Dendritic Cells Are Both Targets and Initiators of Peripheral Immune Tolerance to Self

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ABSTRACT

Mucin 1 (MUC1) is a highly glycosylated membrane-bound protein normally expressed on the apical surface of ductal epithelial cells. During malignant transformation MUC1 acts as a Tumor-Associated Antigen (TAA) by virtue of its overexpression, loss of polarity, and hypoglycosylation, allowing for T cell and antibody recognition of cryptic peptide epitopes derived from its extracellular domain. Almost all adenocarcinomas express abnormal MUC1 making it an attractive target for cancer vaccines. However, vaccination of MUC1.Tg mice with a synthetic, unglycosylated MUC1 peptide (MUC1p) that mimics one tumor form of the molecule results in a weak anti-MUC1p immune response. WT mice receiving the same vaccine generate robust immunity to MUC1p, suggesting that it is viewed as a "self" antigen in MUC1.Tg mice, and apparently subject to peripheral tolerance. To globally query these distinct programs of immunity and tolerance induced by MUC1p in WT and MUC1.Tg mice respectively, we conducted whole transcriptome analysis of splenic RNA 24h and 72h after i.v. immunization of both mouse strains with MUC1p. We found that a new cohort of "pancreatic" enzymes (e.g. trypsin and CPB1) were expressed by splenic dendritic cells (DC) and regulated such that immunization with self-antigen suppressed their expression while foreign-antigens induced it within 24h post-vaccination. The relative expression of trypsin and CPB1 was highly correlated with the immunogenicity of the DC. Suppressed expression marked DC that were highly tolerized as demonstrated by low costimulatory molecule expression, limited motility, production of Aldh1/2, and preferential priming of naïve $CD4^+$ T cells into $Foxp3^+$ Treg versus $IFN\gamma^+$ cells, while enhanced expression identified immunogenic DC. Deficient NFκβ pathway activation and enhanced STAT3 phosphorylation transcriptionally underlie tolerized DC along

with sustained expression of *zDC*. Trypsin was required for efficient degradation of the extracellular matrix, while CPB1 was required by DC to induce optimal, antigen-specific proliferation of $CD4^+$ T cells. Importantly, these vaccine-induced changes in DC phenotype affected the entire splenic DC compartment, revealing an underappreciated role for endogenous DC in the transmission and amplification of vaccine-induced immunity or tolerance. These results underscore the importance of vaccine antigen choice and will contribute to rational vaccine design.

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PREFACE

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1.0 INTRODUCTION

1.1 IMMUNE TOLERANCE TO SELF

A major challenge for the developing immune system is to preclude the release of self-reactive lymphocytes into the periphery, thereby avoiding autoimmunity. Seminal work by Medawar and Burnet in the 1950's proposed the clonal selection theory of tolerance to self-antigen (Ag) based, in part, on experiments demonstrating that immunization of fetal mice and chickens with cells derived from allogeneic animals conferred resistance against the rejection of grafts from the donor species later in life (1, 2). This hypothesis presumed that self-reactive lymphocytes were inactivated during embryonic development, resulting in an exclusively foreign antigen specific lymphocyte repertoire at the time of immunological maturity. The theory of central tolerance is now understood to ensure the development of T, B, NKT, and γδ T cells that recognize foreign peptides derived from pathogens and tumor cells rather than self-peptides. Central tolerance occurs in the thymus for developing T cells, and in the bone marrow for B cells. However, central tolerance is not completely efficient, and many potentially self-reactive lymphocytes escape to the periphery. Here, multiple checks and balances, referred to as peripheral tolerance, act to prevent an effector response against the host's own antigens (3). Central and peripheral tolerance present challenges for the development of cancer vaccines, the goal of which is to induce immunity against tumor-associated antigens (TAA), many of which resemble the self-

antigens against which mechanisms of central and peripheral tolerance act to preclude. We are interested in generating vaccine-induced immunity against the TAA Mucin 1 (MUC1). However, multiple layers of tolerance, including the presence of MUC1-specific regulatory T cells (Treg), and their preferential expansion after vaccination relative to that of MUC1-specific effector T cells (Teff) results suboptimal adaptive responses to MUC1 in both mice and humans (4-7).

1.1.1 Central Tolerance

After immature thymocytes leave the BM and traffic to the thymus they undergo two major rounds of selection: positive selection, and negative selection, or clonal deletion. After egress from the BM, $CD4^+CD8^+$ double positive thymocytes enter the thymic cortex where they rapidly scan cortical thymic epithelial cells (cTEC). Those that have moderate affinity for self-peptide:MHC (pMHC) complexes receive a survival signal from the cTEC, while those with very low affinity for self-pMHC undergo so-called "death by neglect" (8, 9). The precise mechanisms by which cells are positively and negatively selected are incompletely understood, but may involve differences in both the conformational change and duration of signaling through the thymocyte TCR (9, 10). Cells with moderate affinity for self-pMHC upregulate expression of extracellular-signal-regulated kinase (ERK) and migrate to the thymic medulla in a CCR7-dependent manner (9, 11). Because a subset of these positively selected cells are strongly autoreactive, medullary thymic epithelial cells (mTEC) and thymic DC (tDC) mediate negative selection via the presentation of diverse, self, peripheral tissue antigens (PTA). The presentation of extra-thymic antigens is mediated by the autoimmune regulator (AIRE), which allows mTEC to express a plethora of PTA through an imprecisely defined mechanism, but one that may involve the ability of *Aire* not to transactivate multiple PTA promoters, but to prevent stalling of

RNA Pol II on those promoters (12-14). Thymocytes with high affinity for self-antigen are deleted from the repertoire by apoptosis, initiated in part by decreases in mitochondrial anti-apoptotic proteins (e.g.Bcl-2) and increases pro-apoptotic proteins (e.g. Bim) (15), while concurrently signaling through JUN-amino-terminal kinase (JNK) and p38 (16). While mTEC both express and present PTA on MHC I and MHC II, tDC are critical for cross-presentation of mTEC-derived PTA, and both populations require B7:CD28 interactions to mediate negative selection (17, 18). Underscoring the role of DC in mediating negative selection of self-reactive thymocytes, medullary tDC are capable of deleting autoreactive $CD4^+$ T cells without contribution from mTEC or macrophages, and plasmacytoid DC (pDC) transport PTA to the thymus from the periphery and function as canonical mTEC (19, 20). Aire-mediated expression of PTA and ensuing tolerance is not limited to the thymus. Interestingly, lymph node stromal cells present PTA in the periphery and induce tolerance against gut-associated self-antigens independent of DC, demonstrating a functional overlap in maintaining self-tolerance between the thymus and the periphery (21). The efficiency of central tolerance in preventing the release of auto-reactive T cells is highlighted by experiments demonstrating that TCR.Tg $CD8^+$ H-Y T cells recognizing the male antigen are detected in the periphery after transfer into female mice, but are deleted after transfer into males (22). Similarly, the importance of central tolerance is highlighted by the severe autoimmune syndrome polyendocrinopathy-candidiasis-ectodermal-dystophy (APCED) accompanying defects in *Aire* in humans (23).

Natural, or thymic Treg (tTreg), defined as $CD4^+CD25^{hi}Foxp3^+$ T cells assist in the contraction of an immune response after the elimination of a pathogen (24). However, they also preclude the development of spontaneous autoimmunity, as evidenced by the scurfy mouse that contains a mutation in the *Foxp3* gene resulting in a non-functional Treg compartment (25). The

mechanisms by which Treg bearing high affinity TCR for self-antigen escape negative selection are imprecisely defined. Two recent studies demonstrate that CD70:CD27 interactions between tDC, mTEC, and thymocytes are required for the inhibition of apoptosis in Treg precursors, but not $CD4^+Foxp3^-$ conventional T cells (26, 27). The mTEC in human MUC1.Tg mice (discussed in Section 1.5) express human MUC1, resulting in a higher frequency of MUC1-specific Treg compared to WT mice (6, 28). Therefore, MUC1 is a self-antigen and inducing immunity against it in MUC1.Tg animals must first overcome specific, Treg-mediated tolerance.

1.1.2 Peripheral Tolerance: DC-Independent Mechanisms

Thymic selection effectively deletes T cells with high-affinity to self-antigen, but both $CD4^+$ and $CD8^+$ T cells bearing TCR with low to moderate avidity for self peptides escape to the periphery where they can induce autoimmunity within a conducive inflammatory context (29, 30). Therefore, peripheral mechanisms exist to keep potentially auto-reactive T cells in check. The segregation of naïve T cells from most non-lymphoid tissues, referred to as immunologic ignorance, establishes a physical barrier precluding the interaction between an autoreactive TCR and the parenchyma that may express its cognate antigen. Naïve T cells traffic from the blood into secondary lymphoid organs through high endothelial venules (HEV). If they do not encounter a DC presenting cognate antigen, they drain into the efferent lymphatics, and are subsequently returned to the circulation via the thoracic duct, never having encountered antigens concentrated in non-lymphoid tissue (31).

