Regulation by IL-10 of innate immune responses to MUC1 as a self-antigen in MUC1 transgenic mice

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Immune responses against a peptide derived from the MUC1 extracellular domain are inhibited in mice which transgenically express the human tumor antigen MUC1 (MUC1.Tg mice). One of the hallmarks of this tolerance is an inability to generate robust CD4 T cell responses. However, this tolerance is not due to a lack of MUC1 specific effector T cells in these animals, as it is evident even after naïve MUC1 specific CD4 T cells have been adoptively transferred in prior to vaccination. Here we show that immediately following intravenous MUC1 vaccination in MUC1.Tg mice, splenic dendritic cell (DC) activation is suppressed. This is measureable both by reduced levels, compared to DC from vaccinated WT mice, of MHC Class II, CD40, and CD86 on the surface of these DC, as well as by the level of a new marker of DC activation: expression of traditional pancreatic enzymes. These enzymes, exemplified by trypsin 1 and carboxypeptidase B1, are up-regulated in splenic DC following MUC1 vaccination in WT, but not MUC1.Tg mice. Their suppression in MUC1.Tg mice requires the activity of both regulatory T cells and IL-10. IL-10's role in this system appears to be antigen specific as it is produced in the spleens of MUC1 vaccinated MUC1.Tg mice at higher levels than in the spleens of similarly treated WT animals. Furthermore, removal of IL-10 signaling from the system by pretreating animals with an antibody against the IL-10 receptor prior to MUC1 vaccination increases the MUC1 specific CD4 T cell response in MUC1.Tg, but not WT mice. The cellular source of this IL-10 was identified by flow cytometry as being natural killer (NK) cells. In addition to producing IL-10, NK cells from the spleens of MUC1.Tg mice post MUC1 vaccination are more cytotoxic and poorer at maturing DC in co-culture than NK cells from similarly treated WT mice. Depletion of these NK cells improves the quality of the MUC1 specific CD4 response in MUC1.Tg mice. Together, this data identifies a number of previously unidentified early factors

which are responsible for the observed inability of MUC1.tg mice to generate robust MUC1 specific CD4 T cell responses.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

This thesis is dedicated to my parents, who have given me more to be proud of than I have given them; to my wife Anna, who has supported and loved me through one of the oddest first three years of marriage imaginable; to my friend and former mentor, Duane Sewell, who's nurturing influence helped me rediscover my love for science, and whose impact on my life and on the lives of others shines bright even is his absence; to Olja, who has taught me that being a great scientist is not incompatible with being a great spouse, friend, parent, child, etc.; and to Sammy, because meow.

1.0 INTRODUCTION

1.1 THE IMMUNE RESPONSE TO CANCER

The power of the immune system to combat cancer has been recognized for over a century now. In 1891, William Coley conducted what could be considered one of the first trials designed to boost the immune response against cancer, in which *Streptococcus Pyogenes* and *Serratia marcescens* were injected intratumoraly. The work, which was attempting to reproduce a phenomenon observed when some sarcoma patients would undergo spontaneous remission following certain *Streptococcus* infections, showed sporadic efficacy despite little understanding of the underlying mechanism. (1, 2) We now have a much stronger grasp on how the immune system and cancer interact, and how tumors can be targeted for successful eradication by the immune system.

1.1.1 **Antigen Specific Targeting of Tumors**

The specific targeting of cancer cells by the immune system represents a unique challenge when compared to pathogens (e.g. bacteria and viruses). Tumors can be classified into two broad categories on the basis of antigenicity. The first category is tumors that express a foreign antigen, not encoded in the host organism's DNA. Cancers expressing antigens of this type are typically

virally induced. Human examples include human papilloma virus associated cancers, predominately of the cervix and head and neck (3, 4), Merkel cell polyomavirus associated Merkel cell carcinomas (5), Hepatitis B associated hepatocellular carcinoma (6), and Epstein-Barr induced nasopharyngeal carcinoma (7), Specific targeting of these cancers is possible via viral specific proteins expressed by the cancer cells. For instance, HPV associated tumors may be targeted via the viral proteins E6 and E7 (8). These viral products are both specific to the virus and necessary to maintain the transformed phenotype of the tumor cells (9).

The second category with regards to antigenicity is tumors that do not express a foreign antigen. In these tumors, all cellular proteins are encoded by the host organism's DNA. The initial assumption was that these tumors could not be eliminated via specific immune recognition. However, the advent of inbred rodent strains demonstrated that protection from genetically identical transplanted tumors was feasible through vaccination (10-12). Antigens of this type typically derive from proteins that have undergone significant alterations in comparison to their expression in normal cells. These changes include increases in quantity, post-translational abnormalities, germ mutations, and re-expression of proteins not typically expressed in adult tissue, such as cancer testis anitgens and oncofetal proteins, which are normally expressed only in the germline cells of the adult testis or during fetal development, respectively, but which are expressed in a number of tumors (13). The specificity of targeting these different types via the immune system and the level of unwanted off-target reactivity varies amongst subtype (Table 1).

Overexpressed tumor antigens, of which Her2/neu and EGFR are examples, can be targeted because their level of expression surpasses the threshold required for T cell activation (14-16), whereas it does not in normal, non-transformed, cells. However, the expression of these

antigens in normal tissue does raise two important issues. There is still a chance of vaccine or immunotherapy related autoimmunity and the presence of these antigens on normal tissues serves to tolerize the immune system against them (17).

Post translational changes include changes in glycosylation (18). The ability to target these, as well as a prominent example of an antigen possessing such alterations is discussed in detail in the "MUC 1" section of this dissertation.

Mutated genes, of which p53 and β-catenin are common examples, are targetable based on their unique mutations creating new epitopes which are no longer seen as self proteins. The drawback with antigens of this type is that their uniqueness requires that immunotherapies against them be patient- and tumor-specific, although there are a number of common mutations against which vaccination is possible. For instance, EGFRvIII is a common mutation of the EGFR gene, in which exons 2-7 are deleted. (19) It is constitutively active and contributes to the growth and survival of cancer cells through activation of the MAPK pathway and up-regulation of BCL-$X_{L,}$ respectively (20). Vaccination with a unique peptide generated by this mutation (PEP-3) has shown efficacy in rejecting tumors containing this mutation (19, 21).

Oncofetal and Cancer testis antigen are distinct, but will be considered together here because they share several important qualities. Both can be targeted based on tumor specificity. Oncofetal proteins are specific to tumors as they are not expressed in adult tissues. Cancer testis antigens include MAGE-1, the first gene identified to encode a tumor specific protein that can be recognized by T cells (22). These antigens are expressed in spermatocytes/spermatogonia in the testis (23), tissues that do not express MHC Class I molecules (24), thereby making these antigens "tumor-specific" with regards to how the immune system perceives them.

A final category of antigens not yet mentioned are lineage restricted antigens. Prominent examples include the melanoma antigen tyrosinase and prostatic acid phosphatase (PAP), against which the first active immunotherapy was recently approved (25). Lineage-restricted antigens are present in normal tissues, but their expression is limited to certain tissues. Immune reactions against antigens of this type commonly affect normal tissues, but are considered acceptable in light of the benefit provided by the immune response. For example, vitiligo, caused by autoimmune destruction of normal melanocytes, is a common and manageable side effect of successful melanoma vaccines against lineage restricted proteins such as tyrosinase and its related proteins, gp100, and MART-1, and is a positive prognostic marker (26, 27).

Table 1: Cancer antigen categorization

Antigen category	Examples	Tumor Specificity	Expression in Normal Cells
Viral	E6, E7	+++	N/A
Overexpressed	Her2/neu, Cylcin B1	-	On a wide variety of tissues, however at lower levels
Post-translational abnormality	MUC1	+	Low to none
Mutated	β-catenin, p53	+++	N/A
Oncofetal/Cancer Testis	CEA, MAGE, NY-ESO-1	+/-	Expression during fetal development/in the testis (which lack MHC Class I)
Lineage Restricted	Tyrosinase, PAP	-	Lineage restricted expression in normal tissue

Ref (17, 23)

1.1.2 **Active Immunotherapy/Vaccination**

Aside from proper antigen targeting, successful active tumor immunity also requires engagement of the cellular immune response. In its most basic form, this requires the acquisition of tumor antigen from tumor site by dendritic cells (DC), their activation and migration to the draining lymph node, priming of tumor specific T cells in the draining lymph node, and finally the migration of these activated T cells to the tumor site, where they can directly lyse the tumor and support its destruction through innate activation (1). Tumor antigen acquisition by DCs is usually facilitated through necrotic or apoptotic cell death (28-31). Tumor antigen can also be provided exogenously in a therapeutic setting. Defined tumor antigens or lysates from the tumor can be provided via immunization in order to boost the immune response. The major challenge in these vaccinations is providing the antigen in such a way that it is immunogenic and which allows antigen uptake by maturing dendritic cells, as they are necessary for the initiations of a strong cellular response (32).

Exogenous antigens can be delivered in several ways. The easiest is through administration of immunogenic peptide. The advantage of this method is that it is cheap, stable, and the immunodominant epitopes can be selectively administered. However, peptide binding to MHC molecules is allele-specific, so the utility of a given peptide is highly variable in a large patient population (33). Additionally, providing CD8 T cell epitopes alone can actually induce tolerance and promote tumor outgrowth (34, 35). Longer peptides are preferable because the uptake and processing required for their presentation allows these peptides to be presented almost exclusively on dendritic cells, and not on less immunogenic T and B cells (36, 37). Full-length proteins are another option for immunization. Unlike vaccination with peptide, full-length protein includes multiple epitopes that could bind a wider range of MHC molecules. However,

full-length proteins are expensive to produce and require targeting to DC to optimize T cell responses. This can be achieved by conjugating the protein to an antibody against the DC endocytosis receptor DEC-205. Such a strategy results in improved CD4 and CD8 T cell responses and improved tumor protection that outperforms non-conjugated vaccines containing a 1000 fold higher dose of antigen (38, 39). Similar strategies have been explored utilizing CD11c, DC-associated C-type lectin-1, mannose receptor, CD36, and DC-SIGN as targets for delivering antigen to DC (40-45). Viral expression vectors are another strategy to provide antigen for tumor vaccination. These methods take advantage of natural immune activation in response to viral infection. A major advantage of this system is the ability to include immunomodulatory molecules in the vaccine vector, such as GM-CSF, IFNγ, costimulatory molecules like 4-1BBL and CD80/86, etc., all which can greatly increase antitumor immune responses (46, 47). A major concern with viral vectors is that the vectors themselves are so immunogenic and in some cases natural exposure is so common (48) that vaccine responses against the vector will preclude immunity against the inserted tumor antigen. (46) Hence, the most straightforward way to ensure proper delivery of antigen to dendritic cells is to load them directly *ex vivo*.

Preloading DCs requires production of autologous DC from CD34+ hematopoietic progenitors or from peripheral blood monocytes from the patient to be vaccinated (49, 50). DC are then loaded with antigen and matured *ex vivo* and transferred back into the patient for vaccination. This method allows for exquisite control over DC loading and maturation, creating DC capable of priming distinct immune responses. For instance DC matured in the presence of type I interferon are highly capable of inducing TH1 cell differentiation and proliferation (51-54) while IL-15 leads to more efficient CTL responses and confers DC with a qualities associated with Langerhans Cells (55). The ability to control the activation status of the DC presenting the

antigen is a great advantage of using *in vitro*-expanded, matured and activated DC for vaccine delivery. Presenting antigen on improperly activated DC is detrimental to vaccine efficacy, as will be discussed in detail in the "Peripheral Tolerance" section below. The main drawback of DC vaccination is that it is labor intensive, expensive, and patient specific.

Once antigen has been acquired by DC and the DC have been properly matured, either *in vitro* or through inclusion of a vaccine adjuvant, they must traffic to lymph nodes and activate CD8 and CD4 T cells. Because of their natural ability to lyse target cells, CD8 T cells have been the focus of many immunotherapeutic efforts. Tumor-specific CD8 T cells recognize tumor-antigen derived peptides presented on MHC Class I. They can then directly lyse the tumor cells through perforin/granzyme and FasL mediated induction of apoptosis (56, 57) and can increase the immunogenicity of the tumor microenvironment. This can be achieved through secretion of IFNγ, which can further activate CTL function (58, 59), activate macrophages (60), up-regulate DC MHC Class II and costimulatory molecule expression (61-63), activate NK cells (64), and up-regulate MHC Class I expression while inhibiting growth of the tumor (65). Tumor cell lysis by CD8 T cells further promotes tumor immunity by increasing the pool of tumor antigens available for immune priming, while DC activation induced by IFNγ secretion provides an immunostimulatory environment that propagates proper immune activation. Furthermore, both macrophages and NK cells are capable of contributing to tumor immunity by directly lysing tumor cells. Macrophages and NK cells can also produce TNFα, IFNγ (66), and IL-12 (67), which polarize type I helper responses (68) and recruit NK cells (69).

Although often overlooked due to their relative inability to directly lyse tumor cells and concerns raised about the potential induction of regulatory T cells, CD4 T cells are also required for optimal anti-tumor immunity. CD4 T cells can directly lyse tumor cells in some models (70)

and they are required for CD8 T cell priming (71, 72) and formation (73-75) and maintenance of memory (76). Therefore optimal CD8 T cell activation, which can reject tumors, requires concomitant CD4 help. One mechanism involved in this help is CD40:CD40L initiated activation of dendritic cells, which "licenses" them to prime optimal CD8 T cell responses (77-79). CD40-matured dendritic cells will also prime responses against antigens acquired in the tumor microenvironment. CD40 priming can also activate macrophages, which when activated via CD40:CD40L ligation, can control cancer growth even in the absence of T cells (80). Indeed the cytotoxic activities of macrophages have been shown in multiple systems to be an integral part of CD4 induced tumor immunity, especially by directly killing tumor cells via nitric oxide and superoxide (81, 82). It should be noted as well that all functions associated with CD8-produced IFNγ are also mediated by CD4 T cells, which are likewise capable of making this cytokine.

1.1.3 **Passive Immunotherapy**

The type of immunotherapy that is not predicated on de novo generation of tumor-specific immune responses is passive immunotherapy. The prime example is administration of monoclonal antibodies against surface expression of multiple tumor antigens. Typically, these antibodies are humanized and directed against antigens overexpressed in cancer, such as Her2/neu (Trastuzumab) and EGFR (Cetuximab) (83, 84). Originally, there was some controversy over how these antibodies worked in controlling tumor growth. As a number are against growth receptors with function in cell proliferation, direct inhibition oft downstream signaling was proposed as a mechanism (85, 86). However, mice deficient in Fc receptor expression fail to show significant protection in response to monoclonal antibody treatment and

mice lacking the inhibitory receptor FcγRIIB show improved responses (87). Analysis of clinical trial participants treated with Trastuzumab showed that patients with a FcγIIIa polymorphism that allows for tighter binding of IgG1 (88, 89) had improvements in response rate and progression free survival, compared to similarly treated patients without this allele. (90) Similar results were seen with Cetuximab (91) and Rituximab (92, 93). These finding implicated NK-mediated ADCC as an important mechanism of action for monoclonal antibody therapies, specifically through lysis of antibody tagged tumor cells (94). Complement mediated killing has likewise been implicated both directly in preclinical models (95, 96) and correlatively through an association between C1qA polymorphisms and clinical response to rituximab (97).

Passive immunotherapy is not completely passive however. Once again, the introduction of tumor lysate improves the pool of available tumor antigens for processing and presentation by dendritic cells, and the production of activating cytokines such as IFNγ and TNFα from NK cells creates an immunogenic environment capable of priming macrophages, DC, and through DC CD4 and CD8 T cells specific for tumor antigens. Furthermore, antibody coated tumor antigens allow for efficient antigen uptake, IL-12 production, and cross-priming by dendritic cells (98-100). Together, these effects contribute to a vaccine like effect caused by treatment with monoclonal antibodies that takes months to reach maximal effect (101).

1.1.4 **Immune Evasion and Suppression**

Early studies indicated that elimination of solid tumors by the immune system was feasible, but objective clinical successes of therapies that depend on the immune system have been few. This is largely attributed to the process of cancer immunoediting, which occurs in solid tumors prior to their clinical diagnosis (102). Fundamentally, the development of solid tumors is dependent on

and shaped by the immune system. Therefore, in most cases, attempts to induce an immune response post cancer diagnosis are limited by mechanisms that these tumors have necessarily acquired in order to evade immune destruction. A striking observation in support of this theory comes from the fact that carcinogen-induced tumors grown in RAG-/- mice, which lack adaptive immunity, grow progressively when transplanted to RAG-/- hosts, but are largely rejected in WT hosts. In contrast, tumors induced in WT mice are not rejected, owing to the fact that they have acquired resistance to immune destruction in the first immunocompetent host (103) Similar results were also seen using the immunocompromised nude and SCID mouse models in similar experiments (104, 105). Tumor immune suppression and escape involves many other factors in addition to the intrinsic resistance of the tumor itself. By the time the tumor has established itself in the host, the whole tumor microenvironment facilitates its survival and progression, including the recruitment of a number of immunosuppressive cells.

1.1.4.1 Intrinsic mechanisms of immune evasion

One of the hallmarks of cancer is the ability of tumor cells to avoid detection and elimination by the immune system (106). Tumors develop resistance to IFNγ signaling (107), and can downregulate expression of MHC class I itself (108-113), as well as MHC class I peptide processing machinery (TAP1, tapasin, LMP2, β_2m, etc.) (109, 114), in order to avoid recognition and lysis by CD8 T cells. Depending on the necessity of the target antigen to tumor function, antigenic loss can also prevent CD8 T cell mediated lysis (115-117). Normally, loss of MHC Class I expression would trigger recognition and removal by NK cells (118). However, tumors may resist to killing by NK and CD8 T cells by gaining resistance to killing mediated by TRAIL (119), perforin/Granzyme B (120), and FasL (121, 122). Tumors can also directly alter the

cytotoxic capacity of NK cells by secreting soluble ligands for NKG2D. NKG2D, normally responsible for activating and directing NK mediated killing, is down-regulated upon constitutive activation as is the case here. Tumors can also activate killer inhibitory receptors (KIR) (123, 124).

In addition to direct resistance to immune mediated recognition and cytotoxicity, tumors are also able to modulate their microenvironment to prevent immune rejection. Some tumors constitutively express indoleamine 2,3-dioxygenase (IDO), which metabolizes tryptophan and suppresses T cell responses (125, 126). Tumors can secrete VEGF, which inhibits T cell responses by stimulating endothelial cells to produce IL-10 and PGE_2 (127, 128). Tumors can also secrete TGFβ. TGFβ inhibits T cell growth and IL-2 production (129, 130), skews CD4 T cells to induce the generation of regulatory T cells (131-133), and inhibits CTL effector generation and function (134, 135). It is also capable of reducing NK cell IFNγ, GM-CSF, and TNFα production and killer activity (136-138), and can lower MHC Class II expression and IL-12 production in response to TLR mediated DC maturation (139). Tumors also mitigate immune destruction by creating a physical barrier against immune cell migration and can down-regulate the expression of T cell attracting cytokines to prevent homing of T cells to the tumor site (140), or induce post-translational modifications on said cytokines to the same end (141). Tumor vasculature is less conducive to T cell adhesion and migration (142-144) and often expresses molecules like Tim-3, PD-L1, and FasL (145, 146), which actively suppress infiltrating T cells.

1.1.4.2 Extrinsic mechanisms of immune evasion

The ability of tumor cells to prevent immune recognition and destruction is not all mediated by the tumor and its environmental restrictions. Perhaps the most effective and versatile arm of

tumor immune evasion is the rich tapestry of immune suppressor cells which are induced in, or recruited to, the tumor microenvironment.

Dendritic cells

Dendritic cells are well known as the most important cell type for presenting antigen to T cells and for initiating strong effector and memory T cell responses (32). However, in the tumor microenvironment, DC take on a regulatory role, preventing proper immune effecter generation and function (147). DC from tumor sites or those treated with tumor supernatents display lower MHC Class II expression, reduced B7 family expression, and a reduced capacity for generating tumor specific CTL responses (148, 149), but an increased ability to induce T cell anergy (150). Tumor-derived IL-10, TGFβ, and PGE_2 all contribute to the regulatory properties of tumor infiltrating DC, such as inducing low expression of MHC Class I and II, low costimulatory molecule expression, and low IL-12p70 production (150-154). In addition to their low stimulatory capacity, regulatory DC also actively contribute to immune suppression via production of IL-10, TGFβ, IDO, and COX-2 (155).

Regulatory T cells

Increases in the number of regulatory T cells (Treg) are seen in patients with a number of different cancers (156-160) and their presence in the tumor microenvironment is correlated with a reduction in overall survival (161). The presence of regulatory T cells in tumors is caused by an increase in regulatory T cell recruitment through local production of CCL22 (161), and through de novo generation of new regulatory T cells by priming naïve T cells with DC whose maturation has been inhibited by the presence of anti- inflammatory cytokines (i.e. IL-10 and TGFβ) at the tumor site (162, 163). Treg inhibition in tumor sites can be mediated by IL-10,

TGFβ, CTLA-4, and IL-2 deprivation. IL-10, produced by Tr1 cells and intratumoral Foxp3+ Tregs, can inhibit MHC Class II expression on and IL-12 secretion by DC, as well as expression of the costimulatory molecules CD80, CD86 and CD40 (164-167), therefore stimulating the generation of new Treg. TGFβ can inhibit killing by both CTL and NK cells at the tumor site. (168, 169) CTLA-4 interacts with B7 molecules on DC surfaces, induces the production of IDO (170), and has been shown to physically remove costimulatory molecules from the surface of DC (171). IL-2 deprivation occurs through high expression of CD25 on Treg surfaces and has recently been shown to inhibit NK cell activation *in vivo* (172-174).

Myeloid derived suppressor cells

Myeloid derived suppressor cells (MDSCs) are identified as CD11b+MHC II-/lowLy6C+Ly6G- and CD11b+MHC II-/lowLy6ClovLy6G+ cells in mice and CD11b+CD14+CD15-HLA-DR-/low and CD11b+CD14lowCD15+HLA-DR-/low cells in humans. The two different lineages represent monocytic-MDSC (Mo-MDSC) and granulocytic-MDSC (PMN-MDSC) respectively (175). The level of circulating MDSCs in cancer patients is directly correlated with tumor burden and stage (176) and is associated with less effective immune responses (177-179). MDSCs are induced *in vivo* by tumor derived factors such as VEGF, PGE_2, GM-CSF, IL-1β, and IL-6 (180-182) and are recruited to tumors sites by CCL2. (183) MDSCs directly inhibit T cells through the production of reactive oxygen species (ROS) and NO (184), by IL-10 (185), by depletion of L-arginine via high expression of arginase (186, 187), and by inducing the differentiation of suppressive regulatory T cells (188, 189).

Tumor associated macrophages

Macrophages are recruited to tumor sites by CCL2, M-CSF, and VEGF produced in the tumor (190-193). Once at the tumor site, macrophages encounter locally produced IL-10, TGFβ, PGE_2, and IL-4, which skews macrophages towards an M2 like phenotype (194, 195) characterized by IL-10 and IL-1β production (196). Tumor associated macrophages (TAM) then suppress immune responses through secretion of their own IL-10, TGFβ, PGE_2 and VEGF. (194, 195, 197, 198) TAMs also play a role in the angiogenesis and tissue remodeling required for the sustained growth of large tumors by producing EGF, FGF family members, and VEGF (195, 199) and by secreting proteases such as MMP-9 (200, 201).

1.1.5 **Prophylactic Vaccination**

In light of such extensive immune inhibition, certain considerations have to be made in the design of proper anti-cancer immunotherapies. The extensive network of immune suppression common to cancer represents a barrier between the idea and the practice of tumor immunology. There are at least two solutions to this problem. The first is to address the immune suppression during vaccination. For instance, given the role of regulatory T cells in tumor immune suppression, some studies have explored depleting this population of cells or blocking its effector functions, either alone or concurrent with immunotherapy, in an attempt to boost responses. This approach has shown both preclinical and early clinical success (202-206). Examples of similar strategies are being applied to many aspects of the suppressed and diminished immune response against established tumors (207).

Another solution, and the one favored by our group, is to avoid immune suppression by vaccinating in the pre-malignant stage or even earlier, boosting the immune response against developing tumors which have not yet acquired the ability to escape it. The precedent for such

treatment has been established. Somewhat recently, approval was granted to Gardasil®, a quadrivalent human papilloma virus-specific vaccination intended to prevent cervical cancer in women (208-210). Along with the success of the HBV vaccine, which has, through prevention of hepatitis B infection, reduced the incidence of hepatocellular carcinoma (211), this emphasizes the impact that cancer prevention through vaccination can have.

The difference between a true prophylactic cancer vaccination and the two mentioned above is that the latter are not truly targeting tumors, but instead viruses with well-known tumorigenic properties. Vaccines against true cancer antigens as discussed above have yet to be approved, although preclinical success is certainly promising and spontaneous tumors can be prevented using multiple antigen systems (212). A major consideration moving forward with tumor vaccines is that the antigen must be carefully selected in order to optimize cancer prevention while limiting autoimmunity (213). An ideal vaccine antigen should be fairly ubiquitous in its expression on multiple tumor types. Immune responses against the antigen must be able to mediate rejection. Ideally, the vaccine would also target an antigen that drives, or is in some way necessary, for tumor growth to limit the possibility of the tumor escaping immune recognition by deleting or mutating said antigen. Most importantly for regulatory approval, immune responses against the antigen must not be associated with autoimmunity, as a healthy population is to be vaccinated and such events will not be as tolerated when they are not weighed against the risk of active cancer.

1.2 MUC1

MUC1 satisfies all of the criteria described above for an ideal prophylactic cancer vaccine antigenic target. Recent attempts to prioritize cancer vaccine antigens based on their therapeutic effect, immunogenicity, oncogenicity, specificity, expression level, stem cell expression, number of patients with antigen-positive cancers, number of epitopes, and cellular localization identified MUC1 as a high priority vaccine antigen (214). These qualities, as well as recent clinical and preclinical findings regarding MUC1 vaccination are discussed below.

1.2.1 **MUC1 Biology**

Epithelial mucins, of which MUC1 is a family member, serve as a protective barrier between epithelial cells and the harsh environment of the aerodigestive tract. They protect against mechanical distress, diffusion of low pH solutions towards epithelial barriers (215-217), microbial colonization, and they serve to lubricate and hydrate the epithelial layer (218-220). All mucins are characterized by a variable number tandem repeat (VNTR) motif which contain between 5 and 100 O-gylcosylation sites (serine, threonine, and proline residues), with the number of domain repeats falling somewhere between 5 and 500 (218). Mucins come in two variations: secreted and non-secreted. Secreted mucins are released from cells and form a complex matrix that contributes to the gel barrier functions listed above. Non-secreted mucins extend from the cell surface, sometime reaching lengths of 100nm or more, and contribute to this same barrier (219). In addition to the secreted and/or extracellular domain whose characteristics and function are described above, mucins also contain a transmembrane C-terminal domain that is responsible for addition cellular functions. Cleavage of the C-terminal domain and the large

glycosylated N terminal domain is achieved via the auto-proteolytic SEA domain in most mucins (221), followed by non-covalent re-association (222). Depending on the family member, the C terminal domain may also play a significant role in cell signaling and survival, inflammation, and cancer (218, 219).

MUC1 belongs the membrane-bound family of mucins. However, shedding of the extracellular domain is common and mediated by intracellular proteases, as MUC1 is highly resistant to cleavage by extracellular protease addition (223-225). Despite the fact that MUC1 appears to have evolved from the secreted mucin MUC5B, MUC1 shares very little sequence homology with other mucins, aside from the presence of a SEA domain. However, in MUC1 this domain is unique in that it evolved from the heparin sulfate proteoglycan of basement membrane (HSPG2), which is a known tumor driver which activates the Sonic Hedgehog pathway (226-228).

