lymph nodes were mechanically disrupted with a syringe plunger and single cell suspensions in cDMEM obtained by passing them through a 70µm cell strainer. Splenocytes only were then centrifuged, and the cell pellets resuspended and triturated in 1ml RBC lysis buffer (Sigma, MO) for 1min, after which 9mL cDMEM was added to dilute the lysis buffer. Cells were washed three times in DMEM, counted and used in the assays described below.

2.2.1.3. T cell culture and *in vitro* stimulation

LN/spleen cells were seeded at $3x10^6$ cells per well of a in 24-well tissue culture plates (Linbro) in cDMEM containing 20U/mL muIL-2 (Immunex, WA). On day0 of the culture, 100mer pulsed BMDC were added at $3x10^5$ cells per well to stimulate the proliferation of MUC-specific T cells. Cultures were fed every 2 days by replacing half of the media with fresh cDMEM containing 20U/mL mIL-2. T cell function was tested for cytotoxicity on day 5 and cytokine production on day 7, as described below. As a positive control for stimulation, cells were treated with Phorbol myristate acetate (PMA) and Ionomycin (Sigma, MO).

2.2.2. Flow Cytometry

LN/spln cells were prepared as described above and maintained on ice for the duration of the staining procedure. Cells were washed and resuspended in FACS buffer (2% FBS in PBS) at 5 to 10 million cells/ml and transferred to a 96-well Greiner microtiter plate (Fisher, PA) at $5x10^5$ to 10^6 cells (100µl) per well. Surface FCR was blocked for by incubating cells for 15mins with anti-CD16/CD32 MAb, diluted 1:50 in FACS buffer. Without washing, cells were then stained for 30mins using antibodies specific for various surface antigens, as indicated (e.g. CD3, CD4…). All commercial antibodies used for flow cytometry were purchased from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA) and were added to the blocked cells at 2:50 dilution in FACS buffer (final dilution was therefore 1:50). After washing three times in FACS buffer, cells were re-suspended in 300µl FACS buffer and analyzed on an LSR II Flow Cytometer (BD Biosciences), running FACSDiva software. FACSorting was performed with a FACSAria (BD Biosciences). If necessary, cells were stored overnight at 4^oC in FACS buffer containing 1% PFA before analysis the next day.

2.2.2.1. FoxP3 stain.

LN/splenocytes were stained for surface CD3, CD4 and CD25 as described above. Cells were stained intracellularly for FoxP3 using a mouse FoxP3 staining kit (eBioscience, CA), as per the instructions, except with the omission of the PBS wash after the Fix/Perm step. The FoxP3 antibody was applied to cells at 1:50 dilution for 30mins. Cells were then washed three washes in Perm/Wash buffer and resuspended in FACS buffer for analysis.

2.2.2.2. Intracellular Cytokine Staining

LN or spleen cells were, prepared as above, were stimulated for 6-12 hours in the presence of Golgi Plug (Brefeldin A) with either MUC1-pulsed DC (10:1 ratio of lymphocytes to DC) or with PMA/Ionomycin. After stimulation, cells were washed with PBS containing 2% FBS and stained for 30mins for surface markers using antibodies specific for CD3, CD4 or CD8, as described above. Cells were washed three times with FACS buffer. ICS was performed using a Cytofix/Cytoperm kit (BD Biosciences), as per the manufacturer's instructions. Cells were permeabilized by resuspending in cytofix/cytoperm solution for 20mins. After two washes with PermWash solution, intracellular FCR was blocked with anti-CD16/CD32. Without washing, cells were stained intracellularly for 30mins with antibodies specific for IFNγ, TNFα, IL-10 or IL-5 (antibodies at this stage were diluted 1:50 in PermWash). After intra-cellular staining, cells were washed thrice with PermWash and then resuspended in FACS buffer.

2.2.3. IFNγ ELISA

All cytokine ELISA were performed using an OptEIA ELISA set (BD Biosciences, CA), following the manufacturer's instructions. In brief, 10^5 LN or spleen cells were stimulated in

triplicate by 10^4 BMDC in a Costar sterile 96-well U-bottom micro-titer plate (Corning, NY) for 24-48 hours, as indicated, in a total volume of 200µl/well. Immulon 4HBX plates (Fisher) were pre-coated overnight with IFNγ capture antibody and pre-blocked for one hour with PBS containing 10% FBS. 100µl of culture supernatant was transferred to the pre-coated ELISA plate and incubated for 2hr at room temperature. After washing the plates with 0.05% TWEEN20, 100ul working detector (biotinylated IFNγ detection antibody plus avidin-HRP) solution was added for 1hr. Cells were washed again and 100µl TMB substrate (BD Biosciences, CA) added and incubated for up to 30 min in the dark. The reaction was stopped by adding 50µl 2N H_2SO_4. Absorbance at 450nm was recorded using an MRX Revelation ELISA plate reader (ThermoLabSystems, MA).

2.2.4. 2.3.5 Serum Ig ELISA

Mice were bled from the carotid artery immediately after sacrifice. Serum was separated by centrifugation for 5 minutes at 5,000g and stored at 4^oC. ELISA was performed at room temperature. Immulon 4HBX plates (Fisher) were coated overnight at with MUC1 100mer (1µg/50µl in PBS) or 2.5%BSA. After coating, plates were washed with 200µl PBS and then blocked for 1hr with 100µl 2.5% BSA. Sera (diluted appropriately in 2.5% BSA) were added to the plate in triplicate at 50µl/well for 1hr. Plates were washed several times with 200µl Tween20 (0.1% in PBS). 50µl HRP-conjugated anti-mouse Ig detection antibody (Southern Biotech, AL) diluted 1:500 was added for 1hr. Plates were washed again in Tween20, then 100µl TMB substrate (BD Biosciences, CA) added for 30mins in the dark, after which the reaction was stopped by adding 50µl 2N H_2SO_4. Absorbance at 450nm was read using an MRX Revelation ELISA plate reader (ThermoLabSystems, MA), with kind permission from Dr Karen Norris.

2.2.5. ^{51}Cr-release cytotoxicity assay

10^6 RMA and RMA-MUC1 target cells were labeled for 1hr with radioactive ^{51}Cr (vendor), then washed three times with DMEM, resuspended in cDMEM at 10^6 cells/ml and seeded in a 96-well V-bottom microtiter plate at 2,000 cells per well in 100µl. Responder LN/spleen cells were added in triplicate in cDMEM in the indicated quantities ($2.4x10^4$ to 10^5) for 4-6 hours. Supernatants containing released ^{51}Cr were harvested and analysed on a Cobra II auto-gamma counter (Perkin-Elmer, MA). Spontaneous lysis was determined by testing supernatants from wells containing target cells only. Total lysis (i.e. complete lysing of all target cells) was determined from well in which HCl or H_2S0_4 was added to the target cells. Specific lysis was calculated as (lysis – spontaneous lysis)/(total lysis – spontaneous lysis). Averages of triplicates were plotted.

2.2.6. Adoptive T cell Transfers

Donor mice were immunized with 100mer-pulsed DC, as described above. Two weeks after immunization, LN and spleen were removed from donor mice and single cell suspensions prepared as described above. $CD4^+$ T lymphocytes were positively selected by magnetic antibody cell sorting (MACS) using CD4 (L3T4) Microbeads (Miltenyi Biotech, CA). Purified $CD4^+$ T cells were washed and resuspended in sterile PBS. Prior to injection of T cells, mice were placed under a heat lamp to induce vasodilation. Then $1 x10^6$ - $5 x10^6$ $CD4^+$ T cells in 200µl PBS were injected into mice via the lateral tail vein using a syringe with a 27½ gauge needle.

2.3. Results

2.3.1. MUC1-Tg mice respond only weakly to immunization with MUC1, compared to WT mice

In a previous study conducted in our laboratory (50), a DC-based immunization protocol highlighted the vast difference in the magnitude of MUC1-specific $CD4^+$ T cell responses between MUC1-Tg and WT mice, the basis for which was not elucidated. When designing experiments to investigate this problem and in general the effect that MUC1-specific $CD4^+$ T cells have on other arms of the MUC1-specific immune response, we reasoned that using this same DC vaccine would be an appropriate method of generating MUC1-specific CTL responses that are dependent on T cell help. These Th-dependent CTL would reveal any intrinsic differences in MUC1-specific CD4 cells from MUC1-Tg and WT mice.

To confirm in an independent study, that WT and MUC1-Tg mice exhibit the markedly disparate immune responses to the MUC1/DC vaccine as previously shown, WT and MUC1-Tg mice were immunized subcutaneously with dendritic cells that had been pulsed *in vitro* with synthetic MUC1 100mer peptide. Two weeks after vaccination, inguinal lymph nodes, spleens and sera were collected and analyzed for the presence of MUC1-specific immune responses.

In keeping with previous reports, WT mice mounted strong humoral and cellular immune responses to MUC1 immunization, whereas only weak CD8 T cell responses were observed in MUC1-Tg mice (fig2-1). The MUC1-DC vaccine induced potent $CD4^+$ T cell responses in WT mice, but virtually undetectable numbers of responding $CD4^+$ T cells in MUC1-Tg mice (fig2-1a). The absence of MUC1-specific Th cells in MUC1-Tg mice is associated with weak MUC1-

specific CD8 T cell responses, measured by both IFN-γ production (fig2.1a) and inefficient lysis of MUC1^{+} tumors (fig2.1b). The most striking difference between WT and MUC1-Tg mice is in antibody class switching to the IgG isotype (fig 2.1c). As this process is normally initiated via interaction of the B cells with antigen-specific Th cells (158), the lack of MUC1-specific serum IgG in MUC1-Tg mice confirms the absence of MUC1-specific T cell help.

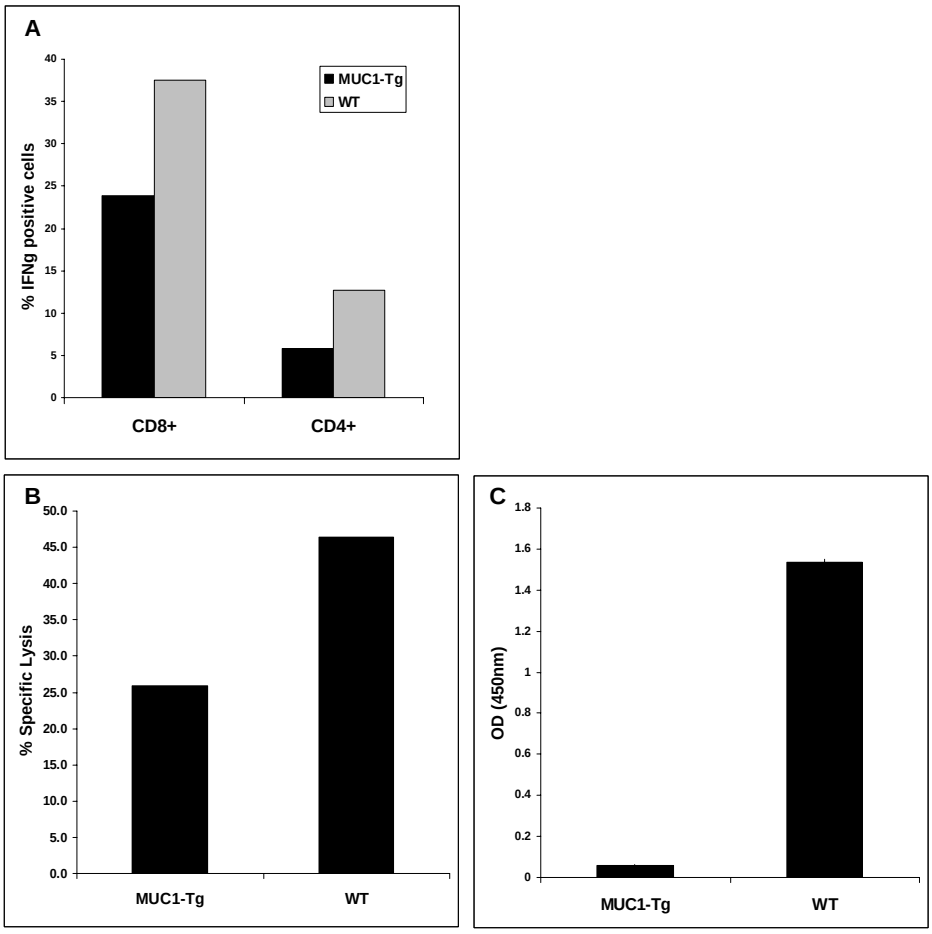


Figure 2-1. MUC1-Tg mice are tolerant to immuzation with MUC1-loaded DC

WT and MUC1-Tg mice (4 each) were immunized and boosted sub-cutaneously with 10^5 MUC1-loaded DC. Two weeks post-boost, DLN and sera were isolated, pooled and tested. A) IFNγ production. LN cells were stimulated for 6hr with MUC1-loaded DC, PMA and Ionomycin in the presence of brefeldin-A, then stained for surface CD4 and CD8 and intracellular IFNγ. B) Specific lysis of the MUC1$^{\pm}$ tumor RMA-MUC1. LN cells were incubated with ^{51}Cr-labelled RMA or RMA-MUC1 targets for 4 hours in triplicate. C) MUC1-specific serum IgG. Sera from immunized mice were incubated on ELISA plates coated with MUC1 100mer then blotted with anti-mouse IgG.

2.3.2. Transfer of WT CD4$^+$ T cells suppresses MUC1-specific immune responses in MUC1-Tg mice

Since the previous experiments pointed to the CD4$^+$ T cells as the root cause of poor MUC1-specific immunity in MUC1-Tg mice we reasoned that MUC1-Tg mice should mount improved immune responses if provided with fully functional MUC1-specific CD4$^+$ T cells from WT donor mice. We also expected that immunizing the WT donor mice prior to adoptive transfer would ensure that the transferred CD4$^+$ T cells were primed to MUC1 in the absence of any tolerizing factors that might be present in the MUC1-Tg mouse, and could then effectively help the recipient mice mount effective anti-MUC1 immunity.

Two weeks prior to adoptive transfer, WT donor mice were vaccinated with MUC1-pulsed DC. As shown in fig2-1, this immunization protocol induces IFNγ-producing CD4$^+$ T cells, which would be expected to provide help to MUC1-specific B cells and CD8 T cells. Two weeks after DC-immunization, CD4$^+$ T cells were isolated from the WT donors and adoptively transferred via the tail vein into naïve MUC1-Tg mice, which were subsequently immunized and boosted with MUC1-loaded DC on days 1 and 15 post-T cell transfer. MUC1-specific immune responses were tested two weeks after the boost.

Contrary to expectations, adoptive transfer of primed CD4$^+$ T cells from WT mice further reduced the ability of MUC1-Tg mice to respond to subsequent immunization (fig 2-2). The transferred CD4$^+$ T cells suppressed both cellular and humoral immune responses to the DC-MUC1 vaccine (fig 2-2). Cells isolated from the LN of adoptively transferred MUC1-Tg mice contained reduced numbers of IFNγ producing CD8 and CD4$^+$ T cells (fig 2-2a), and exhibited a

reduced capacity to specifically lyse the MUC1^{+} tumor RMA-MUC1 (fig 2-2b). In addition, there were significantly lower levels of MUC1-specific IgG in the sera of these mice (fig 2-2c), indicating that antibody isotype class switching by MUC1-specific B cells was also suppressed. No detectable IL-10 or IL-5 was produced by these T cells (not shown).