A second mechanism of peripheral tolerance, the efficient clearance of apoptotic cells, also involves compartmentalization or ignorance of self-antigens from potentially autoreactive T cells. Macrophages are chemoattracted to dying cells and phagocytose cellular corpses after

recognition of phosphatidyl serine on the dead cell's membrane (32). Defects in multiple stages of this clearance process can lead to "leaky" apoptotic bodies, the proinflammatory release of DNA and self-proteins, and the pathogenesis of autoimmune disorders such as lupus and rheumatoid arthritis (33). In addition to shielding the immune system from intracellular contents, clearance of dying cells actively induces IL-10 and TGF-β production by macrophages, acting preemptively to counter the release of potentially immunogenic contents (34, 35).

A third mechanism of peripheral tolerance is the induction of T cell anergy, or functional unresponsiveness to subsequent TCR ligation after the initial recognition of cognate pMHC occurs in a homeostatic or sub-immunogenic context. The priming of a naïve T cell into an effector cell requires the T cell to recognize its specific pMHC complex on an antigen-presenting cell (APC), referred to as Signal 1, followed by engagement of activating or inhibitory costimulatory molecules, referred to as Signal 2. (36). TCR engagement of pMHC in the absence of appropriate costimulation results in clonal anergy that prevents an autoreactive T cell from differentiating into an effector cell. The absence of costimulatory molecules on APC occurs in the steady state, or in a sub-inflammatory milieu. There are numerous costimulatory molecules that can inhibit both naïve and effector T cells at various points during, and after encounter with self-antigen. Naïve T cells constitutively express CD28, and the failure to receive signals from CD80 and CD86 (B7-1 and B7-2) on APC results in anergy (3, 37). Expression of CTLA-4, a higher affinity homolog of CD28 expressed by T cells after activation, acts to inhibit proliferation, IL-2 production, and induces anergy, preventing the prolongation of the effector response (38, 39). Finally, PD-1:PD-L1 interactions maintain anergy in previously activated autoreactive T cells after subsequent encounters with their self-antigens in parenchymal tissues (40).

Treg provide critical suppressive capacity when ignorance and anergy fail to prevent the induction of an autoimmune reaction. Originally identified as a $CD4^+CD25^{hi}$ population with suppressive capacity (41), the importance of Treg in maintaining peripheral tolerance to self is illustrated by the extensive autoimmunity that develops in both mice and humans with a defect in *Foxp3* (25, 42). Treg can develop in the thymus as discussed above, or can be primed from naïve $CD4^+$ T cells in the periphery (iTreg) (43). While these two populations both express Foxp3 and possess a suppressive function, tTreg are mainly responsible for preventing T cell responses to self-antigens, while iTreg are generated *de novo* from naïve $CD4^+$ T cells, and require IL-2 and TGF-β in conjunction with chronic, low-dose exposure to environmental antigens such as food-derived antigens and the commensal microbiota (44-46). Knowledge of the precise mechanisms by which Treg mediate their suppressive effects is limited, however they appear to be diverse and involve both cell-contact as well as elaboration of suppressive cytokines. Constitutive expression of the high-affinity IL-2Rα (CD25) may function as a "sink", depriving neighboring T cells of this crucial cytokine (47), while constitutive expression of CTLA-4 has been proposed to mediate trans-endocytosis of CD80 and CD86 on APC as well as preventing costimulation by those molecules via competitive occupancy (48). Treg express high levels of the adenosine ectonucleotidases CD39 and CD73 that decrease the availability of extracellular cyclic adenosine monophosphate available to effector cells (49). Treg-derived IL-10, TGF-β, and IL-35 all have suppressive functions in various mouse models of autoimmunity, mucosal tolerance, and lymphpoenic proliferation (43, 50). Interestingly, Treg also have direct, cytolytic activity both *in vitro*, and *in vivo* where they are required for the maintenance of skin allografts (51, 52).

1.2 DENDRITIC CELLS

DC were identified in a series of seminal papers by Steinman and Cohn between 1973 and 1974 (53-55). Steinman referred to them as "dendritic" because: "These novel cells can assume a variety of branching forms, and constantly extend and retract many fine cellular processes. The term 'dendritic cell' would thus be appropriate." (53). While the precise mechanisms underlying DC function would not be elucidated for several years, based on their radiosensitivity, endocytic capacity, and lack of expansion following immunization with sheep red blood cells, Steinman postulated that, "These effects suggest a cooperative role for dendritic cells in the immune response" (54). Steinman was also the first to describe the ability of DC to induce T cell proliferation *in vitro* based on their stimulatory capacity in the mixed lymphocyte reaction (MLR) (56). However, it was not until Charles Janeway's 1989 lecture at Cold Spring Harbor that the notion of co-stimulation, and its induction by microbial pattern recognition receptors (PRR) on APC attempted to unify the innate and adaptive mechanisms of host defense (57). Lanzavecchia and Mantovani categorized DC as innate based on their chemotaxis and calcium flux following exposure to complement and bacterial formyl peptides (58.) Following the discovery of TLR4 and its function as an endotoxin receptor, and its expression on DC, DC have been established as the most potent APC capable of bridging innate and adaptive immunity by sensing both microbial, and self-derived danger signals and priming the ensuing T cell response (59-61).

1.2.1 Induction of Immunity by DC

Conventional DC (cDC) originate in the BM and can be subdivided into two major groups: migratory and lymphoid resident DC. Migratory DC undergo the canonical DC lifecycle, characterized by patrolling non-lymphoid tissue in an immature state, followed by maturation after detection of microbial and self-derived "danger" signals, and subsequent trafficking to draining lymph node (LN). During immaturity they are highly phagocytic, sampling their environments for pathogens and tumor cells. After detection of "danger" signals by PRR they begin to mature and process internalized proteins into MHC II and MHC I-restricted peptides (62). DC possess a plethora of PRR specialized for the detection of bacterial cell wall components and DNA, viral RNA, and fungal polysaccharides as well as molecules released from necrotic or physically damaged host cells. These receptors include the TLR, RLR, NLR, and CLR families amongst others (reviewed in (63)). Maturation is characterized by upregulation of a variety of costimulatory molecules including CD80, CD83, CD86 and CD40, as well as MHC II (61). These maturing DC upregulate expression of CCR7 and migrate through the lymphatics to the draining LN in response to gradients of CCL19 and CCL21 (64). After entering the LN through high endothelial venules, they present processed antigen to cognate $CD4^+$ and $CD8^+$ T cells in specialized T cell areas of secondary lymphoid tissues (65). Epidermal Langerhans cells, $CD103^-$ connective tissue DC and $CD103^+$ DC of the dermis, lung, and gut are all prototypic migratory DC. In contrast, lymphoid tissue resident DC are not migratory and spend the entirety of their lifespan in LN, spleen, or thymus presenting both lymph-born self and foreign Ags, however they follow the same mechanisms of maturation and T cell priming as migratory DC. Tissue resident DC are less mature and more phagocytic compared to migratory DC (66). These lymphoid tissue resident DC can be phenotypically classified into three separate

populations, each with specific functionality. $CD11c^+CD4^+CD8\alpha\alpha^-$ DC, $CD11c^+CD4^-CD8\alpha\alpha^+$ DC, and $CD11c^+CD4^-CD8\alpha\alpha^-$ DC (67). The $CD11c^+CD4^-CD8\alpha\alpha^+$DC are a distinct lineage characterized by expression of the transcription factor *Batf3*, and are more efficient at cross-presentation, the display of exogenously derived antigen on MHC I molecules to prime $CD8^+$ T cells, while $CD11c^+CD4^+CD8\alpha\alpha^-$ DC are more efficient in presenting antigen to $CD4^+$ T cells (68-70).

Following pMHC:TCR engagement with a cognate T cell (Signal 1), and ligation of costimulatory molecules (Signal 2), the DC is responsible for delivering all or part of Signal 3 which comprises the cytokine signals required for T cell fate determination. DC are the earliest source of the T cell growth factor IL-2, and the absence of DC-derived IL-2 results in severely impaired T cell proliferation (71). DC provide IL-12 to skew naïve $CD4^+$ T cells towards a Th1 phenotype as well IL-6 and IL-23 to differentiate and stabilize cells towards the Th17 lineage (72, 73). DC also play an important role in the generation peripheral iTreg via the production of the suppressive cytokines IL-10, TGF-β, and retinoic acid (RA) (74-76).

In addition to cDC, plasmacytoid DC (pDC) and monocyte-derived DC (moDC) comprise two additional populations. pDC are hematopoietically distinct from cDC and are a major sensor of viral infection and source of subsequent Type I IFN. pDC have recently also been described as important for the maintenance of self-tolerance, in part due to their low expression of MHC II and costimulatory molecules and inefficient phagocytosis (77, 78). Monocyte-derived DC are found in the circulation and are rapidly recruited to sites of infection and inflammation where they produce chemoattractants, inflammatory cytokines, and can participate in tissue repair, (reviewed in (79)).

1.2.1.1 Antigen Processing and Presentation

Antigen processing and presentation are critical processes for DC function and are also important for tolerance or immunity to a given antigen. As discussed below, processing that results in MHC-restricted presentation of a peptide can induce immunity, while the complete degradation of an antigen during processing can result in a lack of thymic education and resultant autoimmunity. Similarly, the amount of peptide presented by a DC affects the type of T cell response that is primed (80).