The C terminal domain of MUC1 (MUC1-C) possesses a number of functions in addition to formation of the extracellular mucinous barrier by the N terminal domain. MUC1-C can induce the expression of galectin-3, which subsequently acts as a bridge for the interaction of MUC1 with EGFR, which mediates increased nuclear localization and chromatin binding of the latter (229, 230). EGFR can phosphorylate MUC1-C, increasing its interactions with both c-Src and β-catenin (231). Interactions with MUC1-C can subsequently block phosphorylation of β-catenin by GSK3β and prevent its degradation (232). Furthermore, MUC1-C/β-catenin heterodimers can translocate to the nucleus and directly activate Wnt pathway genes (233). MUC1 can interact with p53 in such a way that cell cycle arrest functions are maintained but apoptotic induction of BAX is inhibited (234). MUC1 can also transcriptionally repress p53 by recruiting HDAC1 and HDAC3 directly to the p53 promoter region (235). MUC1 can bind

directly to BAX to prevent its dimerization and localization to the mitochondria (236). MUC1 can also bind to the DNA binding domain of ERα and enhance transcription of ERα target genes (237). Both MUC1-C and the VNTR region have been shown to function in NF-κB signaling by directly interacting with p65 and by activating IKKβ (238-240). Overall, MUC1 has a diverse signaling profile. Given the role of many of these pathways in cell survival and in oncogenesis, it is easy to see why aberrant MUC1 expression is such a common feature in cancer.

1.2.2 **MUC1 as a Tumor Antigen**

MUC1 is estimated to be aberrantly expressed on about two thirds of the cancers diagnosed in the US each year (219). MUC1 expressing tumors encompass a wide variety of cancer types, including most carcinomas and a number of haematological malignancies. The level of circulating MUC1 acts as a prognostic indicator for treatment responsiveness. MUC1 and β-catenin co-expression at the invading edge of colorectal cancer is associated with reduced survival (241). Increases in MUC1 correlate with progressive cancers whereas decreases in MUC1 indicate treatment-responsive disease in breast and a number of other cancers (242, 243). MUC1 expression in thyroid cancers is correlated with decrease in 5 year relapse free survival (244). Gene signatures and lipid metabolism signatures identified in MUC1 transfected 3Y1 cells predict poor prognosis in breast and lung cancer and increases in death and distant metastasis in breast cancer, respectively (245, 246).

The prognostic value of MUC1 is likely due to its extensive activity as a driver of tumor development and survival. In support of this, a mouse model of spontaneous pancreatic cancer shows greatly reduced tumor burden and metastasis in MUC1-/- mice with concurrent increases in survival (247). Transfection of 3Y1 cells with a MUC1 expressing vector is sufficient to allow

anchorage-independent cell growth and enhancement of tumorogenicity in nude mice (248). This effect is mediated in part by β-catenin as mutation of the serine rich motif required for association of these two molecules attenuates this effect (232, 249). MUC1 activation of the β-catenin pathway, known to be important in cancer development (250-252), has also been shown to promote epithelial to mesechymal transition (EMT) in pancreatic dunctal adenocarcinoma, characterized by up-regulation of Snai1 and Slug, which transcriptionally repress E-cadherin expression (253). β-catenin activation by MUC1 also affects E-cadherin mediated adhesion by disrupting the β-catenin-α-catenin-E-cadherin complex, which is also affected by steric inhibition by MUC1 of E-cadherin junctions in a manner which is dependent on the size of the VNTR domain (254, 255). Disruption of these junctions is important for the loss of polarity and epithelial cell integrity associated with MUC1 associated tumors. Additionally, MUC1 interactions with HIF-1α were shown in some models to up-regulate PGDFA, a poor prognostic marker in pancreatic ductal adenocarcinoma. MUC1 can also up-regulate prolyl hydroxylase 3 (PHD3) and promote HIF-1α degradation, thereby decreasing hypoxia induced apoptosis (256, 257). In fact nearly all of the pathways with which MUC1 interacts (detailed in the "MUC1 Biology" section) are known to be co-opted by cancers.

Given the diverse functions MUC1 can play in tumor development and the association of its overexpression with poor clinical prognosis, it is a promising target for immunotherapy. Indeed, detectable MUC1-specific immune responses are associated with good clinical prognosis in a number of different malignancies. In pancreatic cancer, serum MUC1 specific IgG levels were significantly correlated with survival time (258). In breast cancer anti-MUC1 antibodies were associated with reduced incidence, increased time to metastasis, and increased disease specific survival with predominantly local recurrences (259, 260). In ovarian cancer, elevated

anti-MUC1 antibody levels and increases in insults known to raise the levels of such antibodies, are associated with lower overall risk of occurrence (261, 262). In gastric cancer, IgG antibodies against MUC1 or the MUC1 related TF glycotype were both associated with increased survival (263). All of these studies suggest that a MUC1 specific immune response may improve survival in cancer patients, and perhaps prevent cancer in healthy individuals or those with preclinical disease.

MUC1 specific CTLs have been found in the tumor draining lymph nodes of patients with pancreatic, breast, and ovarian cancer (264-266). The immunodominant region to which this reactivity is directed is the VNTR region of the extracellular portion of MUC1. The heavily repeated nature of this domain, and its crucial role in tumorogenesis and survival (reviewed above), make the likelihood of mutational escape to avoid immune detection low. In addition to being the immunodominant region of MUC1, this region allows for tumor-specific targeting. Changes in glycosylation allow binding of tumor MUC1 specific antibodies and lysis by CTL specific for the tumor associated glycoform (265, 267). Of interest, processing and presentation of this region is blocked in healthy cells. The heavily glycosylated nature of the antigen hinders its ability to prime CD4 and CD8 T cells (268, 269). The mechanism behind this is a block in intracellular processing caused by high avidity interactions with the mannose receptor on dendritic cells, which causes the antigen to recycle back to the cell surface instead of trafficking to the late endosome (270). This mechanism is highly dependent on the level of glycosylation, which is greatly reduced in the tumor form of MUC1. Another level of tumor specificity is evident in the finding that tumor-specific glycosylation motifs are not necessarily removed during antigen processing. This allows both for their presentation on the antigen presenting cell surface, and for the priming of CD4 T cells that specifically recognize the tumor glycosylated

species (271, 272). These glycoforms are seen as foreign and can break tolerance to MUC1 in preclinical models (272-274).

1.2.2.1 Clinical Trials of MUC1 Vaccines

MUC1 based immunotherapy has been used in clinical trials for nearly two decades and has been tested in breast, lung, pancreatic, prostate, ovarian, kidney, and colorectal cancer. A recent review of the outcome of these trials has highlighted several promising findings. The first of these is that MUC1 vaccination is well tolerated. As would be expected by the tumor specific nature of MUC1 targeting, no overt autoimmunity has been observed (220). Importantly, this is true even when MUC1 specific CTL are cultured *ex vivo* and transferred into patients, indicating that the lack of autoimmunity is not due to failure to generate a MUC1 specific response (275-277). Of the trials where clinical outcomes were measured, several showed signs of improvement with MUC1 vaccination. Stage II breast cancer patients had a significant reduction in relapse at 5 and a half years (27% in the control group versus 0% in the treated group) after vaccination with MUC1 conjugated to oxidized mannan. Additionally, 9 out of 13 people patients in the vaccinated group had MUC1 specific IgG and 4 out of 10 had MUC1 specific T cells, whereas neither of these was present in the unvaccinated group (278). One of the adoptive therapies mentioned above was able to induce a complete response in a stage IV pancreatic cancer patient with lung metastases and stable disease in 25% of the trial group (277). A liposomal based MUC1 vaccine was shown to increase median survival time by 4.2 months and 3 year survival rates from 17% in the control group to 31% in the treated group. The effect was more pronounced in patients who only had loco-regional disease, as the median survival increased by 17.3 months with a 3 year survival rate of 49%, compared to 27% in the control group (279). Two studies conducted by our lab, involving the vaccination of pancreatic cancer patients post

resection with either MUC1 peptide admixed with SB-AS2 adjuvant or with MUC1-loaded autologous DC, yielded 2 out of 15 and 4 out of 12 patients with long term, recurrence-free survival, respectively (280, 281).

As a whole, MUC1-specific immune responses show potential in controlling and eradicating disease, but these responses are rare in trials. Often only a fraction of the patients assayed have detectable MUC1 specific immunity (220). Limitations on our ability to assay such immunity may play some role in this low rate of responsiveness (281). However, the advanced nature of the disease in clinical trials and the known immune-suppressive effects of tumors (reviewed above) are also likely responsible. In support of this, a recent trial conducted by our group in which patients with advanced adenoma of the colon were vaccinated with MUC1 peptide admixed with poly-ICLC showed measureable MUC1-specific IgG in 43.6% of vaccinated individuals. However, a majority of the non-responding population had high levels of circulating CD33+/lowCD11b+HLA-DR− MDSCs in their peripheral blood, whereas the responding individuals did not, indicating that in the absence of active immunosuppression, MUC1 vaccination is highly immunogenic (179). This study is the first of its kind, examining the ability of MUC1 specific immune responses to prevent cancer in the premalignant stage. Although the efficacy of the vaccine for its intended purpose cannot yet be measured, it does highlight the high immunogenicity of a MUC1 based vaccine in the absence of immune suppression and suggests a brighter future for MUC1 based immunotherapies in preventing disease.

1.2.2.2 Preclinical Data on MUC1 Immunity in MUC1 Transgenic Mice

While MUC1 has shown demonstrable immunogenicity in clinical trials, animal models, specifically the MUC1.Tg mouse, which expresses full length human MUC1 under the control of

its natural promoter (282, 283), suggest that immune responses against the peptide backbone of the VNTR region of MUC1 are limited. MUC1 positive tumors grow faster in these mice when compared to WT mice, and those tumors that do grow in WT mice are more likely to lose MUC1 expression (283, 284). Of interest, MUC1 specific CTLs can be generated in these mice with similar frequency to what is seen in WT mice. This has been shown both in the context of spontaneous CTL generation in response to a MUC1 positive tumor (284), and in response to DC vaccination (285). However, in both cases, MUC1 specific CD4 T cell generation is limited and despite being functionally competent (286), CD8 T cells generated in MUC1.Tg hosts in the absence of properly activated CD4 T cells are unable to prevent the growth of MUC1 expressing tumors (287).

MUC1 specific CD4 T cells are rarely detected in patients. One of the proposed reasons for their absence was the observed block in MUC1 processing and presentation on MHC Class II molecules (269, 270). However, animal models show that spontaneous CD4 responses are possible in WT mice exposed to MUC1 positive tumors (284), suggesting that their absence is not solely attributable to a block in antigen processing. The absence of a CD4 T cell response is also not the result of absolute deletional tolerance as they can be boosted with certain vaccine preparations (288-290). Further supporting this, when irradiated MUC1.Tg mice are reconstituted with bone marrow from MUC1 specific CD4 TCR transgenic mice, these CD4 T cells reach the periphery in equal number and with similar phenotype to the same cells in WT mice (291). The most likely explanation is that CD4 T cells responses are limited by an increased number of MUC1 specific CD4 regulatory T cells in MUC1.Tg mice, compared to WT animals (292). In support of this, increasing the ratio of effector to regulatory T cells through adoptive transfer increases the magnitude of the MUC1 specific IgG response to vaccination (293).

Given the crucial role of CD4 T cells in optimal CD8 T cell activation and memory CD8 T cell formation and maintenance (76, 77, 294), several strategies have shown promise in improving CD4 T cell responses. The inclusion of a non-MUC1 helper epitope to activate non-self CD4 T cells which can support the immune response has shown promise in controlling MUC1 positive tumor growth (295), as has using genetically engineered B cells that express both a MUC1-epitope and a non-self helper epitope, which increases CD4 helper cell cooperation and activation (288). Another strategy is to vaccinate with a MUC1 specific helper epitope which is more foreign and not subject to self-tolerance. The hypogylcosylated form of MUC1, which is most often seen in tumors and not normal tissue (18, 296), has been shown to meet these qualifications and can boost immune responses to the naked MUC1 peptide backbone in mouse models (272, 291).

The focus of this project to better understand the mechanism of tolerance towards MUC1-specific CD4 T cells. Previous studies have identified an early immune inhibition following vaccination that is present regardless of the MUC1-specific CD4 T cell precursor frequency (291). Our goal is to identify the early immune regulation responsible for this inhibition and if appropriate, identify avenues for intervention which may improve vaccine responses.

1.3 IMMUNE REGULATION

1.3.1 **Central Tolerance**

Central T cell tolerance occurs in the thymus. Circulating T cell precursors home to the thymus and undergo a multi stage maturation process before returning to the periphery. TCR rearrangement is the first stage of this process. Focusing on αβ T cells, the α and β chains of the TCR undergo random rearrangement to produce T cell receptors (TCR) with diverse specificities. Before reaching the periphery, these cells must first undergo two rounds of selection (297). The first of these is positive selection, which occurs in the thymic cortex during a phase in which developing T cells express both the CD4 and CD8 co-receptors. T cells with no affinity for self MHC molecules die by neglect, while those with low-to-medium affinity survive. Although this is not the primary site of negative selection, T cells with high affinity can be deleted at this point. This is seen in transgenic TCR models where the TCR recognizes a ubiquitous self antigen (298, 299). At this point, either the CD4 or CD8 co-receptor is down-regulated to form single positive T cells, which undergo negative selection in the medulla of the thymus.

The medulla of the thymus contains two distinct cell populations which are responsible for mediating negative selection: medullary thymic epithelial cells (mTEC) and dendritic cells. mTECS are unique in that they express a protein called autoimmune regulator (AIRE), which promotes the expression of a wide array of tissue specific antigens (300, 301). The expression of these antigens on mTECs and their cross-presentation on medullary DC allows for the negative selection of T cells which bear specificities against tissue specific self-antigens. Patients with mutations in AIRE or mouse models in which AIRE expression is absent or deficient show

multi-organ autoimmune disorders (302-304). T cells in the medullary thymus typically undergo one of four distinct fates. If the T cells possess low-to-moderate reactivity against the locally presented self-antigens, they fully mature and traffic to the periphery as naïve T cells. Those that do have strong reactivity are either deleted through induced apoptosis, are made anergic, or undergo phenotypic skewing to become regulatory T cells. The mechanism by which the fate of self-reactive T cells is determined is not completely understood, although the strength of TCR signaling appears to be a factor, with stronger signals favoring deletion (305, 306). This does not however mean that low affinity TCRs are favored, as high affinity TCRs are more efficiently skewed towards Treg induction. (307). TGFβ and IL-2 are necessary for Treg induction and TGFβ appears to promote the survival of these cells, as Tregs in mice deficient in TGFβ show up-regultion of the pro-apoptotic proteins Bim, Bax, and Bak (308, 309).

1.3.2 **Peripheral Tolerance**

T cell tolerance acquired in the thymus is not a sufficient safeguard against self-reactive T cells. Such T cells do escape into the periphery (311), and T cells specific for innocuous foreign antigens, such as food antigens, are also present in the mature T cell repertoire. The expansion and auto-reactivity of these T cells must be controlled to maintain the health and longevity of an organism. In the periphery, the primary cell type responsible for this action is the dendritic cell. Although primarily thought of for their powerful role in inducing T cell responses (32), DC can also cause T cell deletion and anergy, and they are capable of both expanding existing Tregs and inducing new ones from naïve precursors.

1.3.2.1 Dendritic cells

DC normally act as immune sentinels. They are located throughout the body and constitutively acquire and process antigens for presentation on MHC I and II molecules at their surface. Upon exposure to microbial products, host-derived 'danger signals', or certain immune modulators, DC's up-regulate surface expression of MHC (32). Antigen uptake and presentation undergo a short burst of activity and are then suspended to maintain presentation of antigens acquired at the site of activation (312-315). This is accompanied by surface up-regulation of co-stimulatory molecules such as CD40, CD80, CD86, and CD83. These DC then traffic to T-cell rich areas of the draining lymph nodes, where cognate interactions between the TCR and MHC molecules containing acquired antigen allow the expansion of antigen specific T cells. The outcome of this process is dependent both on the level and origin of the antigen presented and on the activation state of the dendritic cell presenting it.

Stimulation of T cells through TCR-cognate antigen:MHC in the absence of co-stimulatory signaling leads to T cell anergy. This mechanism prevents antigens acquired under steady state conditions from activating potentially dangerous T cell responses and prevents recall responses against innocuous antigens. On a molecular level, this is controlled in part via the level of mTOR activation. TCR signaling, in conjunction with CD28, promotes up-regulation of IL-2 expression which then fully activates mTOR (316). Without this signal, is the case with sub optimally activated DC, which do not express costimulatory ligands, insufficient activation leads to upregulation of factors responsible for inhibiting T cell activation. These negative regulators include Deltex1 and GRAIL, which ubiquinates CD3ζ, preventing TCR derived signals (317, 318).

DC can also induce tolerance through secretion of immunoregulatory factors, such as IDO, whose function in suppressing T cell responses has been discussed, retinoic acid, and IL-10 (319). In addition to IL-10's previously described effect on DC, IL-10 can directly induce an anergic state on CD4 T cells stimulated in its presence, but in the absence of DC (320). IL-10 also appears to be necessary to stabilize Foxp3 expression in Tregs (321). IL-10 does not, however, appear to affect CD8 T cell responses in the same manner, with some studies even showing greater expansion and cytotoxicity in the presence of IL-10 (322). The impact of this on long term immunity is not well understood, however, as optimal CD8 T cell responses also require CD4 help and DC maturation, which are both inhibited in the presence of IL-10 (71-75, 164). And retinoic acid can directly induce regulatory T cell induction over effector generation (323, 324).

1.3.2.2 Regulatory T cells

In addition to their induction from T cells undergoing central tolerance, regulatory T cells can also be induced in the periphery. Similar to the induction of peripheral T cell anergy, regulatory T cell induction is dependent largely on the level of signaling through mTOR. Rapamycin, which inhibits mTOR, promotes the generation of regulatory T cells in the presence of antigen (325, 326). The mTOR complex mTORC2 is specifically important as T cells deficient in it, but not mTORC1, undergo conversion into regulatory T cells after TCR stimulation even in the presence of polarizing cytokines like IFNγ (327). This is caused by phosphorylation and inhibition of the transcription factors FOXO1 and FOXO3a, as downstream targets of mTORC2 which are necessary to promote the expression of the Treg specific transcription factor, Foxp3 (328). Extracellular factors responsible for the induction of regulatory T cells include low costimulatory

ligand interactions and exposure, during priming, to TGFβ (329), which signals through Smad3 and cooperates with NFAT to promote Foxp3 expression (330). Low density of cognate antigen is also an important factor in maintaining low mTOR activity and promoting Treg generation (331).

Once generated, regulatory T cells can suppress immune activation through a number of different mechanisms, many of which have already been discussed. Tregs produce the anti-inflammatory cytokines IL-10, TGFβ, and IL-35. Treg production of IL-10 is essential for preventing inflammation at environmental surface such as the lungs and intestines (332, 333) and is an important component in tumor resident Treg activity (334). Mice deificient in TGFβ show similar pathology, such as enhanced IBD and failure to control islet reactive CD8 T cells in experimental diabetes models (335, 336). Although a much more recent discovery, IL-35 is similarly necessary for homeostatic Treg functions and directly inhibits T cell proliferation (337). Tregs also have a number of contact-dependent functions, including direct cytolysis of target cells (338, 339). They express CTLA-4, which can induce IDO expression in DC (340), as well as LAG-3 and LFA-1, the latter of which permits their preferential aggregation on DCs which promotes the down-regulation of co-stimulatory molecules (341, 342). Regulatory T cells can also indirectly suppress T cell activation by acting as an IL-2 “sink”. The express much higher levels of the IL-2Rα chain CD25, allowing them to bind and internalize large amounts of IL-2. This has been recently reported as an important mechanism in preventing NK activation (172-174) and can induce apoptosis in effector CD4 T cells (343).

1.3.2.3 NK cells

A large amount of research has been dedicated to the ability of natural killer (NK) cells to lyse diseased or infected cells and to produce large amounts of IFNγ and TNFα, however NKs are also capable of negatively regulating immune responses. For instance, NK cells can alleviate diabetes in NOD mice in a TGFβ-dependent manner (344) and their absence in models of transferred colitis and experimental autoimmune encephalitis actually accelerates disease progression (345, 346). Two mechanisms predominate in the literature. The first is direct lysis of immune cells. This may include either effector T cells (347, 348) or dendritic cells (349, 350). NK cells show specificity for lysis of activated T cells specifically (348). Furthermore, under some conditions, specifically IL-10 exposure, DC can gain unique susceptibility to NK-mediated lysis such that immature DC are protected while mature DC are lysed (351). Therefore, NK cells would not only be able to limit the ongoing immune response, but could shape the immune response in a way that promotes T cell anergy and Treg generation, thereby affecting long term immune memory.

The second way that NK cell can inhibit immunity is through secretion of IL-10 and TGFβ. The importance of TGFβ in some models has been mentioned (344), but IL-10 secretion by NK cells is widely recognized as one of the most common and potent mechanisms by which NK cells can control immune responses. A number of cytokines can induce IL-10 production by NK cells, including, IL-12, IL-2, IL-15, and IL-27 (352-354). In addition, “regulatory’ NK cells, identified by and functionally dependent on IL-10 secretion, inhibit CD4 T cells responses *in vitro* (355) and can decrease systemic IL-12 levels *in vivo* (356).

1.4 INTRODUCTION TO THE PROJECT

MUC1-specific immunotherapy holds promise for the treatment and prevention of cancer and could impact a wide variety of tumor types (219). Early clinical trials have yielded promising, if not yet consistent, results. Several factors likely contribute to this inconsistency, the largest at this point being the overall immunosuppressive environment created by the late stage tumors that have been the target of most trials up until this point (220). However, a recently initiated study using MUC1 vaccination as a preventative therapy (179) represents an interesting and new future, not just for MUC1 specific therapies, but for cancer immunotherapy at large.

It is likely that this new direction for the field will yield as many obstacles as it removes at first. Successful immunotherapy is largely defined by clinical outcome. This will have to be changed, as patients receiving prophylactic immunotherapy may never develop clinically identifiable disease. We will therefore need reliable early biomarkers that predict robust, protective immune responses that will be functional years later. We will also need to fully understand the limitations associated with self- and altered self-antigen vaccinations in the absence of tumor mediated suppression, as these will need to be overcome in order for long-lasting immunity to be generated. Such long lasting immunity will be necessary for effective responses against cancer, a disease which may appear decades after vaccination and one in which the incidence increases with age.

These issues were explored in this thesis aiming to define one or more of the mechanisms controlling immune responses to a vaccine based on the tumor associated antigen MUC1. Using the MUC1.Tg mouse, which has well documented tolerance to certain MUC1 vaccines (283, 291, 293), I elucidated early events following vaccination which distinguish low level responses generated in these mice from the more robust responses generated in their wild type counterparts.

These studies will help identify early predictors of successful anti-tumor vaccinations and will pinpoint potential avenues for concurrent interventions aimed at improving immune responses against tumor antigens derived from self-antigens. This work will also add to our understanding of peripheral tolerance to self-antigens in the face of immunologic insult, especially where central tolerance is incomplete (291).

2.0 ANTIGEN CHOICE DETERMINES VACCINE-INDUCED GENERATION OF IMMUNOGENIC VERSUS TOLEROGENIC DC THAT ARE MARKED BY DIFFERENTIAL EXPRESSION OF PANCREATIC ENZYMES

Chapter 2 is adapted from "Antigen choice determines vaccine-induced generation of immunogenic versus tolerogenic dendritic cells that are marked by differential expression of pancreatic enzymes". Farkas, AM*, Marvel DM*, and Finn, OJ. J Immunol. 2013 Apr 1;190(7):3319-27. doi: 10.4049/jimmunol.1203321.

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2.1 ABSTRACT

Dendritic cells (DC) elicit immunity to pathogens and tumors while simultaneously preserving tolerance to self. Efficacious cancer vaccines have been a challenge to develop because they are based on tumor antigens, some of which are self-antigens and thus subject to self-tolerance. One such antigen is the tumor-associated mucin MUC1. Preclinical testing of MUC1 vaccines revealed existence of peripheral tolerance to MUC1 that compromises their efficacy. To identify mechanisms that act early post-vaccination and might predict vaccine outcome, we immunized human MUC1 transgenic mice (MUC1.Tg) i.v. with a MUC1 peptide vaccine against which the mice usually generate weak immunity, and WT mice that respond strongly to the same peptide. We analyzed differences in splenic DC phenotype and function between the two mouse strains at 24 and 72 hours post-vaccination, and also performed unbiased total gene expression analysis of the spleen. Compared to WT, MUC1.Tg spleens had significantly fewer DC and all of them exhibited significantly lower expression of co-stimulatory molecules, decreased motility and preferential priming of antigen-specific Foxp3+ regulatory T cells (Treg). This tolerogenic DC phenotype and function was marked by a new putative biomarker revealed by the microarray: a cohort of pancreatic enzymes (trypsin, carboxypeptidase, elastase and others) not previously reported in DC. These enzymes were strongly upregulated in splenic DC from vaccinated WT mice and suppressed in all splenic DC of vaccinated MUC1.Tg mice. Suppression of the enzymes was dependent on Treg and on signaling through the IL-10 receptor and correlated with global down-regulation of DC immunostimulatory phenotype and function.

2.2 INTRODUCTION

Dendritic cells (DC) are potent inducers of antigen-specific T cell responses and are the major cell type responsible for priming naïve T cells (32, 357). As such, they have been central to vaccination strategies aimed at inducing immunity to both pathogens and tumors (358, 359). However, DC are also important in the maintenance of homeostatic tolerance to self-antigens (Ag) (360). A large body of literature has established the ability of DC to actively induce immunological tolerance against self-Ag, and those closely related to self, thus preventing autoimmunity but also compromising effective anti-tumor immune responses (163, 361). DC utilize diverse mechanisms to mediate T cell tolerance including low expression of costimulatory molecules (362), expression of SOCS1/3 (363, 364), activation of regulatory T cells (Treg) (365), and production of immunosuppressive factors such as IL-10, TGFβ, IDO and retinoic acid (366-369). Significant effort has been devoted to manipulating the phenotype and function of *in vitro* cultured DC used for vaccination (370), as well as to targeting Ag *in vivo* to specific DC populations (371). However, modulating and evaluating the ability of a vaccine to alter the phenotype of endogenous DC populations and the type of immune response they prime is still a significant challenge. Specifically, little data exist regarding the influence of the choice of vaccine Ag on the phenotype and function of endogenous DC. It has been well established that exogenous DC used for immunization are generally short-lived in the host after transfer (372), and that transfer of Ag from vaccine DC to endogenous DC is necessary for optimal $CD4^+$ and $CD8^+$ T cell responses (373, 374). Therefore, understanding the impact of the choice of Ag, specifically the importance of its relative similarity to antigens against which the host is already tolerized, on endogenous DC warrants further study. Additionally, because gauging a vaccine's efficacy often requires waiting several weeks to determine resultant antibody titers and vaccine-

induced T cell function, reliable, early signatures or biomarkers of both the endogenous DC response and the ensuing immune response would be of utility.

We and others have previously shown that a long peptide (MUC1p) corresponding to five tandem repeats in the human tumor antigen MUC1 variable number of tandem repeats region is seen as a self-antigen by the human MUC1 transgenic mouse (MUC1.Tg), and that MUC1p vaccination results in hypo-responsiveness compared to a strong immune response in WT mice where MUC1p is a foreign antigen (273, 284, 293). Now we show that the outcome of the MUC1p vaccine that currently requires several weeks after immunization to be evaluated can be predicted as early as 24h-72h post-vaccination by the change in expression levels in DC of a group of catabolic enzymes, including trypsin, amylase, elastase, and carboxypeptidase B1, previously thought to be pancreas-restricted in expression. These enzymes are significantly up-regulated in the splenic DC of WT mice following i.v. administration of the MUC1p vaccine, but not in MUC1.Tg mice. Failure to up-regulate pancreatic enzyme expression was seen in the entire splenic DC population and was correlated with low co-stimulatory molecule expression, a decreased number of DC in the spleen, preferential priming of $Foxp3^+$ Treg over $IFN\gamma^+$ $CD4^+$ T cells and impaired motility. Mechanistically, this DC phenotype was regulated by Treg and IL-10. The unexpected expression of pancreatic enzymes in DC and correlation with DC immunogenicity or tolerogenicity following vaccination provides a new early biomarker of vaccine efficacy.