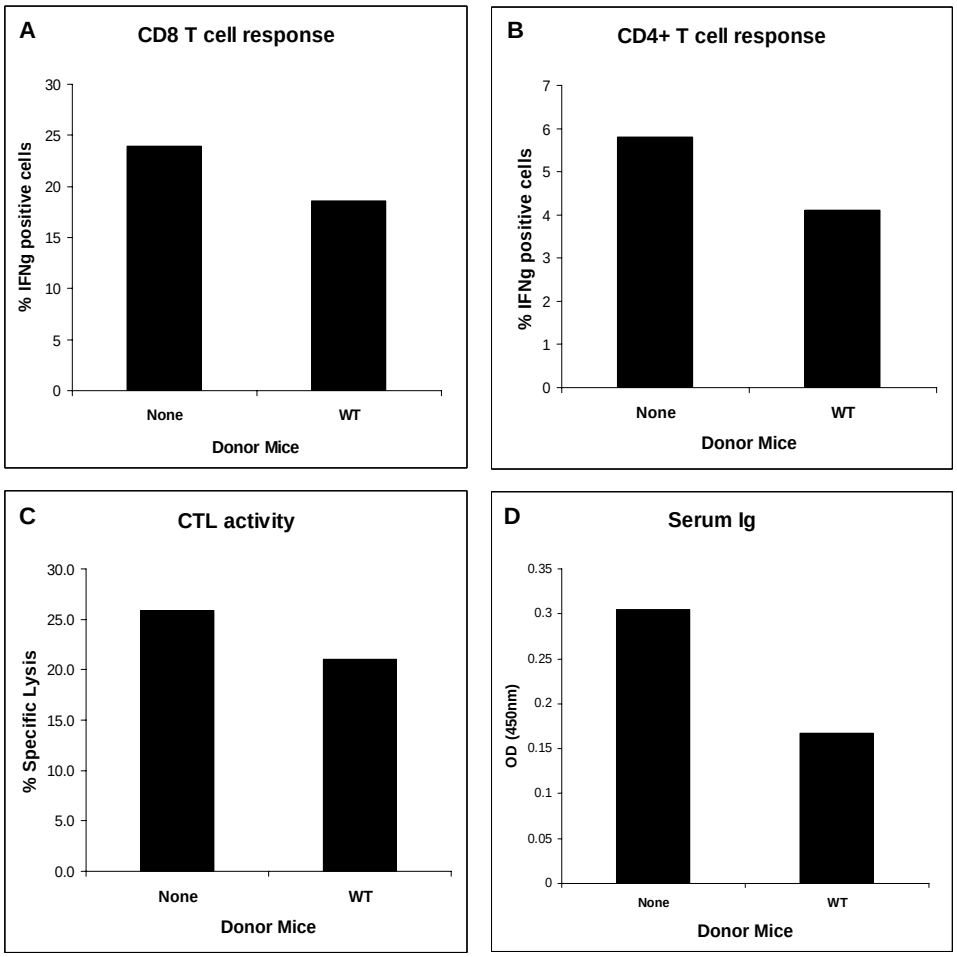


Figure 2-2. Primed WT CD4^{+} T cells suppress MUC1-specific immune responses

WT donor mice were immunized with MUC1-DC. Two weeks later, CD4^{+} T cells were transferred to MUC1-Tg mice. Recipients and untreated controls were then immunized with MUC1-DC. Two weeks after immunization, LN and sera were pooled (4 mice/group) and tested. A & B; IFNγ production LN cells were stimulated *in vitro* for 6hrs in the presence of brefeldin A, then stained for surface CD8, CD4, and intracellular IFNγ. C; Lysis of RMA-MUC1 tumor cells. LN cells were incubated with ^{51}Cr-labelled RMA or RMA-MUC1 targets for 4 hours in triplicate. D; MUC1-specific serum Ig ELISA. Sera from immunized mice were incubated on ELISA plates coated with MUC1 100mer then blotted with polyclonal anti-mouse Ig.

2.3.3. Phenotype of DC used for immunizations

In an attempt to determine why this adoptive immunotherapy had suppressed the immune response in MUC1-Tg recipients, we first decided to investigate the phenotype of the BMDC used to vaccinate the mice. As immature DC have been reported to cause immunological tolerance (35,159), we wished to confirm that our standardized method of generating DC from bone marrow precursors did indeed generate mature DC, as intended. A sample of the DC used for immunization was stained for the presence of various surface markers of DC activation and maturity: CD11c, CD80, CD86, CD40, I-A^b, and analysed by flow cytometry. All of these molecules were expressed by the DC (fig2-3), indicating that those DC used to immunize our mice were mature DC that provided the co-stimulatory molecules necessary for priming of naïve T cells. However, the DC population was heterogeneous, and contained both high and low expressors of these costimulatory molecules.

2.3.4. Mature DC induce FoxP3$^+$ Regulatory T cells

Given the well-documented immuno-suppressive effect of $CD4^+$ regulatory T cells (Tregs) (62,64,70), we wondered if the immune suppression observed after adoptive transfer was due to the presence of Tregs, the hypothesis being that vaccination of the WT donor mice had caused an increase in the numbers of MUC1-specific Tregs in the population of transferred $CD4^+$ T cells. In the first attempt to test this hypothesis, we took advantage of a remaining WT 'donor' mouse that had been vaccinated at the same time as the donor mice in the previous experiment. The number of $FoxP3^+$ $CD4^+$ T cells in lymphoid organs was determined by flow cytometry and compared to a naïve mouse.

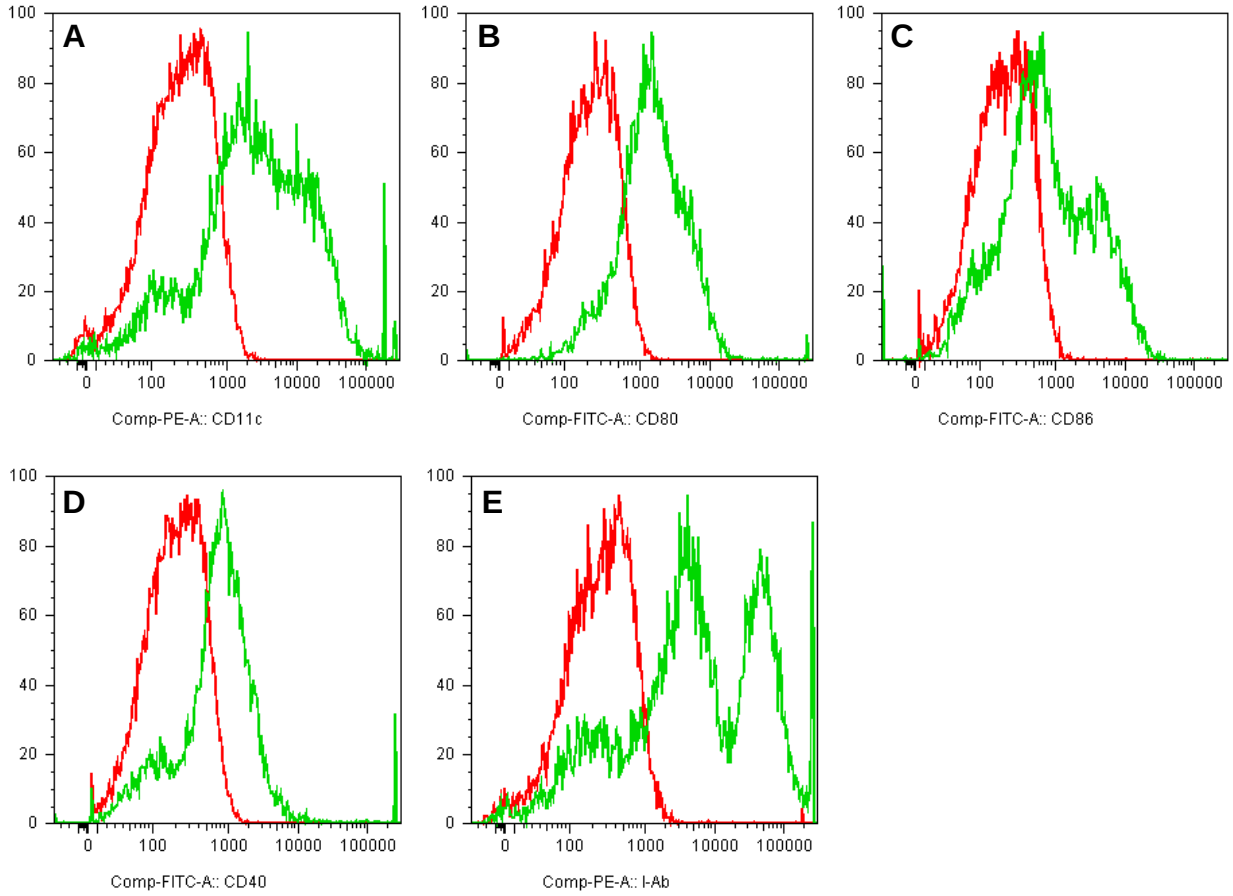


Figure 2-3. Phenotype of DC used for immunization

BMDC were cultured in cDMEM containing GM-CSF and IL-4 for 7 days. DC were then purified by centrifugation over Nycoprep and stained with MAbs specific for CD11c (A), CD80 (B), CD86 (C), CD40 (D) or I-A^b (E). $CD11c^+$ cells were gated and analysed for expression CD80, CD86 and I-A^b. Red lines, isotype control antibody; Green lines, specific staining.

As the original donor mice had been boosted prior to isolation of the transferred $CD4^+$ T cells, this 'donor' mouse was given another boosting injection of MUC1-loaded DC. Two weeks after the boost, inguinal lymph nodes were removed and analysed by flow cytometry for the presence of $CD3^+CD4^+FoxP3^+$ Tregs. Not only were Tregs present in the population of $CD4^+$ T cells,

their numbers were two-fold higher than in a non-immunized control mouse (fig 2-4). This suggested that FoxP3$^+$ Tregs were present in the population of CD4$^+$ T used in the previous adoptive transfer study and that vaccination of the donor mice with MUC1-loaded DC had induced the expansion of Tregs.

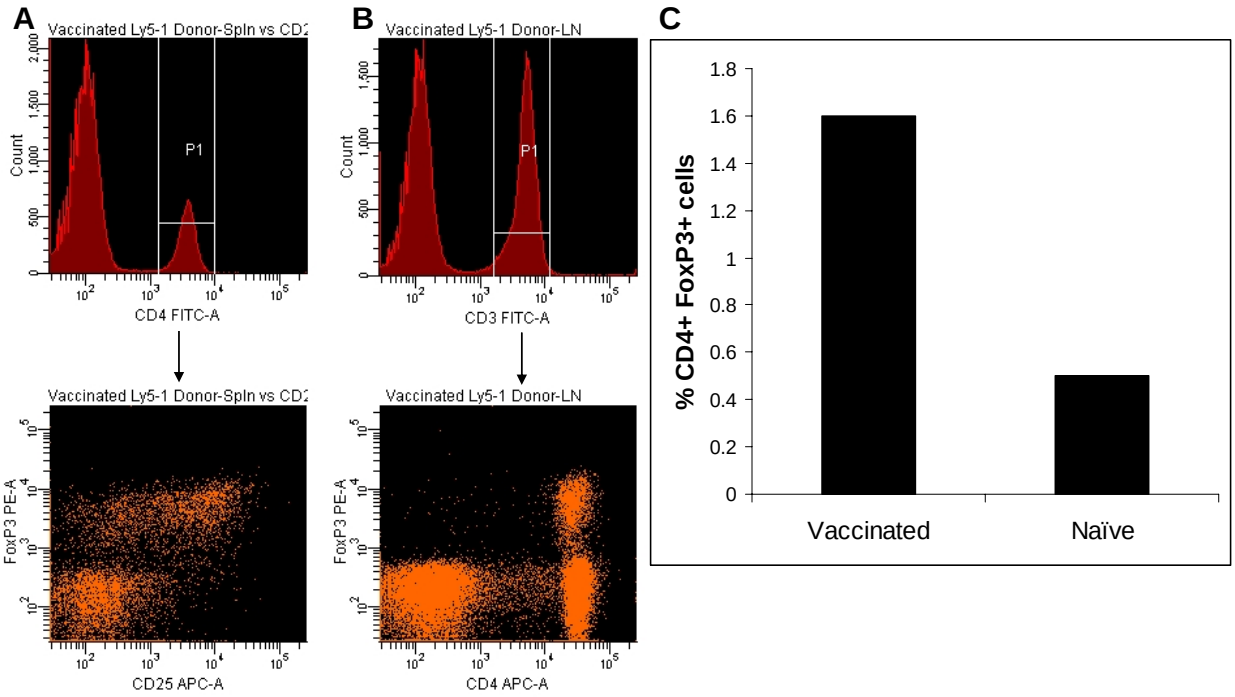


Figure 2-4. FoxP3$^+$ Tregs in DC-immunized WT donor mice

A) LN cells were isolated from WT mice two weeks after immunization with MUC1-DC and stained for surface CD4, CD25 and FoxP3. Gating on CD4$^+$ cells (upper panel), CD25 was plotted against FoxP3 (lower panel).
B) LN cells were stained for surface CD3, CD4 and FoxP3. Gating on CD3$^+$ cells (upper panel), CD4 was plotted against FoxP3 (lower panel).
C) The number of CD3$^+$CD4$^+$FoxP3$^+$ cells was enumerated and is represented as percentage of total LN cells.

This preliminary experiment shows a clear difference in the proportion of Tregs in the lymph nodes of naïve and immunized mice. However, a repeat experiment was warranted to test a more significant number of mice. To this end, WT mice were immunized with MUC1-loaded DC and

the numbers of lymphoid Tregs compared with those in age-matched naïve mice. As before, an increased proportion of FoxP3$^+$ Tregs were observed in the spleens and LN of DC-immunized mice, compared with naïve controls (fig 2-5), confirming the original conclusion that vaccination of donor mice caused an expansion of Tregs. The presence of increased numbers of Tregs in the pool of adoptively transferred CD4$^+$ T cells would explain why the recipient MUC1-Tg mice exhibited a reduced response to subsequent vaccination.

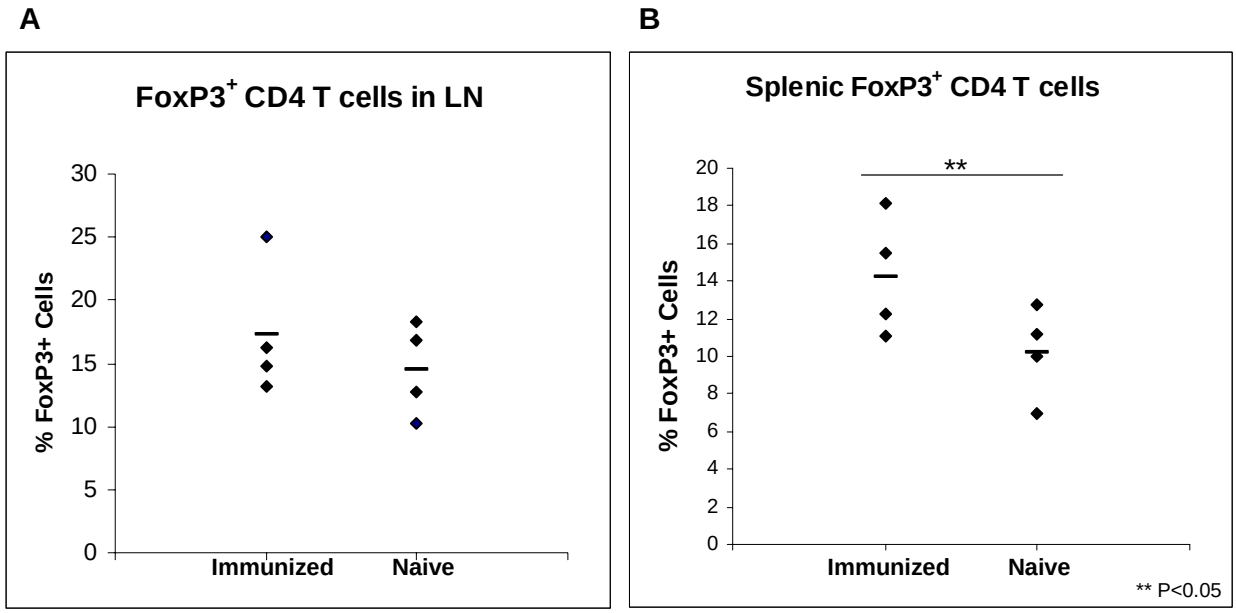


Figure 2-5. DC-induced expansion of Tregs in age-matched WT mice

6 month old WT mice were injected sub-cutaneously with MUC1-pulsed DC. Two weeks after immunization, LN and spleens were isolated and cells stained on the surface for CD3 and CD4, and intracellularly for FoxP3. The number of FoxP3$^+$ cells is expressed as a percentage of CD4$^+$CD3$^+$ cells.

In the first experiment, Tregs were increased 4-fold in the immunized mouse (fig2-4), whereas only a 1.5-fold increase was observed in the repeat experiment (fig2-5). One explanation for this discrepancy is that all of the mice tested in the repeat experiment were six months old, whereas

those used in the first experiment were not age-matched; the original immunized 'donor' mouse was 10months old and was compared with a 6 month old naïve control mouse. Given that age has a significant effect on the magnitude of immune responses (160,161), it is possible that the age difference between the first two mice may have contributed to the observed change in numbers of Tregs. To address this issue, we set out to separate the potential influences that old age and DC-immunization might respectively exert on the conversion of naïve $CD4^+$ T cells into Tregs. Mice were immunized with MUC1-loaded DC at 5 and 9 months of age, and the numbers of lymphoid Tregs enumerated two weeks after vaccination. LN from old mice contained almost twice as many $FoxP3^+$ Tregs than those from younger mice (fig 2-6), indicating that DC-mediated expansion of Tregs is exacerbated in older donor mice.

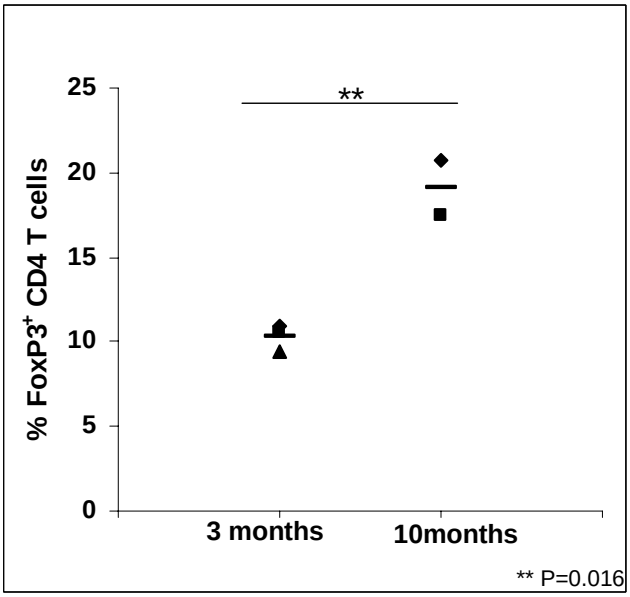


Figure 2-6. Induction of Tregs in old vs young mice

WT mice aged 3 or 6 months were immunized and boosted subcutaneously with MUC1-pulsed DC at two week intervals. Two weeks after the boost, inguinal LN were removed and stained for surface CD3 and CD4, and intracellular FoxP3. Relative numbers of $FoxP3^+$ cells are expressed as % of $CD3^+CD4^+$ cells.