MHC I processing of self-peptides occurrs in most somatic cells, and in DC via cross-presentation. Briefly, misfolded, viral, and tumor-associated proteins are ubiquitylated and degraded by the cytosolic proteasome. Proteasomal catalytic subunits, β1, β2, and β5 hydrolyze peptides after acidic, basic, and hydrophobic residues respecitvely (81). IFNγ induces assembly of the immunoproteasome (expressed constituitively by APC) via expression of the βi subunits LMP2/7/10 that produce peptides tailored for priming a $CD8^+$ response (82) Further N-terminal trimming can occur via cytosolic peptidases such as tripeptidyl peptidase II (TPPII), before transporter associated with antigen processsing (TAP) mediated translocation into the ER. Additional N-terminal processing by the endoplasmic reticulum-associated amino peptidase (ERAAP) precedes chaperone-mediated (tapasin/Erp57/calnexin) loading of peptides onto MHC I molecules for transport to the cell surface (83) (84) (85) (Fig. 1). The diversity of proteases responsible for generating MHC I peptides is beginning to be further elucidated as evidenced by the identification of insulin-degrading enzyme (IDE), a cytosolic metallopeptidase, being involved in generating TAA MAGE-A3-derived epitopes in a proteasome-independent fashion (86). MHC II processing is conducted predominantely by professional APC (DC/macrophages/B cells), and results in presentation of exogenously or endogenously derived Class II restricted antigen via classical or autophagic pathways respectively. In the classical pathway, antigen moves from phagocytic

compartments through a series of progressively more acidic endosomal vesicles that process the protein and facilitate its loading onto MHC II. Briefly, internalized antigen is transported to the late endosomal/lysosomal-like MHC II compartment (MIIC), where the MHC II chaperone invariant chain (Ii, CD74) is cleaved by cathepsin S (CatS) (87) to the shorter, class II associated invariant chain peptide (CLIP). After proteolytic cleavage by a multitude of proteases including serine proteases Cat A/G, aspartyl proteases Cat D/E, cysteine proteases Cat B/S/L and asparaginyl endopeptidase (AEP), the chaperone HLA-DM mediates exchange of CLIP for antigenic peptides (88) (89) (90) with subsequent targeting of peptide-MHC II complexes to the cell membrane via endolysosomal tubules (Fig.1). There is both redundancy and specificity in cathepsin-mediated antigen processing. CatB or CatD deficiency does not impair presentation of the model antigens OVA or HEL to Class II restricted hybridomas (91). However, AEP is required for B cell processing and immunity against tetanus toxin (92), while the absence of CatD *in vitro* improves IL-2 production by myoglobin-specific $CD4^+$ cells (93). Cat activitiy *in vivo* also appears to posess specificity as AEP can destroy myelin basic protein (MBP) in mTEC and thymic DC, precluding negative selection against MBP-reactive T cell clones leading to autoimmunity. (94). This accounts for data suggesting that control of vesicular acidification, and therefore lysosomal enzymatic activity, are important in preventing destruction of immunogenic epitopes (95). Manipulation of antigen processing enzymes for therapeutic benefit has been used with sucess in mouse models of experimental autoimmune encephalomyelitis (EAE) (96) and Sjögren syndrome (97) with inhibitors of CatS. Conversely, induction of the processing/presentation machinery in tumors by IFNγ increases the efficacy of immunotherapy in both mice and humans, illustrating the potential of manipulation of this pathway for clinical benefit (98, 99).

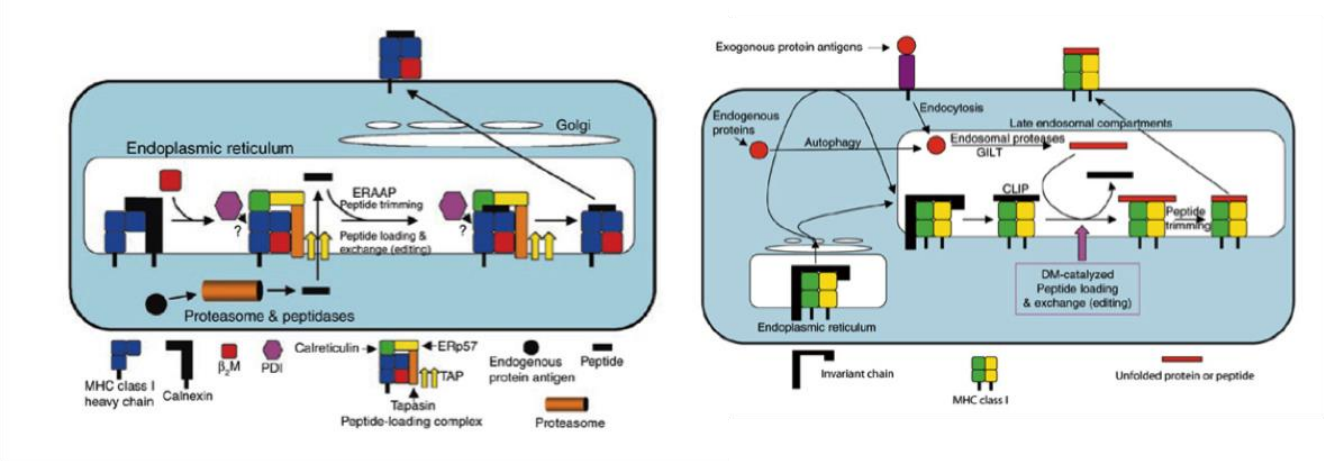

Figure 1 MHC I and MHC II antigen processing and presentation pathways.

(Left Panel) Endogenously or exogenously (through cross-presentation)-derived proteins destined for presentation on MHC I are processed predominantly by the cytosolic proteasome complex before chaperone-mediated translocation and loading in the ER. (Right Panel) Peptides to be presented on MHC II molecules originate extracellularly and are processed and loaded via the endosomal pathway. Reprinted with permission from: Jensen, P.E. *Nature Reviews Immunology*. 2007.

1.2.2 Peripheral Tolerance: DC-Dependent Mechanisms

As discussed above, DC play important roles in central tolerance to self. In the periphery, DC are indispensible for precluding autoimmunity, in large part by inducing anergy or deletion of T cells, promoting the differentiation of iTreg, and producing suppressive factors (100). The importance of the DC contribution to the maintenance of homeostatic tolerance is highlighted by the autoimmunity, mediated by $CD4^+$ T cells, that occurs after their ablation (101), although this role is still somewhat contentious (102). Immature DC (iDC) promote tolerance in the steady state via their low expression of the costimulatory molecules CD80, CD86 and CD40, as well as MHC II (103, 104). T cells that recognize pMHC complexes on iDC in the absence of sufficient

costimulation are rendered anergic or are deleted. Similarly, the amount of antigen presented by a DC can determine the direction of the ensuing T cell response, independent of maturational state, with low doses of antigen favoring Treg differentiation and high doses favoring effector cells (105).

In addition to iDC, several DC subsets have been described as being tolerogenic in the absence of inflammation or infection. In mice, $CCR9^+$ pDC express low levels of MHC II and costimulatory molecules and mediate tolerance to allografts and oral antigen by priming Treg (106, 107). Splenic $CD11c^+CD11b^-CD205^+CD8\alpha\alpha^+$ DC are specialized to induce tolerance against self-antigens, in part through their enhanced production of TGF-β, and *in vivo* targeting of antigen to this population using peptide conjugated to anti-CD205 antibodies induces antigen-specific Treg and tolerized $CD8^+$ T cells (75, 104, 108, 109). The DC associated with mucosal barrier sites such as the gut must maintain tolerance to commensal and food-derived antigens in the context of chronic exposure to these beneficial, yet foreign molecules. As a result, the $CD11c^+$CD103+ DC in the intestinal lamina propria are specialized to induce Treg via a TGF-β and retinoic acid (RA)-dependent mechanism (76). Finally, human $CD123^+$ DC and mouse $CD19^+$ DC expressing indoleamine 2,3-dioxygenase (IDO), an enzyme responsible for tryptophan catabolism, can inhibit T cell proliferation *in vitro* have been detected *in vivo* as well where they may provide important suppressive functions (110, 111). Therefore, during homeostasis, the primary role of iDC is to prevent the priming of autoimmune responses, and these non-inflammatory cells are assisted by additional, specialized tolerogenic DC populations. It is important to note that iDC, pDC, $CD11c^+CD11b^-CD205^+CD8\alpha\alpha^+$ DC, and $CD103^+$DC all have the potential to induce potent, Th1, Th17, or Type I IFN responses during inflammatory insult or infection, yet in the steady state are uniquely equipped to preclude those responses that

would like be directed against the host. For example, $CD103^+$DC from colitic mice induce IFNγ production rather than $Foxp3^+$ Treg when transferred into naïve animals, highlighting the importance of the microenvironmental milieu (resulting from infection or vaccination) in conferring DC with tolerogenic versus immunogenic properties (112).

DC express a wide variety of innate immune receptors that generally induce maturation and immunogenicity upon recognition of microbial or host-derived danger signals. However, signaling through some of these receptors maintains a quiescent state, or actively induces DC tolerance. Signaling through TLR2 by yeast zymosan and phosphatidylserine from *Schistosoma sp.* induce Treg differentiation, while ligation of the TAM receptor tyrosine kinase Mer by ligands on the surface of apoptotic cells actively inhibits DC maturation (Fig. 2) (100, 113).