2.3 MATERIALS AND METHODS

2.3.1 **Mice**

C57BL/6, RIP.OVA, and OT-II mice were purchased from the Jackson Laboratory. MUC1.Tg mice were purchased from Dr. Sandra Gendler (Mayo Clinic) (282) and/or bred in the University of Pittsburgh animal facility. VFT mice were generated at the University of Pittsburgh Transgenic Mouse Facility. All colonies were subsequently bred and maintained at the University of Pittsburgh under specific pathogen free conditions. Experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

2.3.2 **Peptides**

A 100mer MUC1 peptide (MUC1p) represents 5 repeats of the 20- amino-acid sequence HGVTSAPDTRPAPGSTAPPA from the MUC1 VNTR region. It was synthesized as described previously (375) by the University of Pittsburgh Genomics and Proteomics Core Laboratories. $OVA_{323-339}$ peptide and ovalbumin protein were purchased from Sigma.

2.3.3 **DC culture and vaccines**

BMDC were generated according to established protocol (376). Briefly, female C57BL/6 mice (Jackson) were sacrificed and their femurs and tibiae removed. Marrow was extracted using a 25G needle and flushed with RPMI (2% FCS, 1% Penn-Strep and 2-ME). Cells were passed through a 70μM strainer and pelleted before RBC lysis using ACK buffer. Cells were

resuspended in AIM-V (Gibco), counted and plated at 1.5-2×10^6/mL in AIM-V containing 10-20ng/mL GM-CSF (Miltenyi). On d3 and d5 half the media was replaced with fresh AIM-V and GM-CSF. On d6 of culture, DC were harvested with 2mM EDTA, counted and (when indicated) loaded with either 30ug/mL MUC1 100mer or 100μg/mL ovalbumin and matured with 25ug/mL of Poly-ICLC (Hiltonol), a generous gift from Oncovir, overnight. On d7, cells were harvested as above. For immunizations, d7 DC were washed and resuspended in sterile PBS. Mice were immunized i.v. via the lateral tail vein with .5-1×10^6 DC. Soluble peptide immunizations consisted 100ug of MUC1 100mer peptide or ovalbumin and 50ug of Poly-ICLC in 100uL of PBS.

2.3.4 **Microarray**

Whole spleen from WT and MUC1.Tg mice (n=3/group) was obtained at 24h and 72h post-immunization with DC loaded with MUC1 100mer peptide. RNA extraction was performed using Trizol (Invitrogen). RNA from mice within groups was pooled followed by hybridization onto Illumina WG6 arrays. Data analysis was conducted by the University of Pittsburgh GPCL Bioinformatics Core facility using the Efficiency Analysis method of identifying differentially expressed genes (377).

2.3.5 **PCR and qRT-PCR**

RNA was isolated from splenic tissue or CD11c+ bead isolated (Miltenyi) splenocytes using either an RNeasy mini kit (Qiagen) or Trizol (Invitrogen) according to manufacturer's protocol). RT-PCR was performed using $Oligo_{(dT)}$ primers and SuperScript III reverse transcriptase

(Invitrogen). cDNA was amplified using the following primers: trypsin (forward 1: 5'-GGCCCTTGTGGGAGCTGCTG-3'; reverse 1: 5'-GCAGGTGCACAGGAGCTGGG-3'; forward 2: 5'-GCTCTGCCCAGCTCCTGTGCACCT-3'; reverse 2: 5'-TCAGCCTGAGGCAGCAGTGGGGCAT-3'), CPB1 (forward 1: 5'-TGGTGAGTGTGGCCCTGGCT-3'; reverse 1: 5'-TCCACTTGCACGGGTGTGGC-3' forward 2: 5'- GCCCTGGTGAAAGGTGCAGCAAAGG -3'; reverse 2: 5'-AGCCCAGTCGTCAGATCCCCCAGCA -3'), Elastase (forward: 5'-TTCCGGAAACTGACGCCCGC-3'; reverse: 5'-TGGGCCAGCTCCCCATTGGT-3'), GAPDH (forward 1: 5'-TTGGCCGTATTGGGCGCCTG-3'; reverse 2: 5'-TCTCCAGGCGGCACGTCAGA-3'; forward 2: 5'- AGACGGCCGCATCTTCTTGTGCAGT-3'; reverse 2: 5'- TGGTGACCAGGCGCCCAATACGGC-3'), and IL-10 (forward: 5'-CTTCCCAGTCGGCCAGAGCCA-3'; reverse: 5'- CTCAGCCGCATCCTGAGGGTCT-3'). qPCR was done using a QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's protocol. Reactions were run on a StepOne Plus instrument (Applied Biosystems) and data was generated using the ΔC_T method.

2.3.6 **Western blotting**

Cells were lysed and run on a 10% Tris-HCL Mini-PROTEAN TGX precast gel (BioRad), followed by transfer onto a PVDF membrane. After blocking for 1hr in 5% milk, the membrane was incubated with one of the following antibodies: Rb X-CPB1 (M-134), Rb X-trypsin (M-60) (both Santa Cruz) or β-Actin (AC-74, Sigma). Blots were then incubated with the appropriate HRP-conjugated secondary antibodies (Santa Cruz) and developed using SuperSignal West chemiluminescent substrate (Pierce) before imaging on a Kodak Image Station 4000MM.

2.3.7 **DC/T cell co-cultures**

CD4 effector and regulatory T cells were isolated from C57Bl/6 mouse splenocytes using the CD4+CD25+ Regulatory T cell Isolation Kit (Miltenyi) and preactivated overnight with 1µg/ml plate-bound anti-CD3 and .5µg/ml soluble anti CD28. Bone marrow derived dendritic cells (BMDC) were generated using above described procedure used to culture vaccine DC. On day six, semi-adherent cells, which represent semi-mature dendritic cells, were removed by gentle agitation. DC were added to preexisting T cell cultures at DC:T cell ratios of 2: 5, except when both regulatory and effector T cells were added, in which case the ratio was 2:5:5. Where indicated, LPS was added to the culture along with the DC at a final concentration of 1ng/ml. At 24 hours post co-culture, DC were isolated based on plate adherence and RNA was extracted and analyzed as described.

2.3.8 **Depleting and/or blocking antibody experiments**

All antibodies were purchased from Bio-X-Cell. Mice received an i.p. injection containing 200µg of an anti CD25 antibody (clone PC-61.5.3) to deplete CD4+ regulatory T cell. 6 days following this treatment, mice were vaccinated as described and sacrificed 24 hours following vaccination. In the case of IL-10R blockade, mice were given 250µg of on an anti IL-10R antibody (clone 1B1.3A), IP. These mice were then vaccinated as described at 48-72 hours post antibody treatment along with an additional dose of 250µg of anti IL-10R antibody. Mice were sacrificed 24 hours following vaccination and second antibody dosing. An equal concentration and volume of Rat IgG1 specific for horseradish peroxidase (HRPN) was injected as a control for the depleting/blocking antibodies where indicated.

2.3.9 **Flow cytometry**

Anti-CD11c-PacificBlue, anti-CD80-FITC, anti-CD3-PerCP, anti-CD25-PE (BD Bioscience), anti-I-A^b-PeCy7, anti-CD40-APC, anti-CD86-PerCP, anti-Foxp3-PacificBlue (BioLegend), anti-IFNγ-APC, and anti-CD4-FITC (eBioscience) antibodies were used. Cells were analyzed on an LSR II (BD) and data were analyzed using FacsDiva software (BD).

2.3.10 ***Ex vivo* motility assay**

Pooled splenocytes were recovered from MUC1p- immunized WT and MUC1.Tg mice 48h post-immunization (n=2/group). DC were isolated with CD11c beads (Miltenyi) and plated at 2×10^5 cells into Poly-D-Lysine coated 35mm dishes (MatTek). Cells were labeled according to protocol with Cell Tracker Red (Invitrogen) and imaged at 10X in DIC and TRITC channels on a Nikon Eclipse live cell system at 5min intervals for 24h. Motility was analyzed using the Imaris Track algorithm in Imaris (Bitplane).

2.3.11 **Statistics**

Data show mean ± the standard error of the mean (SEM). Statistical significance between groups was defined as $p\leq.05$ using an unpaired, 2-tailed Student's *t* test (GraphPad Prism).

2.4 RESULTS:

2.4.1 DC from MUC1p-immunized MUC1.Tg mice exhibit decreased expression of co-stimulatory molecules, preferentially induce Foxp3$^+$ Treg cells and have reduced motility

Multiple factors contribute to or limit the ability of DC to prime T cells. These include the number of antigen-loaded DC (378), expression of co-stimulatory molecules on DC and production of stimulatory or suppressive cytokines (362), and the ability of DC to move to T cell areas within lymphoid tissue (379). We found that immunization of MUC1.Tg mice with MUC1p resulted in a decrease in the absolute number of CD11c$^+$ cells in the spleen at 24h, while the same protocol in WT mice resulted in an increase in DC number (Fig. 1A). The same immunization also resulted in differential expression of co-stimulatory molecules, with significantly fewer DC from MUC1.Tg mice expressing CD40 and MHC II (Figs. 1B and 1C), as well as a reduction in the amount of CD86 expressed by those DC (Fig. 1D), relative to immunized WT mice.

To examine the ability of DC that have been exposed to a self Ag induced environment to prime naïve CD4$^+$ T cells, we again immunized WT and MUC1.Tg mice with MUC1p and isolated total splenic DC 24h post-immunization. The DC were immediately loaded with OVA and co-cultured with naïve, CFSE-labeled OT-II CD4$^+$ T cells that recognize an I-A^b-restricted OVA peptide. After 7 days, T cells from those co-cultures were analyzed by flow cytometry. DC recovered from immunized MUC1.Tg mice primed a significantly higher percentage of Foxp3$^+$ (Fig. 1E) and fewer IFNγ producing OT-II T cells compared to DC recovered from immunized WT mice (Fig. 1F). DC can induce antigen-specific Treg proliferation (380) so we examined the relative proliferation of CD4$^+$Foxp3$^+$ Tregs. DC recovered from MUC1p vaccinated MUC1.Tg

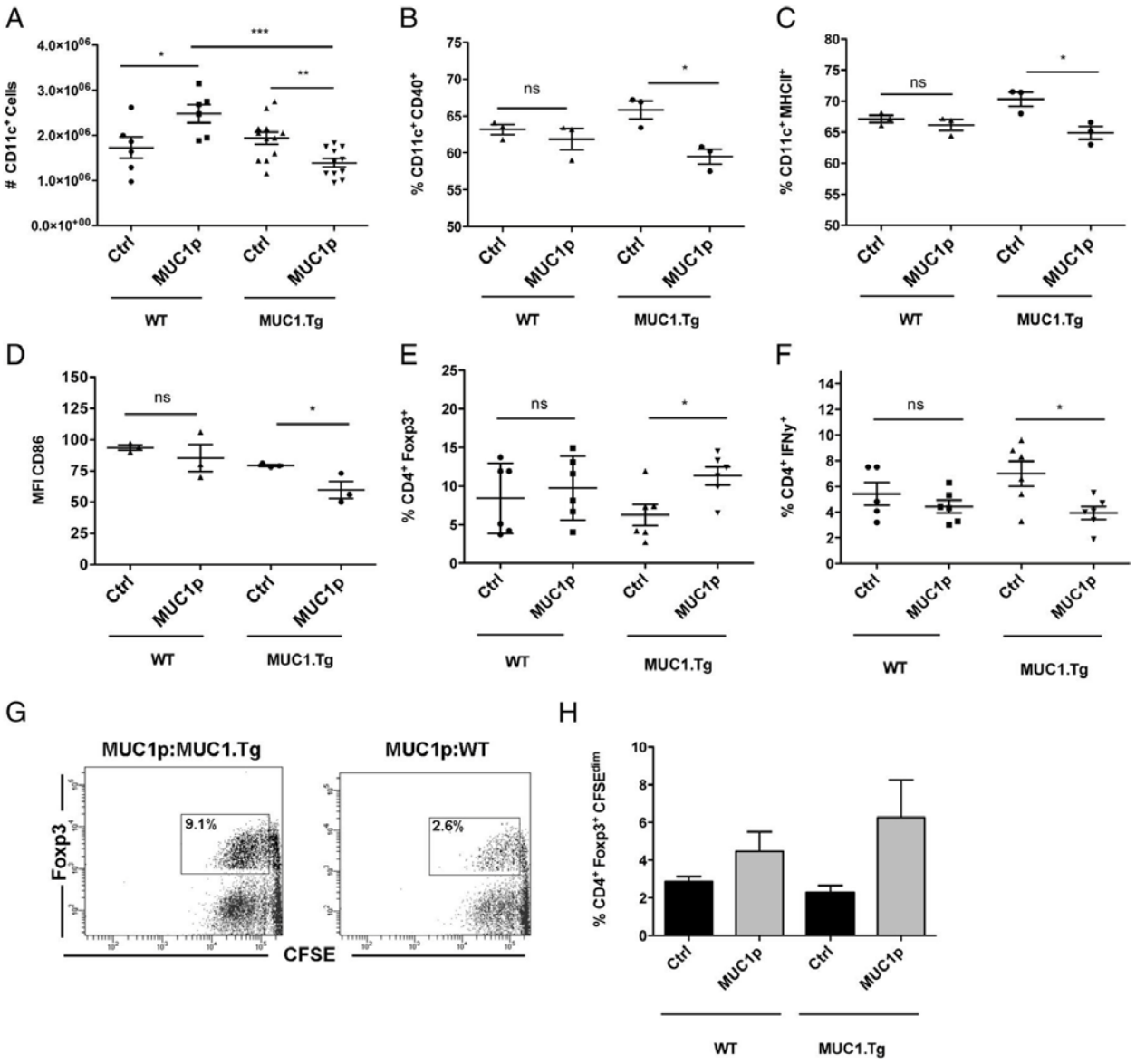

Figure 1: Immunization of MUC1.Tg mice with MUC1p results in decreased splenic DC number and costimulatory molecule expression and preferential priming of Foxp3+ Treg.

(A) WT and MUC1.Tg mice were immunized with unloaded DC (Ctrl) or DC loaded with MUC1p. Twenty-four hours postimmunization, total splenic DC numbers were analyzed. Each symbol represents one mouse, with bars showing mean ± SEM from three pooled independent experiments and each experiment including two to four mice per group. (**B**–**D**) WT and MUC1.Tg mice were immunized as in (A). Forty-eight hours postimmunization, bulk splenocytes were stained for FACS analysis. Data represent percentage of positive cells within the $CD11c^+$ gate (B, C) or the mean fluorescence intensity (MFI) of cells within the $CD11c^+$ gate (D). Symbols represent individual mice, with bars showing mean ± SEM, and are representative of two independent experiments. (**E**–**H**) WT and MUC1.Tg

mice were immunized as in (A). Twenty-four hours later, splenic DC were bead isolated, loaded with OVA, and cocultured with OT-II CD4 T cells for 7 d. On day 7, OT-II cells were treated with PMA/ionomycin and analyzed by FACS. Each symbol represents an individual mouse, with bars depicting mean ± SEM. Data are pooled from two independent experiments. (G) OT-II $CD4^+$ T cells were labeled with CFSE and cultured as in (E)–(H). On day 7, CFSE dilution was assessed in $CD4^+Foxp3^+$ cells. Representative dot plots from MUC1p-vaccinated WT and MUC1.Tg mice are shown (G). (H) Bars represent mean percentage proliferation ± SEM of OT-II $CD4^+Foxp3^+$ cells. Data are representative of two independent experiments. $*p \leq 0.05$, $**p \leq 0.001$, $***p \leq 0.0001$.

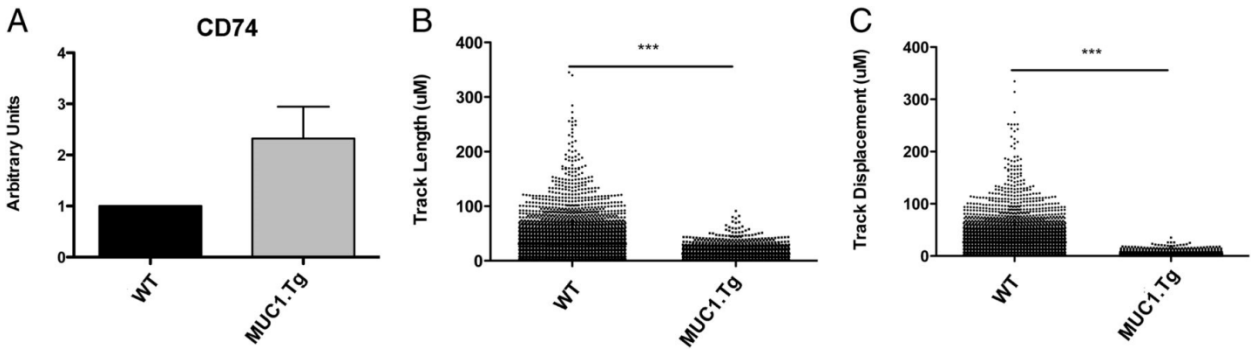

Figure 2: Immunization of MUC1.Tg mice with MUC1p results in decreased DC motility.

(A) WT and MUC1.Tg mice were vaccinated i.v. with DC loaded with MUC1p. RNA was extracted from pooled splenic DC 72 h postvaccination for qRT-PCR. Bars represent mean ± SEM. Data are representative of three independent experiments. (B and C) WT and MUC1.Tg mice were vaccinated as in (A). At 72 h postimmunization, splenic DC were bead isolated for live cell imaging. The track length (B) and displacement (C) were analyzed after 20 h in culture. Each dot represents a single DC, and bars depict mean ± SEM. Data are from two mice comparing 6 × 103 DC/group. $***p \leq 0.0001$

mice induced higher OT-II Treg proliferation compared to DC from MUC1p vaccinated WT animals (Figs. 1G and 1H).

While costimulatory molecule expression was decreased in DC recovered from mice that received immunization with self peptide, we found that immunization of MUC1.Tg mice with MUC1p surprisingly resulted in increased expression of CD74 (the MHC II invariant chain) in DC at 72h, compared to DC from MUC1p immunized WT mice (Fig. 2A). Previous studies have shown that expression of CD74 is inversely correlated to *in vivo* motility of DC (381). We purified splenic $CD11c^+$ cells from WT and MUC1.Tg mice 72h post-MUC1p immunization and analyzed them immediately *ex vivo* using live cell microscopy. DC isolated from MUC1.Tg mice traveled shorter distances (Fig. 2B) and had smaller net displacements (Fig. 2C) than DC from WT mice.

2.4.2 **Differential expression *in vivo* of pancreatic enzymes in DC in response to vaccination with a foreign versus a self-antigen**

We were interested in comparing early (24h-72h) post-immunization events in the spleens of WT versus MUC1.Tg mice that might reveal one or more new mechanisms induced by the presence of a self-antigen that could mediate antigen-specific peripheral tolerance. Accordingly, we immunized i.v. WT and MUC1.Tg mice with DC loaded with MUC1p as previously and conducted whole transcriptome analysis of total splenic RNA at 24h and 72h post-immunization.

We identified 189 genes differentially expressed at both time points, with the most unexpected being a group of seven pancreatic catabolic enzymes and several of their isoforms that had not previously been reported to be expressed in lymphoid tissue (Table I). Significantly

Table 2: Pancreatic enzymes are expressed at a significantly lower level in the spleens of MUC1p-vaccinatedMUC1.Tg mice compared to MUC1p-vaccinated WT mice

Gene	Accession Number	Fold Change (24h)	Fold Change (72h)
Trypsin 1	XM_001477976.1	-9.736	-20.824
Elastase 1	NM_033612.1	-11.531	-27.754
Carboxypeptidas B1	NM_029706.1	-14.302	-30.478
Trypsin 10	NM_001038996.1	-24.006	-36.193
Trypsin 4	NM_011646.5	-32.199	-48.856
Elastase 2A	NM_007919.2	-44.824	-81.952
Amylase 2	NM_001042711.2	-85.541	-88.073

WT and MUC1.Tg mice (n=3/group) were immunized i.v. with DC loaded with MUC1p. At 24h and 72h, spleens were harvested, total splenic RNA isolated, pooled within groups, and whole transcriptome analysis was conducted. A cohort of catabolic enzymes with previously characterized pancreatic expression were significantly under-expressed in the spleens of immunized MUC1.Tg mice, relative to immunized WT mice. Data reflect expression of genes in immunized MUC1.Tg mice relative to immunized WT mice.

lower levels (between 10-80 fold) of transcripts for these enzymes were found in the total splenic RNA from MUC1p-vaccinated MUC1.Tg mice relative to WT mice

Since the expression of each of these enzymes mimicked the entire cohort, we used trypsin 1 and carboxypeptidase B1 (CPB1) as representatives for more detailed analysis. qPCR analysis of total splenic RNA recapitulated the microarray data, showing a lack of up-regulation of trypsin and CPB1 transcript in spleens from MUC1.Tg mice post immunization with MUC1p relative to significant up-regulation in WT mice (Fig. 3A). Because there was little information about pancreatic enzymes in hematopoietic cells, we analyzed their baseline expression in different WT spleen cell populations: purified $CD11c^+$ DC, T cells, bone marrow-derived macrophages (BMDM) and CD11c-depleted bulk splenocytes which included, among other cell types, B cells. $CD11c^+$ DC expressed trypsin and CPB1 (Fig. 3B) as well as all the other enzymes identified in the gene array (not shown). BMDM expressed CPB1 but not trypsin, while purified T cells and CD11c depleted spleen cells were negative for both. Further dissection of the DC compartment into plasmacytoid DC, $CD8\alpha^+$ DC and $CD8\alpha^-$ DC revealed that all DC subpopulations express these enzymes post vaccination while $CD11c^-$ cells do not (Fig. 4). Furthermore we show that these same pancreatic enzymes found in murine DC are also found in human monocyte-derived DC (Fig. 5).

To confirm that the enzyme's expression profiles observed in the whole spleen after immunization of WT and MUC1.Tg mice with MUC1p reflected primarily what was occurring in $CD11c^+$ DC, we repeated the immunizations and 24 hours later examined changes in trypsin and CPB1 expression in purified DC. The failure to up-regulate expression post-immunization was recapitulated in DC recovered from MUC1.Tg mice, while DC isolated from immunized WT mice dramatically increased these transcripts (Fig. 3C). This was also observed at the

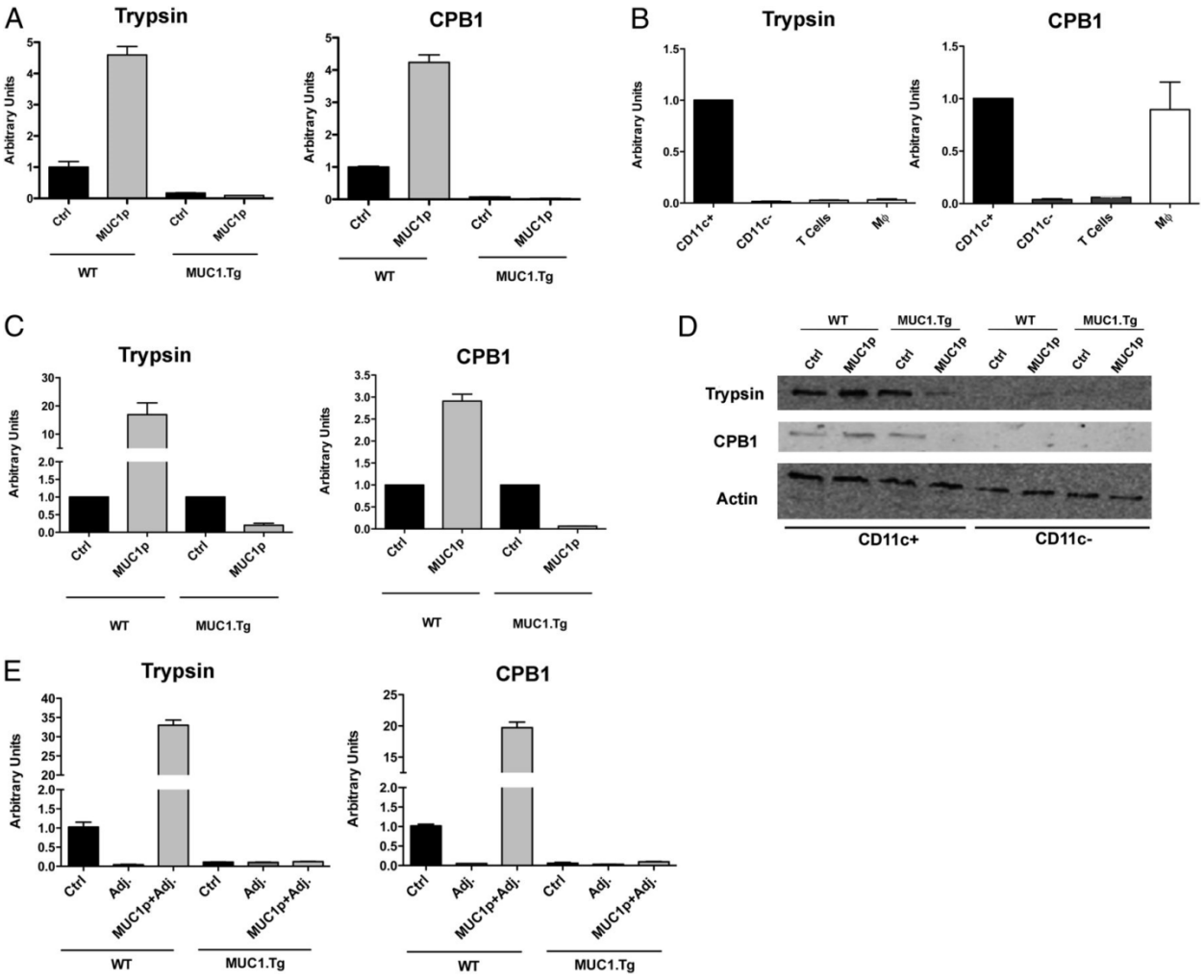

Figure 3: Immunization of WT but not MUC1.Tg mice with MUC1p results in upregulation of pancreatic enzymes in splenic DC.

(A**) WT and MUC1.Tg mice were injected i.v. with unloaded BMDC (Ctrl) or BMDC loaded with MUC1p. Twenty-four hours later, spleens were harvested, pooled according to group, and RNA extracted for qRT-PCR. Arbitrary units were normalized to WT mice given the Ctrl vaccine. Bars represent mean ± SEM. Data are representative of two independent experiments. (**B**) Splenic DC from unvaccinated mice were isolated with CD11c$^+$ beads (*n* = 3), total splenic T cells were isolated using negative selection via MACS depletion of CD3$^-$ cells, and BMDM (MΦ) were cultured for 8 d in the presence of L-cell supernatant as a source of M-CSF. RNA was isolated from all populations for qRT-PCR analysis. Units were normalized to expression levels in CD11c$^+$ cells. Bars represent mean ± SEM. Data representative of two independent experiments. (**C**) WT and MUC1.Tg mice were immunized as in (A). At 24 h, splenic DC were isolated using CD11c$^+$ beads for**

analysis by qRT-PCR or Western blotting for trypsin and CPB1 (D). Bars represent mean ± SEM after normalization to control vaccination. Data are representative of two (C) and three (D) independent experiments. (E) Mice were immunized i.v. with PBS (Ctrl), Poly-ICLC (Adj.), or soluble MUC1p admixed with Poly-ICLC (MUC1p+Adj.). Twenty-four hours later, spleens were harvested for qRT-PCR analysis. Bars represent mean ± SEM normalized to PBS control and are representative of four independent experiments.