2.3.5. DC-mediated expansion of Tregs is MUC1-specific

We next set out to confirm that the DC-induced Tregs do respond to MUC1-specifically and are not induced by other antigens presented by the DC (e.g. bovine serum proteins in the culture medium), or even by DC independently of antigen presentation. To this end, WT mice were immunized with MUC1-pulsed DC, un-loaded DC, or PBS. As before, increased numbers of FoxP3$^+$ Tregs were observed in the DLN of mice receiving MUC1-loaded DC. Mice receiving unloaded DC had the same baseline level of Tregs as control mice injected with PBS (Figure 2-7). This confirms that the expansion of Tregs observed after immunization with DC is dependent upon the presence of MUC1, suggesting that those responding Tregs are MUC1-specific.

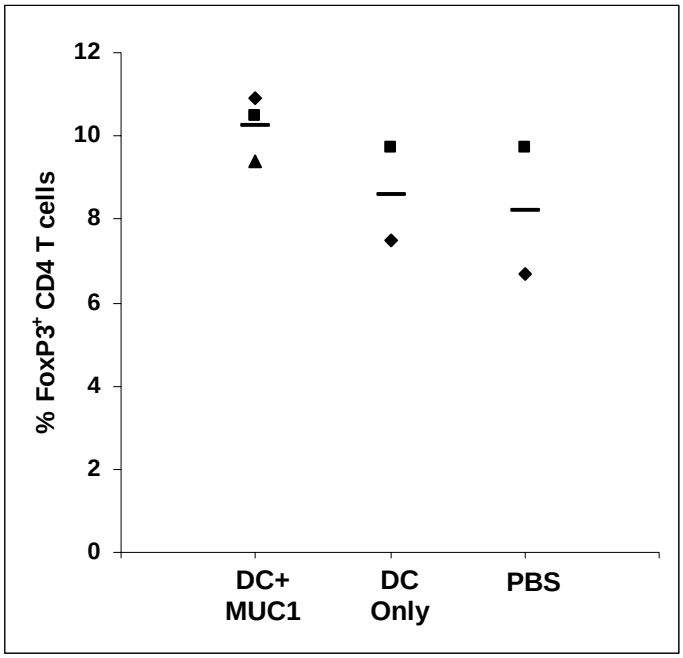


Figure 2-7. MUC1-specific expansion of Tregs

Mice were immunized with unloaded DC or DC that were loaded *in vitro* for 4 hours with 50µg/ml MUC1 100mer,. Control mice were injected subcutaneously with PBS (the vehicle for the DC vaccine). Two weeks after immunization, DLN were harvested and stained for CD3, CD4 and FoxP3.

2.3.6. MUC1-expanded Tregs suppress MUC1-specific T cells *in vitro*

The data shown so far indicate that $CD4^+$ T cells from DC-immunized WT donors suppress MUC1-specific immune responses *in vivo* and that the pool of adoptively transferred $CD4^+$ T cells contains $FoxP3^+$ $CD4^+$ T cells that were expanded in a MUC1-specific fashion. It therefore seems reasonable to assume that the $FoxP3^+$ T cells are Tregs and that they are the cause of the immune suppression observed after the adoptive transfer.

In order to state this conclusively, it was necessary to prove in a functional assay that the $FoxP3^+$ T cells, when isolated from the other adoptively transferred $CD4^+$ T cells, maintain the ability to suppress MUC1-specific T cell responses. As FoxP3 is an intracellular molecule, the putative Tregs could not be purified based on FoxP3 expression. However, as all of the $CD4^+CD25^+$ cells detected in the immunized donor mice are $FoxP3^+$ (fig 2-4), isolation of $CD3^+CD4^+CD25^+$ triple-positive cells provides a pure population of the putative $FoxP3^+$ Tregs.

Accordingly, $CD3^+CD4^+CD25^+$ T cells were FACSorted from lymph nodes and spleens of immunized WT mice. Their ability to suppress MUC1-specific immune responses was tested by adding them in varying amounts to MUC1-primed $CD8^+$ responders and stimulating the co-culture with DC that were pre-loaded with MUC1. These CD4+CD25+ T cells isolated from MUC1-immunized WT mice effectively suppressed the MUC1-specific production of IL-2 by $CD8^+$ T cells (fig 2-8). This suggests that the $FoxP3^+CD4^+CD25^+$ T cells, induced MUC1-specifically by DC, are indeed Tregs.

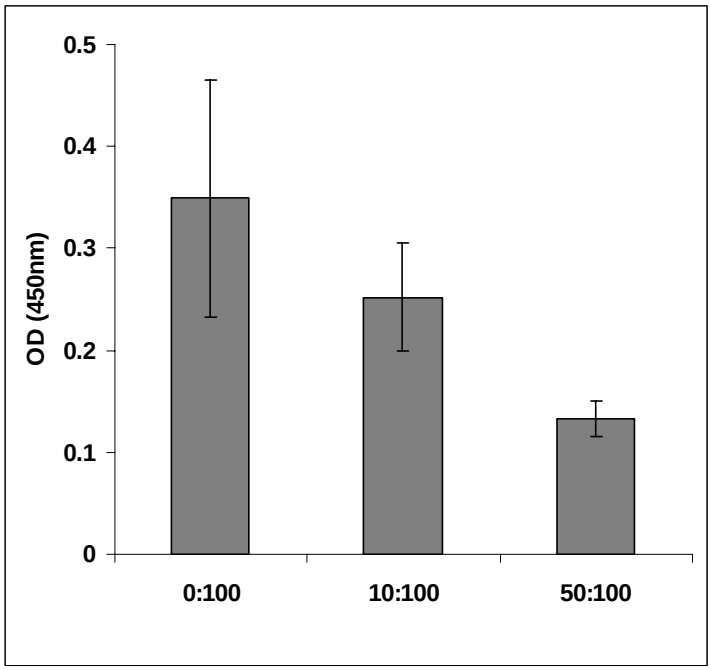


Figure 2-8. Putative Tregs suppress MUC1-specific T cells *in vitro*

10^5 responder CD8 T cells were purified from MUC1-DC-immunized WT mice and stimulated *in vitro* for 24hrs with 10^4 MUC1-loaded DC in the presence or absence of $CD4^+CD25^+$ T cells purified from the same immunized WT mice. IL-2 production was measured in culture supernatants by ELISA.

The data presented so far collectively indicate that mature DC presenting MUC1 epitopes induce the expansion of MUC1-specific Tregs. This is surprising, as MUC1-loaded DC also prime naïve T cells to become effective Th cells, which form part of an effective MUC1-specific immune response capable of rejecting $MUC1^+$ tumors (Fig 2-1) and (50).

2.3.7. MUC1-specific Tregs predominate in MUC1-Tg mice

In the MUC1-Tg mouse, MUC1-specific Th responses are virtually undetectable (fig2-1) (50,51,137). We wondered whether, in the absence of MUC1-specific Th responses, MUC1-loaded DC will still induce MUC1-specific Tregs and, if so, what the implications are for the other arms of the immune system. To answer these questions, MUC1-Tg mice were immunized with MUC1-loaded DC and their spleens analysed for the presence of $FoxP3^+$ Tregs. Surprisingly, immunization of MUC1-Tg mice with MUC1-loaded DC causes a similar expansion of $FoxP3^+$ Tregs as seen in WT mice (fig 2.9).

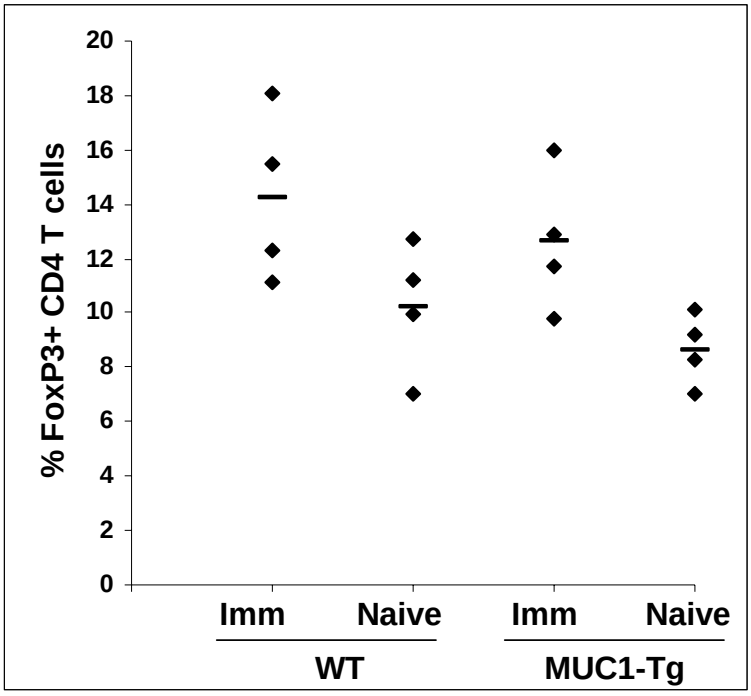


Figure 2-9. Expansion of Tregs in MUC1-Tg mice

WT and MUC1-Tg mice were immunized and boosted with MUC1-loaded DC. Two weeks after the boost, splenocytes were isolated and stained for FoxP3. Numbers of $FoxP3^+$ cells are expressed as a percentage of splenic $CD4^+$ T cells.

When $CD4^+$ T cells from immunized MUC1-Tg mice were adoptively transferred to WT mice, they suppressed MUC1-specific immune responses by the same degree as $CD4^+$ T cells from WT mice (fig 2-10). Therefore, MUC1-specific Tregs respond normally in MUC1-Tg mice and are potent suppressors of MUC1-specific immune responses.

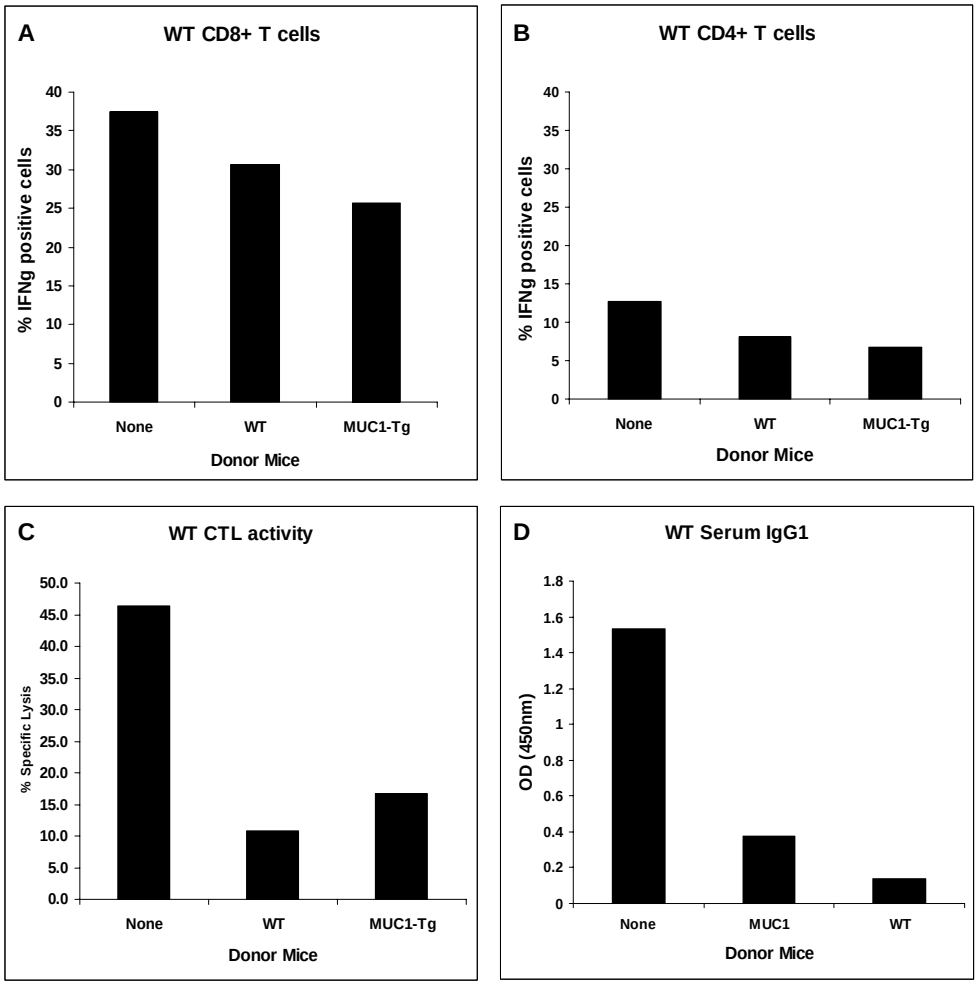


Figure 2-10. MUC1-Tg $CD4^+$ T cells suppress WT MUC1-specific immune responses

WT and MUC1-Tg donor mice were immunized with MUC1-loaded DC. Two weeks later, their $CD4^+$ T cells were purified and adoptively transferred to WT recipients ($5x10^6$ cells/mouse). Recipient mice were immunized and boosted with MUC1-DC. Two weeks post-boost, LN and blood samples were tested: A and B) IFNγ production by $CD8^+$ and $CD4^+$ T cells, measured by Intracellular Cytokine Staining. C) MUC1-Specific lysis of RMA-MUC1 tumor. D) MUC1-specific serum IgG1 ELISA.

2.3.8. Adoptive immunotherapy with Treg-depleted WT Th cells improves MUC1-specific immunity in MUC1-Tg mice

The presence of MUC1-specific Tregs and concomitant absence of MUC1-specific Th cells in MUC1-Tg mice suggests that there is an imbalance in the $CD4^+$ T cell compartment such that the MUC1-specific Treg component outweighs the Th. This might account for the poor CTL seen in these mice (fig2-1). We modified our initial hypothesis that transfer of $CD4^+$ T cells from WT mice would improve MUC1 specific responses in MUC1 Tg mice to the new hypothesis that shifting the balance in favor of MUC1-specific T cell help should improve the $CD8^+$ T cell response to MUC1.

To test this modified hypothesis, $CD4^+CD25^-$ Th cells from MUC1-immunized $Thy1.1^+$ congenic WT donors were purified by MACS-depletion of $CD4^+CD25^+$ Tregs and adoptively transferred into MUC1-Tg mice. Recipient MUC1-Tg mice were subsequently vaccinated with MUC1-loaded DC and then evaluated for MUC1-specific immune responses.

After transfer of CD25- CD4+ Th cells, MUC1-Tg mice exhibited improved MUC1-specific immune responses, of similar magnitude to those seen in WT mice (Fig 2-11). IFN-γ production by both CD8 and CD4 cells was increased in MUC1-Tg mice that received WT Th cells, whereas MUC1-Tg mice that received total $CD4^+$ T cells exhibited suppressed responses, as shown earlier (fig2-2). The IFNγ-producing $CD4^+$ T cells observed in the spleens of MUC1-Tg recipients of WT Th cells must be endogenous T cells, as $Thy1.1^+$ donor T cells could not be detected in the recipient mice (data not shown). This indicates that the adoptive transfer of

functional Th cells provided help for both CD8 and $CD4^+$ T cells in MUC1-Tg recipients, such that tolerance of the host $CD4^+$ T cells to MUC1 was broken.