After a DC is rendered tolerogenic, inherently by virtue of immaturity, after ligation of tolerizing receptors, or by belonging to a $CD11c^+$ compartment specialized to maintain tolerance as discussed above, the DC can effect that tolerance through the production of immunosuppressive cytokines, or by the lack of production of immunogenic cytokines. The pulmonary DC of mice exposed to airway allergens produce suppressive IL-10 versus Th1-skewing IL-12p70, as do DC isolated from the peyers patches in the gut to prevent the development of airway hypersensitivity and contribute to oral tolerance respectively (74, 114). Similarly, the suppressive cytokine TGF-β produced by DC is required for control of experimental autoimmune encephalitis in mice (115) while DC-derived retinoic acid (RA) is a key driver of Treg development in the gut and has broad immunosuppressive effects on diverse cell types (Fig. 2) (116). Arginase, an enzyme that metabolizes L-arginine to L-ornithine and urea, has immunosuppressive effects on T cells and is produced by DC. Specifically, DC

production of arginase has been shown to be directly induced by RA, suggesting a feed-forward mechanism exploited by the suppressive, $CD103^+$ DC in the gut (117).

The transcriptional programs that underlie DC tolerance and immunity are not completely characterized, though some important players have emerged. In common with many cells, activation of the NFκβ pathway, specifically translocation of phospho-p65 into the nucleus, is essential for the upregulation of costimulatory molecules and MHC II in DC (118) Recently, the transcription factor *Zbtb46* (zDC) has been shown to be constitutively expressed by iDC in the steady state and rapidly downregulated after TLR-induced maturation, however the promoters it occupies are currently unknown (119, 120). The highly tolerogenic DC (and macrophages) found in the lamina propria of the intestine require the constitutive activity of β-catenin to produce RA and IL-10, as well as for maintenance of $Foxp3^+$ Treg from naïve $CD4^+$ T cells (121). Similarly, a novel role for histone deacetylase 11 (HDAC11) had been described in negatively regulating transcription of IL-10 in human DC and murine macrophages (122). The signal transducer and activator of transcription (STAT) proteins signal downstream of multiple cytokine receptors. STAT3 is phosphorylated in response to IL-10 signaling, amongst other cytokines, and its deletion in DC confers a mild autoimmune phenotype in mice demonstrating its role as a negative regulator of DC immunogenicity (123). Important negative regulators of cytokine signaling in DC and other hematopoietic cells are the suppressor of cytokine signaling proteins (SOCS) that bind to janus tyrosine kinases (JAK) inhibiting signaling after a cytokine binds to its receptor (124). Accordingly, tolerized DC upregulate expression of SOCS1 resulting in decreased production of the proinflammatory cytokines TNF-α, IFNγ, and IL-12 (125).

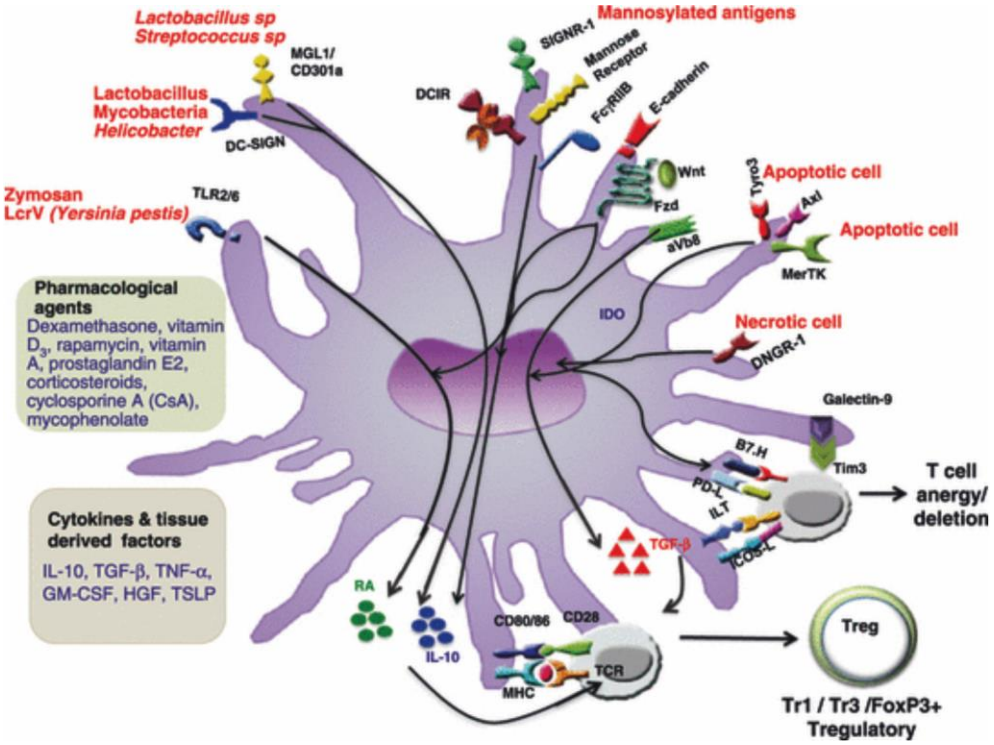

Figure 2 Mechanisms of DC tolerization and tolerogenicity

Recognition by DC of microbial and self-derived molecules can induce tolerance depending on the inflammatory milieu in which it occurs, while some tolerance (e.g. the clearance of apoptotic bodies and tolerance to oral antigen) occurs independently of inflammation. Reprinted with permission from Manicassamy, S. Immunological Reviews. 2011.

1.2.3 DC Vaccines

Given their central role in priming antigen-specific T cell responses, DC are uniquely suited to be used as both prophylactic and therapeutic vaccines for cancer, pathogens, autoimmunity, and transplantation. Recently, the first DC vaccine for clinical use was approved by the FDA for the treatment of metastatic prostate cancer (126). The vaccine, Sipuleucel-T, consists of the patient's autologous PBMC cultured for three days with a prostatic acid phosphatase-GM-CSF fusion protein, and is administered bimonthly for six weeks. Sipuleucel-T increased the median survival

time of patients by 4.1 months, a notable result given the relative immunogenicity of many prostate tumors compared with other malignancies. There are 3 general approaches to DC vaccination that are currently in use: the use of autologous (clinical) or syngeneic (preclinical) DC that are loaded with the antigen(s) against which the ensuing T cell response is to be directed, and then matured, and reinfused into the patient/animal, the targeting of antigen to specific DC populations *in vivo* using chimeric antigen-antibody complexes, and the manipulation of DC function by antibody/ligand-mediated engagement of DC stimulatory/inhibitory molecules (127). Autologous, peptide-pulsed DC vaccines for cancer, HIV, and hepatitis C have been demonstrated to be safe, effective at inducing antigen-specific $CD4^+$ and $CD8^+$ T cells, and in some cases, eliciting objective clinical responses (128-131). "Negative vaccination", or the use of autologous, tolerized/tolerizing DC to prevent autoimmune pathologies such as rheumatoid arthritis and type 1 diabetes, as well as to prevent transplant rejection have also been shown to be safe, but with varying degrees of efficacy (132-134). Some tolerance-inducing vaccines rely on the ability of iDC, either peptide loaded or unloaded, to prime Treg rather than effector T cells. Others rely on pharmacologically manipulated DC (e.g. using rapamycin, dihydroxyvitamin D_3, or prostaglandin E_2) to induce tolerogenic DC by preventing maturation and/or IL-12p70 production (135).

Targeting antigen to DC *in vivo* is an attractive strategy by which to take advantage of different DC subsets and their specialized functions. For example, $CD8\alpha^+$DEC-205^+ DC are specialized to cross-present antigen to $CD8^+$ T cells (68, 109), while $CD8\alpha^-$DEC-205^-DCIR2^+ DC preferentially present antigen to $CD4^+$ T cells (69). Targeting ovalbumin specifically to DEC-205^+ DC using an antigen-conjugated antibody *in vivo* leads to improved rejection of OVA^+ tumors as well as viral clearance (136). Similarly, targeting the TAA Her-2/neu to

$CD11c^+$ DC *in vivo* resulted in both protection against subsequent challenge with a Her-2/neu^+ transplantable tumor, as well as a delay in the growth of spontaneous tumors in Balc-*neu*T mice (137). The ability to inhibit tumor growth by targeting antigen to DC, in the context of tolerance to transgenically expressed Her-2/neu, suggests that vaccines that target DC *in vivo* v can break self-tolerance.

The plethora of PRR expressed by DC to detect molecules derived from microbes and damaged host cells can also be exploited to induce DC maturation, and thus enhance their immunogenicity. These include synthetic TLR ligands such as Poly:ICLC (TLR 3), Imiquimod (TLR 7/8), and CpG dsDNA (TLR 9) (138). Many of these adjuvants used to mature vaccine DC *ex vivo* can also be administered systemically to mediate global DC maturation. Similarly, agonistic antibodies directed against activating costimulatory molecules such as CD40, or inhibitory molecules such as ASGPR1 can be administered to the host before or after infusion of matured DC (139, 140).

1.3 IMMUNOSUPPRESSION AND CANCER

1.3.1 Tumor-Mediated Immune Suppression

Portions of Sections 1.3.1 and 1.4.2 have been adapted from "Vaccines based on abnormal self-antigens as tumor antigens: Immune regulation". Farkas, AM and Finn OJ. *Seminars in Immunology*. 22:125-131. 2010. Reprinted with permission from Elsevier, Amsterdam. Copyright permission is kept on file with Adam M. Farkas.