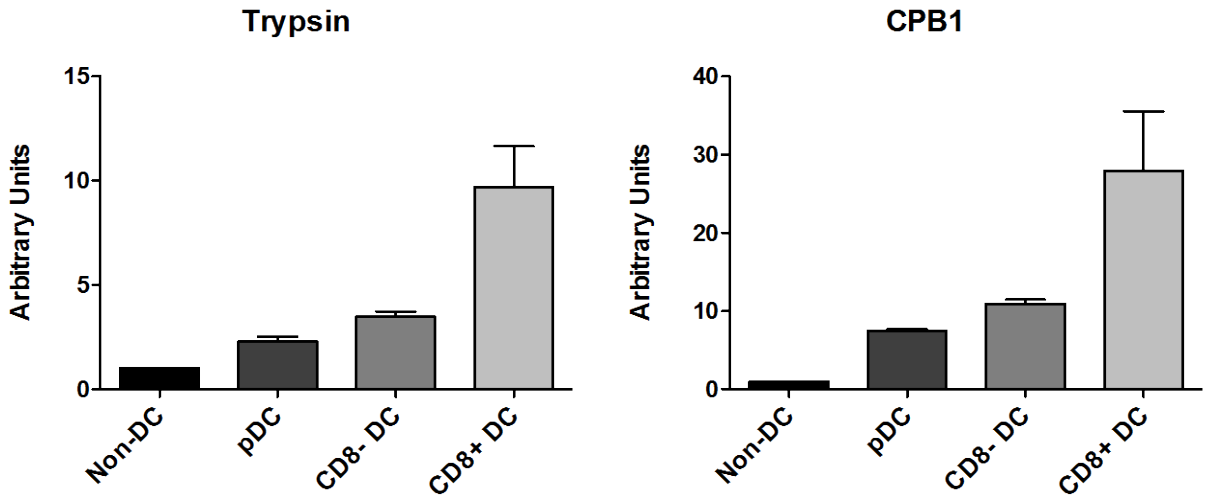

Figure 4: All major DC subpopulations express higher levels of pancreatic enzymes than CD11c- splenocytes WT mice (n=3) were give 100µg of MUC1p admixed with 50µg poly:I-C in a total volume of 100µL PBS via tail vein. 24 hours post injection, spleens were removed, pooled, and total splenic DC were bead isolated. DC were further separated via FACS into CD8α+ DC (CD11c+CD8α+B220-), CD8α- DC (CD11c+CD8α-B220-) and pDC (CD11c+ B220+). mRNA was extracted from these purified populations as well and DC depleted whole splenocytes and analyzed via qRT-PCR for trypsin and CPB1 expression. Bars represent mean ± SEM Data are representative of two independent experiments.

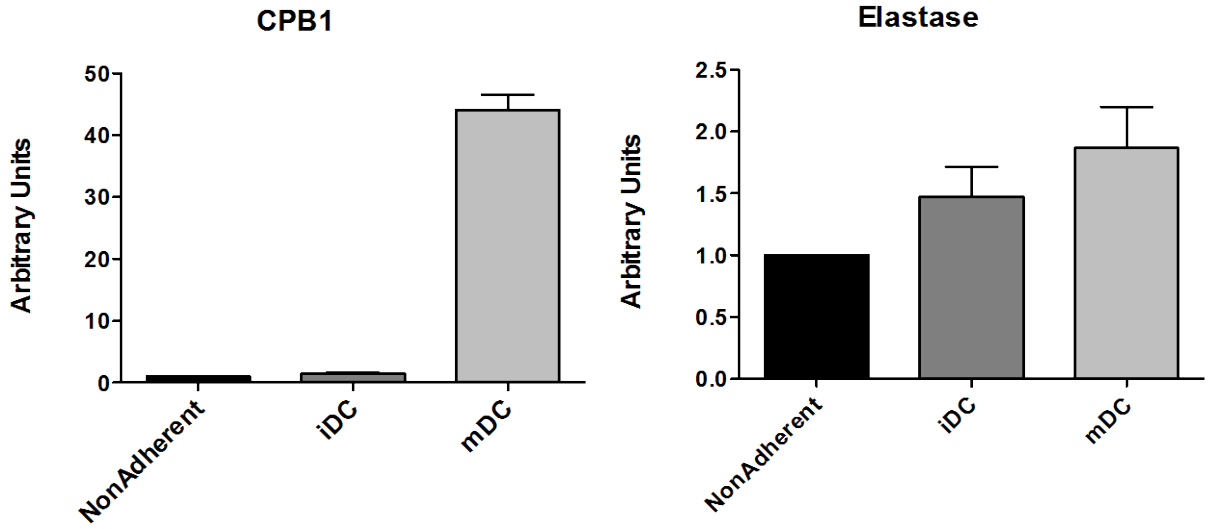

Figure 5: Human monocyte-derived DC up-regulate pancreatic enzyme expression upon TLR3 stimulation

Peripheral blood mononuclear cells (PBMC) obtained from the leukopaks of healthy donors were cultured for 5 days in the presence of 100U/mL of GM-CSF and 200U/mL IL-4. On d5, immature DC were harvested, RNA extracted and qPCR conducted for CPB1 and Elastase. Remaining DC were matured overnight with 30ug/mL of Poly-I:C, and CPB1 and Elastase levels examined the following day to query differences in mDC.

protein level by Western blotting of whole cell lysates from DC purified from MUC1p-immunized WT and MUC1.Tg mice. CD11c-depleted splenocytes were negative confirming that DC are the main cell population that expresses these enzymes (Fig. 3D).

Finally, we show that immunization with soluble MUC1p admixed with Poly-ICLC adjuvant (a TLR3 agonist) also led to up-regulation of trypsin and CPB1 in WT mice but not in MUC1.Tg mice (Fig. 3E). Adjuvant alone had no effect on these enzymes in either mouse strain. Thus the process is antigen dependent rather than delivery system or adjuvant dependent and it is regulated in all DC rather than only in the exogenous DC delivering the antigen.

To show that differential regulation of these enzymes in WT and MUC1.Tg mice was driven by exposure to foreign versus self Ag rather than by a physiologic difference between WT

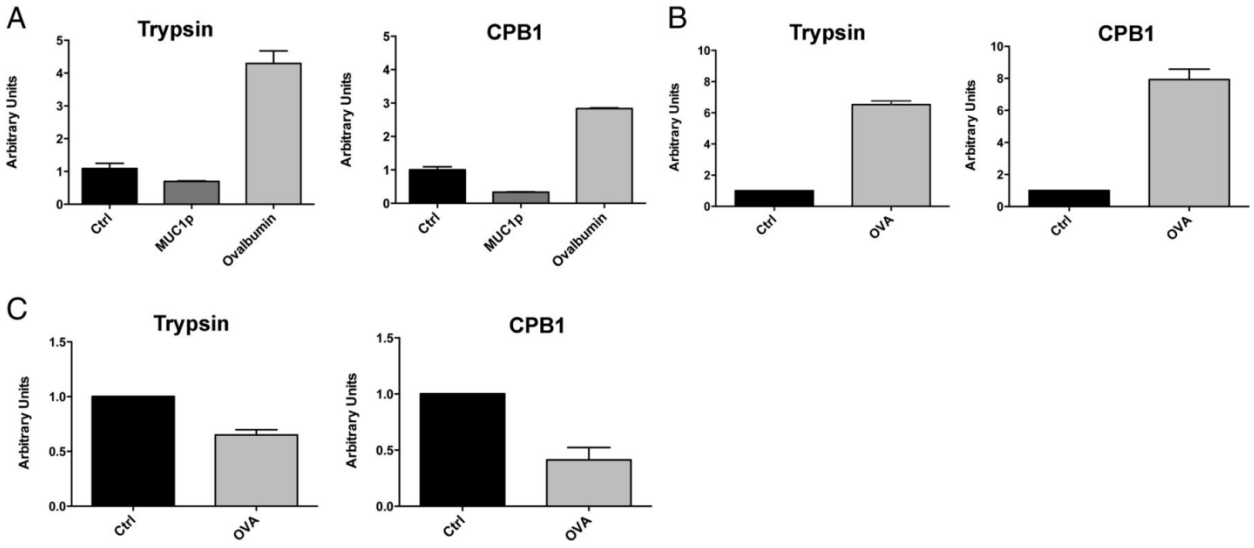

Figure 6: Failure of DC to upregulate pancreatic enzymes following immunization with MUC1p as a self-Ag is recapitulated in the OVA model of self-tolerance.

(A) MUC1.Tg mice were immunized i.v. with PBS (Ctrl), soluble MUC1p, or OVA (Ovalbumin) admixed with Poly-ICLC. Spleens were harvested at 24 h postimmunization and pooled for qRT-PCR analysis. Bars represent mean ± SEM normalized to PBS control. Data are representative of three independent experiments. (B) MUC1.Tg mice were immunized i.v. with unloaded DC (Ctrl) or DC loaded with OVA. Twenty-four hours postimmunization, splenic DC were MACS purified for qRT-PCR analysis. Bars represent mean ± SEM normalized to Ctrl. Data are representative of three independent experiments. (C) RIP.OVA mice were immunized and processed as in (B). Bars represent mean ± SEM normalized to Ctrl vaccination. Data are representative of two independent experiments.

and MUC1.Tg mice, we immunized MUC1.Tg mice with OVA, a foreign Ag in that mouse strain, and examined total and DC-specific splenic RNA 24h later. In contrast to MUC1p and control immunized mice, we found up-regulation of enzymes in the total splenic RNA and DC RNA of OVA immunized MUC1.Tg mice (Figs. 6A and 6B).

We also wanted to show this regulation by a self Ag in another model of self-tolerance to be certain that it was not unique to the MUC1.Tg strain or MUC1p as Ag. We immunized

RIP.OVA mice, which express the ovalbumin gene under transcriptional control of the rat insulin promoter and are tolerant to OVA protein (382), with DC loaded with OVA. The DC recovered from these mice also failed to up-regulate trypsin and CPB1 (Fig. 6C).

2.4.3 **Regulation of expression of pancreatic enzymes in DC is dependent on CD4$^+$ regulatory T cells**

Given the antigen specificity of Treg and their ability to modulate DC phenotype and function (171, 383), we hypothesized that the differential expression of pancreatic enzymes in DC might mark DC that had been acted upon by Treg. We cultured BMDC with bead isolated CD4+ Teff and/or Treg, polyclonally activated with anti-CD3 and anti-CD28 antibodies. After 24 hours of co-culture, we found that DC up-regulated trypsin and CPB1 in the presence of activated Teff, but not in the presence of Treg. Importantly, simultaneous culture of DC with Teff and Treg also resulted in low levels of trypsin and CPB1 in DC, demonstrating that Treg actively suppress the ability of Teff to induce enzyme up-regulation. LPS alone had no effect on enzyme levels. (Fig. 7A)

To determine if Treg played a similar role *in vivo*, MUC1.Tg mice were depleted of Treg by injection of anti-CD25 antibody and subsequently vaccinated with soluble MUC1p admixed with Poly-ICLC adjuvant. In control Treg competent mice, we observed the anticipated DC phenotype with suppressed enzyme expression, while DC from immunized Treg-depleted MUC1.Tg mice up-regulated the enzymes (Fig. 7B).

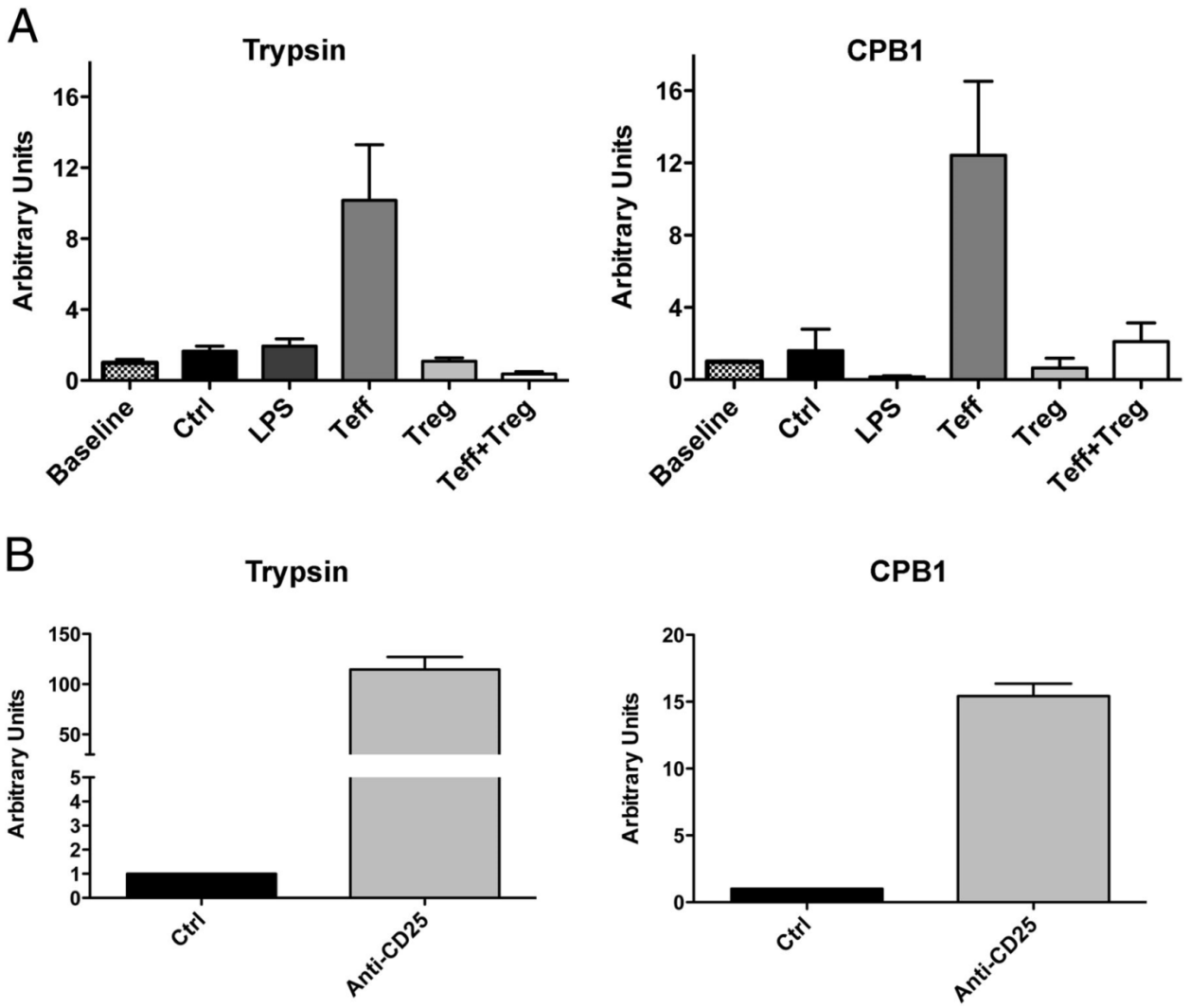

Figure 7: Interactions between DC and CD4 T cells regulate expression levels of pancreatic enzymes in DC. **(**A**) DC were cultured alone (Ctrl), with LPS, or with polyclonally activated $CD25^{-}CD4^{+}$ T cells (Teff) and/or $CD25^{+}CD4^{+}$ T cells (Treg). After 24 h of coculture, DC were separated, and mRNA was extracted for qRT-PCR analysis. Units were standardized against levels preculture (baseline). Bars represent mean ± SEM. Data are representative of two independent experiments. (**B**) MUC1.Tg mice were treated with an Ab against CD25 to deplete regulatory CD4 T cells (Anti-CD25) or with an isotype control (Ctrl). Two days following depletion, mice were vaccinated with soluble MUC1p plus Poly-ICLC i.v. Splenic RNA was extracted 24 h postvaccination for qRT-PCR analysis. Units were standardized against isotype control–treated mice. Bars represent mean ± SEM, respectively. Data are representative of three independent experiments.**

2.4.4 **IL-10 is required *in vivo* for suppression of pancreatic enzyme expression in DC**

One of the few transcripts in the gene array data that was dramatically higher at 24 hours post vaccination in MUC1.Tg mice compared to WT mice was IL-10 (not shown). To confirm, we vaccinated mice with soluble MUC1p admixed with Poly-ICLC and saw a dramatic increase in IL-10 transcript levels (Fig. 8A). Given the known ability of IL-10 to modulate DC phenotype and function in the direction of tolerance versus immunogenicity (384), we hypothesized that it might also be participating in the suppression of DC pancreatic enzyme levels. Accordingly, we treated MUC1.Tg and WT mice with an antibody against the IL-10 receptor (IL-10R) prior to vaccination with MUC1p (Fig. 8B). Blockade of the IL-10R *in vivo* resulted in DCs that had equal levels of pancreatic enzymes in both WT and MUC1.Tg mice in response to MUC1p vaccination.

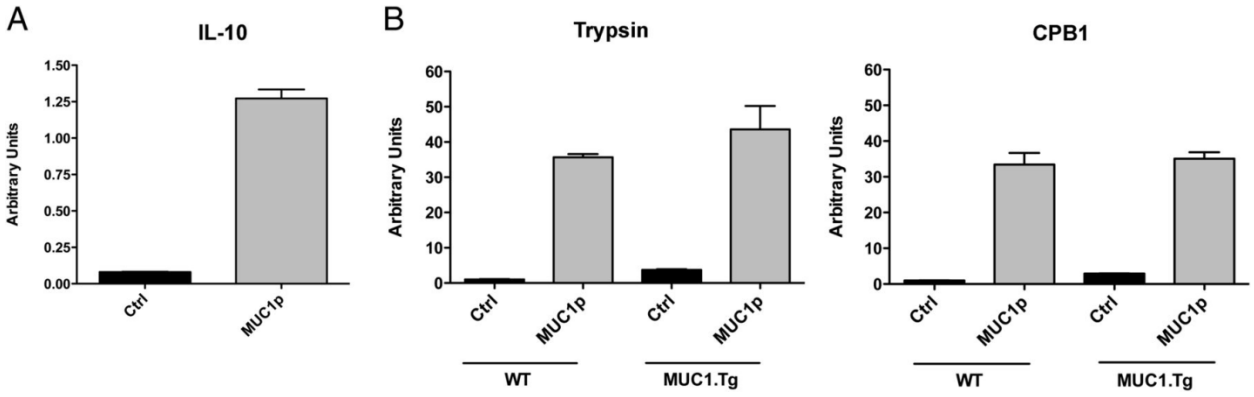

Figure 8: IL-10 is required in vivo for regulation of pancreatic enzymes expression in DC.

(A) MUC1.Tg mice were immunized with PBS (Ctrl) or a soluble MUC1p admixed with Poly-LCIC (MUC1p). IL-10 expression was measured by qRT-PCR on total splenic mRNA 24 h postvaccination. Bars represent mean ± SEM. Data are representative of at least four independent experiments. (B) WT and MUC1.tg mice were treated with an IL-10R blocking Ab followed by i.v. immunization with PBS (Ctrl) or MUC1p as in (A). Twenty-four hours postvaccination, splenic RNA was extracted for qRT-PCR analysis.

Units were normalized to WT Ctrl. Bars represent mean ± SEM. Data are representative of two independent experiments.

2.5 DISCUSSION

Our data reveal the presence of a new pancreatic enzymes signature in DC that may be predictive very early post vaccination, 24-72 hours, of downstream antigen-specific T cell responses. The enzymes comprising this signature (e.g. trypsin, CPB1, elastase) have well-characterized functions in the pancreas but have not been previously reported in DC. Differential expression of these enzymes in DC following immunization with a self or a foreign Ag was associated with dramatic changes in the immunogenicity of the total endogenous splenic DC compartment. A number of other peptidases utilized by DC, especially in the context of antigen processing and presentation, have been characterized (385) and an expanding repertoire of enzymes involved in generating MHCI-restricted peptides is beginning to be elucidated. None of them, however, fall into the category of pancreatic enzymes. Our interest in these enzymes was generated by the observation that their expression levels seen on the total spleen gene array were differentially regulated in response to immunization with a self versus a foreign antigen. They are up-regulated following exposure to a foreign antigen (e.g. OVA in MUC1.Tg mice and MUC1p in WT mice) and suppressed following exposure to a self-antigen (e.g. MUC1p in MUC1.Tg mice and OVA in RIP.OVA mice). As early as 24h post vaccination and until at least 72h, the differential expression pattern of these enzymes was observed in the total $CD11c^+$ splenic compartment. This was independent of whether antigen was presented on exogenous DC that had taken up and processed the peptide prior to immunization, or as soluble antigen plus adjuvant. This showed

that both the initial DC presenting the antigen as well as all other DC in the spleen that either gained access to the antigen or were subject to microenvironmental changes, such as increased IL-10 initiated by the antigen, were suppressed presumably in order to not propagate anti-self responses.

Our data suggest that a DC presenting a self-antigen is rapidly affected by interactions with pre-existing Treg specific for that antigen, as depletion of Treg restores antigen-specific up-regulation of pancreatic enzymes. A large number and repertoire of MUC1p-specific Tregs could arise from thymic expression of MUC1 in MUC1.Tg mice (296), or through prior exposure to antigen in a sub-immunogenic setting. We also show that IL-10 is an important soluble regulatory mediator that is likely elicited either directly or indirectly by Treg upon encounter with self-antigen on DC and is involved in the suppression of pancreatic enzyme expression in addition to its well-characterized effects on DC stimulatory capacity and CD80/86 and MHCII expression (164-166)

The most stimulating question is how are the vast majority of splenic DC (and potentially all) simultaneously either prevented from or stimulated to induce an immune response, the surrogate marker of which is up-regulation or lack of expression of pancreatic enzymes. At least two possibilities exist: 1) highly efficient Ag distribution throughout the spleen such that many DC are presenting self Ag and are therefore individually affected by the action of Treg or T effector cells, and/or 2) highly effective signal transduction to all other DC in the organ from a rare DC that is presenting the antigen and has been affected by Treg or T effector cell. There is support in the literature for both mechanisms (386, 387).

The term “infectious tolerance” has been applied to the process by which one population of leukocytes transfers tolerance to another. In most instances, this involves Treg suppression of

T effector cell generation either through a direct contact or through elaboration of regulatory factors (388). Tolerogenic DC have also been implicated because of their ability to promote the generation of iTreg (389, 390). Most of the studies showing these interactions have been performed *in vitro* and although similar regulation has been postulated *in vivo*, most data in support of it have been generated by pharmacologic manipulations of the system (391). We suggest that our results provide evidence that infectious tolerance occurs *in vivo*. We propose a two-step model of infectious tolerance. The first step is a signal to all DC in the lymphoid organ, and presumably other tissues where self-antigens can be processed and presented by DC, to prevent the up-regulation of pancreatic enzymes. This step is immediate and is initiated by the first encounter of a self-antigen-presenting DC and a Treg. The earliest time point we studied was 24h post-vaccination when expression of the enzyme cohort was already suppressed. However, we suspect that the signal is sent much earlier depending on the route of antigen delivery. With an exogenous DC-based vaccine, the antigen is already processed when the DC enters a lymphoid organ such as spleen, and the suppression signal from Treg may be very quickly generated and propagated. In the case of a soluble antigen entering a lymphoid organ, there is likely a minor delay in suppression due to the time it takes for resident DC to take up, process and present the antigen. The second step is delayed and involves the conversion of the DC into a phenotypically and functionally tolerogenic cell that primarily supports generation of Treg. We show that DC recovered from spleens exposed to self Ag through vaccination expressed low levels of costimulatory molecules and had reduced motility, likely resulting in less efficient traffic into T cell zones, and primed the expansion of more Treg than Teff cells when cultured with antigen specific T cells. *In vivo*, this would assure that self-antigen specific Treg continue to be primed for the duration of antigen exposure, which would likely protect the host

from autoimmunity in non-pathologic conditions, but may also be responsible for preventing effective anti-tumor immunity.

We are reporting a new observation that will require further studies to fully elucidate the exact mechanism involved, especially at the level of the regulated DC. We do not know the exact role of pancreatic enzymes in DC, whether it is in antigen processing or another DC-intrinsic function, nor can we yet postulate how their expression is coordinately regulated. However, the expression levels of trypsin and CPB1 provide an early readout of the effects of self or foreign Ag on the phenotype and function of endogenous splenic DC. The microarray data did not reveal any candidate transcription factors that are differentially expressed in the regulated DC that could be responsible for this enzyme cohort's transcriptional control. We expect that the 24-hour time point may have been too late for identifying such factor(s). Now that our attention is focused specifically on these enzymes and DC, we will look at much earlier time points. We also have not yet fully explored the role of IL-10 and the precise signals it provides to the DC and how those signals relate to enzyme suppression, or other effects on DC phenotype. Nevertheless, pancreatic enzyme expression in DC represents a new finding and suggests an easily accessible signature that can be used to assess almost immediately the effect of a particular immune manipulation designed to either induce tolerance or immunity. This can be particularly helpful in animal models where various immunotherapeutic approaches are being tested and multiple approaches compared. Time could be saved and animals spared if the final outcome (e.g. tissue graft acceptance or a tumor rejection) were not the primary and to date the main endpoints by which the success of the immune manipulation could be evaluated.

Our specific interest is the response to a tumor antigen vaccine and determining how best to evaluate and compare vaccine efficacy early after vaccination, other than waiting for the

results of a tumor challenge in an animal or tumor recurrence in a patient. Our previous studies have emphasized the importance of antigen selection, especially in the case of non-viral tumor associated antigens (392). This study confirms the importance of proper antigen selection that in some cases may outweigh the importance of adjuvants or delivery systems. Among the many tumor associated antigens that have been fully characterized (214), it should be possible to focus on those that are less self and more foreign due to many differences in their post-translational modifications between normal and tumor cells. As we have shown previously, a tumor-specific sugar added to MUC1p to create MUC1.Tn results in strong immunogenicity rather than tolerance in immunized MUC1.Tg mice (376). The wrong antigen or the wrong epitope, on the other hand, leads to DC suppression, infectious tolerance, and further promotion of Treg generation that not only fails to achieve an effective antitumor immune response, but may actually promote tumor growth by selectively expanding tumor-antigen-specific Treg (393). Depletion of Treg with anti-CD25 antibodies or diphtheria toxin have shown a good deal of promise in preclinical models of cancer immunotherapy (394-398). IL-10R blockade has also been shown to improve overall vaccine responses in several models, while IL-10 production, specifically by CD4+CD25+ Treg is negatively correlated with vaccine success (399, 400). We propose that these treatments work because they prevent DC-propagated infectious tolerance.

3.0 GLOBAL INHIBITION OF DC PRIMING CAPACITY IN THE SPLEEN OF SELF-ANTIGEN VACCINATED MICE REQUIRES IL-10

Chapter 2 is adapted from "Global inhibition of DC priming capacity in the spleen of self-antigen vaccinated mice requires IL-10". Marvel DM and Finn, OJ. Frontiers in Immunology 2014 Feb 17;5:59. doi: 10.3389/fimmu.2014.00059.

3.1 ABSTRACT

DC in the spleen are highly activated following intravenous vaccination with a foreign antigen, promoting expansion of effector T cells, but remain phenotypically and functionally immature after vaccination with a self-antigen. Up-regulation or suppression of expression of a cohort of pancreatic enzymes 24-72 hours post-vaccination can be used as a biomarker of stimulatory versus toleragenic DC, respectively. Here we show, using MUC1 transgenic mice (MUC1.Tg) and a vaccine based on the MUC1 peptide which these mice perceive as a self-antigen, that the difference in enzyme expression that predicts whether DC will promote immune response or immune tolerance, is seen as early as 4-8 hours following vaccination. We also identify early production of IL-10 as a predominant factor that both correlates with this early time point and controls DC function. Pre-treating mice with an antibody against the IL-10 receptor (IL-10R)

prior to vaccination results in DC that up-regulate CD40, CD80, and CD86 and promote stronger IFNγ+ T cell responses. This study suggests that transient inhibition of IL-10 prior to vaccination could improve responses to cancer vaccines that utilize self-tumor antigens.