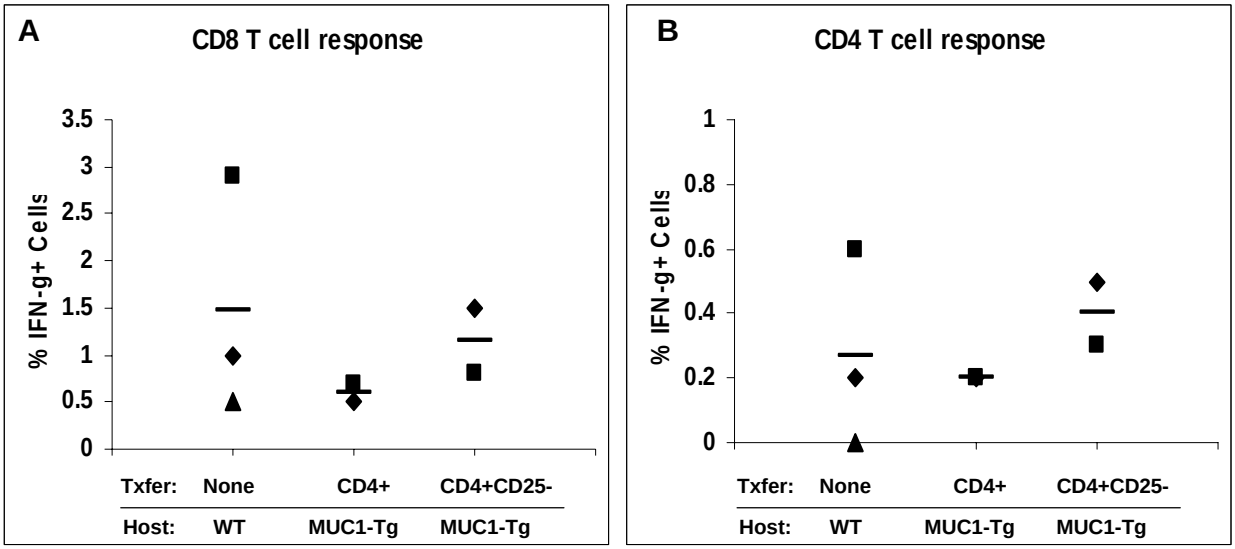


Figure 2-11. Purified WT Th cells break tolerance in MUC1-Tg mice

$CD4^+$ T cells, or Th cells depleted of $CD25^+$ Tregs, were transferred from MUC1-DC immunized $Thy1.1^+$ mice to MUC1-Tg mice. Recipient MUC1-Tg mice, and non-transferred WT controls were immunized and boosted once with MUC1-loaded DC. Ten days after the boost, DLN cells were harvested, restimulated *in vitro* with MUC1-loaded DC. 7days after restimulation, cells were stimulated again with PMA and ionomycin and IFNγ responses measured by intracellular cytokine staining, gating on $CD4^+$ or $CD8^+$ T cells.

2.4. Discussion

2.4.1. Th:Treg imbalance in MUC1-Tg mice maintains MUC1-specific "tolerance"

The data presented here establish an important role for $CD4^+$ T lymphocytes in augmenting and regulating immune responses to the tumor antigen MUC1. The two components of the $CD4^+$ T cell compartment, Th and Tregs, have opposing effects. In WT mice, MUC1-specific Tregs are balanced by intact Th cell compartment, such that the DC-based MUC1 vaccine establishes effective MUC1-specific immunity. However, such balance does not exist in MUC1-Tg mice. When the MUC1-specific Tregs run unopposed by Th cells, MUC1-specific CTL and antibody

responses are suppressed (figs2-1 & 2-2), resulting in susceptibility to $MUC1^+$ tumors (50,51,137). When MUC1-Tg mice are provided with functional Th cells by adoptive transfer, they generate improved MUC1-specific immune responses. This indicates that effective tumor immunity is dependent upon the relative balance of help and regulation.

Although MUC1 expression in the peripheral tissues is restricted to the apical surface of epithelia, it is still a self-molecule. A recent study has shown that expression of peripheral self-antigens by thymic epithelial cells causes the deletion of self-reactive effector T cells in the thymus without affecting the development of Tregs (162), which suggests that the two sub-types of $CD4^+$ T cells may be selected in the thymus by different mechanisms. The Tregs described here were induced by immunizing mice with an unglycosylated form of MUC1, suggest that these Tregs are specific for the unglycosylated MUC1 tandem repeat peptide sequence. It is possible that presentation of unglycosylated MUC1 epitopes in the thymus could cause the deletion of MUC1-specific Th cells from the repertoire, while Tregs would exit into the periphery. This would account for the Th:Treg imbalance observed in MUC1-Tg mice.

One way to avoid this problem would be to immunize with forms of MUC1 that carry tumor-specific glycosylations, such as TF and TN antigens (87,122,123). Even if the MUC1 gene is expressed thymically, these tumor-associated MUC1 glycoforms should not be produced in the thymus, as it does not share the exact repertoire of glycosyl transferases with the tumor. $CD4^+$ T cells specific for TN-MUC1 or TF-MUC1 should not be subjected to mechanisms of central tolerance. Hence, an improved MUC1 vaccine would use forms of MUC1 that carry tumor-specific oligosaccharides, such as Tn or TF antigens.

2.4.2. Adoptive immunotherapy with Th cells breaks tolerance in MUC1-Tg mice

The lack of a MUC1-specific helper T cell response is clearly an important factor in the weak immune responses that MUC1 vaccines elicit in MUC1-Tg mice. MUC1-specific immune responses were improved in these mice by the adoptive transfer of functional Th cells from WT mice (fig2-11). This indicates that generation of MUC1-specific Th responses is fundamental to generating effective anti-tumor immunity. Furthermore, adoptive immunotherapy with WT Th cells resulted in detectable MUC1-specific Th responses in MUC1-Tg mice. Since the responding $CD4^+$ T cells were not the transferred $Thy1.1^+$ Th cells, this result indicates that help was also provided for the endogenous Th cells in the MUC1-Tg mice. This suggests that, if the right signals can be provided exogenously, it is possible to break the MUC1-specific tolerance that exists in the $CD4^+$ T cell compartment of MUC1-Tg mice and, more importantly, cancer patients.

2.4.3. Do Tregs survive adoptive transfer better than Th?

The response of MUC1-specific $CD4^+$ T cells to stimulation by DC involves both helper and regulatory cells. The data presented in this chapter indicate that, in addition to stimulating potent MUC1-specific immunity in WT mice, presentation of MUC1 by DC leads to the induction of $FoxP3^+$ Tregs (fig2-4, 2-7, 2-8). The net outcome of immunizing WT mice with MUC1-loaded DC is effective MUC1-specific immunity (fig2-1) and (50), and the presence of Tregs clearly is not detrimental to this. The concomitant induction of MUC1-specific Th and Tregs by DC is therefore considered to be part of a balanced immune response, whereby Tregs induced in order to bring the ongoing immune response to a quiescent state after the pathogen has been cleared (34).

However, this 'balanced' population of $CD4^+$ T cells suppressed MUC1-specific immunse responses when adoptively transferred into MUC1-Tg mice. This was particularly surprising because the same $CD4^+$ T cell population provided effective helper function in the donor mouse. The fact that Tregs triumph over Th only after adoptive transfer suggests there is a selection pressure *against* the Th cells in the recipient mouse. This was true whether the recipient mouse was WT or MUC1-Tg, so was independent of any tolerogenic mechanisms related to expression of self-MUC1.

Several studies have shown that effector T cells do not survive well after adoptive transfer unless "space" is created in the recipient by non-myeloablative chemotherapy to allow homeostatic proliferation of adoptively transferred T cells (163). Recent evidence indicates that Tregs express higher amounts of the anti-apoptotic molecule Bcl-2, causing them to be less susceptible to apoptosis than their Th counterparts (164). Taken together with the data present here, these lines of evidence suggest that adoptively transferred Tregs may survive better than Th cells after adoptive transfer, and therefore suppress immune response of the recipient.

2.4.4. Timing of vaccination with respect to age

The preliminary observation that older mice appear to have even higher numbers of $FoxP3^+$ Tregs has obvious implications for the timing of anti-cancer vaccines with respect to the age of the individual. Further studies will need to be performed to determine whether this was due to impaired activation of MUC1-specific Th cells, enhanced proliferation of MUC1-specific Tregs, or whether it is simply reflective of a higher background number of Tregs, regardless of antigen-

specificity. Whatever the outcome of these future experiments, there is a clear link between old age and increased numbers of Tregs. Given that cancer is predominantly a disease of the aged, these preliminary data support the case for administering prophylactic cancer vaccines to high-risk individuals at a young age.

3. GENERATION OF A MUC1-SPECIFIC TCR-TRANSGENIC MOUSE, "VFT"

3.1. Requirement for a MUC1-specific TCR-Tg mouse

In the past few decades a wealth of knowledge has been amassed regarding tumor-specific $CD8^+$ T cells and their essential role in anti-cancer immunity. Much less attention has been paid to tumor-specific $CD4^+$ T cells. The data in the previous chapter indicate that the inability of MUC1-Tg mice to mount effective MUC1-specific immune responses is caused by a dominance of MUC1-specific $CD4^+$ Tregs over their Th counterparts. The suppression of MUC1-specific immune responses caused by these Tregs was relieved by providing WT Th cells that had been primed to MUC1. In order to conclusively prove that the improved immune response was due to the provision of MUC1-specific $CD4^+$ Th cells, it was necessary to confirm that the responding $CD4^+$ T cells (both Th and Tregs) are actually MUC1-specific. Given the antigen-independent manner in which Tregs suppress other T cells, it is especially important to be sure of the antigen specificity of the $CD4^+$ T cells under investigation.

To focus our studies on *MUC1-specific* $CD4^+$ T cells, we created a transgenic (Tg) mouse in which all of $CD4^+$ T cells are specific for MUC1. In the following chapter, we describe the cloning of an MHC Class II restricted T cell receptor (TCR) that is specific for a 12 amino acid peptide derived from the tandem repeat region of unglycosylated MUC1 100mer. The genes encoding the TCR alpha and beta chains from the MUC1-specific, CD4+ T cell hybridoma, VF5 (165) were cloned into TCR expression cassettes, pTα and pTβ (166). In these cassette vectors,

expression of the cloned TCR is controlled by the natural TCRα and -β promoters. The MUC1-specific TCR constructs were then used to generate the MUC1-specific TCR-Tg mouse strain, VFT. Two initial experiments were performed with the VFT mouse to confirm the two main conclusions from the previous chapter:

1) Presentation of MUC1 peptides by DC causes the induction of MUC1-specific Tregs

2) Adoptive transfer of MUC1-specific Th cells restores MUC1-specific immune responses in MUC1-Tg mice.

3.2. Generating the VFT mouse: Issues and Methodology

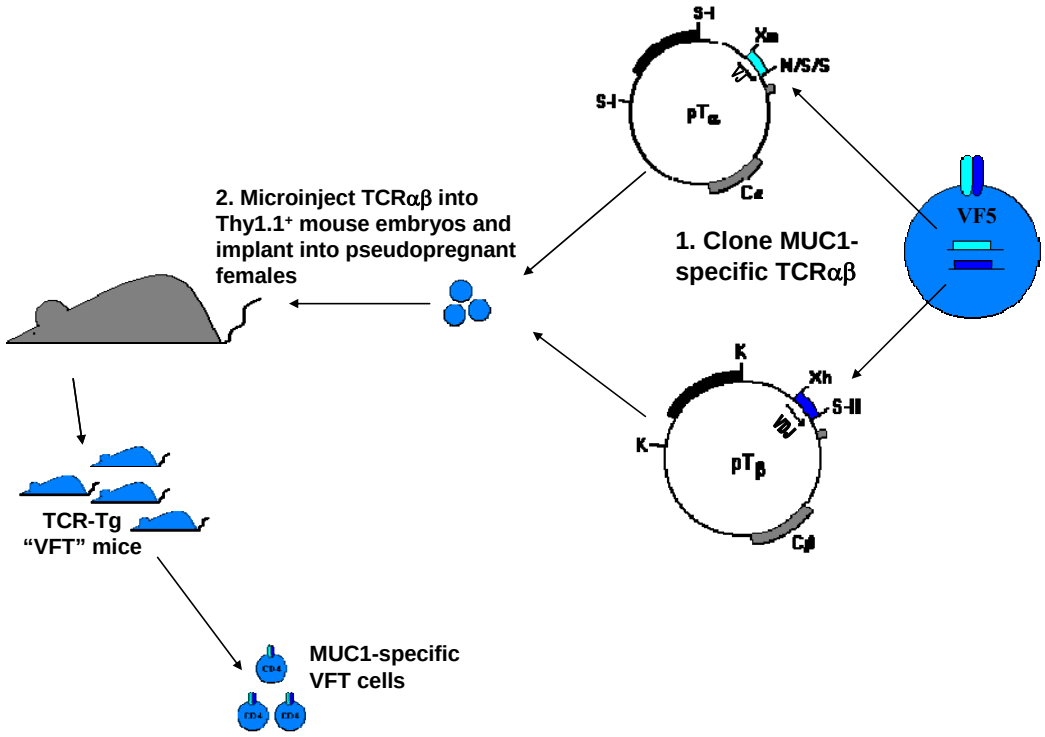


Figure 3-1 Making the VFT TCR-Tg mouse

The VJα and VDJβ genes encoding the variable regions of a MUC1-specific TCR were amplified by PCR from the CD4+ T cell hybridoma, VF5 (165), and cloned into the TCR expression cassette vectors pTα and pTβ (166), respectively. These TCR expression constructs were injected into embryos of Thy1.1$^+$ mice to generate the MUC1-specific TCR-Tg mouse strain, VFT, on a C57BL/6 Thy1.1$^+$ congenic background.

3.2.1. "Natural" Expression of the transgenic TCR: pTα/β expression cassettes

We wanted to generate a mouse in which all $CD4^+$ T cells express a T cell receptor specific for MUC1. As these MUC1-specific T cells would be used to answer fundamental questions regarding T cell tolerance and immunity to the tumor antigen MUC1, it was important that expression of the TCRα and β transgenes is regulated correctly with respect to T cell development and function. To achieve this, the alpha and beta chains of the MUC1-specific TCR were cloned into two TCR cassette vectors, pTα and pTβ respectively. These vectors contain large fragments of genomic DNA derived from the TCRα and-β loci, which flank multiple restriction enzyme sites into which the TCR of interest is cloned (166) and (figs3-5 and 3-8). The genomic fragments of the plasmids contain the cis-acting promoter and enhancer elements necessary for correct developmental and tissue-specific expression of the cloned TCR chains. In addition, pTα and pTβ already contain the corresponding TCR constant gene segments (Cα and Cβ, respectively). It was therefore possible to clone just the rearranged V(D)J gene segments without the constant gene segment, and insert them into the vector. This avoids having to clone several kb of intron that separate the V(D)J and C exons. When cloning the VDJ genes from genomic DNA, inclusion of the splice donor site 3' to the J gene segment allows correct splicing of the cloned VDJ segments to the constant gene segment encoded by the vector.

3.2.2. VF5 hybridoma: source of the MUC1-specific TCR

The source of the MUC1-specific TCR is an MHC-class II restricted, CD4+ T cell hybridoma, VF5, which was generated in our laboratory and described previously (165). VF5 is

specific for a 12aa peptide GVTSAPDTRPAP derived from the tandem repeat region of unglycosylated MUC1. The VF hybridomas were generated by fusing the AKR thymoma BW5147 (167) with $CD4^+$ T cells isolated from WT mice immunized with unglycosylated synthetic MUC1 100mer. Hybridoma clone VF5 displayed the highest avidity for MUC1 100mer, as tested by IL-2 production in response to 100mer-loaded DC. The high-affinity MUC1-specific TCR expressed by VF5, hereafter called "VF5 TCR" or "VF5αβ", is therefore representative of the TCRs that are expressed by peripheral MUC1-specific effector $CD4^+$ T cells in WT mice.

3.2.3. Expression of BW5147 TCR genes in the VF5 hybridoma

Cloning of the MUC1-specific TCR was confounded by the presence of multiple TCR re-arrangements in the VF5 hybridoma. We found that the TCR loci of both fusion partners, the original MUC1-specific T cell and the BW5147 thymoma, carried several productive VDJ rearrangements, all of which are expressed in the VF5 hybridoma. As will be described later in more detail, the MUC1-specific TCR was identified by the variable gene segments of its alpha and beta chains, Vα2.7 and Vβ6, respectively. The BW5147-derived TCR contains Vα1.1 and Vβ1.1 (168).

Adding a further level of complexity, the BW5147 fusion partner actually carries V(D)J rearrangements in all of its TCR loci (i.e. both TCRα loci and both TCRβ loci). Of these rearrangements, only two are productive, resulting in protein expression of Vα1.1 and Vβ1.1. The two non-productive TCR re-arrangements, involving Vα16 and Vβ5.2, are expressed at the mRNA level only due to in-frame stop codons which prematurely terminate protein translation.

The VF5 hybridoma, which was the result of fusing BW5147 with the MUC1-specific T cell, contains 4N chromosomes and carries all of the TCR rearrangements from the parent cells. Thus, the VF5 hybridoma expresses three TCRα chain mRNAs and three TCRβ chain mRNAs, (see table 2). Of these TCR mRNA species, two TCR alpha chains (Vα1.1 and VF5α) and two beta chains (Vβ1.1 and VF5β) are translated into full-length protein and expressed at the cell surface. Either of the TCRα chains can associate with either of the TCRβ chains, potentially resulting in the expression of *four* different heterodimeric TCRαβ combinations on the cell surface of VF5: Vα1.1/Vβ1.1; Vα1.1/VF5β; VF5α/Vβ1.1 and VF5α/VF5β. The latter combination was assumed to be the MUC1-specific TCR of interest, to be identified and cloned.