Cancer vaccines must overcome the strongly immunosuppressive milieu created by the tumor itself. Tumor cells can prevent an effective immune response through a multitude of mechanisms (141). Firstly, transformed cells can produce soluble immunosuppressive factors such as IL-10, Transforming Growth Factor β (TGF-β), gangliosides and Prostaglandin E2 (PGE_2) that all diminish the potency of effector cells. Tumor cells can also directly mediate the killing of T cells through expression of Fas-L, Tumor Necrosis Factor Apoptosis-Inducing Ligand (TRAIL), and Indolamine 2 3-Dioxygenase (IDO) production, as well as provide negative co-stimulation to tumor-specific T cells through B7-CTLA-4 and Programmed Death Ligand (PD-L1) receptor (PD-1) interactions. These interactions recruit SHP family members to de-phosphorylate the Immunoreceptor Tyrosine-Based Activation Motif (ITAM) responsible for signal transduction through the TCR (142). Tumor cells are also able to downregulate MHC I expression, leading to their sub-optimal recognition by effector cells through the loss of TCR-MHC avidity and impaired tumor antigen processing and presentation (143).

Expansion of Myeloid-derived suppressor cells (MDSC) is another mechanism by which tumor-specific effector T cell immunity is dampened. MDSC, characterized as a heterogeneous population of $Gr\text{-}1^{+}CD11b^{+}$ cells in mice (144) and $CD11b^{+}CD33^{-/lo}HLA\text{-}DR^{-/lo}$ cells in humans (145) are present at higher frequencies in the blood of tumor-bearing mice and humans (146). MDSC arise from common myeloid progenitors in the bone marrow (BM) that in healthy animals differentiate into macrophages, dendritic cells and granulocytes under the influence of GM-CSF, FMS-related tyrosine kinase 3 (FLT3) and IL-3 (147). However, the cytokine and growth factor milieu produced by tumors, including VEGF, TGFβ, IL-10 and COX-2, supports the maintenance of an immature phenotype and antigen-specific suppressor function. MDSC express MHC I but little MHC II or co-stimulatory molecules, such that presentation of Class I

restricted, tumor-derived peptides to $CD8^+$ cells results in inhibition of IFNγ production and lack of CTL activity (148). MDSCs mediate their suppressive effect through additional mechanisms including high level arginase expression (thus depleting the pool of L-arginine requisite for effector cell proliferation), iNOS (increasing T cell apoptosis via nitric oxide), peroxynitrite, and reactive oxygen species (ROS) (149) (147). MDSC have also been demonstrated to elicit peripheral Treg (150).

1.3.2 Tumor-Mediated Immune Suppression and DC

Many of the tumor-derived mechanisms of immunosuppression that affect tumor recognition and T cell effector function also inhibit the immunogenicity of DC, and thus preclude the priming of robust anti-tumor immunity (146, 151). Tumor-derived IL-10, TGF-β, and PGE_2 can all render DC refractory to maturation by the danger signals released from dying tumor cells as well as vaccination, leading to low expression of CD80, CD86 and MHC II (152) and reviewed in (141). As discussed above, this immature DC phenotype results in the priming of Treg rather than $IFN\gamma^+$ anti-tumor $CD4^+$ and $CD8^+$ T cells (153) and often mechanistically involves the induced expression of TGF-β by DC (154). IL-10 produced by the tumor as well as DC appears to play a critical role in the ability of DC to prime cytotoxic lymphocytes (CTL) as $IL\text{-}10^{-/-}$ mice are resistant to UV-induced melanoma, and vaccination of mice using BMDC derived from $IL\text{-}10^{-/-}$ mice induces tumor rejection and specific memory (155, 156). Additionally, the tumor microenvironment is generally hypoxic, acidic, and contains byproducts of anaerobic metabolism such as lactate. This milieu affects the ability of DC to infiltrate solid tumors, thereby reducing the antigen presentation capacity within the tumor, and also predisposing the priming of a Th2-biased T cell response that has little anti-tumor effect (157, 158). A final, major contributor to

DC immunosuppression induced by tumors is altered myelopoeisis resulting in inefficient production of mature DC in the BM in favor of immature DC precursors, and a corresponding defect in the number of tumor-infiltrating DC (146). The reduction in DC number and tumor localization, combined with the inability to functionally mature and prime T cells results in a highly immunosuppressive milieu for therapeutic cancer vaccines to overcome.

1.4 MUC1 AS A TUMOR-ASSOCIATED ANTIGEN (TAA)

1.4.1 MUC1 Background

MUC1 is a highly O-glycosylated, trans-membrane protein expressed on the apical surface of ductal epithelial cells (Fig. 3). It is a constituent of the mucous layer in the gut and respiratory tracts along with the secreted mucins, thus protecting epithelial cells from pathogens, ROS, and toxins (159, 160). It is comprised of an extracellular domain consisting of 20-120 repeats of a conserved 20mer sequence (PDTRPAPGSTAPPAHGVTSA) called the variable number of tandem repeats (VNTR) region, that remains silent to the immune system during homeostasis, due to inaccessibility to APC, lack of efficient processing by DC (161), and the masking of peptide epitopes by the long, branched sugars attached at Ser and Thr residues within the VNTR. During malignant transformation and chronic inflammatory disorders, MUC1 loses the polarity of its expression, becomes overexpressed, and is extensively hypoglycosylated. These shorter, O-linked glycans represent conserved, neo-antigens such as the Tn antigen (GalNAcα-*O*-S/T) and the Tf antigen (Galβ1-3GalNAc-*O*-S/T) (162). Hypoglycosylation reveals previously masked peptide epitopes that potentially represent “foreign” or “abnormal self” antigens on MUC1.

Because these cryptic peptide epitopes are selectively expressed on transformed epithelial cells, they represent an attractive target antigen for cancer vaccines (162-164). In addition to its role as a TAA, MUC1 also functions as an oncoprotein via its cytoplasmic tail. The inducible expression of MUC1 by proinflammatory cytokines such as IFNγ, TNF-α and IL-6 results in direct activation, by the cytosolic tail of MUC1, of p65, p53 and β-catenin (165-167). More recently it has been demonstrated that the extracellular domain of MUC1 can induce transcription of IL-6 and TFN-α by associating with NFκβ (168). Through these interactions, MUC1 can enhance tumor progression in a feed-forward loop driven by its overexpression on transformed cells.

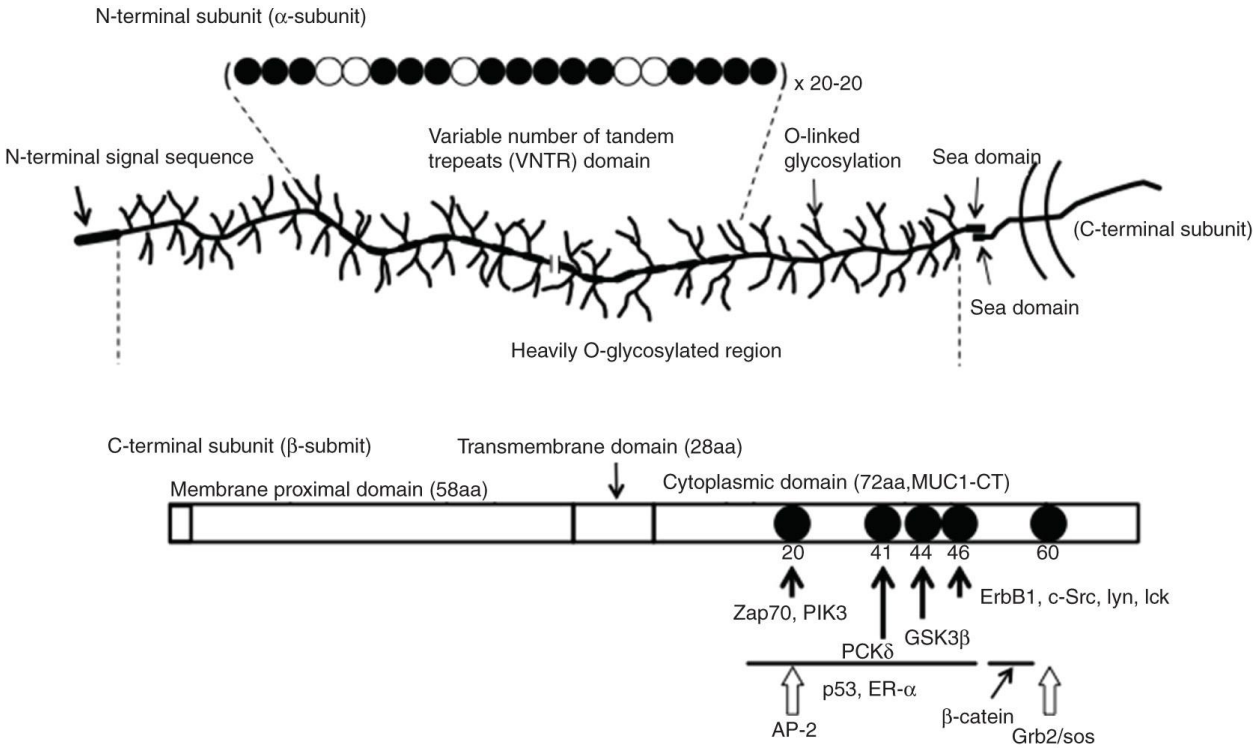

Figure 3 Biochemical structure of MUC1.