3.2 INTRODUCTION

The impact of IL-10 on the cells of the immune system is well studied and varied. Originally identified as cytokine synthesis inhibitory factor, IL-10 can play a role in the development and maturation of almost all immune cells. (401, 402) Signaling through the IL-10 receptor (IL-10R) occurs through a STAT3 intermediate and is known to induce SOCS-3 expression, to suppress IFN signaling by blocking STAT1 phosphorylation, and to inhibit NF-κB signaling by preventing its nuclear translocation as well as inhibiting its binding to DNA. (167, 401) In dendritic cells (DC), known for being the most important professional antigen presenting cells, IL-10 can reduce expression of MHC Class II and the costimulatory molecules CD80/86 and CD40, as well as reduce IL-12 secretion. (164-167) This is true even for DC previously activated with IFNγ. IL-10 can also prevent monocyte differentiation into DC. (401)

IL-10 has a profound effect on T cells as well. For example, reduced IL-12 production by DC affected by IL-10 antagonizes the development of T helper type I (TH1) responses while reduced MHC II levels on DC result in presentation of low density antigen that preferentially stimulates differentiation of regulatory CD4 T cells. (331, 403) IL-10 can also act directly on T cells to inhibit synthesis of cytokines like IL-2 and IFNγ in CD4 T cells or to inhibit their

proliferation. (167) The effect of IL-10 on CD8 T cells is less clear although some studies have shown that IL-10 can favor activation of CD8 T cells. (322, 404, 405)

Recently, our group implicated IL-10 in controlling in part the function of dendritic cells post vaccination with antigens derived from self-proteins. We showed that 24 hours following vaccination, there is an IL-10 dependent suppression of DC activation that is detectable via suppression of expression of a newly discovered biomarker, a cohort of pancreatic enzymes. These enzymes, represented by trypsin 1 and carboxypeptidase B1, are up-regulated post vaccination with a foreign but not a self-antigen and identify a DC population that has higher MHC Class II, higher costimulatory molecule expression, and a higher T cell stimulatory capacity. (406)

In this study we present new evidence of an important role for IL-10 in the suppression of splenic DC following intravenous vaccination with a self-antigen. We show an early (4-8hr) up-regulation in IL-10 levels in spleens of self-antigen vaccinated mice that is not seen in mice that see that same antigen as foreign, that coincides with the time when we also see differences in biomarker enzyme expression. Furthermore, DC in the spleens of self-antigen vaccinated mice have an increased sensitivity to IL-10. When the effect of IL-10 is blocked by pre-vaccination treatment of mice with an anti-IL-10R blocking antibody, there is a significant increase in the activation level and stimulatory capacity of DC at 24 hours post vaccination and a significant increase in CD4 T cell responses 7 days post vaccination. These data implicate IL-10 in the regulation of antigen specific immunity versus tolerance at a previously underappreciated early time post vaccination, and suggest that manipulating its function at the time of vaccination might overcome tolerance and improve responses to cancer vaccines that utilize self-antigens..

3.3 MATERIALS AND METHODS

3.3.1 **Mice**

Human MUC1.Tg mice (283) on the C57Bl/6 background were a generous gift from Dr. Sandra Gendler (Mayo Clinic) and were bred and maintained in the University of Pittsburgh Animal Facility. C57Bl/6 (WT) mice were purchased from The Jackson Laboratory. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

3.3.2 **MUC1 Vaccination**

A 100-aa peptide containing of 5 repeats of the MUC1 VNTR motif, HGVTSAPDTRPAPGSTAPPA, was synthesized as previously described (407) by the University of Pittsburgh Genomics and Proteomics Core Laboratories. For soluble peptide vaccinations, 100µg of this 100mer peptide, admixed with 50µg polyinosinic-polycytidylic acid and poly-L-lysine (Poly-ICLC; Hiltonol) was brought up to 100µL with PBS and injected via tail vein. For DC based vaccinations, vaccine DC were prepared as previously described. (291) Briefly, RBC lysed bone marrow cells were put into culture for 6 days in AIM-V supplemented with 10ng/ml GM-CSF (Miltenyi), feeding once on day 3. On day 6, semi-adherent cells were collected by gentle agitation and put into culture overnight in AIM-V containing 33µg/mL MUC1 100mer peptide and 25µg/mL Poly-ICLC. The next day, mature DC were collected and resuspended in PBS at a final concentration of .5-1x10^6 cells/ml. 100µL of this solution was then injected intravenously via tail vein.

3.3.3 **IL-10R Blockade**

Where indicated, mice were given 250μg of an antibody against the IL-10 receptor (Bio X Cell, Clone 1B1.3A) or an isotype matched control antibody (Bio X Cell, Clone HPRN), intraperitoneally. 24-48 hours following treatment, mice were vaccinated as described in *MUC1 Vaccination* above and analyzed as described.

3.3.4 **Quantitative RT-PCR**

RNA was extracted from whole spleen using TRIzol (Invitrogen) according to the manufacturer's protocol. Following extraction, cDNA was generated using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen). qPCR was performed using QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's protocol. Reactions were run on a StepOne Plus instrument (Applied Biosystems). The following primer pairs were used: Trypsin 1 (forward: 5'-ACTGTGGCTCTGCCCAGCTC-3'; reverse: 5'-AGCAGGTCTGGTTCAATGACTGT-3'), CPB-1 (forward: 5'-GCCCTGGTGAAAGGTGCAGCAAAGG-3'; reverse: 5'-AGCCCAGTCGTCAGATCCCCCAGCA-3'), IL-10 (forward: 5'-CTTCCCAGTCGGCCAGAGCCA-3'; reverse: 5'- CTCAGCCGCATCCTGAGGGTCT-3'), and HPRT (forward: 5'-TGAGCCATTGCTGAGGCGGCGA-3'; reverse: 5'-CGGCTCGCGGCAAAAAGCGGTC-3').

3.3.5 **Intracellular Cytokine Staining/Flow Cytometry**

For *ex vivo* T cells assays, 7-9 days post MUC1 vaccination, mice were sacrificed and spleens were removed. Single cell suspensions were made by mashing the spleens through a 40μm filter. Total T cells were then bead isolated (Miltenyi) and cultured with day 6 MUC1 loaded BMDC (prepared as described in *MUC1 Vaccination*) for 4-6 hours in the presence of GolgiStop (BD biosciences). Cells were then stained with the indicated antibodies using the BD Cytofix/Cytoperm^{TM} kit (BD Bioscience) according to the manufacturer's protocol. All samples were run on a Fortessa (BD bioscience) flow cytometer and analyzed using FACSDiva (BD Bioscineces) and FlowJo software (Tree Star, Inc.). Antibodies used: CD3-PerCP, CD11c-BV421, CD80-FITC, CD86-APC/Cy7, CD40-APC, CD3-PeCy5, CD4-V450, CD8-AF700, IFNγ-PeCy7, TNFα-PE, IL-2-APC, CD44-FITC, CD3 APC/Cy7, and CD8 PerCP.

3.3.6 **Phosphoflow**

24 hours following MUC1 vaccination, splenocytes were harvested as above. Post isolation, cells were put into AIM-V with or without 30ng/mL IL-10 (PeproTech) for 20 minutes. At the end of culture, cells were immediately fixed in 1.6% PFA for 10 minutes at room temperature. After ten minutes, four volumes of ice cold methanol were added and samples were stored at -80°C. At the time of staining cells were put at room temp for 10 minutes and then immediately spun down and resuspended in flow buffer (PBS containing 1%BSA, .02% sodium azide, and 2nM EDTA). After ten minutes incubation at room temperature, cells were spun down and washed with flow buffer twice. Samples were then stained with antibodies against cell surface antigens CD11c, NK1.1 and CD3 and phospho-specific anti-pSAT3 antibody for 1hr at room temperature and

prepared for analysis via standard protocol and as described above. Antibodies used: CD11c-Pacific Blue, pSTAT3-AF647, NK1.1-PE, and CD3-APC/Cy7.

3.3.7 **Ex vivo DC Stimulatory Capacity Analysis**

MUC1.Tg mice were pretreated with antibodies and vaccinated as in the "IL-10R Blockade" section. Post vaccination, DC were bead isolated (CD11c MicroBeads, Miltenyi) from the spleens of the vaccinated animals. These DC were put into culture with bead isolated (CD4 T cell Isolation Kit II, Miltenyi) CFSE stained MUC1 specific VFT CD4 T cells (Ryan et al., 2010) at a ratio of 1 DC to 5 VFT cells in complete DMEM. 24 hours after the start of culture half of the media was removed and saved for cytokine analysis. IL-2 was analysed by ELISA (BD OptEIA Mouse IL-2 ELISA set, BD) according to the manufacturer's protocol. The media was replaced with fresh cDMEM and the cultures were allowed to incubate for three more days. T cell proliferation was then analyzed by CFSE dilution.

3.3.8 **ELISPOT**

Millipore MultiScreen® Filter Plates (Millipore) were pretreated according to the manufacturer's instructions using the Mouse IFNg ELISPOT kit (Mabtech). Bead isolated CD4 and CD8 T cells (Miltenyi) were cultured as above (see *Intracellular Cytokine Staining/Flow Cytometry*) with MUC1 pulsed BMDC and analyzed according to the established protocol. DC alone, media alone and T cells alone were used to establish background cytokine production.

3.3.9 **Statistical Analysis**

When appropriate, statistical significance was determined by performing an unpaired Student's t-test. * denotes a p value <.05 and ** denotes a p value of <.01. When indicated, to allow for pooling of data from multiple experiments, values have been transformed to account for minor variations in instrument settings and other potential sources of variation (i.e. minor batch to match variance in dendritic cell vaccine prep, etc.). Briefly, all experimental values were divided by the mean value of the control group from the experiment in which they were run. "Relative" values therefore represent a standardized deviance from control.

3.4 RESULTS

3.4.1 **IL-10 expression in the spleen is increased 4-8 hours post vaccination with self-antigen and correlates with DC suppression**

In order to determine how quickly post-vaccination with a self- versus a foreign antigen DC phenotype and function begin to diverge and obtain a more accurate picture of what factors might be responsible for supporting this divergence, we vaccinated intravenously WT and MUC1.Tg mice with the MUC1 100mer peptide (MUC1p) admixed with the Poly-ICLC adjuvant. MUC1.Tg mice express the human tumor antigen MUC1 under the control of its endogenous promoter and therefore MUC1p is seen as a self-antigen in these mice, whereas it is seen as a foreign antigen in WT animals. Mice were sacrificed 4, 6, 8, and 16 hours post vaccination and the spleens removed for mRNA isolation and analysis. As early as 4 hours post

vaccination, two newly discovered biomarkers of DC activation, trypsin 1 and carboxypeptidase B1 (CPB1)(406), were up-regulated in the spleens of WT mice but suppressed in MUC1.Tg mice (Figs. 9A-B). In addition to differences in the levels of these enzymes, which our previous study showed to be expressed only in DC, we also detected at this early time point higher levels of IL-10 mRNA in the spleens of vaccinated MUC1.Tg mice compared to WT mice. At 24 hours post vaccination and later, IL-10 production was at equal levels in self- and foreign antigen vaccinated mice (Figure (9C and data not shown).

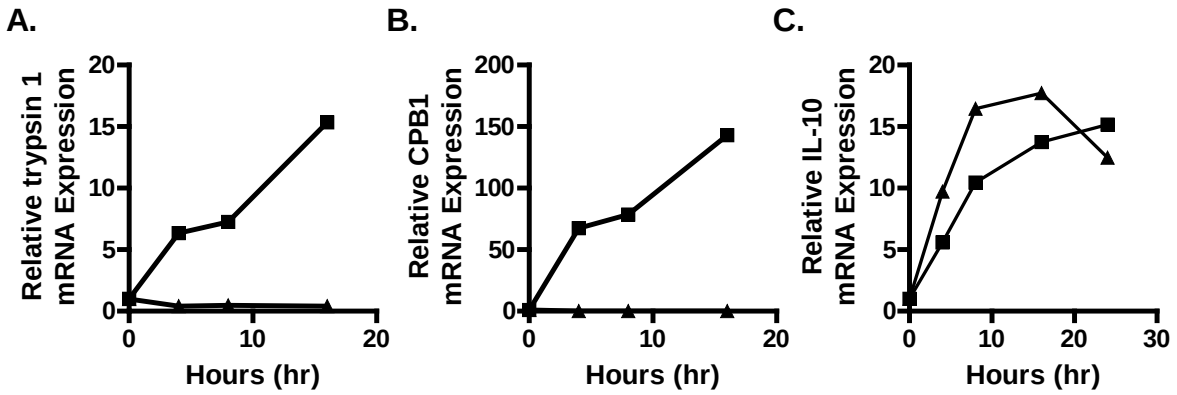

Figure 9: Splenic DC activation is suppressed as early as 4-8 hours post vaccination with a self-, but not a foreign antigen and correlates with early IL-10 production in the spleens of these animals.

WT (squares) and MUC1.Tg mice (triangles) were vaccinated with MUC1p plus Poly-ICLC via tail vein. Spleens were removed at indicated hours postvaccination and total splenic mRNA levels of trypsin 1 (A), carboxypeptidase B1 (CPB1, B), and IL-10 (C) were determined relative to the control gene HPRT. Values shown represent expression relative to the baseline expression in mice of that genotype (WT and MUC1.Tg) at 0 hours post vaccination. Data are representative of 3 pooled mice were group per timepoint shown. Data points show mean ±SEM of three technical replicates and are representative of two independent experiments.

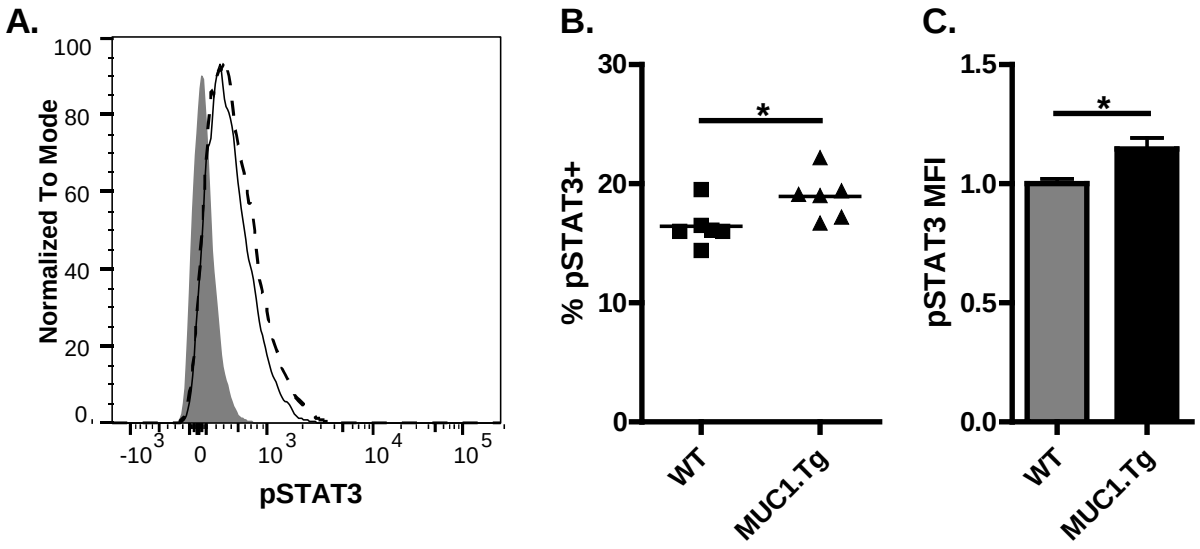

Figure 10: DC from spleens of mice vaccinated with self-antigen have higher levels of phosphorylated STAT3 after IL-10 treatment than DC from spleens of mice vaccinated with foreign antigen.

WT (solid line) and MUC1.Tg (dashed line) mice were vaccinated with MUC1p via the tail vein. 24 hours post vaccination splenocytes were removed and treated with 30ng/mL of IL-10 for 20 minutes. Following incubation, cells were fixed and phospho-STAT3 expression in CD11c+NK1.1- splenocytes was analyzed via phoshpoflow. (A) A representative flow plot is shown. The shaded histogram represents the fluorescence level when cells are treated with standard surface markers and an isotype matched control instead of the phoshpospecific antibody. pSTAT3 positivity (B) and MFI (C) were analyzed. In (B) symbols correspond to individual animals and are representative of two independent experiments. (C) Values shown have been normalized to the expression level of the control group (WT) in order to allow for pooling of data from separate experiments run on multiple days. Bars are representative of 9 mice from 2 combined experiments and show the mean ±SEM. *indicates a p-value of <.05

3.4.2 **DC from the spleens of self-antigen vaccinated mice are more sensitive to IL-10 than those from mice vaccinated with a foreign antigen**

The above data showing differences in IL-10 levels early post vaccination but no difference at 24 hours and later would indicate a modest and transient effect by IL-10 on DC. This was, however, inconsistent with our previous observations that functional differences between DC post self-antigen versus foreign-antigen vaccine were evident as late as 72 hours post vaccination. (406) We considered the possibility that the early action of IL-10 on DC, along with other factors, might increase their sensitivity to IL-10 at the later time points. To query this, DC were removed from the spleens of WT and MUC1.Tg mice 24 hours post MUC1p vaccination and exposed to IL-10. As signaling through the IL-10R is known to occur through a STAT3 intermediate, the sensitivity of DC to IL-10 was assessed by phosphoflow measuring phospho-STAT3 levels post *ex vivo* exposure to IL-10. As hypothesized, there was a significant increase in the number of DC showing STAT3 phosphorylation as well as higher levels of pSTAT3 in the spleens of MUC1p vaccinated MUC1.Tg mice (Fig. 10A-C), indicating that DC in the spleens of MUC1.Tg mice are not only exposed to more IL-10 early on, but are also more sensitive to it at the later time points.

3.4.3 **IL-10R blockade increases costimulaltory molecule expression on DC following vaccination with self-antigen**

Given the inverse correlation between IL-10 production and DC pancreatic enzyme expression in the first 24 hours following vaccination, and previously published data showing that IL-10 is necessary for suppression of the trypsin 1 and CPB1 following vaccination with a self-antigen

(406), we hypothesized that blocking IL-10 signaling in self-antigen vaccinated mice would improve DC activation and costimulatory molecule expression. We injected MUC1.Tg mice with an antibody against IL-10R and vaccinated intravenously 24-48 hours later with MUC1p plus Poly-ICLC. At 24 hours post vaccination, the surface phenotype of splenic DC was analyzed by flow cytometry. As hypothesized, there was an increase in the level of cell surface expression of CD40, CD80, and CD86 in DC from mice pretreated with the antibody to IL-10R, but not from mice treated with the isotype control antibody (Fig. 11A-C). Increases in CD40 and CD86 were statistically significant, which is of interest because these two molecules were shown previously to be specifically inhibited in mice vaccinated with a self- but not a foreign antigen. (406) There was also an increase in the number of splenic DC post vaccination (Fig. 12), which is again of interest as our previous study showed the number of splenic DC to be decrease post vaccination with a self-, but not a foreign antigen. In addition to being less active as measured by surface marker expression and in fewer numbers, these DC are also less capable of stimulating MUC1 specific CD4 T cells *in vitro*. DC isolated from MUC1.Tg mice pretreated with an antibody against the IL-10 receptor prior to MUC1 vaccination and put into culture with MUC1 specific CD4 T cells induce higher levels of IL-2 (Fig. 13A) and a higher level of CD4 T cell proliferation (Fig. 13B-C), compared to DC from MUC1.Tg mice pretreated with an isotype matched control antibody.

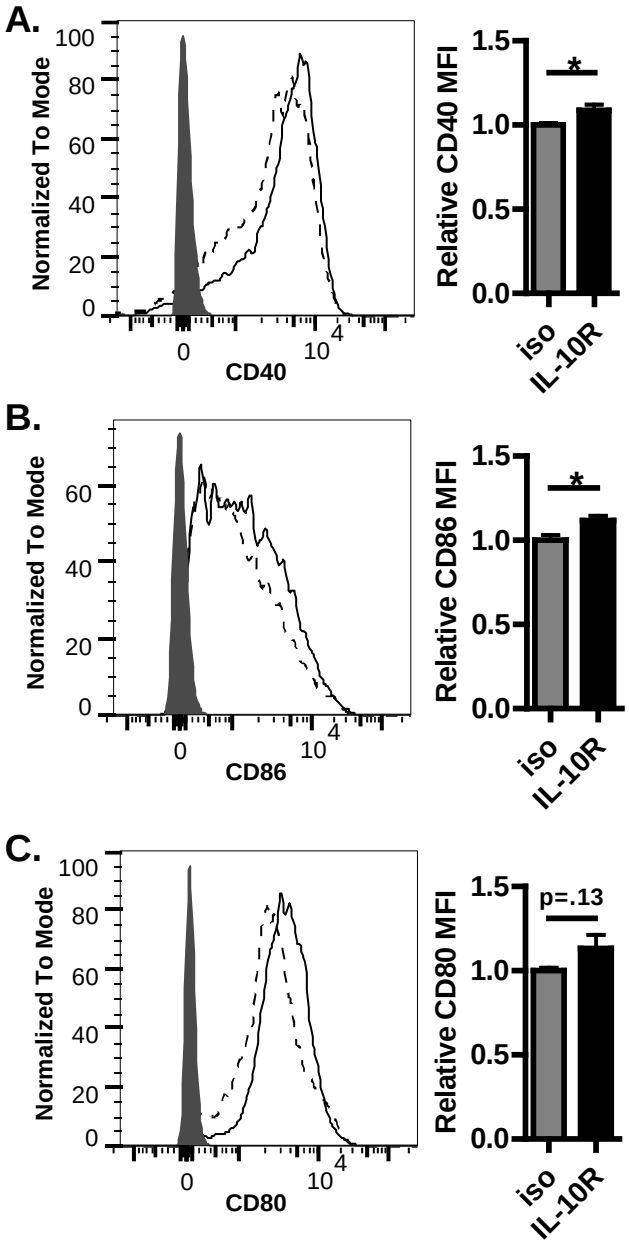

Figure 11: Pretreatment with an antibody against the IL-10 receptor increases the level of costimulatory molecule expression on DC in the spleens of self-antigen vaccinated mice.

MUC1.Tg mice were pretreated with an antibody against the IL-10 receptor (IL-10R, solid lines) or were given a nonspecific isotype control (iso, dashed lines). 1-2 days later they were vaccinated as in Figure 1 and 24 hours post vaccination, splenocytes were removed and analyzed via flow cytometry. The expression level of CD40 (A), CD86 (B), and CD80 (C) on splenic DC (CD11C+, MHC-Class II+) was determined. Shaded

histograms represent fluorescence in samples stained with isotype alone. Bar graph values shown have been normalized to the expression level of the control group (iso) in order to allow for pooling of data from separate experiments run on multiple days. (A, C) Data are combined from two independent experiments and representative of 6 mice. (B) Data are combined from 3 independent experiments and are representative of 10 mice. Bars represent mean ±SEM. P values are as stated unless designated by a *, which indicates a p-value of <.05.

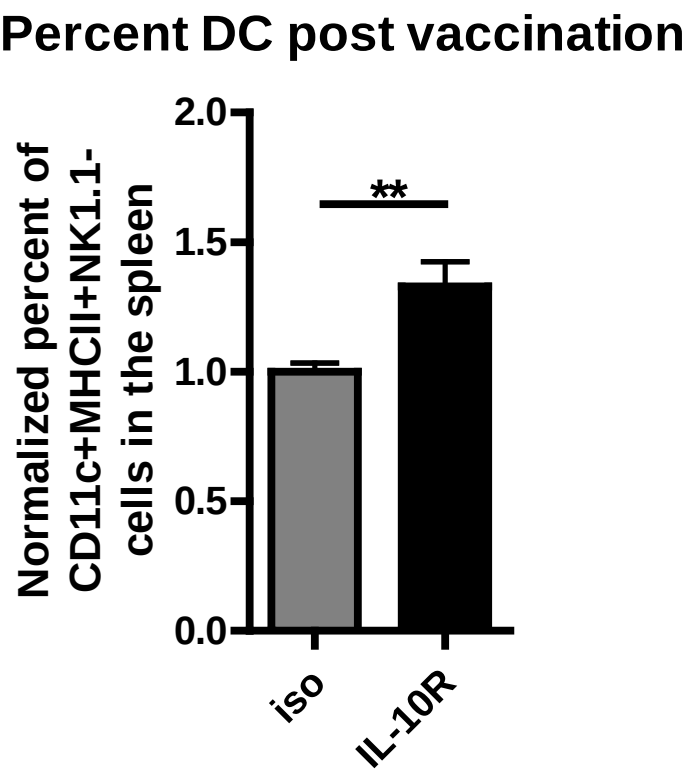

Figure 12: Blocking IL-10 prior to MUC1 vaccination increases the number of splenic DCs 24 hours post vaccination.

MUC1.Tg mice were pretreated with an antibody against the IL-10 receptor (IL-10R, black bar) or were given a nonspecific isotype control (iso, grey bar). 1-2 days later they were vaccinated as in Figure 1 and 24 hours post vaccination, splenocytes were removed and analyzed via flow cytometry. The percentage of CD11c+MHC Class II+NK1.1- cells was calculated. In order to allow for the pooling of multiple experiments, this percentage was normalized, with the percent in the control group being set to 1. Data are representative of at least 4 experiments. Bars represent mean ±SEM. ** indicates a p-value of <.005.

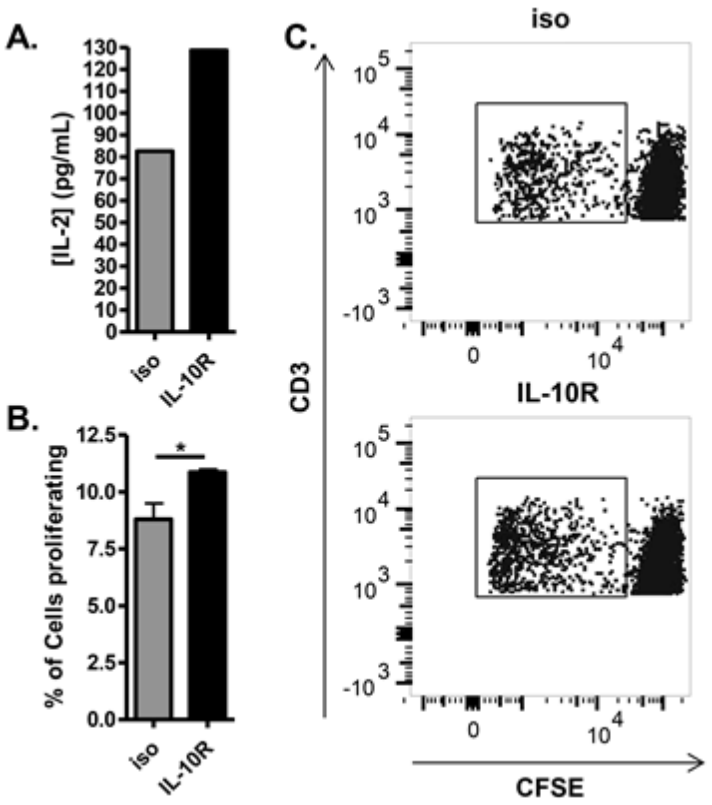

Figure 13: Blocking of the IL-10 receptor prior to intravenous MUC1 peptide vaccination increases the ability of splenic DC from MUC1.Tg mice to stimulate MUC1 specific CD4 T cells ex vivo

MUC1.tg mice were treated as in Figure 3. 24 hours post MUC1 vaccination, splenocytes from 3-4 mice per treatment group were pooled and bead isolated DC from these pooled splenocytes were put into culture with CFSE labelled MUC1 specific CD4 T cells (VFT cells) at a ratio 1DC:5VFT. 24 hours after the start of culture, half of the culture media was removed and the concentration of IL-2 was measured by ELISA (A). Cultures were allowed to incubate 3 more days for a total of 4 and VFT proliferation was analyzed by CFSE dilution (B-C). (B) Bars represent the mean percentage of CD3+CD4+ T cells that had proliferated at 4 days of 3 technical replicates ±SEM. (C) A representative flow plot is shown. Data are representative of 2-3 independent experiments. * indicates a p-value of <.05.