Table 2. TCR Re-arrangements in the VF5 hybridoma

Original Source	TCRα gene segments	TCRβ gene segments	Productive Rearrangement?	mRNA expression?	Protein expression?
MUC1-specific T cell	Vα2.7-Jα49	Vβ6-Jβ2.5	Yes	Yes	Yes
BW5147	Vα1.1	Vβ1.1	Yes	Yes	Yes
BW5147	Vα16	Vβ5.2	No	Yes	No

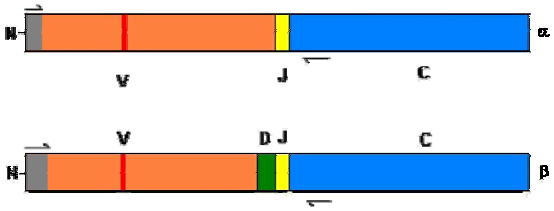
3.2.4. Designing Methods for identifying the VF5 TCR

Cloning of the VF5 TCR was more complicated than simply performing PCR amplification of the TCR with 5' and 3' flanking primers, as this would indiscriminately amplify all of the TCR mRNA species. Primers were needed that could specifically amplify the MUC1-specific TCR but not the fusion partner TCR. In order to design such primers, it was first necessary to identify the variable and joining gene segments that are incorporated in the VF5 TCR.

Due to the paucity of Vα-specific antibodies, it was not possible to use flow cytometry to identify the VF5 TCR alpha chain. Similarly, the commercially available Vβ antibody kits did not cover all of the known Vβ gene segments. Therefore, a molecular approach was used to identify the V and J gene segments that are incorporated into the MUC1-specific TCR. This methodology involved RT-PCR amplification of the unknown TCRα and β chains, followed by sequencing of the amplicon and alignment with a database of known murine TCR V/J gene sequences (169).

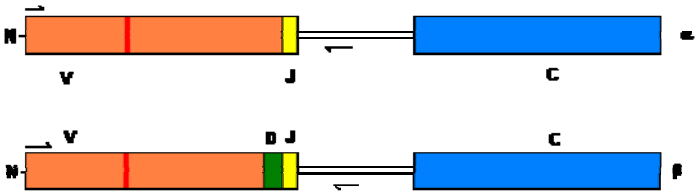
The major challenge was to specifically amplify and clone the *MUC1-specific* TCR chains without actually knowing their sequence. To this end, two slightly different RT-PCR-based techniques were used for identification of the unknown VF5α and VF5β chains. They will be described in detail in the following section. The VF5 TCRα chain was identified by 5'RACE. The VF5 TCRβ was identified using degenerate primers that bind to all the Vβ gene segments.

1. Amplify V(D)J from mRNA by 5’ RACE or degenerate primers:



2. Clone the PCR product and check the DNA sequence to identify the V and J gene segments

3. Design appropriate primers to clone VDJ from ***genomic*** DNA:



4. Clone the genomic fragment into TOPO vector.

5. Sub-clone into expression vector (pTα/pTβ)

Figure 3-2. Overview of the identification and cloning of the VF5 TCR

3.3. Materials & Methods

Unless otherwise specified, all PCR primers were synthesized in the DNA synthesis facility at the University of Pittsburgh. All DNA sequencing was performed in the DNA sequencing facility at the University of Pittsburgh. All mouse cells were cultured in complete DMEM (cDMEM) containing 10%FBS, Penicillin & Streptomycin, L-Glutamine, Na Pyruvate, Non-Essential Amino Acids, Hepes Buffer, and β-2 Mercaptoethanol.

3.3.1. TCRα 5' RACE

Total cellular RNA was isolated from the VF5 hybridoma using an RNeasy mini kit (Qiagen, CA). 5' RACE was performed using a Generacer Kit (Invitrogen, CA). Total cellular DNA was synthesized using the supplied oligo dT primers, as per the manufacturer's instructions. The TCRα chains were then amplified by nested PCR using forward primers specific to the ligated 5' Oligo (supplied in the Generacer Kit): 5' CGACTGGAGCACGAGGACACTGA 3' and 5' GGACACTGACATGGACTGAAGGAGTA 3'; paired with a reverse primer specific for the TCRα constant gene segment: 5' ACAGCAGGTTCTGGGTTCTGG 3'. After electrophoreses in a 1% agarose gel containing ethidium bromide, the RACE product was eluted using a GenElute gel extraction kit (Sigma, MO), then TA cloned into the pCR4-TOPO vector and transfected into chemically competent TOP10 *E. coli* using a TOPO-TA cloning Kit (Invitrogen, CA). Transfected bacteria were grown on selection overnight in LB broth containing 100μg/mL ampicillin (Sigma, MO) and then cloned by spreading on a plate of LB agar containing 100μg/mL ampicillin. Individual colonies were screened by PCR for the presence of a TCRα

RACE product using the Cα-specific primer (see above), coupled with T7 or T3 primers specific for the vector (supplied in the TOPO-TA kit). TCRα$^+$ clones were then screened by a second PCR to eliminate those clones that contained either of the fusion partner TCRα fragments, Vα1.1 (primer sequence, 5' GCACTCTGGGAAAAGCCCC 3') and Vα16 (primer sequence, 5' GGAAGAACAGAAAGTTTTCACGCTCC 3'). Recombinant plasmids containing the unknown VF5α chain were isolated from TCRα$^+$ Vα1$^-$ Vα16$^-$ clones using a plasmid mini kit (Qiagen, CA) and were subsequently sequenced in the DNA sequencing facility at the University of Pittsburgh School of Medicine.

3.3.2. Cloning the VF5α chain into pTα

Genomic DNA was isolated from the VF5 hybridoma using a DNeasy kit (Qiagen, CA). The VF5α VJ fragment was amplified by PCR primers specific for Vα2.7 (5'CATCTCCCGGGATGGACAAGATCCTGACAGCGC 3') and the downstream intron of Jα49 (5' GCGGTGGCGGCCGCAATTAGGACAGAGCTTTAGC 3'), using Pfu turbo Hotstart DNA polymerase (Stratagene, CA). These primers introduced flanking restriction enzyme sites for XmaI and NotI, respectively. PCR was performed at 3mM $MgCl_2$ for 30 cycles with an annealing temperature of 55^{o}C. The 769bp VF5 VJα genomic fragment was eluted from a 1% agarose gel using an EthBr Minus kit (Sigma, MO) and terminal overhanging adenine nucleotides added using Taq polymerase (Applied Biosystems, CA). The VF5 VJα genomic fragment was TA-cloned into pcDNA3.1/V5-His (Invitrogen, CA) and propagated in TOP10 *E.coli* (Invitrogen, CA) in LB media containing 100μg/ml ampicillin (Sigma, MO). VF5α$^+$ clones were identified by PCR using the vector-specific primers T7 and BGH (Invitrogen, CA), paired with a Jα49-specific primer (5' CCTTCCCAAATGACAGCTTAGA 3'). The integrity of

the cloned sequence was confirmed by the DNA sequencing facility at the University of Pittsburgh. pcDNA3.1-VF5α and pTα were digested with XmaI and SacII and the resulting fragments resolved by agarose gel electrophoresis. The 825bp VF5α fragment and ~19kb pTα were eluted from the gel with Etr Minus columns (Sigma, MO) and ligated with T4 ligase (New England Biolabs, MA). The ligation product was transfected into TOP10 *E.coli* (Invitrogen, CA) and transfectants selected in LB media containing 100μg/ml ampicillin. Colonies were screened by PCR for pTα containing VF5α inserted in the correct orientation, using primers specific for Vβ2.7 (5' CACAATCTTCTTCAATAAAAGGGAGAA 3') and pTα (5' TACACTGGCCAGTCACGGA 3').

3.3.3. RT-PCR with degenerate Vβ primers

Total cellular RNA was isolated from the VF5 hybridoma using an RNeasy mini kit (Qiagen, CA) and cDNA synthesized with Oligo-dT using a GeneAmp RNA PCR kit (Applied Biosystems, CA), according to the manufacturer's instructions. PCR amplification of TCRβ cDNA was performed using a reverse primer specific for TCR Cβ (5' AATCTCTGCT TTTGATGGCT 3') and degenerate Vβ-specific primers, as previously published (170) at a [$MgCl_2$] of 2mM. The TCRβ cDNA fragment was eluted from a 1% agarose gel using a Genelute kit (Sigma, MO) and TA cloned into pCR4-TOPO (Invitrogen). pCR4-VF5β was transfected into Top10 *E.coli*, which were grown in LB plus 100μg/ml ampicillin and screened for the presence of Cβ using T7 and T3 primers (Invitrogen) paired with a Cβ-specific primer (5' GAGACCTTGG GTGGAGTCAC 3'). Plasmids isolated from VF5β^+ clones using a plasmid

mini kit (Qiagen, CA) were sequenced by the DNA sequencing facility at the University of Pittsburgh.

3.3.4. Cloning the VF5β chain into pTβ

The VF5 VDJβ fragment was amplified from genomic DNA by PCR using the Pfu turbo Hotstart DNA polymerase (Stratagene, CA) and primers specific for Vβ6.1 (5' CAGTATGTCGACATGAACAAGTGGGTTTTCTGCTGGGTAA 3') and the downstream intron of Jβ2.5 (5' ACAAACCCGCGGAACCCTGTGACTCCCAAGAGAAA 3'). These primers introduced flanking restriction enzyme sites for SalI and SacII, respectively. PCR was performed at 3mM $MgCl_2$ for 35 cycles with an annealing temperature of 55^{o}C. The 631bp VF5 VDJβ PCR product was eluted from a 1% agarose gel using an EthBr Minus kit (Sigma, MO) and terminal overhanging adenine nucleotides added using Taq polymerase (Applied Biosystems, CA). The VF5 VDJβ genomic fragment was TA-cloned into pcDNA3.1/V5-His (Invitrogen, CA) and propagated in Stbl IV *E.coli* (Invitrogen, CA) at 30^{o}C in LB media containing 100μg/ml ampicillin (Sigma, MO). VF5β^{+} clones were identified by PCR using primers specific for Vβ6 (5' TCAATAACTGAAAACGATCTT 3') and Jβ2.5 (5' TAACACGAGGAGCCGAGT 3'). The integrity of the cloned sequence was confirmed by the DNA sequencing facility at the University of Pittsburgh. The cloned VF5β genomic fragment was excised from pcDNA3.1 with SalI and SacII. pTβ was digested with XmaI and SacII. The resulting fragments were resolved by electrophoresis in a 1% agarose gel, eluted with Etr Minus columns (Sigma, MO) and ligated with T4 ligase (New England Biolabs, MA). The pTβ-VF5β ligation product was transfected into TOP10 *E.coli* (Invitrogen, CA) and transfectants selected in

LB media containing 100μg/ml ampicillin. Colonies were screened by PCR for the presence of VF5β, using the same primers specific for Vβ6 and Jβ2.5 described above.

3.3.5. Propagation of pTβ-VF5β in Stbl4 *E.coli*

Restriction analysis indicated that the pTβ-VF5β expression construct is unstable and susceptible to self-recombination events, leading to the accumulation of spurious recombinants of varying sizes. To combat this, pTβ-VF5β was propagated in Stbl4 *E.coli* (Invitrogen, CA), which lack several DNA recombination enzymes and therefore maintain large and unstable plasmids more faithfully.

3.3.6. ELISA on DO.11.10 transfectants

1 x 10^6 DO.11.10 cells were co-transfected with 5μg of pTα-VF5α, 5μgpTβ-VF5β, and/or 0.5μg pEF6 (Invitrogen, CA), by electroporation at 280mV and 960μF. Electroporated cells were rested overnight in cDMEM then grown for two weeks in cDMEM supplemented with 5μg/ml Blasticidin (Invitrogen, CA). DO.11.10 cells transfected with pTα-VF5α were stained with anti-Vα2 MAb (BD Biosciences, CA) and sorted on a FACSaria (BD Biosciences, CA). Presence of pTβ-VF5β was confirmed by RT-PCR using the primers specific for Vβ6 and Jβ2.5 described above. 10^5 DO.11.10 cells were stimulated for 24 hours with 10^4 day6 BMDC loaded with 50ug/mL MUC1 100mer. Supernatants were harvested and analysed for presence of IL-2 using an IL-2 OptEIA ELISA set (BD-Pharmingen, CA), as per the manufacturer's instructions.

3.3.7. Generation of VFT Mice

Founder VF5α and VF5β mice were generated in the transgenic mouse facility at the University of Pittsburgh School of Medicine by microinjection of C57BL/6 Thy1.1 embryos with the pTα-VF5α and pTβ-VF5β constructs, respectively. Injected embryos were implanted into pseudopregnant C57BL/6 Thy1.1 females. VF5α and VF5β founder mice were identified by PCR of tail DNA using primers specific for the Vα2-Jα49 and Vβ6-Jβ2.5 rearrangements. Founders were bred with Thy1.1 mates to confirm that the transgenes were in the germline. Then VF5α and VF5β F_1 progeny were cross-bred to produce double-transgenic VFT mice, carrying both VF5α and VF5β transgenes.

3.3.8. Detection of VF5 TCR expression by flow cytometry

Expression of the transgenic TCR chains on the surface of T cells was confirmed by flow cytometry. Cells were stained with monoclonal antibodies specific for Va2 and Vβ6. The PE-conjugated Va2 MAb (clone B20.1, BD Biosciences, CA) was used at 1:50 dilution. The Vβ6-specific antibody was produced by the hybridoma clone 44-22-1, a generous gift from Dr Pamela Ohashi (University of Toronto), and was applied to cells in the form of raw hybridoma culture supernant. The hybridoma was grown to confluence for up to 1 month in Iscove's Modified Eagle Media (Cellgro, Mediatech, VA), containing 10%FBS, Penicillin & Streptomycin, L-Glutamine, Na Pyruvate, Non-Essential Amino Acids, Hepes Buffer, and β-2 Mercaptoethanol. Supernatant was harvested and stored at 4^oC in the dark and used, unpurified to stain cells for Vβ6. FitC-conjugated anti-mouse IgG2a secondary antibody (BD Biosciences, CA) was then used to detect surface-bound 44-22-1 MAb. Due to cross-reactivity of the secondary antibody

with the Vα2 MAbs (Rat IgG2a isotype), the three antibodies were applied in succession in the following order: 44-22-1 supernatant…FitC-anti-mouse IgG2a…PE-anti-Va2.

3.3.9. Generation of Bone Marrow DC

BMDC were generated as described earlier in chapter 2. Briefly, BM cells isolated from mouse femurs were cultured for 7days in cDMEM containing 10ng/ml each of GM-CSF and IL-4 (a generous gift from Immunex, WA) and fed every two days by adding 5ml cDMEM containing 10ng/ml GM-CSF and IL-4. On day 7 of culture, cells were harvested and purified by density centrifugation using Nycoprep 1.068 (Accurate Chemical, NY).

3.3.10. Vaccinations and adoptive T cell transfers

Purified day7 DC were loaded for 6 hours with 20-50ug/mL synthetic MUC1 100mer, washed twice with PBS and resuspended in PBS at $5x10^5$ cells/mL. Soluble 100mer was added at 500µg/ml. Mice were injected subcutaneously in the right flank with 200µl PBS containing 10^5 loaded DC and 100µg soluble 100mer.

T cell donor mice were immunized with 100mer-pulsed DC, as described above. Two weeks after immunization, LN and spleen were removed from donor mice and single cell suspensions prepared as described above. $CD4^+$ T lymphocytes were positively selected by magnetic antibody cell sorting (MACS) using CD4 (L3T4) Microbeads (Miltenyi Biotech, CA). Purified $CD4^+$ T cells were washed and resuspended in sterile PBS. Prior to injection of T cells, mice were placed under a heat lamp to induce vasodilation. Then $1 x10^6$ - $5 x10^6$ $CD4^+$ T cells in 200µl PBS were injected into mice via the lateral tail vein using a 27½ gauge syringe.

3.4. Making the VFT mouse: Results

3.4.1. Identification of the VF5 TCR VJα by 5'RACE

The MUC1-specific VF5α chain was amplified by 5'RACE (Rapid Amplification of cDNA Ends) PCR. This method is ideal for the amplification of a gene in which the sequence of the 3' end is known, but the 5' sequence is yet to be determined. This exact situation exists in TCR chains of unknown identity; the Variable and Joining regions could be encoded by any of the available V and J gene segments, but the constant regions is, by definition, always encoded by the same gene segment. In 5' RACE, a short oligonucleotide was ligated to the 5' end of cellular mRNA prior to its reverse transcription into cDNA. As the reverse transcriptase proceeds from the 3' poly-A tail, synthesizing a complementary strand to the mRNA encoding the TCRα, it continues through the oligonucleotide ligated to the 5' end. The resulting TCRα cDNA contained the unknown VDJ fragment, flanked by two known sequences (i.e. the 5' ligated oligo, and the 3' constant gene segment). This was then amplified by PCR using primers specific for the 5' oligo and the TCRα constant gene segment. The Cα reverse primer ensured that only TCRα cDNA would be amplified. However, as the Cα primer does not distinguish between the MUC1-specific TCRα or the fusion partner TCRα chains, all three TCRα species are amplified and represented as a single band upon electrophoresis in an agarose gel (fig 3-3).