The extracellular VNTR region of MUC1, as well as detail of the membrane-proximal domain, transmembrane domain, and cytoplasmic tail are depicted. The 20 circles represent 1 repeat of the repetitive 20mer with open circles depicting Ser and Thr residues that can be glycosylated

with O-linked sugar moieties. Filled circles in the cytoplasmic tail represent Tyr residues with the adaptor/signaling molecules shown to interact with each Tyr depicted below. Figure reprinted with permission from: Kimura, T. and Finn, OJ. Expert Opinion on Biological Therapy. 2013.

1.4.2 MUC1 Vaccines in Clinical Trials

The first clinical trial (Phase I) using a MUC1 based vaccine enrolled 22 patients with advanced adenomas of the breast, pancreas, and colon (169). The vaccine was composed of a 105 amino acid synthetic MUC1 peptide admixed with BCG adjuvant and administered subcutaneously. While there was no survival benefit, the vaccine was safe and there was a 2-4 fold increase in MUC1-specific CTL's in 7 patients as well as an increase in anti-MUC1 IgM in a fraction of patients. The inability of this vaccine to induce effective anti-tumor immunity likely involved multiple immune system intrinsic and tumor intrinsic suppressive mechanisms given the advanced stage of disease in all patients. Low levels of MUC1-specific IgM illustrate a failure to activate $CD4^+$ T cells required for isotype class switching and the IFNγ production requisite for efficient CTL expansion.

Another trial (Phase I/II) used a MUC1 peptide 100mer in combination with the SB-AS2 adjuvant (monophosphoryl lipid A in alum and water) in patients with locally advanced or surgically resected pancreatic cancer with no prior chemotherapy (7). The vaccine was administered i.m. at 3 week intervals, and was again well tolerated. Two of 16 patients were disease free at 32 and 61 month follow-ups. Notably, there was a significant increase in the number of $CD8^+$ T cells in the peripheral blood of the vaccine group able to make IFNγ in response to polyclonal activation, perhaps representing a vaccine-mediated effect on reducing

systemic immunosuppression. Importantly, these patients initially had less advanced disease compared with the trial discussed above, and the induced immunity was likely not subject to the same suppressive mechanisms as are present in more advanced cancers. There was also an increase in T cell infiltrate post-vaccination in skin biopsies, and the first observed induction of MUC1-specific IgG in 5 patients. The improved response as far as clinical outcome and specific IgG might represent the enhanced efficacy of vaccination in earlier stage disease before tumor-intrinsic mechanisms of immune regulation become more entrenched.

A more recent MUC1 vaccine (PhaseI/II) was tested in patients with surgically resected pancreatic or biliary cancers (129). The vaccine consisted of MUC1 peptide-pulsed autologous DC's administered intradermally or subcutaneously. Four of 12 vaccine recipients remained alive and disease free 4 years post-completion of the study, representing significantly improved survival compared to conventional treatments. Immunologically, there was an increased frequency of Treg in patients receiving the vaccine compared with healthy, age-matched controls that was transiently increased following each vaccination as previously observed in mice (4). However, the vaccine did not reduce Treg, and the ability to examine MUC1-specific IFNγ production from CD4 and CD8 cells in the peripheral blood was difficult to assess against a high basal level of T cell activation. This may illustrate the vaccine's ability to non-specifically reduce suppression in these patients, which translated into the positive clinical outcomes that were observed. No increase in MUC1 specific IgG or IgM was observed, though as a cellular vaccine this was not its primary goal (i.e. soluble antigen was not delivered). The lack of detectable change in conventional correlates of protection, combined with favorable outcome leads to the question of how best to monitor the induction of immunity. One hypothesis is that

cells of the peripheral blood may not be representative of the induction of effective immunity due to the presence of micro-metastases that imprint a suppressive phenotype on these cells.

A Phase II trial involving a vaccine against MUC1 in prostate cancer made use of a modified vaccinia virus vector (rMVA) encoding MUC1p and IL-2 (170). The primary endpoint was a 50% increase in PSA doubling times and was not achieved, however 13 of 40 patients had a 2+ fold increase in PSA doubling time and 10 maintained stable PSA levels for 8 months with a median improvement of 1.6 moths. Interestingly, while 7 individuals developed MUC1-speicific $CD8^+$ T cells post-vaccination, over half of the patients had MUC1-specific $CD8^+$'s pre-vaccination. This observation likely reflects the immunoregulatory cancer microenvironment that prevents endogenous TAA-specific T cells from mediating effective immunity. Indeed, the positive results in this trial may have resulted not from the successful priming of new TAA-specific T cells but rather the elicitation of additional IL-2.

MUC1 is also aberrantly expressed in Non-Small-Cell Lung Cancer (NSCLC) and a Phase IIB trial was conducted in individuals with advanced (Stages IIIB and IV) disease following first-line chemotherapy (171). The vaccine used a liposomal vector (BLP25) to deliver MUC1 in conjunction with a monophosphoryl lipid A adjuvant via subcutaneous injection. Vaccine was administered weekly to four anatomic sites to increase the amount of MUC1 present in relevant draining lymph nodes. Although 98.9% of patients receiving the vaccine reported adverse effects (AE), 95.2% of the control subjects did also suggesting that the majority of AE are NSCLC related and not due to vaccination. Once again, no symptoms of autoimmunity were observed. While this trial did not reach statistical significance, individuals who received the vaccine showed a median improvement in survival time of 4.4 months. Interestingly, vaccinated patients with stage IIIB disease had a 2-year survival rate of 60% compared to 36.7% for the

control group. The vaccine induced MUC1-specific T cell proliferation in only 16 of 78 individuals (20.5%). Only 2 of these 16 patients had stage IIIB disease, suggesting that a specific proliferative response is not predictive or mechanistically correlated with the stage-specific survival benefit observed. This highlights the difficulty of identifying immunologic parameters that can be used as hallmarks of vaccine efficacy, however the results of this trial are promising for the use of TAA vaccination for therapy of solid tumors.

Because the immunosuppression present in patients with pre-existing tumors decreases the immunogenicity of targeted, therapeutic vaccination, a recent study used prophylactic vaccination against MUC1 as a new strategy. Thirty-nine patients with a history of pre-malignant colonic adenomas were immunized with MUC1p and Poly:ICLC, a TLR3 agonist. 17/39 developed MUC1-specific IgG while the 22/39 who did not had concurrently high circulating levels of myeloid-derived suppressor cells (MDSC) (172). This study demonstrated both the immunogenicity of MUC1p as an antigen as well as an early immunosuppressive state associated with pre-malignant lesions that affects vaccine efficacy in a manner similar to frank cancer.

1.5 MUC1.TG, VFT, AND RFT MICE

Human and murine MUC1 share just 34% amino acid homology in the VNTR region (160). Therefore, to examine the efficacy of various MUC1-based vaccines, the human MUC1.Tg mouse was created. MUC1.Tg mice express human MUC1 under transcriptional control of its endogenous promoter, with the cellular distribution and transformation-induced hypoglycosylation recapitulating that observed in humans (173, 174). mTEC in MUC1.Tg mice present a variety of MUC1 peptides to developing thymocytes, including peptides recognized by

antibodies specific for unglycosylated and hypoglycosylated MUC1 (28). As a result, MUC1 is a self-antigen in MUC1.Tg mice, and this is reflected in the hyporesponsiveness to vaccination with a synthetic, naked peptide derived from the VNTR region of human MUC1 (MUC1p) in these animals (4, 5, 173, 175). However, tumor-associated, hypoglycosylated MUC1 (TnMUC1) behaves as a foreign, or abnormal-self antigen, and immunization of both WT and MUC1.Tg mice with DC loaded with TnMUC1 elicit comparable immunity (5, 176).

To examine MUC1-specific T cell responses to vaccination with MUC1p and TnMUC1, two $CD4^+$ TCR.Tg mice were generated. VFT mice have an MHC II-restricted TCR that recognizes an epitope derived from the extracellular tandem repeat region of human MUC1 (MUC1p). RFT mice have an MHC II-restricted TCR that recognizes an epitope derived from the extracellular region of human MUC1 decorated with O-linked GalNac residues (TnMUC1) (5, 176).