3.4.4 **Blocking IL-10 signaling improves the CD4 T cell response to self-antigen vaccination without affecting the CD8 T cell response**

The increase of costimulatory molecule expression when IL-10 signaling was blocked just prior to vaccination suggested that there would be a resultant increase in the T cell response. To test this, we again pretreated mice with an anti-IL-10R antibody or an isotype matched control and injected with a vaccine composed of DC loaded with MUC1p. We chose the DC-based vaccine expecting that it will optimally stimulate both CD4 and CD8 T cells, as has been previously shown. (285) 7-9 days post vaccination, splenic T cells were isolated and their production of relevant cytokines analyzed by ELISPOT and intracellular flow cytometry. In MUC1.Tg mice treated with anti-IL-10R, there was a significant increase in MUC1p specific, IFNγ+ CD4 T cells when compared to mice treated with an isotype matched control antibody (Figs. 14A, C). The level of the response was equivalent to the response of WT mice pretreated with the isotype control antibody. (Fig. 14A.) There was no increase over the isotype control of the T cell response in WT mice pretreated with the anti-IL-10R antibody (Figs. 14A,C), indicating that the effect of IL-10 we saw in MUC1.Tg mice was specific for controlling responses to self- but not foreign antigens. There was a small but not significant increase in the CD8 response that was detectable only by the more sensitive ELISPOT. (Figs. 14B,D)

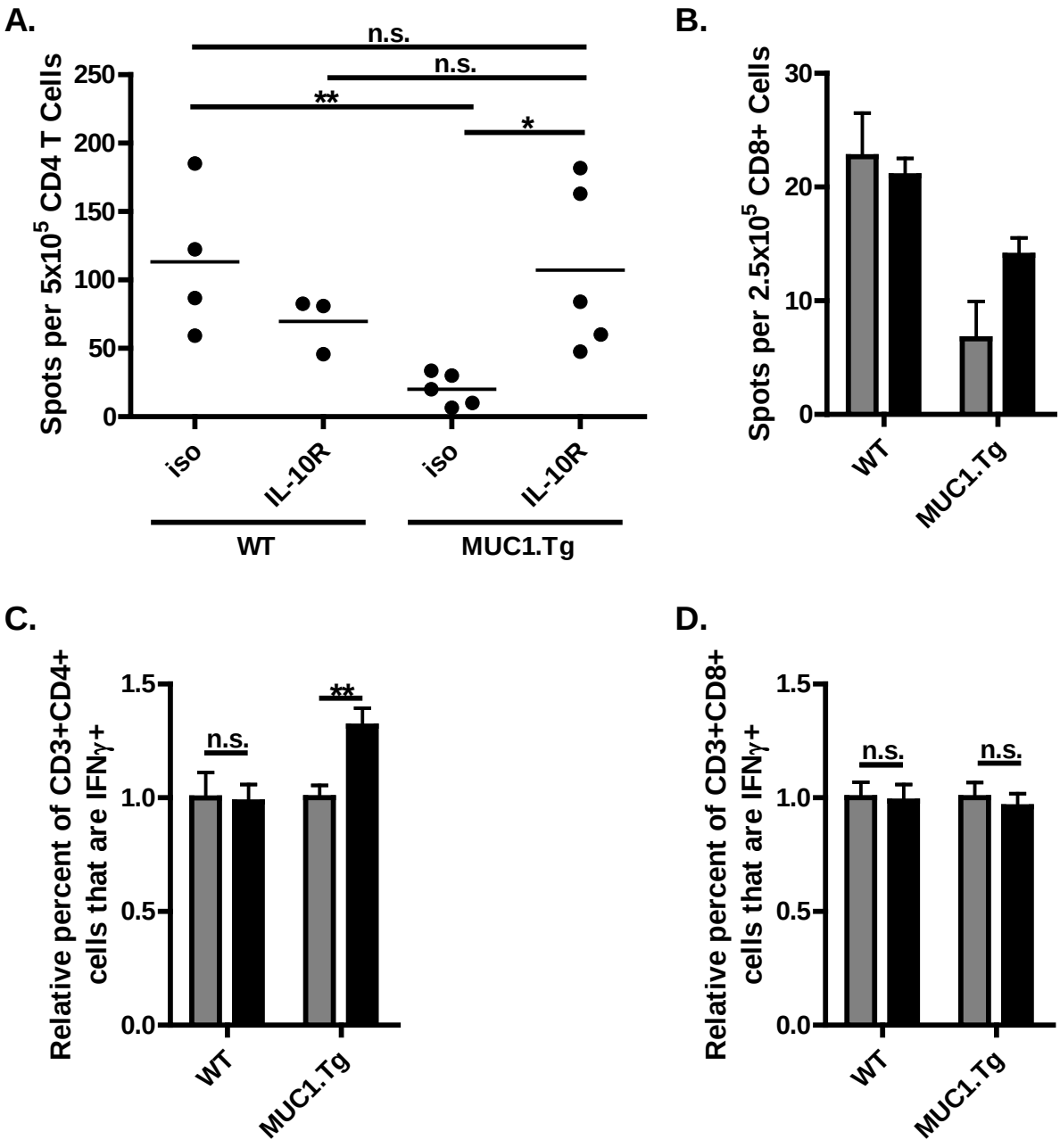

Figure 14: Treatment with anti-IL-10R antibody at the time of vaccination increases the number of MUC1p specific, IFNγ+ CD4 T cells without an effect on CD8 T cells

WT and MUC1.Tg mice were pretreated with an antibody against the IL-10 receptor (IL-10R, black bars) or a nonspecific isotype control (iso, grey bars). 1-2 days following antibody treatment, mice were vaccinated with DC loaded with MUC1p. 7-9 days post vaccination, spleens were removed and bead isolated CD4 (A, C) and CD8 T cells (B, D) were cultured with MUC1p loaded bone marrow derived DC overnight and analyzed

by ELISPOT (A-B), or were cultured for 6-8 hours in the presence of brefeldin-A and analyzed by intracellular flow cytometry (C-D). (A) Data are combined from two independent experiments with each spot indicating an individual animal. Data are representative of 3 independent experiments. (B) Bars indicate the average of 3 technical replicates pooled from 3 individual animals per group. Data are representative of two independent experiments. (C-D) Values shown are normalized to the response of mice of that genotype (WT vs. MUC1.Tg) given the control treatment (iso). Data are combined from 2 independent experiments and are representative of 5-6 mice per group. Bars represent mean ±SEM. * indicates a p-value of <.05. ** indicates a p-value of <.005.

3.5 DISCUSSION

Vaccines against cancer have garnered a lot of attention in recent years. Much of this was sparked by the relatively recent approval of Sipuleucel-T, the first vaccine to show survival benefit in a solid metastatic tumor. (25, 408) Implementation of Gardasil®, a quadrivalent human papilloma virus specific vaccination intended to prevent cervical cancer in women (208, 209) has also sparked new efforts in designing prophylactic cancer vaccines not just for viral cancers but for many tumor types. (179, 220, 409, 410) Most non-viral tumor antigens fall into the category of self- or altered self-antigens. Mounting an effective immune response against them represents a unique challenge. One must design vaccines that overcome the natural tolerizing forces acting on responses to self-antigens, while minimizing adverse autoimmune effects.

Our work with the MUC1 tumor antigen in the MUC1 transgenic mouse model system has shown that hyporesponsiveness to the MUC1 peptide vaccines in these mice is not due to the elimination of MUC1 peptide-specific T cells by central tolerance but rather by the control of

their activation in the periphery. (291) Most recently we showed that tolerization of DC in MUC1p vaccinated MUC1.Tg mice early post vaccination is responsible for this T cell hyporesponsiveness. (406) Here we show that this is likely due to the very early and exaggerated effect of IL-10 on these DC in the first 4-24 hours post vaccination. IL-10 is known to reduce MHC Class II and costimulatory molecule expression on DC (164-166), DC motility (411, 412) and overall T cell stimulatory capacity (413, 414), all of which are characteristics of DC in the spleens of MUC1p vaccinated MUC1.Tg mice. (406)

The effects on IL-10 on vaccine outcome have been observed in several models. In the therapeutic setting, IL-10R blockade alone or along with vaccination can improve Th1 responses and enhance pathogen clearance. (415-417) In a prophylactic setting, mice given the BCG vaccination for prevention of Mycobacterium tuberculosis show improved Th1 responses and enhanced resistance to pathogen challenge when IL-10R is blocked at the time of vaccination. (418) However, the role of IL-10 we describe in this paper is unique in its specificity for responses to self-antigen vaccines. Additionally, in these other studies IL-10 was produced in response to persistent or initial pathogen infection whereas in this case it was either produced specifically in response to the presence of a self-antigen or inhibited specifically in the presence of a foreign antigen.

The role of IL-10 in this system is quite novel and supports IL-10 inhibition as a way of improving the efficacy of vaccines against self-antigens that are candidate tumor antigens. While our major success in this study was in improving CD4 T cell responses, we would hypothesize that CD8 T cell responses generated upon boosting would be improved as well in these animals as a consequence of generation of a larger population of helper CD4 T cells that are required for effective CD8 T cell memory differentiation. (75, 423) The concern remains that any

manipulation leading to enhanced responses to self/tumor antigens might cause adverse autoimmune reactions. However, current research has shown this concern can be addressed by proper antigen selection. For example, vaccines against self/tumor antigens MUC1 and α-lactalbumin have shown clinical and preclinical efficacy with no induction of autoimmunity (179, 212, 424). And vitiligo, caused by successful anti-melanoma vaccines is an autoimmune event that can be easily tolerated. (26, 27, 425) Furthermore, while long term IL-10 deficiency can cause adverse autoimmune effects (426, 427), our data suggests that in order to improve the vaccine response, IL-10 would need to be blocked only transiently at the time of initial vaccination.

It was next important to identify the cell type in the spleen that produced the IL-10 this early IL-10 production in self-antigen vaccinated mice and chapter 4.0 will deal with that topic. Every cell of the immune system can produce IL-10 given proper stimulation. However the kinetics and pattern of IL-10 production in MUC1p-vaccinated MUC1.Tg mice limits the possibilities considerably. The fact that IL-10 production was antigen dependent suggests a cell of the adaptive immune system. Regulatory T cells have previously been shown to be important in preventing MUC1p specific immune responses in MUC1.Tg mice. (292, 406) However, IL-10 producing regulatory T cells were not detected in MUC1.Tg mice at rest or immediately following vaccination (data not shown). Given that regulatory T cells can modulate the function of a wide variety of innate cells, including NK cells (419, 420) and dendritic cells (421, 422), it is possible that through secretion of another cytokine or through direct interactions, they induce IL-10 production either directly or indirectly in another cell population.

4.0 IL-10 PRODUCING NATURAL KILLER (NK) CELLS ARE INDUCED AFTER VACCINATION WITH A SELF ANTIGEN AND ARE POLARIZED TOWARDS INHIBITION RATHER THAN SUPPORT OF THE ADAPTIVE IMMUNE RESPONSE

4.1 ABSTRACT

MUC1 specific vaccine responses are known to be inhibited in MUC1.Tg mice. Recent evidence has implicated early IL-10 production and reduced immunogenicity of dendritic cells in the spleens of these animals in response to MUC1 vaccination as initiating factors in this inhibition. They have not, however, addressed the source of this IL-10. Here we show that splenic NK cells make IL-10 in response to MUC1 vaccination in MUC1.Tg, but not WT mice. Compared to NK cells in spleens of MUC1-vacinated WT mice, NK cells in the spleens of MUC1.Tg mice have low CD127, CD25, and CD69 and are more cytotoxic. They are also less capable of promoting DC maturation in co-culture assays. Furthermore, in MUC1.Tg mice multi-functional CD4 T cell responses specific for the MUC1 peptide are improved by pre-vaccination depletion of NK cells. Together, these data suggest that NK cells, normally required for optimal Th1 responses, can actually inhibit such responses after vaccination with a self-antigen.

4.2 INTRODUCTION

Prophylactic and therapeutic cancer vaccines face challenges that traditional vaccines are not subject to. In the case of therapeutic vaccines, tumor induced immune suppression and the advanced age of the target population remain the largest obstacles. (428) Prophylactic vaccinations, while capable of avoiding these pitfalls, are still limited by the nature of the vaccine antigen. With the exception of virally induced tumors, all tumor antigens are derived from self- or modified self-antigens. In most cases, T cells specific for self-antigens are either deleted or converted to regulatory T cells in the thymus. Those that escape central tolerance are often prevented from proliferating in the periphery, usually as a result of the action of regulatory cells produced in the thymus and bone marrow. (297, 429) Increasing antigen specific T cell precursor frequency can be done through adoptive T cell therapy and TCR gene therapy. However, these treatments are limited by expense and are likely limited by a finite therapeutic window that still needs to be defined. (430) Limiting peripheral tolerance towards tumor specific T cells has equal potential with regards to efficacy, but much greater potential when considering scalability. However, a detailed and comprehensive understanding of peripheral tolerance, especially as it relates to self-antigen vaccination, is necessary in order to maximize the tumor specific effects while limiting effects unwanted autoimmunity.

Work with MUC1.Tg mice, which express the human tumor antigen MUC1 under its endogenous promoter (283), has increased our understanding of how immune responses against foreign and self-antigens differ. It has been known for some time that these mice are hyporesponsive to vaccination with a MUC1 peptide (MUC1p) derived from the extracellular tandem repeat region. (431, 432) There are fewer MUC1p specific T cells induced by vaccination compared to WT mice that don’t have human MUC1, as well as lower amounts of

circulating MUC1 specific antibodies. (407) Similar hyporesponsiveness to the vaccine is seen upon adoptive transfer of MUC1 specific CD4 T cells into MUC1.Tg versus WT mice, suggesting an immunosuppressive influence of the environment rather than an intrinsic defect in the MUC1-specific T cells. (291) As early as 24 hours post vaccination, as shown in Chapter 2, this tolerance is identifiable in splenic dendritic cells (DC). Specifically, when mice are vaccinated with a foreign antigen (MUC1p into WT mice) vs a self-antigen (MUC1p into MUC1.Tg mice) the DC have higher expression of costimulatory molecules, higher T cell stimulatory capacity, and higher motility. (406)

The role of natural killer (NK) cells in this model has not been explored. NK cells and dendritic cells have a well-known system of reciprocal regulation that implicates NK cells as potential initiators/potentiators of the tolerized DC phenotype. NK cells secrete GM-CSF that can differentiate and activate DC. (433) DC can activate NK cells to produce IFNγ through IL-15 production and trans-presentation via IL-15Rα (434), as well as through secreted IL-12 and IL-18. (435, 436) IFNγ produced by NK cells can then activate DC to produce IL-12 and IL-27, and to upregulate surface expression of costimulatory molecules. (437) IL-2, produced by DC or DC activated CD4 T cells is known to activate NK cells as well. (438-440) IL-2 activated NK cells have been shown to induce the maturation of blood plasmacytoid DC (pDC). (441) Activated pDC produce large amounts of type I IFN that is known to activate NK cell cytotoxic functions. (442, 443) Activated NK cells can kill immature DC via a TRAIL dependent mechanism (444, 445) to sculpt and in some cases limit downstream immune responses. (351) Additionally, both DC and NK cells are capable, upon activation, of producing a variety of chemokines to attract one another to the site of activation and encourage these reciprocal interactions. (446, 447)

In this chapter, we show that NK cells in the spleens of MUC1.Tg mice vaccinated intravenously with MUC1p have elevated IL-10 secretion when compared to those in WT mice given the same vaccination. This is of particular interest given the role of IL-10 in inhibiting DC activation in these mice, post MUC1p vaccination. (Described in Chapter 3) In addition to and perhaps because of the impact of this IL-10 secretion on NK cells themselves and on splenic dendritic cells, these NK cells are aberrantly activated, as measured by their cell surface phenotype and killing function. This contributes to reduced DC helper function, and inhibited CD4 T cell responses. These findings suggest that one of the earliest events in post vaccination peripheral self-tolerance is the inhibition of NK cell activation, which is a governing influence on the downstream immune response.

4.3 MATERIALS AND METHODS

4.3.1 **Mice**

MUC1.Tg mice, purchased from Dr. Sandra Gendler (Mayo Clinic), were bred and maintained in the University of Pittsburgh animal facility. C57BL/6 (WT) mice were purchased from The Jackson Laboratory. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

4.3.2 **MUC1 vaccination**

A 100-aa peptide consisting of 5 repeats of the MUC1 VNTR motif, HGVTSAPDTRPAPGSTAPPA, was synthesized as previously described by the University of Pittsburgh Genomics and Proteomics Core Laboratories. For DC vaccines, BMDC were generated as previously described. (291) Briefly, RBC lysed bone marrow from female C57Bl/6 was put into culture at a density of 1-2x10^6 cells per mL of AIM-V supplemented with 10ng/mL GM-CSF (Miltenyi Biotech). On day 3, cultures were fed by replacing half the media with fresh AIM-V containing GM-CSF. At day 6, cultures were harvested by removing and discarding the non-adherent fraction, and harvesting the semi-adherent cells by agitation in 3mM EDTA. Cells were re-plated overnight in AIM-V containing 33µg/mL MUC1 100mer peptide and 25µg/mL polyinosinic-polycytidylic acid and poly-L-lysine (Poly-ICLC; Hiltonol). On the following day, the indicated mice received 5x10^4-3x10^5 MUC1 loaded DC in 100µL PBS via tail vein. For soluble vaccine, 100µg of MUC1 100mer peptide was admixed with 50µg of Poly-ICLC and the resultant solution was brought up to 100µL with PBS and given via tail vein.

4.3.3 **Quantitative RT-PCR**

RNA was extracted from whole spleen using TRIzol (Invitrogen) according to the manufacturer's protocol. Following extraction, cDNA was generated using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen). qPCR was performed using QuantiTect SYBR Green PCR kit (Qiagen) according to the manufacturer's protocol. Reactions were run on a StepOne Plus instrument (Applied Biosystems). The following primer pairs was used: IL-15

(forward: 5'- AACAGCTCAGAGAGGTCAGGAAAGA-3', reverse: 5'-GGACCTCACCAGCAAGGACCA-3')

4.3.4 *In vivo* antibody blockade/deletion

Where indicated, mice were given 250μg of an antibody against the IL-10 receptor (Bio X Cell, Clone 1B1.3A) or an isotype matched control antibody (Bio X Cell, Clone HPRN), intraperitoneally. Likewise, NK cells were depleted by administration of 200μg of an antibody against the NK1.1 surface marker (Bio X Cell, Clone PK136). Control mice were mock depleted with 200μg of an isotype matched control (Bio X Cell, Clone C1.18.4). 24-48 hours following treatment, mice were vaccinated as described in *MUC1 Vaccination* above and analyzed as described.

4.3.5 In vitro cytotoxicity

Bead isolated, negatively selected Natural Killer (NK) cells (Miltenyi Biotech) were isolated and put into culture with YAC-1 cells for 4 hours. Cytotoxicity was measured via LDH release using the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega) according to the manufacturer's suggested protocol.

4.3.6 In vitro NK/DC culture

NK cells were isolated as in the "In vitro cytotoxicity" section above. NK cells were then put into culture with naïve CD11c+, bead isolated (Miltenyi biotech) splenocytes and allowed to

incubate overnight at 37°C in complete RPMI. Cells were harvested by vigorous pipetting and stained for surface marker analysis by flow cytometry.

4.3.7 **Intracellular Cytokine Staining/Flow Cytometry**

All samples were run on an LSR II flow cytometer and analyzed using FACSDiva software (both BD bioscience). For analysis of unstimulated, *ex vivo* cytokine production post vaccination, spleens were harvested at the indicated timepoint directly into serum free RPMI containing BD GolgiPlug (BD Bioscience). Whole spleens were dissociated by mashing through a 70µm membrane and put into culture in complete DMEM containing BD GolgiPlug for 4-6 hrs. At the end of culture samples were staining for surface markers and intracellular cytokine production using the BD Cytofix/Cytoperm^{TM} kit (BD Bioscience) according to the manufacturer's protocol. For analysis of T cell responses, bead isolated (Miltenyi Biotech) CD4+ and CD8+ T cells were put into culture with MUC1 pulsed day 6 BMDC (prepared as in the *MUC1 Vaccination* section above) at a ratio of 5-10:1 T cells to DC for 4-6 hrs in complete DMEM supplemented with BD GolgiPlug and harvested as above.

4.4 RESULTS AND DISCUSSION

4.4.1 **NK cells in the spleens of mice vaccinated with a self-antigen express higher amounts of IL-10 post vaccination than do the same NK cells in foreign-antigen vaccinated mice.**

As shown in chapter 3, IL-10 is up-regulated in the spleens of MUC1.Tg mice vaccinated intravenously with a MUC1 peptide based vaccine compared to WT mice given the same vaccination. Furthermore, IL-10 is involved in inhibiting proper DC maturation and in inhibiting downstream MUC1 peptide specific CD4 T cells responses in these mice. (406) Almost all immune cells are capable of producing IL-10 given proper stimulation, however its production inside of the 24 hour window suggested a previously unappreciated early role for IL-10 in inhibiting self-antigen specific responses. Accordingly, we were interested in what cell population(s) was producing it early, specifically what cell population(s) was producing it at higher levels in mice vaccinated with a self-antigen, compared to a foreign antigen, as that knowledge would likely clue us in to unique therapeutic targets/initiators of antigen specific peripheral tolerance. We vaccinated WT and MUC1.Tg mice with MUC1p plus adjuvant via tail vein. Six hours after vaccination, when IL-10 production is up and DC phenotype has begun to diverge (see Chapter 3), we removed the spleens from these mice and immediately dissociated them in media containing a golgi transport inhibitor. The cells were incubated for 6 additional hours to accumulate protein and analyzed via flow cytometry. Interestingly, not only were NK cells producing IL-10 in this timeframe, but NK cells from the spleens of MUC1.Tg mice were producing higher amounts of IL-10 than their WT counterparts. (Fig. 15A-C) This indicates that

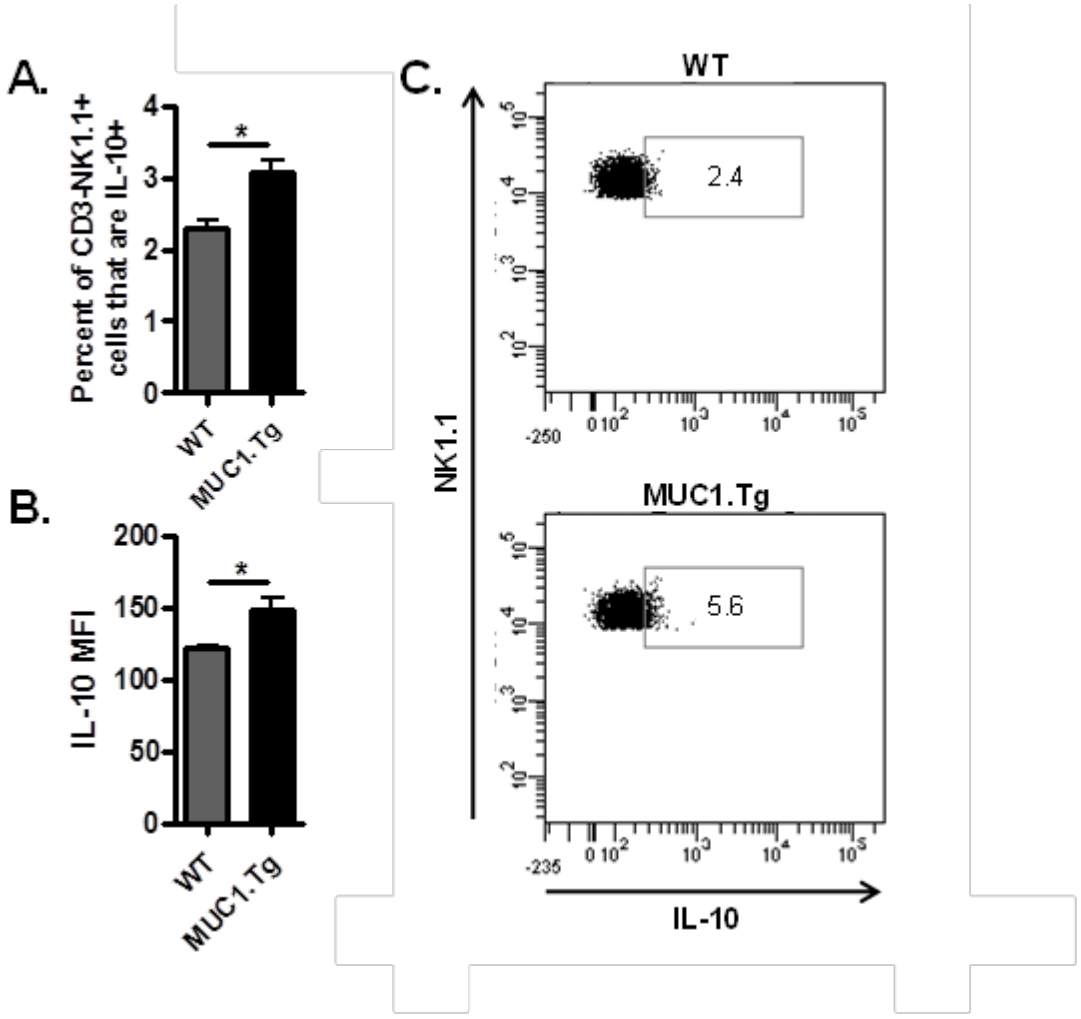

Figure 15: NK cells in the spleens of MUC1.Tg mice produce more IL-10 post vaccination than do those from WT mice, post MUC1 peptide vaccination.

WT and MUC1.Tg mice were vaccinated intravenously with 100µg MUC1 admixed with 50µg PolyICLC. 6-7 hours post vaccination, splenocytes were harvested and immediately put into culture in media containing Brefeldin A. After 6 hours in culture, cells were analyzed via intracellular flow cytometry. IL-10 positivity (A) and MFI (B) were assessed in the CD3-NK1.1+ population. (C) A representative flow plot is shown. Percentages on the flow graphs represent the mean for that experimental group. * indicates a p value of <.05.

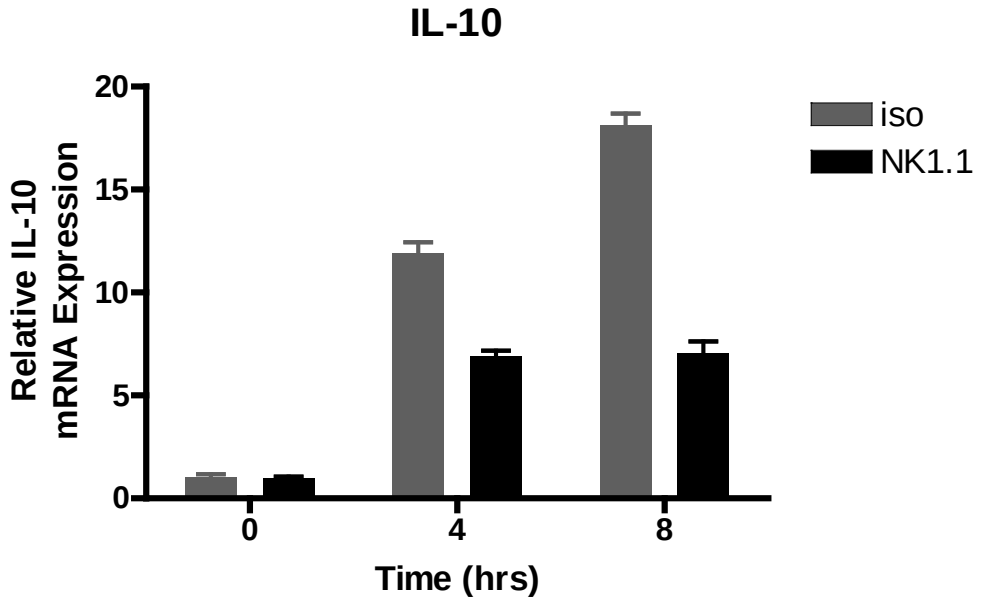

Figure 16: Depletion of NK cells prior to vaccination with MUC1p prevents vaccine induced early IL-10 production in MUC1.Tg mice.