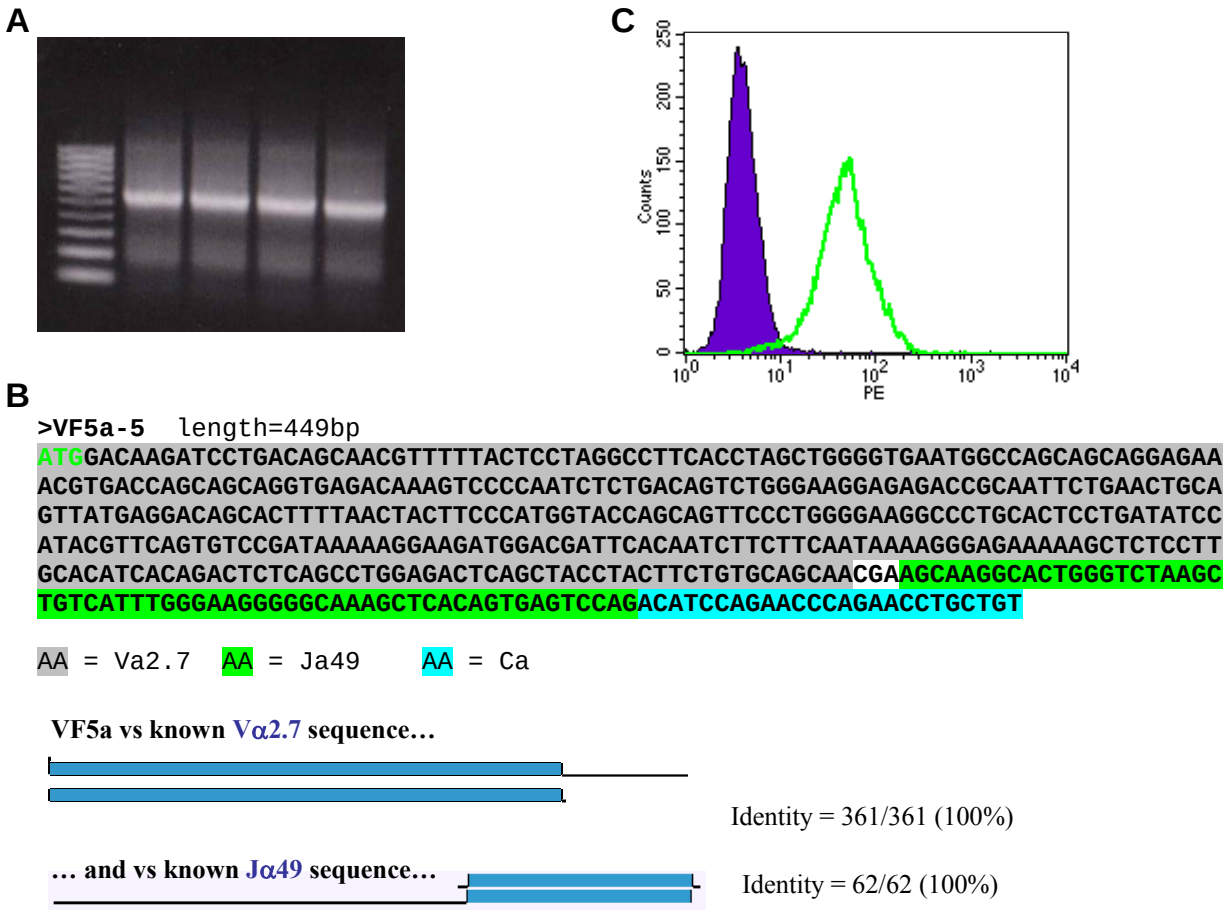


Figure 3-3. VF5 TCRa contains Vα2.7 and Jα49

A) The 550bp VF5 TCR VJα was amplified by 5'RACE eluted from a 1% agarose gel and TOPO-cloned into pCR4-TOPO B) Sequence of the TCRα RACE product matches known sequences of Vα2.7 and Jα49 in the BLAST database. C) Expression of Va2 on the surface of VF5 was confirmed by FACS using anti-Vα2 MAb.

The three species of TCRα chains amplified by 5'RACE therefore needed to be separated before VF5α could be accurately sequenced. To do this, the 550bp TCRα RACE product was eluted from the agarose gel, and TOPO cloned into pCR4-TOPO. Individual clones were screened by PCR for the unknown VF5α, as determined by the presence of a TCRα chain that was not Vα1.1 or Vα16. The VF5 VJα sequence from five clones was aligned with the BLAST database and also with published sequences of all the known murine TCR Vα genes (169) and Jα genes (171).

These analyses revealed that the MUC1-specific TCRα contains Vα2.7 and Jα49 (fig 3-3). Cell surface expression of Va2 was confirmed by FACS using a Va2-specific antibody (fig 3-3). Thus, the MUC1-specific TCRα chain was identified and found to contain the Vα2.7 and Jα49 gene segments.

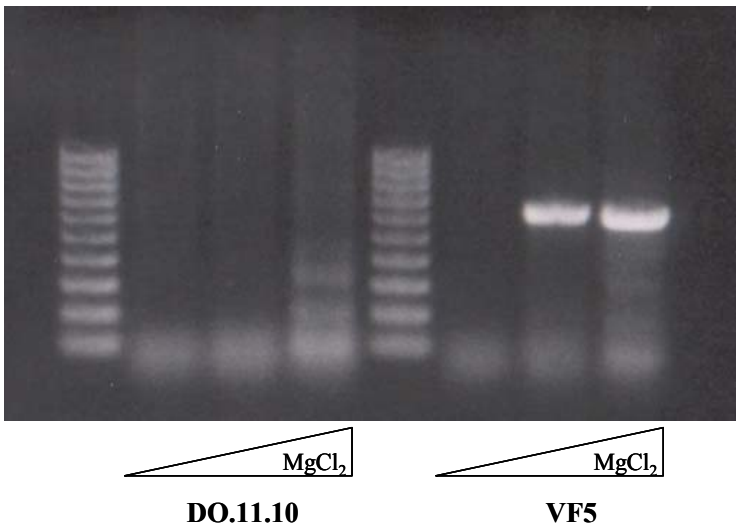


Figure 3-4. PCR amplification of VF5α

VF5a TCR was amplified from genomic DNA using primers specific for the 5' and of Vα2.7 and the 3' Jα49 intron. DO.11.10 (shares the same fusion partner as VF5) was used as a negative control for specificity of PCR amplification.

3.4.2. Cloning the VF5 TCRα into the pTα cassette vector

With the VF5α chain gene segments identified, it was possible to design primers specific for the Vα2.7-Jα49 rearrangement that could be used to specifically amplify the VF5 TCRα chain from genomic DNA (fig3-4): The forward cloning primer was specific for the leader sequence of Va2.7; the reverse cloning primer was specific for the 3' intron of Jα49. A high fidelity DNA polymerase, Pfu turbo, was used to ensure faithful replication of the gene sequence. The genomic TCRα fragment cloned from VF5 contained the complete variable domain of the

MUC1-specific TCR, from the Vα2.7 leader sequence, to a point 150nt downstream of the Jα49 gene segment. We included part of the 3' Jα49 intron, as this contains the splice donor site necessary for splicing of the VJ exons with the Cα exon in pTα. The VF5α fragment was initially TA cloned into the pcDNA3.1/V5-His plasmid, to allow its sequence to be verified, and subsequently sub-cloned into the pTα expression vector by virtue of flanking Xma I and Sac II restriction sites, which were engineered into the TCRα fragment by the cloning primers. Fig 3-5 shows a map of the final expression construct.

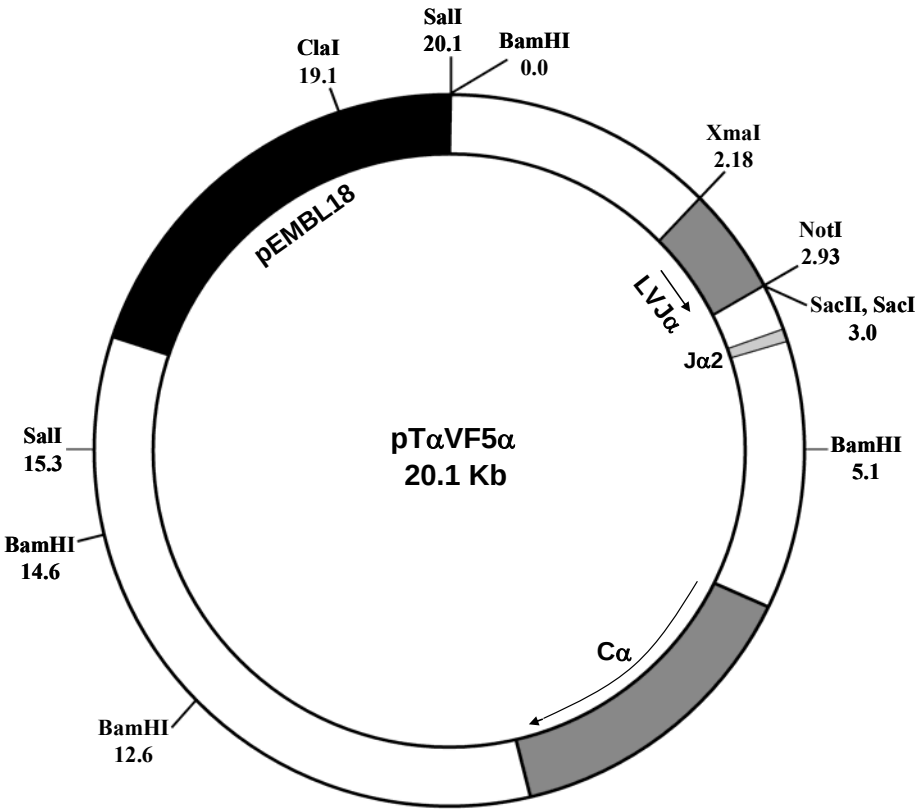


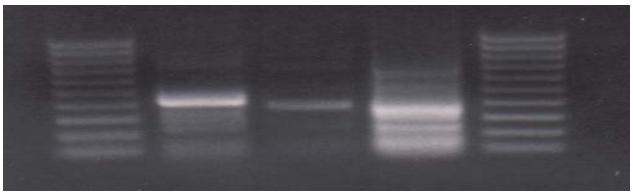
Figure 3-5. Map of the pTαVF5α expression construct

The VJα fragment cloned from VF5 was inserted into the XmaI/NotI cloning site. The VJα exon is spliced to the Cα exon during mRNA processing. The pEMBL18 prokaryotic backbone is necessary for propagation in *E.coli* and provides the *Neo* gene for resistance to G418 (Genticin), to allow selection of transfectants. This prokaryotic sequence was removed by digestion with SalI prior to injection into the mouse embryos.

3.4.3. Identification of VF5 TCRβ using degenerate Vβ-specific primers

Unlike VF5α, attempts to use 5' RACE to identify the VF5 TCRβ chain were unsuccessful; despite modification of various parameters, a TCRβ RACE product was never obtained. Therefore an alternative approach was taken involving RT-PCR amplification of the TCRβ chains using a Cβ-specific reverse primer in combination with degenerate forward primers which will anneal to all of the known Vβ gene segments (170). Similar to 5'RACE, this method allowed amplification across the unknown VDJ region. As for the VF5α chain, the RT-PCR product was eluted from an agarose gel and TA-cloned into pCR4-TOPO to separate the MUC1-specific VFJβ from those derived from the BW5147 fusion partner. BLAST alignment of sequences from several clones indicated that the MUC1-specific TCRβ chain contains Vβ6 and Jβ2.5 (fig 3-6). Owing to the lack of a good Vβ6 MAb at this time, the presence of this rearrangement in VF5 was confirmed by PCR using primers specific for the Vβ6 CDR and Jβ2.5 (fig 3-7).

A



Reverse Primer: Cβ1 Cβ2 Cβ3

B

>pCR4-VF5 β3

CGAATGAATTTAGCGGCCGCGAATTCGCCCTTN CGTACCCAAAATCCT GATTGGTCAGGAAGG G
CAAAAACTGACCTTGAAATGTCAACAGAATTTCAATCATGATACAATGTACTGGTACCGACAG G
ATTCAGGGAAAGGATTGAGACTGATCTACTAT **TCAATAACTGAAAACGATCTT** CAAAAAGGCG A
TCTATCTGAAGGCTATGATGCGTCTCGAGAGAAGAAGTCATCTTTTTCTCTCACTGTGACATC T
GCCCAGAAGAACGAGATGGCCGTTTTTCTCTGTGCCAGCAGTAT ACTGGGGGGCC AAGACACC C
AGTACTTTGGGCCAGGCACTCGGCTCCTCGTGTTAG AGGATCTGAGAAAT GTGACTCCACCCA A
GGTCTC CTTGTTTGAGCCATCAAAAGCAGAGATTAAGGGCGAATTCGTTTAAACCTGCAGGAC T
AGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTG

NN = Vector Sequence
NN = Vβ gene segment
NN = Dβ gene segment
NN = Jβ gene segment
NN = Cβ gene segment

NN = Vβdeg primer site
NN = CβNIII primer site

C

VF5β matches Vβ6:

Identities = 263/264 (99%) Strand = Plus / Plus

…and Jβ2.5:

Identities = 100%

Figure 3-6. Identification of VF5β, containing Vβ6 and Jβ2.5

A) Degenerate forward primers specific for the Vβ gene segments were used in combination with one of three different Cβ-specific reverse primers. B) Sequence results from the "Cβ2" RT-PCR product. C) The VF5β sequence matches Vβ6 and Jβ2.5.

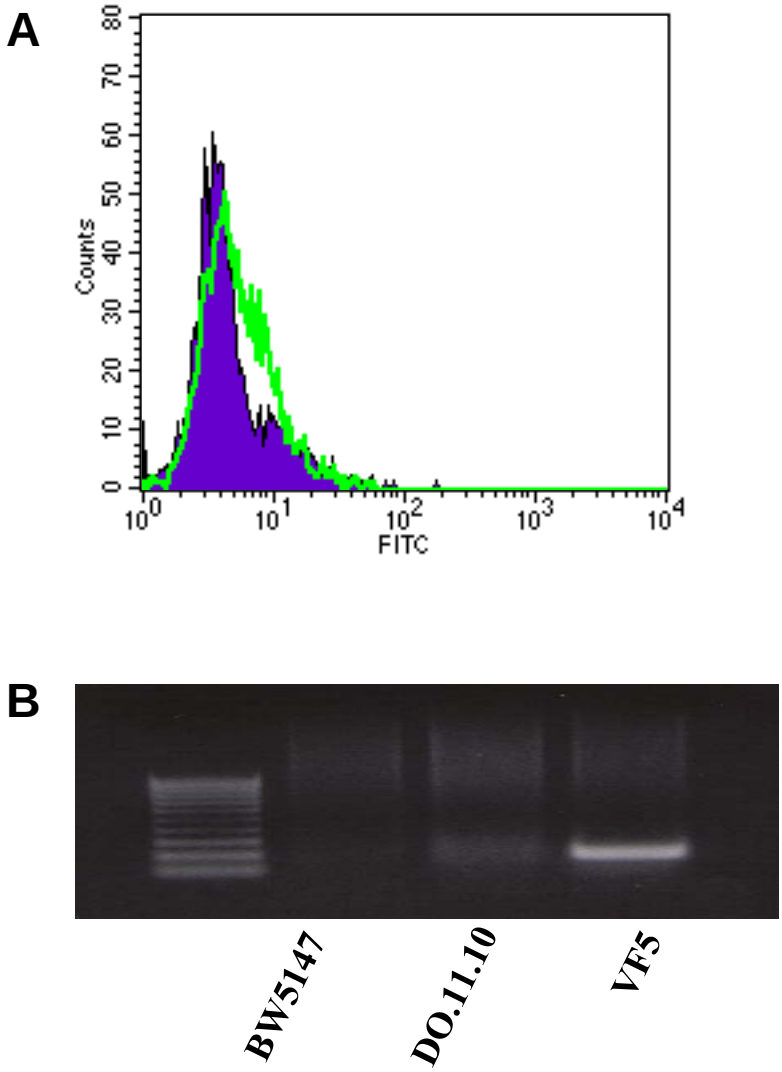


Figure 3-7. Expression of Vβ6 in the VF5 hybridoma

A) Anti-Vβ6 MAb (clone RR4-7) does not recognize the VF5 TCR: Blue histogram, isotype control antibody; green histogram, RR4-7.
B) The Vβ6Jβ2.5 mRNA rearrangement is only expressed in VF5: RT-PCR amplification from total mRNA using primers specific for Vβ6 and Jβ2.5.

3.4.4. Cloning the VF5 TCRβ into the pTβ cassette vector

Using this information, PCR primers specific for Vβ6 and the 3' Jβ2.5 intron were designed to specifically amplify the genomic VF5 VDJβ fragment, including 150bp of downstream intron. The introduction of flanking restriction enzyme suitable for insertion of the VDJβ fragment into pTβ was more complicated than for the VF5α fragment. The only restriction enzyme sites available in the pTβ cassette were the 5' XhoI and 3' SacII. However, the presence of an XhoI site in the middle of the Vβ6 gene segment precluded the use of XhoI at the 5' end of the VDJβ fragment. Therefore, a SalI restriction site, which is compatible with XhoI, was engineered into the 5' cloning primer. The amplified cloned VDJβ fragment was cut with SalI and SacII, pTβ was cut with XhoI and SacII, and the VF5β fragment was ligated into the pTβ cassette. Fig 3-8 shows a map of the final expression construct.