1.6 INTRODUCTION TO THE PROJECT

Background: The ability to induce immunity against the tumor-associated antigen MUC1 has broad implications for cancer vaccines, both therapeutic and prophylactic. However, previous work has identified deficient proliferation of MUC1 peptide-specific $CD4^+$ T cells after immunization of MUC1.Tg mice with MUC1p. This defect is not observed in immunized WT mice suggesting that MUC1p is viewed as "self" in the MUC1.Tg system while being "foreign" in WT (5). Central tolerance was ruled out by previous work in the lab and several mechanisms of peripheral tolerance were suggested. One mechanism was a higher Treg:Teff ratio in immunized MUC1.Tg mice, and could be corrected by transferring Treg-depleted $CD4^+$ T cells into MUC1.Tg mice pre-immunization (4). Similarly, concurrent activation of $CD4^+$ T cells specific for "foreign" peptide antigen (i.e., OT-II) could rescue the proliferation of MUC1p-specific CD4 T cells (5, 176). To globally query early, additional mechanisms of peripheral tolerance to MUC1p we vaccinated WT and MUC1.Tg mice with DC loaded with MUC1p and conducted total gene expression analysis of the spleen at 24 and 72h post-immunization. We found that expression of a number of "pancreatic" enzymes was suppressed in splenic DC from MUC1.Tg mice immunized with MUC1p, while their expression in splenic DC from immunized WT mice was dramatically increased. Based on this data we hypothesize that: *Vaccine-induced upregulation in splenic DC, of a cohort of catabolic enzymes previously characterized as being pancreas-restricted in expression, is an early biomarker of DC that will prime strong immunity (i.e., in response to a foreign antigen), and is highly correlated with an immunogenic DC phenotype and function. Conversely, vaccine-induced suppression of these enzymes is an early biomarker of DC that will promote tolerance (i.e., in response to a self-antigen), and is highly correlated with DC that acquire a tolerogenic phenotype and function.*

Specific Aims

Specific Aim 1: Analyze expression of "pancreatic" enzymes in DC post-vaccination with self versus foreign antigen in two mouse models: MUC1.Tg mice that are hyporesponsive to immunization with MUC1p, and RIP.OVA mice that are hyporesponsive to immunization with ovalbumin. Correlate the pattern of protease expression with the phenotype and function of splenic DC post-immunization with MUC1p or OVA respectively (i.e., cytokine production, co-stimulatory molecule expression, interaction with other cellular compartments, motility, and ability to prime naïve $CD4^+$ T cells into distinct lineages).

Specific Aim 2: Define the intracellular signaling pathways in DC that determine their immunogenic versus tolerogenic phenotype and function in response to immunization of MUC1.Tg mice with MUC1p and ovalbumin as representative self or foreign antigens.

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

Portions of Chapter 2 are 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. 190: 3319-3327. 2013.

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 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 they 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 they 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 the splenic DC from vaccinated WT mice and suppressed in the 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 (61, 177). As such, they have been central to vaccination strategies aimed at inducing immunity to both pathogens and tumors (127, 178). However, DC are also important in the maintenance of homeostatic tolerance to self-antigens (Ag) (179). 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 (20, 180). DC utilize diverse mechanisms to mediate T cell tolerance including low expression of costimulatory molecules (100), expression of SOCS1/3 (181, 182), activation of regulatory T cells (Treg) (183), and production of immunosuppressive factors such as IL-10, TGFβ, IDO and retinoic acid (74,

76, 184, 185). Significant effort has been devoted to manipulating the phenotype and function of *in vitro* cultured DC used for vaccination (186), as well as to targeting Ag *in vivo* to specific DC populations (187). 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 (188), and that transfer of Ag from vaccine DC to endogenous DC is necessary for optimal $CD4^+$ and $CD8^+$ T cell responses (189, 190). 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 (Fig. 4) (4, 5, 191). This hypo-responsiveness results in the inability of the vaccinated mice to control growth of both transplantable and spontaneous tumors (175, 192). Variations in vaccine design have resulted in some instances in a better immune response and better tumor control (5, 175), but they have been empirical, and without the full understanding of the underlying mechanism and early biomarkers of their efficacy, not readily

predictable. 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.

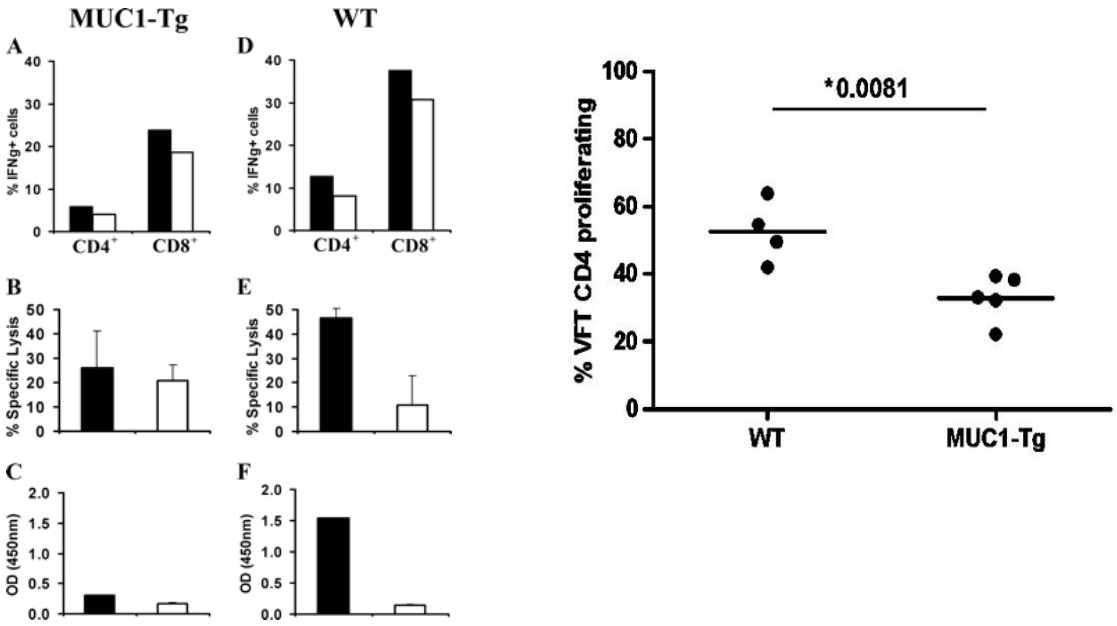

Figure 4 Vaccination of MUC1.Tg mice with MUC1p results in an impaired adaptive immune response compared to WT.

WT and MUC1.Tg mice were immunized s.c. with DC:MUC1p 1d after transfer of $5*10^6$ $CD4^+$ T cells from immunized WT donors (black bars) or PBS Ctrl (white bars). At 2 weeks post-vaccination, LN and sera were pooled and tested for reactivity against MUC1p. (A and D) Re-stimulation of lymphocytes recovered from immunized WT mice resulted in higher IFNγ production in both $CD4^+$ and $CD8^+$ compartments compared to MUC1.Tg mice. (B and E) Specific lysis of RMA-MUC1 tumor cells by pooled LN cells recovered from immunized WT and MUC1.Tg mice. (C and F) MUC1p-specific total IgG in the sera of immunized WT and MUC1.Tg mice. (G) $5*10^6$ MUC1p-specific $CD4^+$ T cells (VFT) were transferred i.v. into WT and MUC1.Tg recipients and mice were vaccinated with DC:MUC1p 1d later. After 4-5d, spleens were harvested and the percentage of VFT proliferation determined by gating on $CFSE^{dim}V\alpha2^+Thy1.1^+CD4^+$ T cells. Figures reprinted with permission from Turner, M.S. *Journal of Immunology*. 2007, and Ryan, S.O. *Cancer Research.* 2010.

2.3 RESULTS

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 (193), expression of co-stimulatory molecules on DC and production of stimulatory or suppressive cytokines (100), and the ability of DC to move to T cell areas within lymphoid tissue (194). 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. 6A). This decrease in DC number in MUC1.Tg mice also included a decrease in the absolute number of the DC used for immunization, as well as those from the endogenous DC compartment (Fig. 5). 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. 6B and 6C), as well as a reduction in the amount of CD86 expressed by those DC (Fig. 6D), 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. 6E) 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 (195) so we examined the relative proliferation of $CD4^{+}Foxp3^{+}$ Tregs. DC recovered from MUC1p vaccinated MUC1.Tg mice induced higher OT-II Treg proliferation compared to DC from MUC1p vaccinated WT animals (Figs. 6G and 6H).