MUC1.Tg mice were treated with 250µg of an antibody against the NK1.1 cell surface antigen (black bars) or were given an isotype matched control (grey bars). 48 hours after antibody treatment, mice were vaccinated with 100µg MUC1 peptide admixed with 50µg PolyICLC via tail vein. At the indicated timepoints, mice were sacrificed and their spleens removed for RNA isolation and subsequent analysis via qRT-PCR. mRNA amounts were normalized to the level in control (PBS) vaccinated mice treated the antibodies indicated.

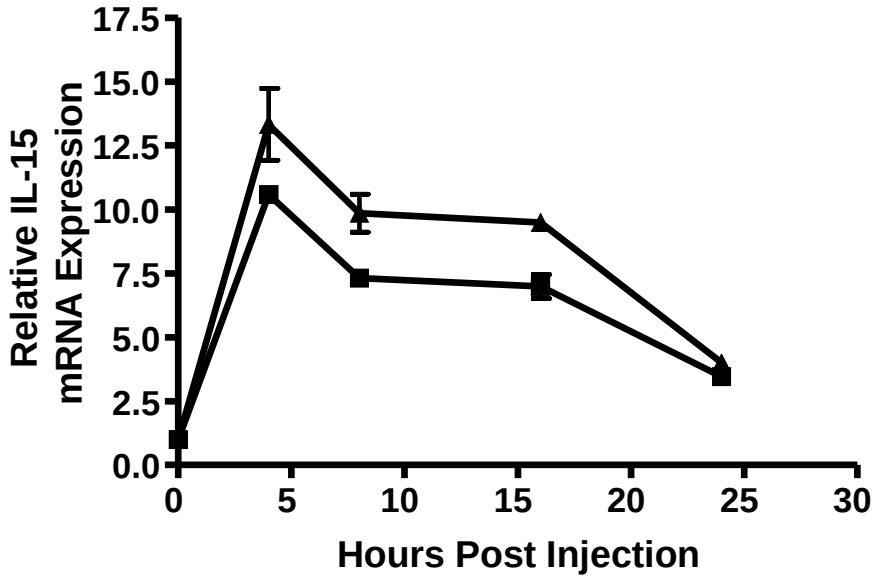

Figure 17: IL-15 is up-regulated in response to MUC1 vaccination at similar levels in both WT and MUC1.Tg mice.

WT (squares) and MUC1.Tg mice (triangles) were vaccinated with MUC1p admixed with Poly-ICLC via tail vein. Spleens were removed at indicated hours post vaccination and total splenic mRNA levels of IL-15 were determined relative to the control gene HPRT. Values shown represent expression relative to the baseline expression in mice of that genotype (WT and MUC1.Tg) at 0 hours post vaccination. Data are representative of 3 pooled mice per group per timepoint shown. Data points show mean ±SEM of three technical replicates.

more IL-10 is produced by splenic NK cells in response to a self-antigen vaccine, as opposed to a foreign antigen vaccine. Furthermore, it appears that NK cells are responsible for the early IL-10 sugre seen in MUC1.Tg mice previously (see Chapter 3) as depletion of NK cells prior to vaccination greatly reduces the amount of early IL-10 produced in these mice. (Fig. 16) Given the hypothesized importance of IL-10 in inhibiting self-antigen vaccine responses specifically, it also potentially puts NK cells center stage in controlling the downstream immune response. Interestingly, the kinetics of IL-10 production in MUC1.Tg mice, which appears to peak around

8 hours post vaccination, closely mirrors what is seen when NK cells are treated with IL-15 *in vitro*. (352) Although there are no observed differences in IL-15 expression between WT and MUC1.Tg mice post vaccination, IL-15 production is increased in the spleens of both groups (Fig. 17) and an undefined number of *in vivo* factors could be contributing to the differential impact of this cytokine post vaccination.

4.4.2 **NK cells in the spleens of self-antigen vaccinated mice are not activated to the extent that is seen in WT mice, but can be rescued via IL-10R blockade.**

NK cells and DC are well known to cross regulate each other. (446) In MUC1.Tg mice vaccinated with MUC1p, there is a reduction in the activation level of splenic DC post MUC1p vaccination, compared to WT mice. (406) However, prior to the discovery of IL-10 production in splenic NK cells, little was known about what effect vaccination with a self-antigen had on their phenotype. To query this, WT and MUC1.Tg mice were vaccinated with a MUC1p plus adjuvant and 24 hours post vaccination, NK cell phenotype was analyzed by flow cytometry. Compared to splenic NK cells from WT mice vaccinated with MUC1p, those from MUC1.Tg mice expressed lower levels of CD127, CD25, and CD69. They did, however, trend towards having higher levels of NKG2D on their surface. (Fig. 18A) When these NK cells were co-cultured with the NK sensitive YAC-1 mouse lymphoma cell line, NK cells from the MUC1.Tg mice were also shown to have a higher cytotoxic capacity than those from WT mice. (Fig. 18B)

Decreases in CD69 and YAC-1 killing suggest a less traditionally activated phenotype in the splenic NK cells of self-antigen vaccinated mice. CD25 and CD127 are interesting because of recent insights into Treg/NK cells interactions. Acute depletion of regulatory T cells results in an accumulation of CD127+ NK cells in the spleen. These CD127+ NK cells had lower levels of

Granzyme B and IL-10, compared to CD127- NK cells and readily up-regulated CD25 expression upon activation. (172) Lowers levels of Granzyme B and IL-10 fit in quite nicely with our current observations that NK cells in foreign antigen vaccinated mice express less IL-10 and show lower cytotoxicity against YAC-1 cells. And CD25 up-regulation suggests a mechanism of gaining resistance to Treg mediated suppression. Specifically, it was recently found that IL-2 deprivation by regulatory T cells is an important, *in vivo* mechanism by which regulatory T cells control NK cell activation. (172-174) While future studies are needed to confirm a link between our observation and previous studies suggesting a larger precursor pool of MUC1p specific regulatory T cells in MUC1.Tg mice (292), the prospect is especially intriguing as direct, post vaccination control of NK cells by regulatory T cells has never been shown in an antigen specific manner. Finally, CD127+ NK cells express more IFNγ after IL-12/IL-18 stimulation, suggesting a higher capacity for DC help in NK cells from the spleens of foreign antigen vaccinated mice. Down-regulation of NKG2D is somewhat contradictory to what would be considered a classically activated NK cell. However, studies have shown that down-regulation can occur in some cases to prevent reactivity against normal cells while preserving cytotoxic capacity in an inflammatory setting (448), while others have suggested NKG2D mediated killing may be a means to dampen immune responses. (347, 348) NK mediated killing of DC in particular is an interesting prospect to consider as IL-10 treatment of DC has been shown in one model to increase the ability of NK cells to kill mature DC specifically. (351) This could explain the recently reported decrease in DC number following MUC1p vaccination of MUC1.Tg mice, and account for the overall decrease in the maturation level of the remaining population. (406)

Given recent work by our group showing that IL-10 is a major factor in dampening self- but not foreign antigen specific vaccine responses (Chapter 3), and the current work showing

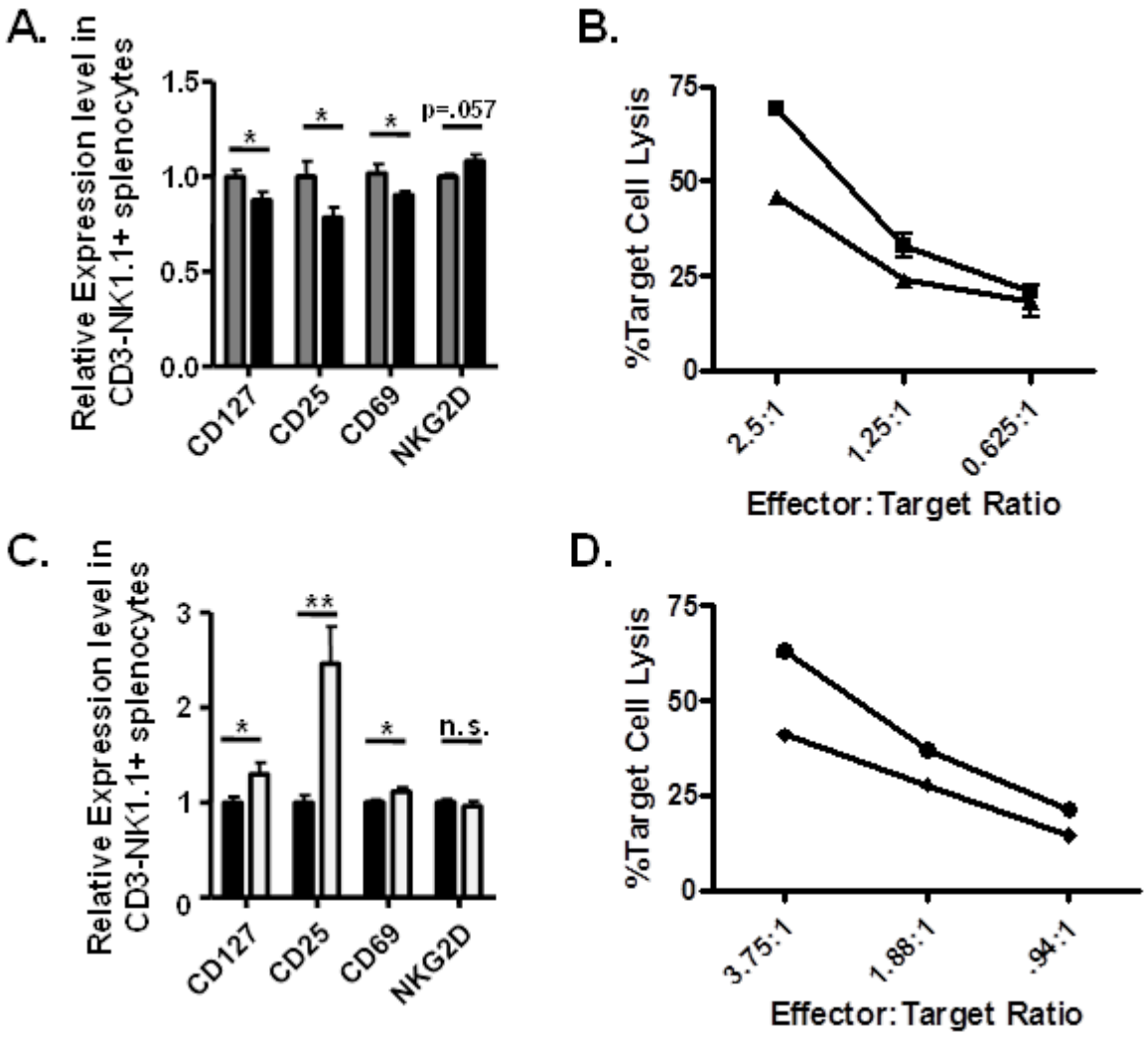

Figure 18: NK cells in the spleens of MUC1 vaccinated WT mice and MUC1.Tg mice treated with an IL-10R blocking antibody have a unique surface phenotype and a higher activity level when compared to untreated MUC1.Tg mice.

(A-B) WT (grey bars, triangles) and MUC1.Tg (black bars, squares) mice were vaccinated as in Figure 1. (A) 24 hours following vaccination, splenocytes were harvested and analyzed via flow cytometry. Values shown represent mean ± SEM for MFI (CD127, CD69, and NKG2D) or percent positive (CD25) and were normalized to the expression level in WT animals to allow for the pooling of data from multiple experiments. (B) 24 hours following vaccination, splenic NK cells from 3-4 mice per group were isolated and pooled, then put into culture with YAC-1 cells at the indicated ratios. Cytotoxicity was determined by LDH release. Samples were run in triplicate, points represent mean ±SEM of technical replicates. (C-D). 1-2 day prior to

vaccination, MUC1.Tg mice were treated I.P. with an antibody against IL-10R (white bars, diamonds) or with an isotype matched control (black bars, circles). Mice were then vaccinated and analyzed as in (A-B). (C) Values were normalized the level of MUC1.Tg mice treated with the isotype control. * indicates a p value of <.05. ** indicates a p value of <.01.

splenic NK cells to be producing higher amounts of IL-10 in MUC1p vaccinated MUC1.Tg, relative to WT, we were interested in whether IL-10, likely produced by the NK cells themselves, had any effect on their post vaccination phenotype at 24 hours. Accordingly, we pretreated MUC1.Tg mice with an antibody against the IL-10R and analyzed splenic NK surface phenotype and killing capacity post MUC1p vaccination as before. On all measured parameters, the NK cell phenotype was reversed. NK cells in the spleens of mice treated with the IL-10 receptor blocking antibody expressed higher CD127, CD25, and CD69, lower NKG2D, and a decreased capacity for killing YAC-1 cells, compared to isotype treated controls. (Fig. 18C-D) This suggests that IL-10 is responsible for the phenotypic skewing that is seen in NK cells post self-antigen vaccination. In support of this being a self-antigen specific induction and effect of IL-10, this pattern change in NK cell phenotype was not seen in similarly treated WT mice. (Fig. 19)

4.4.3 **NK cells in the spleens of self-antigen vaccinated mice have a reduced capacity for DC help, relative to those from foreign antigen vaccinated mice, a trait which is likewise mediated by IL-10.**

Given that NK cells in the spleens of MUC1p vaccinated MUC1.Tg mice are phenotypically distinctive from those from similarly treated WT mice and produce higher amounts of the anti-

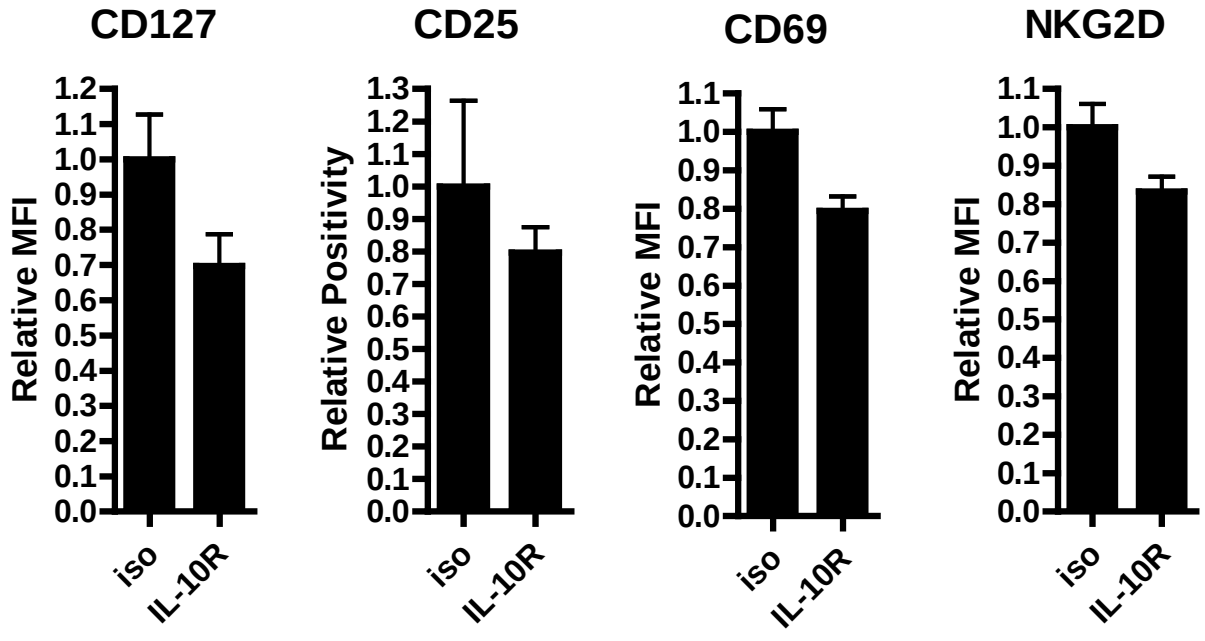

Figure 19: Phenotypic conversion of NK cells in response to IL-10 receptor blockade is specific to NK cells in mice vaccinated with a self-antigen.

1-2 days prior to vaccination, WT mice were treated I.P. with an antibody against the IL-10R (IL-10R) or with an isotype matched control (iso). Mice were then vaccinated and probed for splenic NK surface markers as before. Values were normalized the expression level on NK cells from WT mice treated with the isotype control and vaccinated as described.

inflammatory cytokine IL-10, we hypothesized that they are less capable of activating dendritic cells, and therefore likely contribute to differences seen in DC activation post vaccination. To examine this possibility, we depleted WT and MUC1.Tg mice of NK cells by treatment with an NK1.1 specific antibody prior to MUC1p vaccination. Twenty four hours following vaccination we removed spleens and analyzed DC surface phenotype. In keeping with previous studies, the DC in the spleens of WT mice treated with a control antibody were significantly more mature than DC from the spleens of MUC1.Tg mice treated with a control antibody. This was evident by increases in surface levels of MHC Class II, CD40, and a trend towards higher CD80 (Figs. 20A-

C) However, in the absence of NK cells, there was no significant difference in DC phenotype post vaccination. This suggests that differences seen in the phenotype of post vaccination splenic DC's could be the result of suppressed NK cell activation in MUC1.Tg mice. In support of this theory, when NK cells were removed from WT and MUC1.Tg mice post vaccination, those from WT mice induced higher expression of MHC Class II, CD80, CD86, and CD40 on DC when co-cultured overnight. (Figs. 20D-G) As one might predict based on the reversal that was seen with NK cell phenotype when MUC1.Tg mice were pretreated with an antibody against the IL-10 receptor prior to vaccination, NK cells taken from these mice had an improved capacity for *ex vivo* DC help on all parameters tested, compared to mice given an isotype matched control antibody. (Figs. 20H-K)

4.4.4 **NK depletion improves MUC1p specific CD4 T cell responses in MUC1.Tg mice.**

Given the role of DC in initiating T cell responses, we were interested in what the inhibited NK cell phenotype may be having on downstream CD4 T cell responses. In previous studies, CD4 T cells, more so than CD8 T cells in MUC1.Tg mice, have been shown to be hyporesponsive to MUC1 vaccination, regardless of vaccine formulation. (284, 285) Given the importance of CD4 T cell responses in the generation of memory CD8 T cells (74, 75), this represents a potential roadblock in the development of long term preventative MUC1p based vaccinations. Therefore, understanding the peripheral mechanisms underlying this CD4 hyporesponsiveness may have a significant role in the development of MUC1p based preventative therapies. The link between DC phenotype and the CD4 T cell response is well understood (32), as is the role of IL-10 in inhibiting these same responses. (164-167) Therefore, despite traditionally being thought of as having a positive effect on the T cell response, we hypothesized that in self-antigen vaccinated

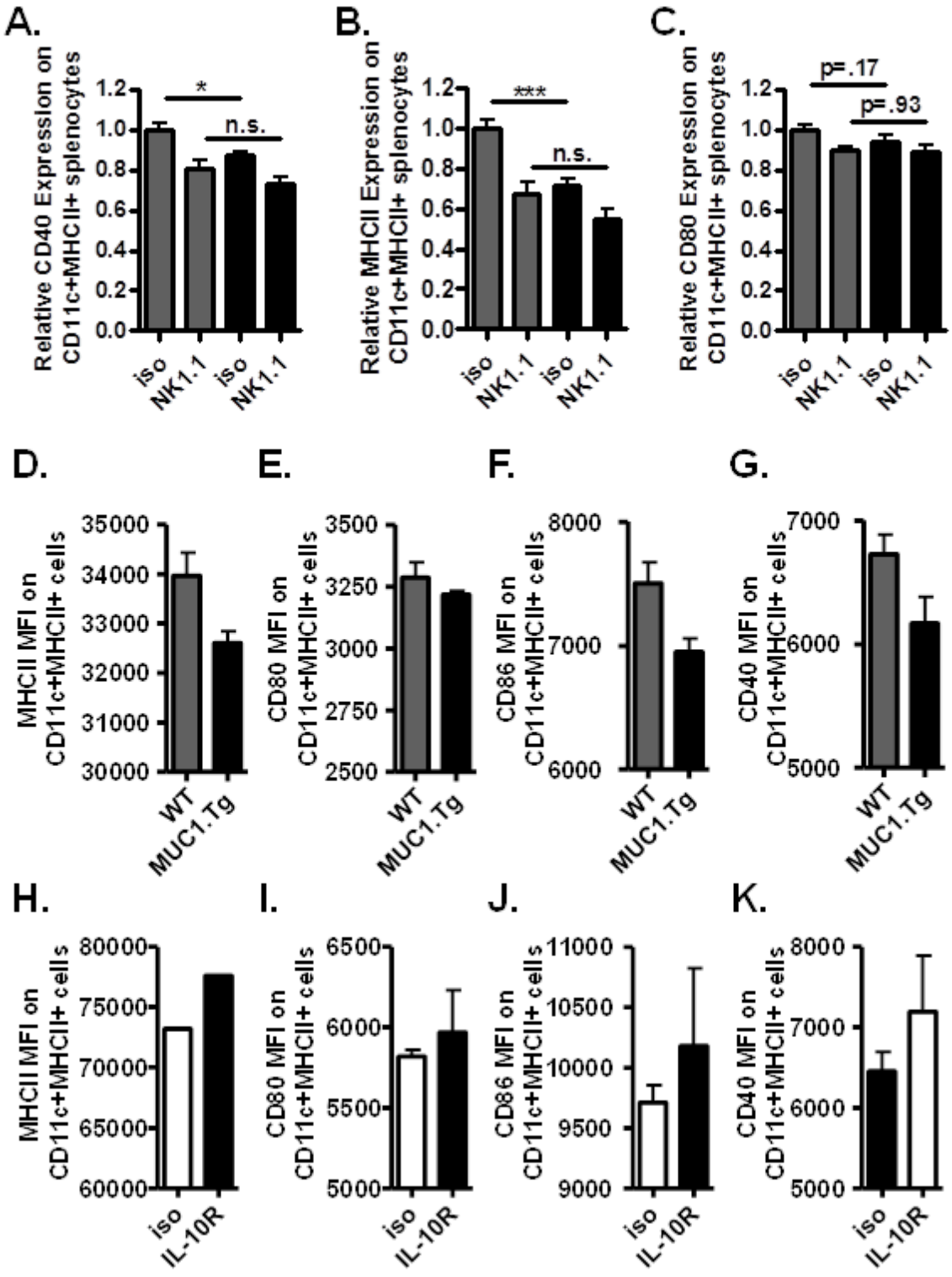
A.
Relative CD40 Expression on CD11c+MHCII+ splenocytes
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
n.s.
iso
NK1.1
iso
NK1.1
B.
Relative MHCII Expression on CD11c+MHCII+ splenocytes
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
n.s.
iso
NK1.1
iso
NK1.1
C.
Relative CD80 Expression on CD11c+MHCII+ splenocytes
1.2
1.0
0.8
0.6
0.4
0.2
0.0
p=.17
p=.93
iso
NK1.1
iso
NK1.1
D.
MHCII MFI on CD11c+MHCII+ cells
35000
34000
33000
32000
31000
30000
WT
MUC1.Tg
E.
CD80 MFI on CD11c+MHCII+ cells
3500
3250
3000
2750
2500
WT
MUC1.Tg
F.
CD86 MFI on CD11c+MHCII+ cells
8000
7000
6000
WT
MUC1.Tg
G.
CD40 MFI on CD11c+MHCII+ cells
7000
6000
5000
WT
MUC1.Tg
H.
MHCII MFI on CD11c+MHCII+ cells
80000
75000
70000
65000
60000
iso
IL-10R
I.
CD80 MFI on CD11c+MHCII+ cells
6500
6000
5500
5000
iso
IL-10R
J.
CD86 MFI on CD11c+MHCII+ cells
11000
10500
10000
9500
9000
iso
IL-10R
K.
CD40 MFI on CD11c+MHCII+ cells
8000
7000
6000
5000
iso
IL-10R

Figure 20: NK cells in the spleens of MUC1 vaccinated WT mice and MUC1.Tg mice treated with an IL-10R blocking antibody activate DC to a higher extent than do those from the spleens of untreated untreated MUC1.Tg mice.

(A-C) WT (grey bars) and MUC1.Tg (black bars) mice were treated with an anti-NK1.1 antibody or an isotype matched control (iso) I.P.. 2 days following antibody treatment, mice were vaccinated with MUC1 peptide as in Figure 1. 24 hours following vaccination, expression of CD40 (A), MHC Class II (B), and CD80 (C) was measured on CD11c+MHCII+ splenocytes. Values represent mean ±SEM and have been normalized to the level seen in WT animals treated with the isotpye control to allow for the pooling of multiple experiments. (D-G) WT and MUC1.Tg mice were vaccinated as in (A-C). After vaccination, NK cells were isolated from each group and pooled. Cells were then put into culture overnight with naïve bead isolated splenic DC at a 1:1 ratio. Surface expression of MHC Class II (D), CD80 (E), CD86 (F), and CD40 (G) were assessed in CD11c+MHCII+ cells post culture. (H-K) NK cells from MUC1 vaccinated MUC1.Tg mice pretreated with an antibody against the IL-10 receptor (IL-10R) or an isotype matched control (iso) were analyzed as in (D-G). Values are from three technical replicates and represent mean ±SEM. * indicates a p value of <.05. * indicates a p value of <.001.**

mice, there may in fact be a deleterious role for NK cells in the CD4 T cell response. To test this we examined T cell responses in MUC1.Tg mice with and without NK cells at the time of initial MUC1p vaccination. In MUC1.Tg mice depleted of natural killer cells, there is a trend towards higher numbers of antigen specific IFNγ+ and IL-2+ CD4 T cells 8 days post vaccination. (Figs 21A-4D) However, there is a significant increase in cells producing both of these cytokines. (Fig 21D) Previous studies using unrelated vaccine and infection models have shown these dual producers to be enriched for early memory cells and correlated with optimal protective responses (449-451). This is confirmed in our model by the fact that these cells overwhelmingly are $CD44^{High}CD62L^{Low}$ (Fig21E). Therefore, there is a significant increase in the quality, if not the quantity of the CD4 response in the absence of NK cells.