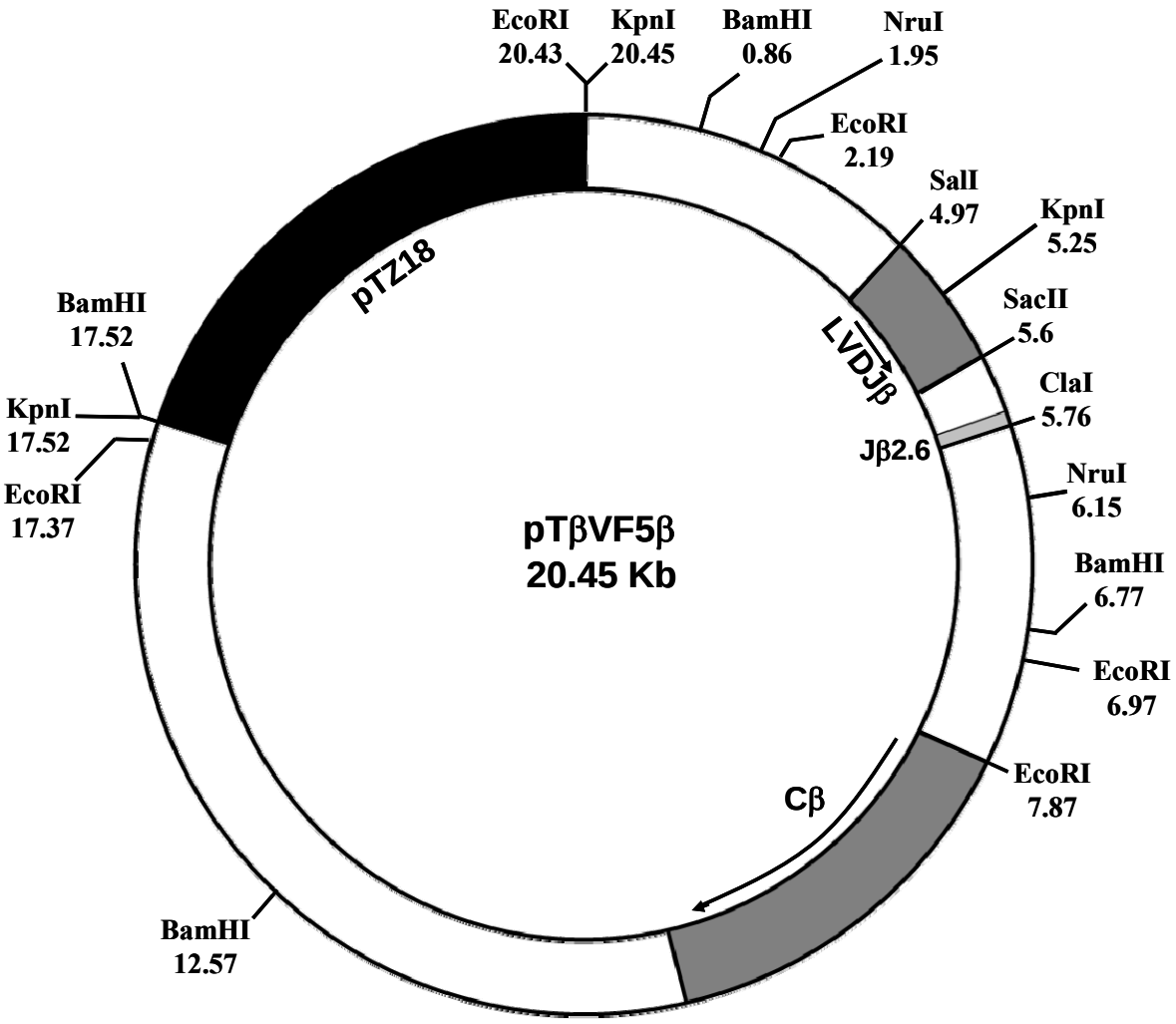


Figure 3-8. Map of the pTβ-VF5β expression construct

The VF5 VDJβ genomic fragment was cut with SalI and SacII and inserted into pTβ via the 5'XhoI and 3' SacII cloning sites. The cloned VDJβ exon is spliced to Cβ during mRNA processing to allow expression of full-length VF5 TCRβ.

Restriction analysis of this final construct revealed an anomaly in the map of the pTβ cassette originally published (166). This map includes the correct positions of restriction sites from 4.97kb SalI through 20.45kb KpnI.

3.4.5. MUC1-specificity of the cloned VF5 TCRαβ

Before proceeding to the generation of the VF5 TCR-Tg mouse, the integrity and MUC1-specificity of the pTα-VF5α and pTβ-VF5β constructs was tested by transfecting them into the DO.11.10 hybridoma. After confirming expression of the VF5 TCR chains in the DO.11.10 transfectants (fig 3-9), their ability to produce IL-2 in response upon stimulation by MUC1-presenting DC was tested by ELISA of culture supernatants.

Concomitant expression of VF5 TCRα and TCRβ constructs conferred specificity for MUC1 upon DO.11.10 (fig 3-9). Control DO.11.10 cells transfected with either VF5α or VF5β individually did not respond to MUC1. This proved that the cloned TCRα and β chains are derived from the MUC1-specific TCR in VF5 and that they are necessary and sufficient for MUC1-specificity. As DO.11.10 shares the same fusion partner as VF5, the fact that either construct alone did not confer MUC1 specificity on DO.11.10 rejects the possibility that the MUC1-specific TCR in VF5 may have been contained one of the TCR chains derived from BW5147.

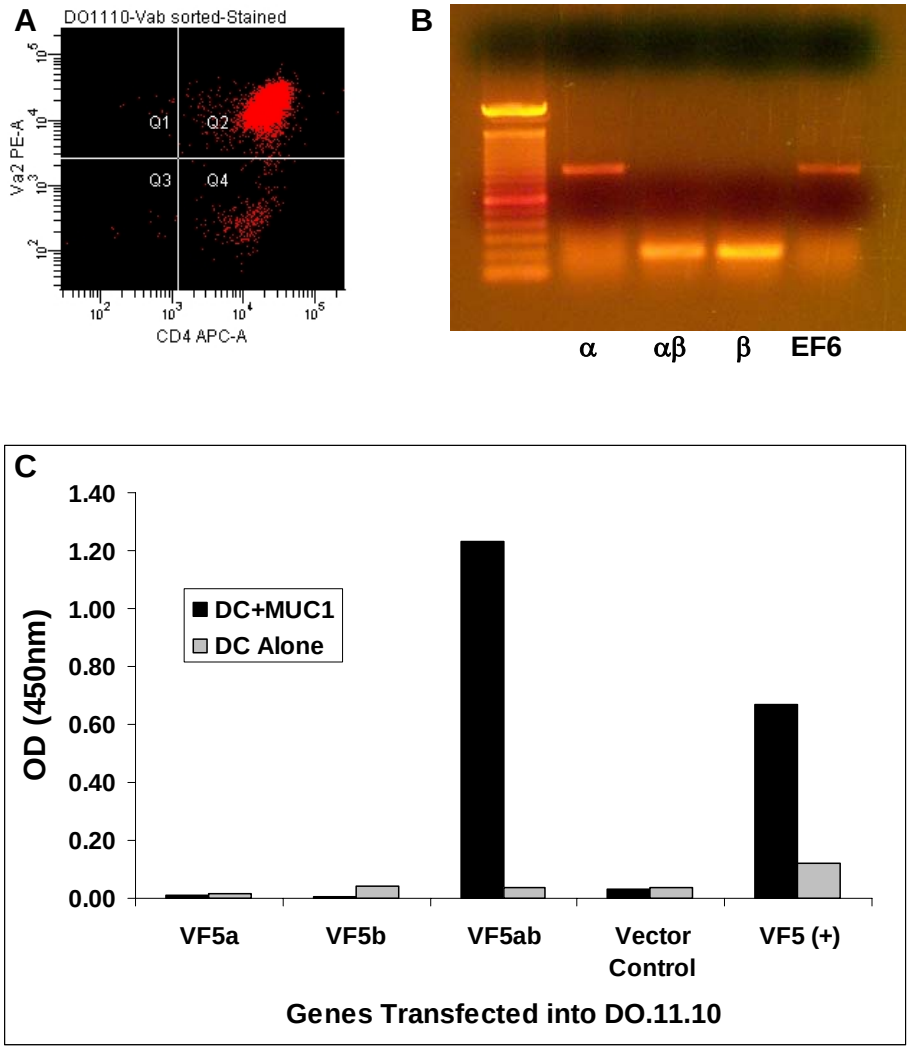


Figure 3-9. The cloned VF5 TCR is MUC1-specific

The VF5 TCR expression constructs pTα-VF5α and pTβ-VF5β were co-transfected into DO.11.10 with pEF6 (encoding the ble resistance gene) and grown in blasticidin selection for 1 week. (A) Transfectants expressing VF5α were stained with a MAb specific for Va2 and sorted by FACS. (B) Due to the unavailability at this time of a Vβ6-specific MAb, VF5β expression was confirmed by RT-PCR using primers specific for Vβ6 and Jβ2.5. The 200bp band indicates expression of VF5β mRNA. (C) Transfectants were stimulated with MUC1-pulsed DC for 24 hours and IL-2 production was measured by ELISA of culture supernatants. The original VF5 hybridoma was included in the stimulation assay as a positive control. (B and C), x-axis labels indicate constructs that were transfected into the cells.

3.4.6. The VFT TCR-Tg mouse

The pTα-VF5α and pTβ-VF5β TCR expression constructs were used to create the TCR transgenic mouse, "VFT", whose $CD4^+$ T cells are specific for MHC Class II restricted MUC1 100mer. Single transgenic VF5α and VF5β founder mice were generated separately on a C57BL/6 Thy1.1 congenic background. Presence of the transgenes in founder mice was confirmed by PCR amplification from tail DNA (fig 3-10). Passage of the TCRα and β transgenes through the germline was confirmed in F1 progeny by breeding the founders with Th1.1 mates. Correct expression of the transgenic receptors in T cells was confirmed by FACS (fig 3-10). The VF5α and VF5β transgenic mice were then cross-bred to produce the double-transgenic mouse, VFT, in which 100% of CD4+ T cells (hereafter called "VFT cells") express the MUC1-specific TCRαβ.

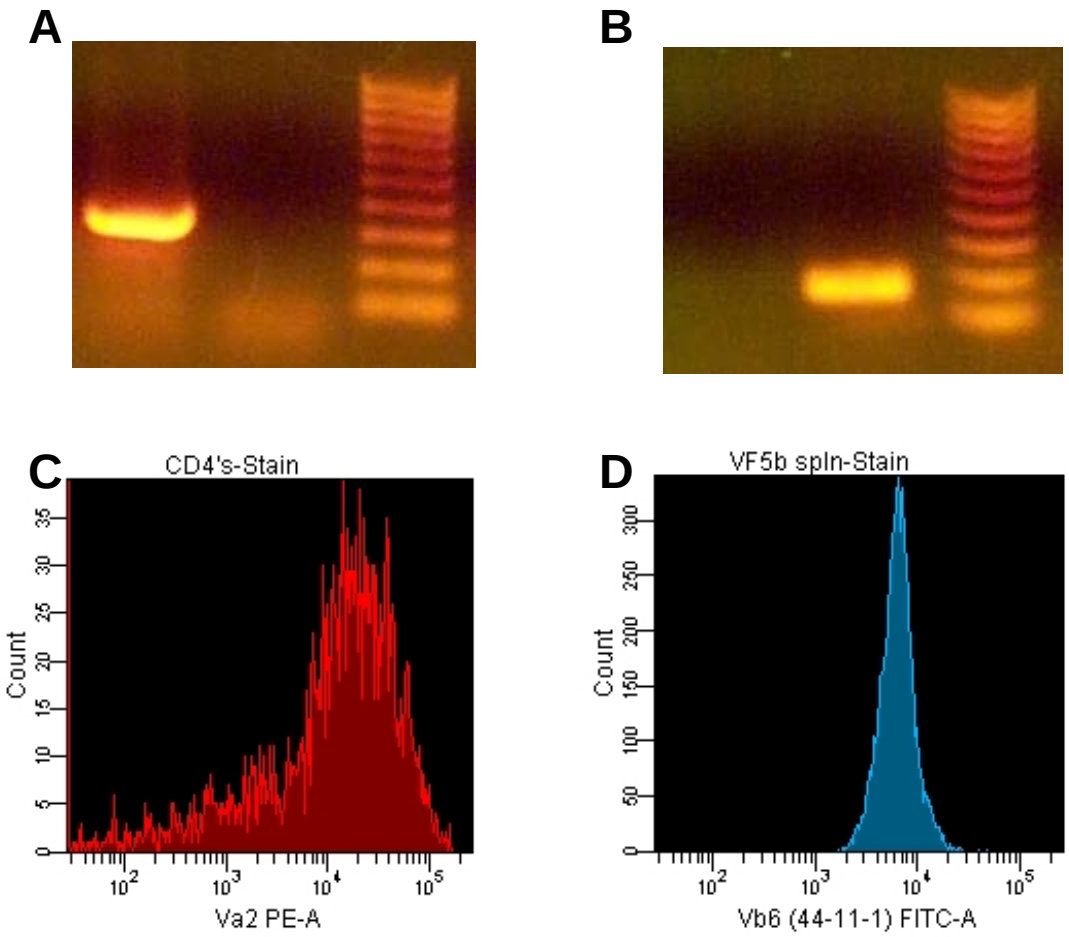


Figure 3-10. Expression of the transgenic TCR in VFT mice

A) Detection of VF5α transgene in tail DNA by PCR. Lane 1, VFTα mouse. Lane 2, WT mouse
B) Detection of VF5β transgene in tail DNA by PCR. Lane 1, WT mouse. Lane 2, VFTβ mouse
C) VFT cells were stained with PE-conjugated anti-Va2 MAb
D) VFT cells stained with 44-22-1 hybridoma supernatant, then FitC-conjugated anti-RatIgG2a

The C57BL/6 Thy1.1 congenic background of the VFT mouse allows the VFT cells to be distinguished from host T cells when adoptively transferred into WT and MUC1-Tg mice, which express the Thy1.2 allele on their T cells.

3.5. $CD4^+$ T cells from VFT mice control MUC1-specific immune responses

The VFT TCR-Tg mouse provides the perfect opportunity to study the fate of MUC1-specific $CD4^+$ T cells after encountering their cognate antigen, and their subsequent effect on the overall immune response to MUC1. Given the dual role of $CD4^+$ T cells in enhancing and suppressing immune responses to MUC1 described in chapter 2, our initial experiments using this novel TCR-Tg mouse were set out to answer two main questions:

1. How do VFT mice respond to the MUC1-DC vaccine, which induces both effector and suppressor $CD4^+$ T cells in WT mice, but predominantly Tregs in MUC1-Tg mice?

2. Does provision of MUC1-specific Th cells (now derived from VFT mice) enhance the immune response of MUC1-Tg mice to the DC vaccine. (i.e. does MUC1-specific help, actually *help*?).

3.5.1. Immune Response of VFT mouse $CD4^+$ T cells to MUC1-loaded DC.

The studies described in chapter 2 indicated that immunization of WT mice with MUC1-pulsed DC resulted in the expansion of $FoxP3^+$ regulatory T cells. To confirm conclusively that the responding Tregs are indeed MUC1-specific, VFT mice were vaccinated with MUC1-loaded DC and then tested for the presence of Tregs. Compared with naïve littermates, increased numbers of FoxP3+ Tregs were observed in vaccinated VFT mice (Fig3-11a), confirming the results obtained previously in WT mice (figs 2-5, 2-7) that DC immunization induces Tregs.

Interestingly, the expansion of Tregs by MUC1-pulsed DC is even more pronounced in VFT mice than in WT mice. This is likely due to the fact that a very small proportion of $CD4^+$ T cells in WT mice are MUC1-specific, compared with 100% in VFT mice. In support of this conclusion, gating on $V\alpha2^+V\beta6^+$ 'MUC1-specific' T cells indicated that the proportion of *MUC1-specific* $CD4^+$ T cells that are $FoxP3^+$ Tregs is the same in WT mice and VFT mice (Fig3-11b). The fact that $FoxP3^+$ cells represented a lower proportion of $Va2^+Vb6^+$ cells than $CD3^+CD4^+$ cells can be explained by the fact that the $Va2^+Vb6^+$ gate includes both CD4 and CD8 T cells (the latter are not expected to be $FoxP3^+$ Tregs).

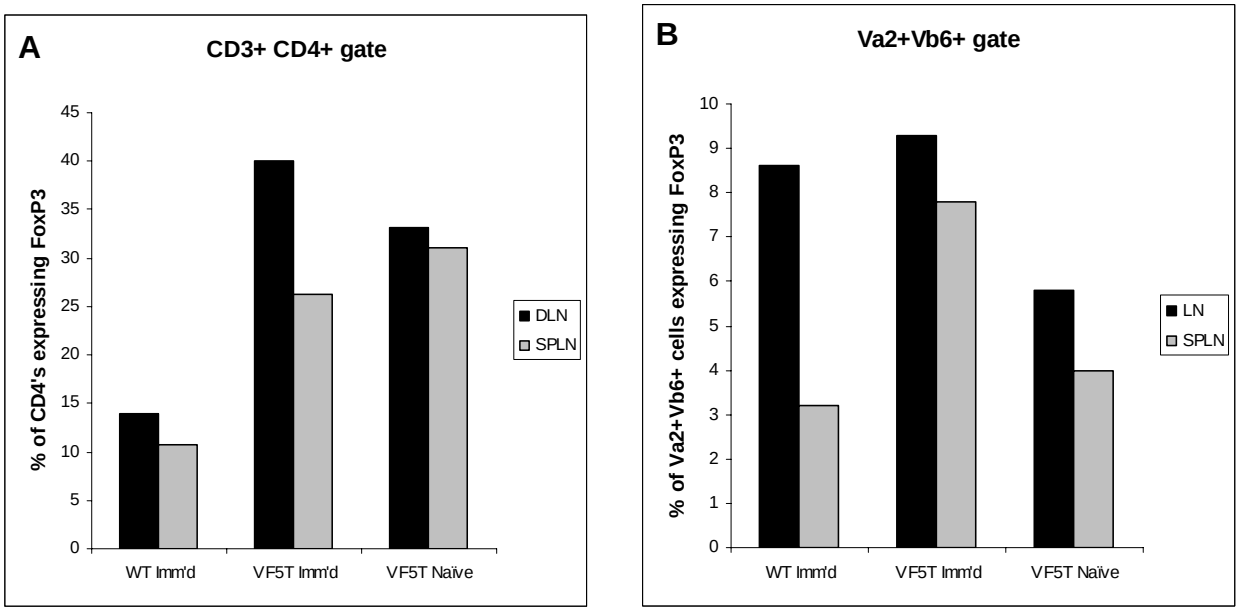


Figure 3-11. MUC1-loaded DC induce Tregs in VFT mice

WT and VFT mice were immunized sub-cutaneously with MUC1-loaded DC. The "Naïve" VFT mouse was injected with PBS. Two weeks after vaccination, inguinal LN were analysed by FACS for presence of $FoxP3^+$ cells.
A) Proportion of $FoxP3^+$ LN cells expressed as a percentage of total $CD3^+CD4^+$ T cells in the iLN.
B) Percentage of $V\alpha2^+V\beta6^+$ cells expressing FoxP3.

3.5.2. Adoptive immunotherapy with MUC1-specific $CD4^+$ VFT cells

Results in chapter 2 describe that, in our attempts to adoptively provide MUC1-specific Th cells for MUC1-Tg mice, immunization of WT donor mice expanded MUC1-specific Tregs, which suppressed immune responses upon transfer into MUC1-Tg mice. As all VFT cells are MUC1-specific, it is not necessary to immunize VFT mice to expand the number of MUC1-specific T cells prior to adoptive transfer. Given the increased number of MUC1-specific T cells in VFT mice, we reasoned that if naïve VFT cells are adoptively transferred into MUC1-Tg recipients, sufficient numbers should survive and become stimulated upon subsequent immunization to provide antigen-specific T cell help. Our hypothesis was that provision of MUC1-specific T cell help in this manner should improve MUC1-specific immune responses in MUC1-Tg mice. The results shown in fig 3-12 indicate that this was indeed the case, confirming our original hypothesis that adoptive transfer of MUC1-specific $CD4^+$ T cells into MUC1-Tg mice restores the MUC1-specific immune response to its full potential. This conclusion comes with the stipulation that Tregs must not be present among the transferred $CD4^+$ T cells. Otherwise they will dominate the Th cells and suppress the immune response to MUC1. Importantly, this experiment proves that naïve MUC1-specific $CD4^+$ T cells can function in the MUC1-Tg mouse, at least in the short-term.

Also, MUC1-Tg recipients of primed VFT cells exhibited marginally reduced immune responses to subsequent vaccination with MUC1-loaded DC. This is in keeping with our previous results from WT donor mice, and supports the claim that the $FoxP3^+$ $CD4^+$ T cells expanded by immunization of VFT mice (fig 3-11) are functional Tregs.

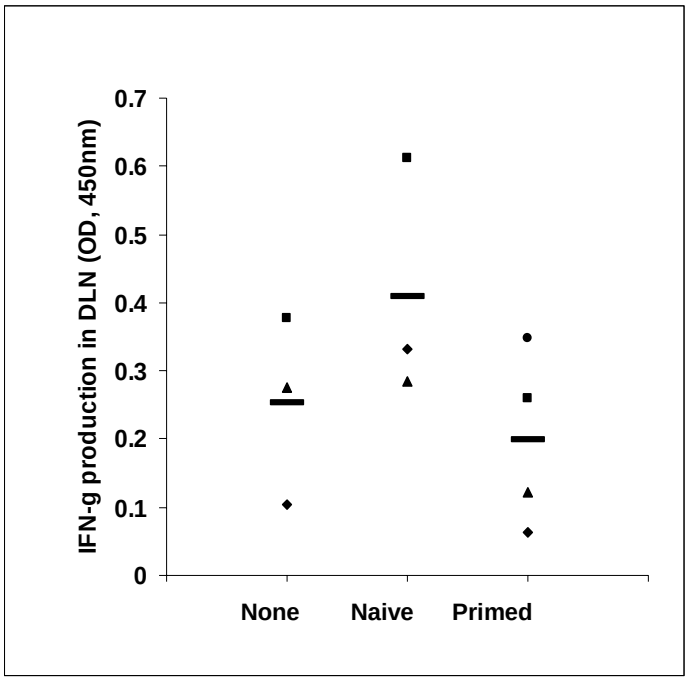


Figure 3-12. Naive VFT cells improve MUC1-specific immunity in MUC1-Tg mice

MUC1-Tg mice were given naïve or primed VFT cells, or no T cells, and then immunized with MUC1-loaded DC. 10 days post immunization, LN were harvested, stimulated in vitro with MUC1-loaded DC, then restimulated on day7. After 24 hours of stimulation, culture supernatants were harvested and tested by ELISA for presence of IFNγ. X-axis labels indicate the cells that were adoptively transferred.

3.6. Discussion

This chapter describes the generation and characterization of a novel TCR-Tg mouse, VFT, whose $CD4^+$ T cells express an MHC-class II restricted, MUC1-specific TCR. MUC1-specific T cells in VFT mice respond to stimulation with MUC1-loaded DC in a similar fashion to T cells from WT mice. VFT mice are therefore a useful tool for studying the behavior MUC1-specific $CD4^+$ T cells in a variety of settings. The first set of experiments performed with these new mice

were designed to confirm our observations from WT and MUC1-Tg mice (described in chapter 2), but in this case with a pure, MUC1-specific T cell population.

3.6.1. VFT cells expressing a WT TCR are effectively activated by MUC1

The OVA-specific TCR expressed endogenously by DO.11.10 hybridoma has a very low affinity for its cognate antigen. When DO.11.10 is stimulated by DC presenting OVA, IL-2 is produced in such low quantities that it can only be detected using IL-2 dependent cell lines (172). However, when DO.11.10 cells were transfected with the VF5 TCR$\alpha\beta$, they responded to MUC1-loaded DC with over 200pg/ml IL-2, which was easily detected by ELISA (fig 3-9). As the rest of the TCR signaling pathway in the hybridomas was the same, this result suggests that the MUC1-specific TCR cloned from VF5 has a high affinity for I-A^b-restricted MUC1, which is in keeping with the fact that the VF5 TCR was cloned from a WT effector T cell. As MUC1 is not expressed in WT or VFT mice, their T cells develop in the absence of any central or peripheral regulation, which most often results in deletion of the high affinity TCRs.

3.6.2. VFT $CD4^+$ T cells enhance MUC1-specific immune responses in MUC1-Tg mice

As all of the $CD4^+$ T cells in VFT mice are MUC1-specific, it is not necessary to immunize the donor VFT mice to expand the numbers of MUC1-specific T cells, so the problem of Treg induction is circumvented. We were therefore able to transfer naïve VFT cells into MUC1-Tg mice, knowing that all of the transferred cells are MUC1-specific, to determine their effect on MUC1-specific immune responses. Restoration of the MUC1-specific Th response in MUC1-Tg in this manner appears to provide the support needed for establishment of effective MUC1-

specific immunity. However, this experiment will need to be repeated with a larger number of mice. If confirmed, this result has important implications for the design of anti-cancer vaccines, as it is now clear that in addition to stimulating CTL, such vaccines must also contain a component for the stimulation of tumor-specific Th responses.

3.6.3. Naïve VFT $CD4^+$ T cells can be primed in MUC1-Tg mice

Naïve MUC1-specific T cells that have not differentiated into either Th or Tregs should be susceptible to any peripheral tolerance mechanisms that might exist in the MUC1-Tg mouse. The fact that naïve VFT cells can be primed in MUC1-Tg mice and function as effective Th cells indicates that, whatever tolerance mechanisms exist in MUC1-Tg mice, they do not appear to affect the activation of naïve $CD4^+$ T cells in the periphery, at least in the short term. This provides hope for a possible immunological treatment for $MUC1^+$ cancers. Assuming that MUC1-specific T cells are present (or provided exogenously), they can be stimulated to provide effective MUC1-specific immunity. This result also indicates that peripheral tolerance mechanisms that may exist in the MUC1-Tg mouse require some time to take effect. With this in mind, it would be prudent to administer MUC1-specific vaccines to young individuals, as this would increase the chances that naïve MUC1-specific $CD4^+$ T cells would be primed before they have been tolerized to self-MUC1. We are currently engaged in additional experiments using VFT and MUC1-Tg mice to test this hypothesis.

4. Conclusions and Implications

We have shown that the balance between $CD4^+$ T cell help and regulation is critical for establishing effective immune responses to MUC1+ tumors. In MUC1-Tg mice, MUC1-specific Th cells are not functional, so MUC1-specific Tregs that respond to MUC-DC vaccine are un-opposed. This results in suppression of MUC1-specific immune responses. In spite of this immunosuppression, naïve MUC1-specific $CD4^+$ T cells can be primed in MUC1-Tg mice to provide adequate help for the establishment of robust MUC1-specific CD8 T cell responses. The challenge now is to design the most effective approach of generating the MUC1-specific T cell help that is a pre-requisite for effective anti-tumor immunity.

4.1. Implications for adoptive immunotherapy of cancers

The Th-Treg balance is critical in the setting of adoptive immunotherapy. The data presented here are in agreement with other studies (71) which indicate that adoptive immunotherapy for $MUC1^+$ tumors involves more than simply transferring a heterogeneous mix of T cells into the patient. In order to dictate the desirable immune responses, the transferred T cells must be of a well-defined specificity and phenotype (i.e. Th cells, not Tregs).

We showed that adoptive transfer of MUC1-primed $CD4^+$ T cells results in selection of the Tregs over Th cells, presumably because Tregs are less susceptible to apoptosis than primed effector T cells (164). So $CD4^+$ T cells depleted of Tregs must be used instead. Assuming that observations from the MUC1-Tg mouse translate into humans, the risk of the MUC1-specific Th

cells causing autoimmunity is minimal because MUC1-specific Tregs are functional and will keep them in check.

Interestingly, whereas primed $CD4^+$ T cells suppressed MUC1-specific immune responses, naïve MUC1-specific $CD4^+$ T cells improved the immune response in MUC1-Tg mice. This suggests that naïve $CD4^+$ T cells, like Tregs, may be less susceptible to apoptosis than primed Th cells. In this case, it would be worth exploring alternative methods, such as TCR gene therapy, for increasing the frequency of MUC1-specific TCRs in the transferred population of naïve T cells. Studies are already underway in our laboratory and others to investigate better methods of delivering and expressing TCR genes to naïve T cells and T cell precursors (173-176).

4.2. DC-induced Tregs: Implications for design of anti-cancer vaccines

$CD4^+CD25^+$ Tregs were originally considered to be anergic, but recent studies, including those described here, indicate that their proliferation can be induced under certain circumstances (177,178). The experiments described here implicate DC in the conversion of naïve MUC1-specific T cells into $CD4^+CD25^+$ Tregs. A similar phenomenon has been reported in other systems (60,74,159,179). The outcome of the DC:T cell interaction is affected by the maturation/activation status of the DC and the milieu of costimulatory molecules it provides to the T cell (35,159,180,181). Originally only immature DC were implicated in the development of Tregs. However, it is now clear that mature DC and semi-mature DC also possess this tolerogenic capacity (182). Given the heterogeneous phenotype of the DC used to vaccinate the VFT mice (fig2-3), it is likely that the DC which converted naïve VFT cells into Tregs were a distinct sub-population from those DC which induced Th cells. If it is possible to tease apart the

maturation requirements for the different subsets of DC that induce Tregs and Th cells, this will aid the design of cancer-specific vaccines that favor the generation of Th responses over Tregs.

4.3. What happens to MUC1-specific T cells during development?

The generation of the VFT mouse in combination with the MUC1-Tg mouse provides a unique opportunity to study the development and behavior of MUC1-specific $CD4^+$ T cells in a setting where MUC1 is expressed endogenously as a self-antigen. The cause of the Th:Treg imbalance in the MUC1-Tg mouse is still unknown. Are high affinity MUC1-specific Th cells deleted in the thymus, while high affinity Tregs are allowed to survive and populate the peripehery? Or do they survive negative selection, only to be subjected to peripheral tolerance mechanisms? From the final experiment described here, one thing seems certain: Naïve MUC1-specific CD4 cells that have developed in the absence of MUC1 can function when MUC1 is expressed peripherally. This is evidenced by the fact that we were able to effectively prime naïve MUC1-specific $CD4^+$ VFT cells in the MUC1-Tg mouse. This provides conclusive evidence in support of a role for MUC1-specific $CD4^+$ T cells in enhancing the immune response to MUC1. All that remains is to find the most efficient way of generating those MUC1-specific Th cells *in vivo*. If this can be achieved, the possibility of a cancer vaccine capable of stimulating effective, MUC1-specific anti-tumor immune responses will become a reality.

APPENDIX A

Restriction enzyme analysis of pTβ-VF5β

The pTβ-VF5β TCR expression construct was made by inserting the cloned VF5 TCRβ fragment into the pTβ expression construct. This is described in detail in chapter 3. The integrity of the expression construct was tested by restriction enzyme analysis. When pTβ-VF5β was digested with KpnI, the resulting DNA fragments did not match the sizes that were predicted from the map of pTβ originally published by Kouskoff *et al* (166). According to this published map (and allowing for the insertion of my cloned TCRβ), digestion of pTb-VF5b with KpnI was expected to produce three bands of sizes 2.9kb, 4.75kb and 12.77kb. However, the observed fragments were 2.9kb, 5.2kb and 12.77kb (see figure below). This unexpected result was obtained repeatedly, using several pTb-VF5b clones.

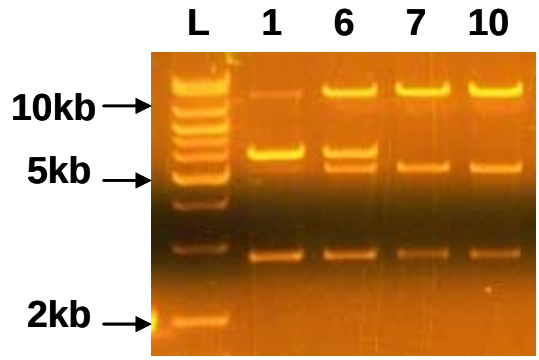


Figure 4-1 Restriction Enzyme Analysis of pTβ-VF5β

The pTβ-VF5β expression construct was propagated and cloned in Stbl4 *E.coli*. Plasmids were purified from several clones and digested with KpnI for 4hours. Resulting DNA fragments were resolved by electrophoresis in a 1% agarose gel containing ethidium bromide. Numbers indicate the Stbl4 clone from which the plasmids were purified. L,1kb DNA ladder. Digestion of clones 7 and 10 produced the correct size bands, as predicted by *in silico* analysis of the contig assembly sequence. pTβ-VF5β#10 was eventually used to generate the VF5β transgenic mouse. The ~6.1kb fragment in clones 1 and 6 is may have resulted from insertion of VF5β without removal of the original TCRβ from pTβ.

To resolve this discrepancy, I determined the predicted sequence of pTb-VF5b and located the exact restriction enzyme sites listed on the published map of pTβ. To do this, I referred to the description of pTβ in the Kouskoff manuscript, which lists the fragments of genomic DNA that

were assembled to produce the pTb expression cassette. Following this description, I obtained the sequences of those genomic fragments from the NCBI database, and made a contiguous sequence assembly of the pTb-VF5b expression construct, which included the sequence of my inserted TCR VF5b fragment. Then I identified the positions of the Kpn1 restriction sites within that construct. Using this *in silico* method, KpnI restiction enzyme sites were identified at positions 0(20.45kb), 5.35kb and 17.52kb. Complete digestion at these sites would produce bands of sizes 5.25kb, 12.17kb and 2.93kb. These predicted band sizes are in corroboration with the bands that I observed after KpnI digestion of the pTb-VF5b expression construct cassette. The published map therefore contains an error, such that the fragment upstream of the cloned VDJ fragment is actually 500bp larger than published. Figure 3-8 shows a predicted map of the pTb-VF5b expression construct used to make the VFTb mouse. This map includes the updated restriction enzyme sites allowing for the 500bp correction.

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