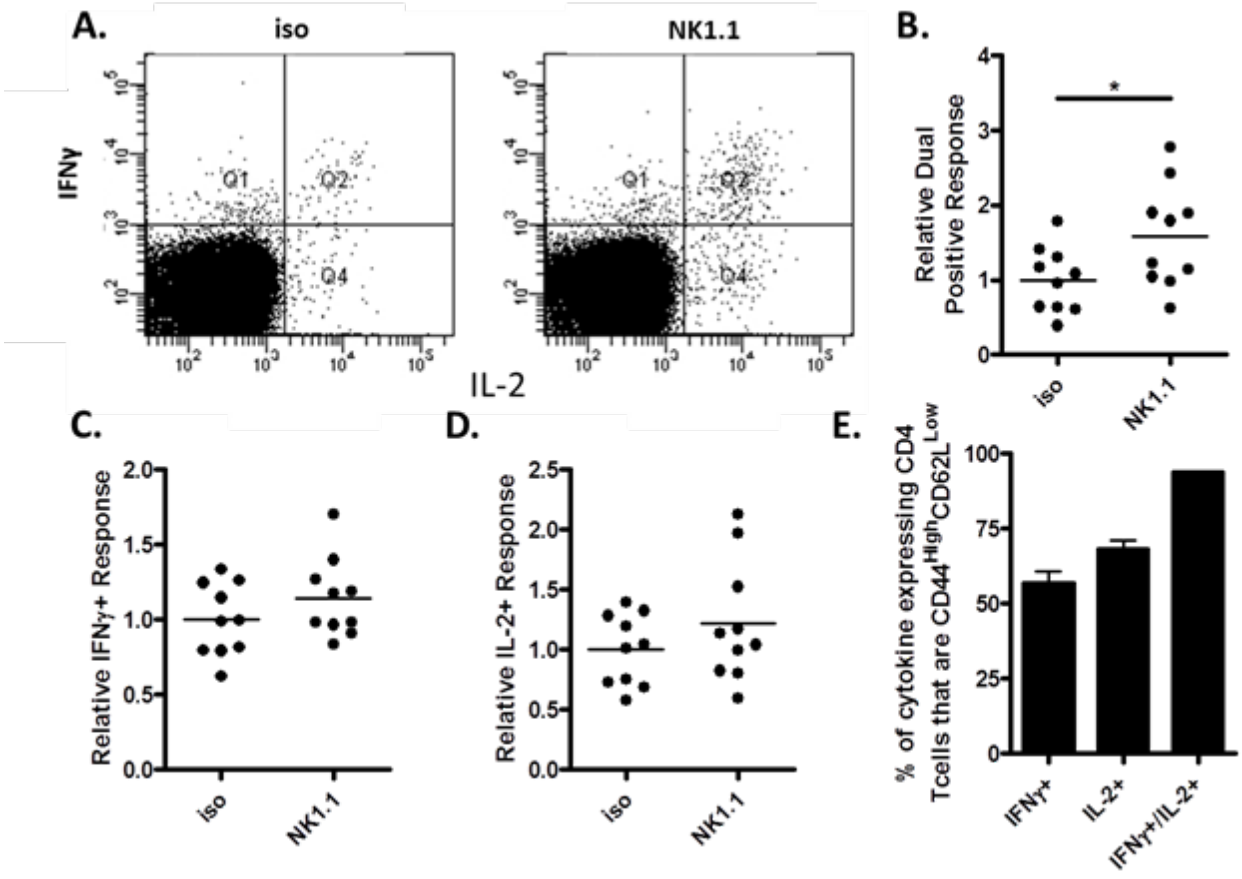

Figure 21: Depletion of NK cells pre MUC1 peptide vaccination improved the MUC1 specific CD4 T cell response in MUC1.Tg mice.

MUC1.Tg mice were injected I.P. with an antibody against NK1.1 or an isotype matched control. 2 days following antibody treatment, mice were vaccinated with .5-1x10^5 MUC1 peptide loaded dendritic cells (DC). 7 days later, mice were sacrificed and their splenic CD4 T cells were cultured with MUC1 peptide loaded DC for 6 hours in the presence of brefeldin A. Cells were then analyzed via intracellular flow cytometry. Representative flow cytometry plots are shown (A). The percentage of CD4 T cells producing IFNγ (B), IL-2 (C), and both (D) was assessed. Data points represent individual mice and have been normalized to the response in isotype control treated animals (E) The percentage of cells with each cytokine profile having high CD44 and low CD62L was assessed. Bars represent mean ±SEM. * indicates a p value of <.05.

4.4.5 **Conclusions**

This data presents a new picture on the role of NK cells in vaccine generated responses. While it is clear that in most vaccine and infection scenarios, NK cells are a crucial part of generating an optimal TH1 response (452, 453), in the case of self-antigen vaccines, their role appears to be much less beneficial and is likely detrimental. This makes understanding how NK cell activation is affected by various vaccine formulations an interesting area for future research. Most of our understanding of this is currently focused around how adjuvants directly and indirectly activate these cells. Here we show that antigen choice may also be an important consideration. While there has been some recent work detailing memory NK cells populations, recognition seems to be hapten based and not peptide specific, as is the nature of the antigen used in this study. (454) It is likely that the NK reaction in our model is governed by an antigen specific intermediary. The most likely candidate is regulatory T cells, as they have been shown to be important in the maintenance of tolerance to MUC1p vaccination in MUC1.Tg mice. (292, 293, 406)

Treg/NK cells interactions have recently sparked a great deal of interest. Treg deficient *scurfy* mice and mice acutely ablated of Treg show increases in NK cell activation and proliferation in several models. (169, 455-457) IL-2 deprivation (172-174) and TGFβ (169, 458) have both been implicated in controlling NK activation. And although Treg inducing vaccinations have been shown to decrease NK cell function and increase the incidence of chemically induced tumors (459), there haven't been any studies thus far indicating a role for regulatory T cells acutely regulating NK cells in response to vaccination, despite a theoretical potential. Our work represents the first step in identifying and understanding this potential. Future studies further elucidating the nature of this potential interaction will likely lead to a new

understanding of the complex network of inhibition initiated by regulatory T cells to restrain self–antigen specific immune responses and will identify new avenues for improving the response against these antigens.

5.0 OVERALL SUMMARY AND DISCUSSION

Previous studies by our group and others have highlighted the reduced capacity for generating MUC1 specific CD4 T cell responses in MUC1.Tg mice. (284, 293) Recently, our understanding of this CD4 specific hyporesponsiveness has grown quite a bit. We know that it is not the result of deletional tolerance, as MUC1 specific CD4 T cells can develop in the thymus and reach the periphery with a normal phenotype. (273) Furthermore we can pinpoint the timing of the tolerance as an early post vaccination event that occurs in the periphery, as transferred MUC1 specific CD4 T cells fail to proliferate at maximal capacity at early timepoints in MUC1.Tg mice, compared to WT mice. As these studies involved the transfer of large numbers of MUC1 specific CD4 T cells, they also suggested an active mechanism of tolerance, as suboptimal immune activation due to reduced availability of MUC1 specific CD4 T cells in the periphery would not be a factor in experiments with such a high precursor frequencies. We are now able to appreciate several previously unidentified components of this active tolerance.

The first is an inhibition of DC. This occurs early. By surface co-stimulatory marker expression, this can be detected as early as 24 hours post vaccination. (Figs. 1, 18)) Using the newly identified biomarker of pancreatic enzyme expression in DC, it can be detected as early as 4 hours. (Fig. 9) We known this effect is dependent on the action of regulatory T cells and IL-10 on some level as removal of either one of these components from the system alleviates this suppression. (Figs. 7, 8, 11) In the future it will be interesting to query the contribution of each of

these mediators in the processes of establishing and maintaining the suppressed DC phenotype. For now, the point and mechanism of first action remains to be elucidated. It is likely that MUC1 specific regulatory T cells recognize the presence of their cognate antigen presented on dendritic cells and that this is the first step in the process of DC inhibition. There is support in the literature for an increased number of MUC1 specific Tregs in MUC1.Tg mice. (292) However, the exact mechanism of action is not yet clear. Direct action of regulatory T cells on dendritic cells is possible. However, the possibility that direct action accounts for all of the differences seen in DC phenotype is difficult to envision.

Based on our data, we would hypothesize that Tregs likely mediate the bulk of their inhibitory functions through indirect mechanisms. This is supported by the fact that regulatory T cells from MUC1.Tg mice do not appear to make IL-10 in response to MUC1, but IL-10 seems to have an important role in the inhibition of the both the phenotype of dendritic cells and the overall immune response. (Figs. 8, 9-13) We hypothesize that the role of regulatory T cells is more likely to be in the establishment of a self-amplifying regulatory circuit. We further suggest that NK cells are likely to play large role in this inhibitory circuit, as they are shown to produce elevated levels of IL-10 post vaccination in MUC1 peptide vaccinated MUC1.Tg mice compared to similarly treated wild type mice and can directly inhibit CD4 T cell responses.

This finding is quite novel. The function of NK cells in response to vaccination is typically thought of as predominantly positive, with these cells generally supporting Th1 cell generation through IFNγ secretion, which up-regulates the Th1 skewing cytokine IL-12 in dendritic cells. The idea of NK cells limiting immune responses in a self-antigen specific manner has not been observed before. Understanding what factors contribute to their optimal activation, and what prevents this in self-antigen vaccinated mice will add a new level to our understanding

of peripheral tolerance and will likely lead to the intelligent design of optimal vaccine concurrent immune modulation strategies to overcome this tolerance.

We report of one such of these strategies here. Specifically, we show that blockade of IL-10 not only converts the phenotype of post vaccination NK cells in MUC1.Tg mice to one that mimics what is observed in WT mice, it also improved downstream CD4 T cell responses. (Figs. 16, 18-19) Though the improvement observed with IL-10R blockade is likely due to reducing the ability of this molecule to directly inhibit DC and CD4 T cell activation, it is intriguing to consider that its effect on NK cells may indeed me a major component of its ability to improve the vaccine responsiveness of these populations. For example, increases seen in DC number and phenotype post vaccination in IL-10R blocked, but not isotype treated MUC1.Tg mice (Fig. 12) could be due to decreased killing of these cells by NK cells, as NK cells from the IL-10R blocked group show a lower cytotoxic capacity. Unfortunately, this mechanism is not supported in the NK depletion model, where DC numbers remain equal between MUC1.Tg mice depleted of NK cells with an anti-NK1.1 antibody prior to vaccination, and those given a non-specific control antibody. (data not shown) One explanation for this disparity (i.e. why does IL-10 blockade but not removal of the IL-10 producing population improve DC numbers) could be that NK cells in IL-10R blocked MUC1.Tg mice post vaccination actively recruit and encourage the differentiation of DC. However, this mechanism will need to be investigated further before any hard conclusion can be made.

Linking the observed NK phenotype to the action of regulatory T cells will be an area of active investigation in the future. Several possibilities exist. The first is that activation of regulatory T cells by recognition of their cognate peptide allows them to exert direct control over NK cell function. This could be through the action of an anti-inflammatory intermediary such as

TGFβ; or their recognition of cognate peptide could sequester them at the DC surface and allow them to locally consume IL-2 produced by the DC and naïve cognate CD4 T cells recognizing antigen, reducing the availability of this molecule to activate NK cells. Another possibility is that Tregs exert direct inhibitory effects on DC, reducing their ability to activate NK cells. These suboptimally activated NK cells would then produce IL-10, further suppressing DC function and establishing a self-amplifying regulatory loop. We favor the latter mechanism as preliminary data appears to be in support of it.

Cytokine profiling at the mRNA level of MUC1.Tg and WT mice post vaccination reveals that there is a decrease in the level of type I interferons produced in the MUC1.Tg mice. (Appendix B) Type I interferons are known to be produced by dendritic cells, particularly plamacytoid DC, however all populations are capable of making them. (446) It is interesting to hypothesize that regulatory T cells are able to prevent the up-regulation of type I interferons. This could potentially link Treg mediated inhibition and decreased NK cell immunogenicity as type I IFNs are well documented in their ability to activate NK cells. Determining the effect of removing type I IFN from the system on IL-10 production and vice versa will likely clarify the relative role of these two cytokines in the other's production.

In total, my data begin to identify the major players, some of which were not previously suspected, responsible for tolerance induction in response to a vaccine containing a self-peptide. In the model below we propose that the presence of a self peptide induces an anti-inflammatory cascade that is dependent largely on IL-10 and NK cells. The IL-10 acts by suppressing the activity of dendritic cells at early timepoints and CD4 T cells responses at later timepoints, possibly through a dendritic cell intermediate. The NK cells, who are large producers of IL-10, act in a similar fashion. Although the sum total of their effect on DC is not inhibitory, autocrine

IL-10 appears to hamper the ability of the NK cells to provide signals to DC that up-regulate their immunostimulatory phenotype. IL-10 also possibly activates the NK cells to directly kill DC. And although the exact mechanism has yet to be elucidated (i.e. direct vs. indirect mechanism), it is clear that NK cells inhibit the generation of robust CD4 T cell responses and have a previously unappreciated role in regulating self-antigen specific vaccine responses.

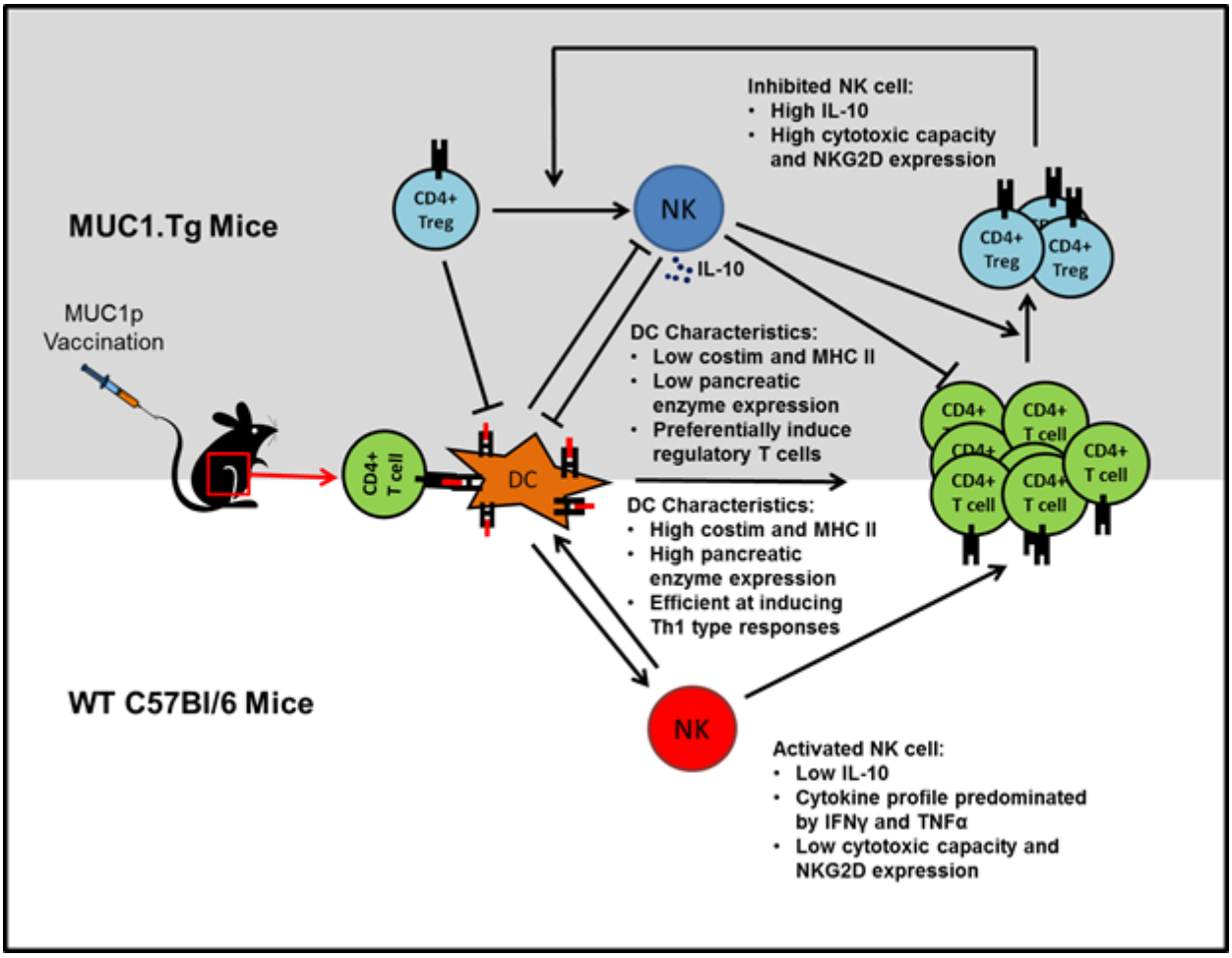

Figure 22: Proposed model of early the immune events responsible for maintaining tolerance to MUC1 as a self-antigen in MUC1.Tg mice.

APPENDIX A

ABBREVIATIONS USED

Abbreviation	Meaning
ADCC	Antibody dependent cell mediated cytotoxicity
AIRE	Autoimmune regulator
costim	Costimulatory molecules, specifically CD80, C86, and CD40
CPB1	Carboxypeptidase B1
DC	Dendritic cell
EGFR	Epidermal growth factor receptor
HER2/neu	Human epidermal growth factor receptor 2
IBD	Inflammatory bowel disease
IDO	Indoleamine 2,3-dioxygenase
IL-10R	IL-10 receptor
MDSC	Myeloid derived suppresor cell
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
NK	Natural Killer cell
TCR	T cell receptor
Teff	Effector CD4 T Cell
Treg	Regultory CD4 T cell

VNTR	Variable number tandem repeat region of the MUC1 extracellular domain

APPENDIX B

EARLY CYTOKINE PROFILING IN WT AND MUC1.TG MICE POST MUC1 VACCINATION

B.1 INTRODUCTION

We identified a subset of pancreatic enzymes which were differentially expressed between MUC1 peptide vaccinated WT and MUC1.Tg mice via gene array of the total spleen at 24 hours post intravenous vaccination. (Table. 1) We chose to query the nature of these enzymes and their implications of downstream immunity (Chapter 2) for several reasons. Firstly, the differences in the levels of expression of these enzymes were quite high between WT and MUC1.Tg mice. Secondly, there was no precedent for their expression in any cell of the immune system, let alone their differential expression as a result of activation status. However, the third reason was a relative paucity in the results of the gene array of traditional immune mediators which were differentially expressed. In fact almost no molecules were identified which could account for the observed differences in immunity. As our understanding of the early, post vaccination events grew, we came to understand that one explanation for the lack of traditional immune mediators was that these event came much earlier than 24 hours. DC enzymes began to diverge as early as 4 hours post vaccination, as did expression of IL-10, a molecule that was not identified in the

original 24 hours gene array. (Fig. 9 and data not shown) This led us to hypothesize that in addition to IL-10, a number of other immune mediators may have been differentially expressed very early after vaccination (4-8 hours), but normalized by 24 hours when we originally queried the system.

B.2 RESULTS

In order to query the cytokine profile early post MUC1 vaccination, we vaccinated WT and MUC1.Tg mice intravenously with MUC1 peptide admixed with Poly-ICLC as an adjuvant. At 4, 8, 16, and 24 hours post vaccination, the spleens of the animals were removed and their RNA content was analyzed via qRT-PCR. Interestingly, type I IFNs and IFNγ were up-regulated at early timepoints in WT mice, relative to MUC1.Tg mice. (Fig. 24) In accordance with these molecules not being identified as differentially expressed in the 24 hour gene array performed on the spleens of vaccinated WT and MUC1.Tg mice, their expression returns to baseline by the time. A number of other cytokines were profiled along with these molecules. Their expression at 8 hours, which was representative in the experiment shown of the time when expression of these molecules was at its most divergent, is shown. (Fig. 25)

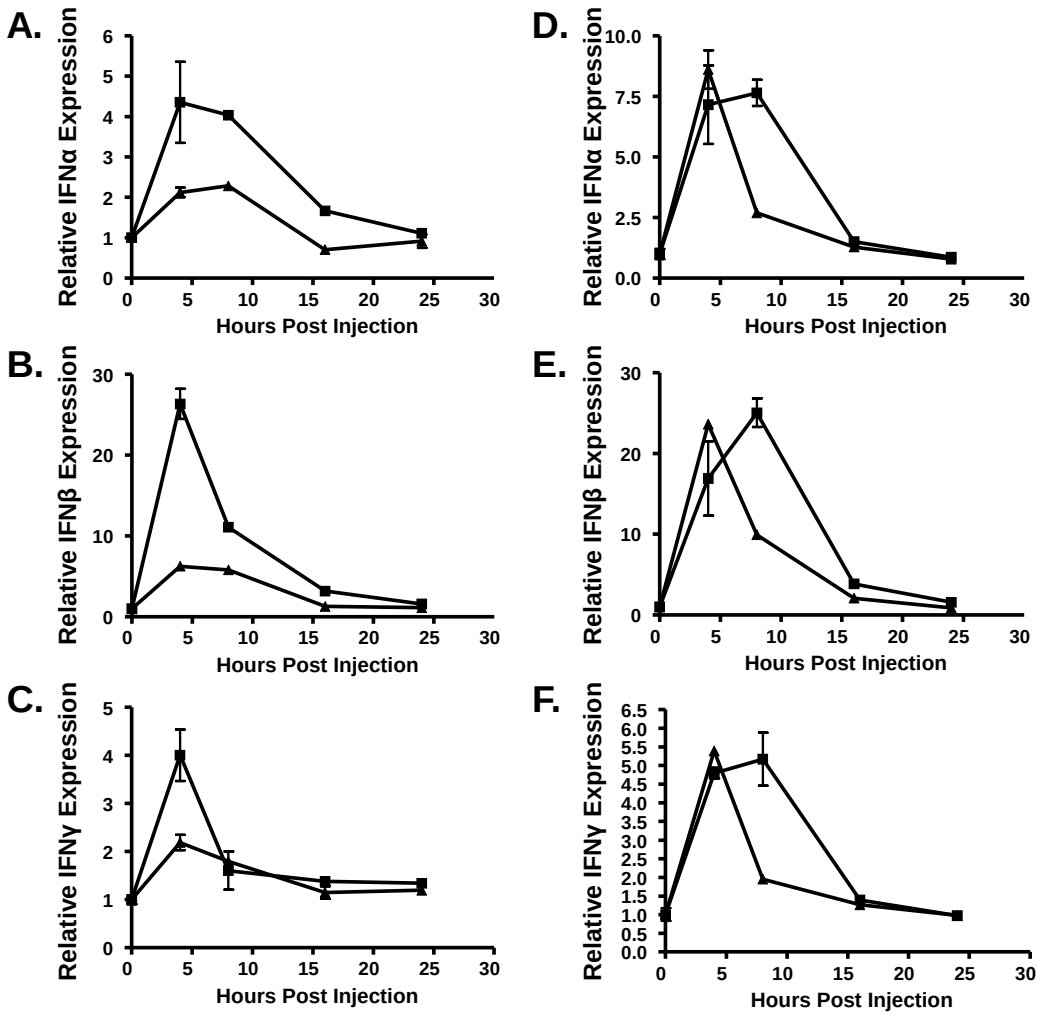

Figure 23: IFNα, IFNβ, and IFNγ are all up-regulated in the spleens of WT mice early post MUC1 vaccination, but not MUC1.Tg mice.

WT (squares) and MUC1.Tg mice (triangles) were vaccinated with MUC1p admixed with Poly-ICLC via tail vein. Spleens were removed at the indicated hours post vaccination and total splenic mRNA levels of IFNα (A, D), IFNβ (B, E), and IFNγ (C, F) were determined relative to the control gene HPRT. Two repeats are shown (A-C vs. E-F) Values shown represent expression relative to the baseline expression in mice of that genotype (WT and MUC1.Tg) at 0 hours post vaccination. Data are representative of 3 pooled mice per group per timepoint shown. Data points show mean ±SEM of three technical replicates.

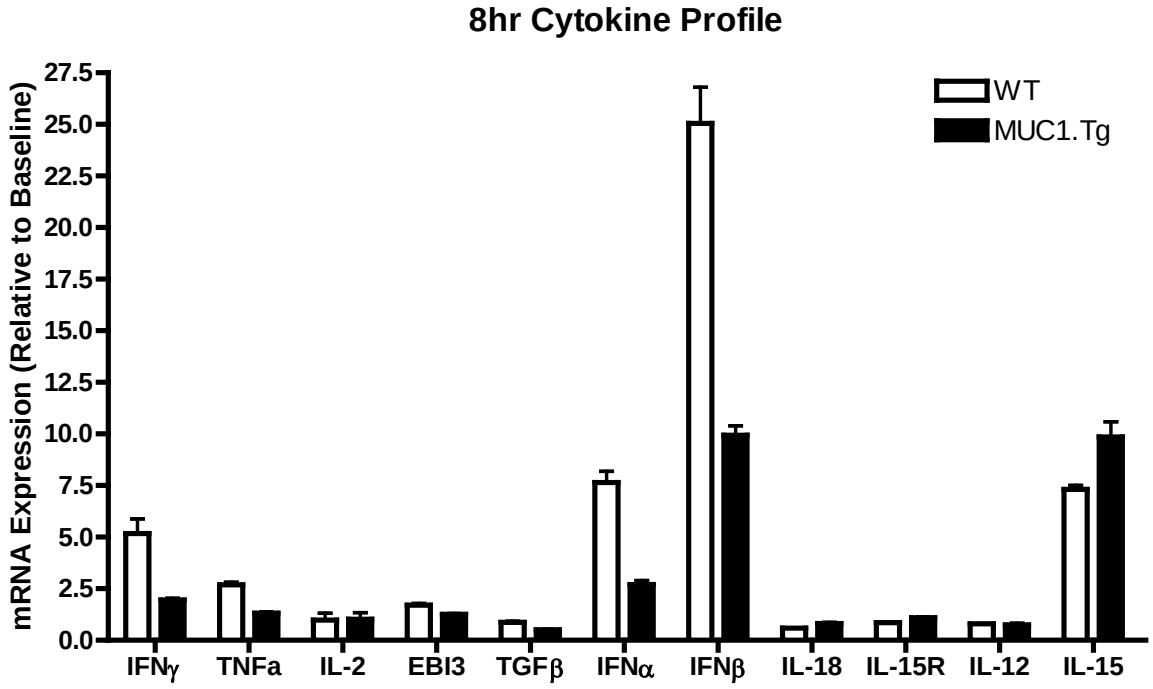

Figure 24: Cytokine profile in the spleens of WT and MUC1.Tg mice vaccinated intravenously with MUC1 at 8 hours post vaccination.

WT (white bars) and MUC1.Tg mice (black bars) were vaccinated as in Figure 22. Spleens were removed at 8 hours post vaccination and the total splenic mRNA levels of the indicated cytokines were determined relative to the control gene HPRT. Values shown represent expression relative to the baseline expression in mice of that genotype (WT and MUC1.Tg) at 0 hours post vaccination. Data are representative of 3 pooled mice per group. Data points show mean ±SEM of three technical replicates.

B.3 DISCUSSION

Given the marked differences we observe in splenic NK cell activation between WT and MUC1.Tg mice vaccinated with MUC1p, the differential expression of type I interferons is quite an interesting results. Type I IFN is well documented in its ability to activate NK cell IFNγ and cytotoxicity (460-463), which both supports and contradicts our finding on the phenotype of NK

cells in our system. Specifically, it supports the induction of IFNγ in the spleens of MUC1 vaccinated WT mice, which we see concurrent with the increases in type I IFN, but it contradicts the decreases we see in the cytotoxicity of these NK cells, relative to those in MUC1 vaccinated MUC1.Tg mice. Further examination of what, if any role it plays on the post vaccination NK cell phenotype will be necessary to resolve this contradiction. It is likely that the combination of other factors present in the microenvironment and the tight window in which type I IFN is produced may be important factors in this consideration.

These studies will be of particular interest as expression of type I IFN in the spleens of WT, but not MUC1.Tg mice post MUC1p vaccination could be responsible for a number of the other post vaccination differences characterized in this thesis as well. Type IFN can support the differentiation (53, 54) and migration (464) of DC, perhaps providing a mechanistic explanation for the increased DC number in WT, but not MUC1.Tg mice post MUC1 vaccination. Type I IFN supports the differentiation of IFNγ producing CD4, but not CD8 T cells, suggesting its absence in MUC1.Tg mice may be a contributing factor to the observed hyporesponsiveness of MUC1 specific CD4 T cells in these mice. (465) And finally, type I IFNs can up-regulate CD40, CD80, CD86, and MHC II expression on DC surfaces (466), perhaps implicating the differential levels of type I IFNs early post vaccination in the differential expression of these molecules later on.

In conclusion, type I IFNs are powerful immune mediators capable modulating the adaptive immune response in a number of ways, and the effect of their differential expression following vaccination with a foreign vs. a self-antigen merits further investigation.

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