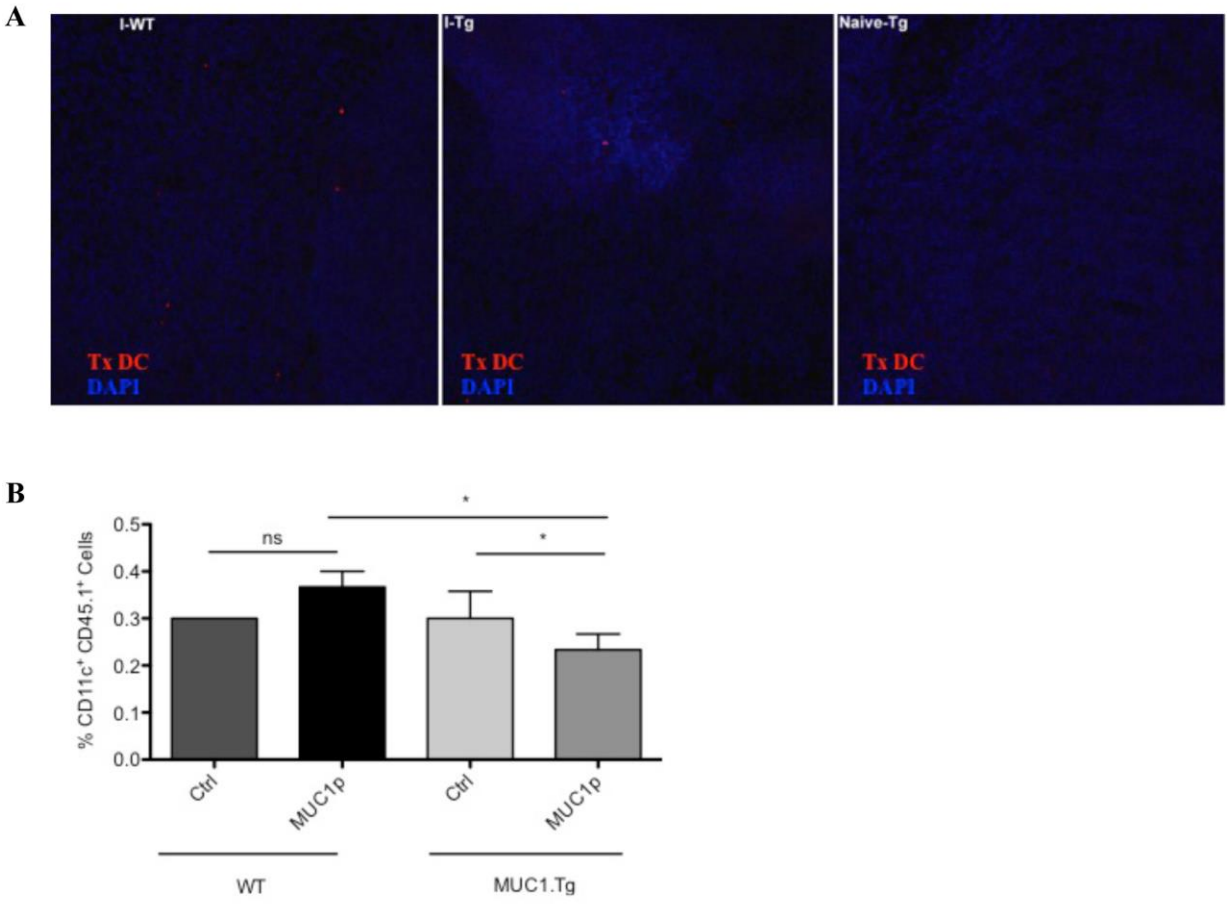

Figure 5 Fewer vaccine-derived DC are detectable in the spleen of immunized MUC1.Tg mice compared to WT.

(A) WT and MUC1.Tg mice (n=2/group) were immunized with Cy3-labeled DC loaded with MUC1p ($1*10^{6}$ cells). 24h later, spleens were sectioned for confocal microscopy. Shown are representative images from immunized WT mice (I-WT), immunized MUC1.Tg (I-Tg), and a non-immunized control (Naïve-Tg). Magnification 20X. Images are representative of 5 sections

examined from 2 mice/group and 2 independent experiments. (B) Mice (n=3/group) were immunized as in (A) but with congenic, CD45.1+ DC.

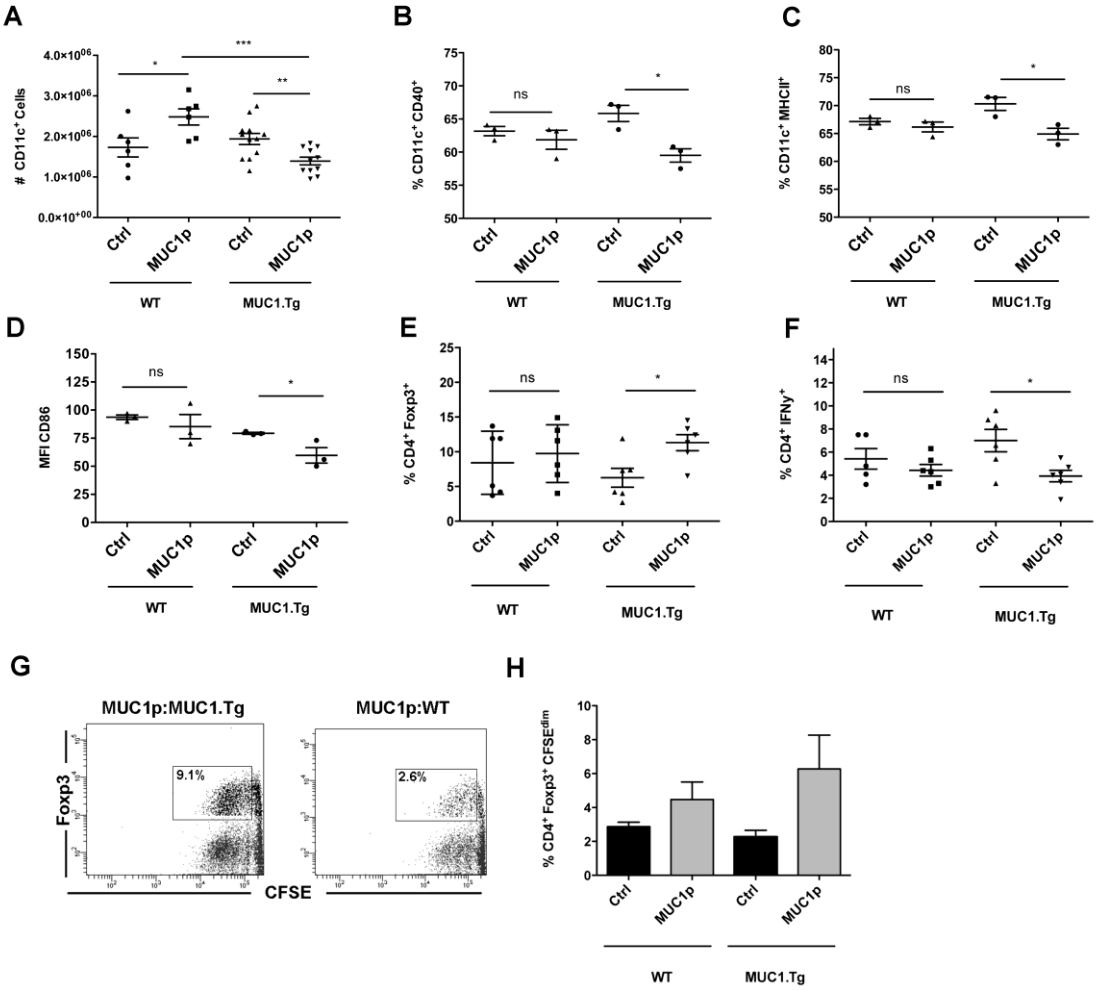

Figure 6 Immunization of MUC1.Tg mice with MUC1p results in decreased splenic DC number, costimulatory molecule expression, and preferential priming of Foxp3$^+$ Treg.

(A) WT and MUC1.Tg mice were immunized with unloaded DC (ctrl) or DC loaded with MUC1p. 24h post-immunization total splenic DC numbers were analyzed. Each symbol represents one mouse with bars showing mean ± SEM from three pooled independent experiments, with each experiment including 2-4 mice per group. (B-D) WT and MUC1.Tg mice were immunized as in (A). 48h post immunization bulk splenocytes were stained for FACS analysis. Data represent percentage of positive cells within the CD11c$^+$ gate (B-C) or the MFI of cells within the CD11c$^+$ gate (D). Symbols represent individual mice with bars showing mean ±

SEM and are representative of 2 independent experiments. (E-H) WT and MUC1.Tg mice were immunized as in (A). 24h later, splenic DC were bead isolated, loaded with OVA and co-cultured with OT-II CD4 T cells for 7 days. 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.

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. 7A). Previous studies have shown that expression of CD74 is inversely correlated to *in vivo* motility of DC (196). 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. 7B) and had smaller net displacements (Fig. 7C) than DC from WT.

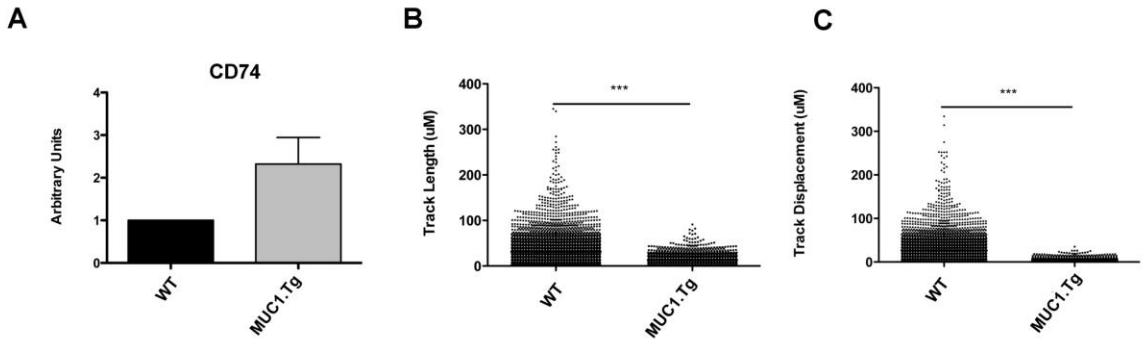

Figure 7 Immunization of MUC1.Tg mice with MUC1p results in decreased DC motility.

(A) WT and MUC1.Tg mice were vaccinated i.v. with DC loaded with MUC1p. RNA was extracted from pooled splenic DC 72h post vaccination 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). 72h post immunization, splenic DC were bead isolated for live cell imaging. The track length (B) and displacement (C) were analyzed after 20h in culture. Each dot represents a single DC and bars depict mean ± SEM. Data are from two mice comparing 6×10^3 DC per group.

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

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

Table 1 Immunization of MUC1.Tg mice with MUC1p suppresses expression of a cohort of "pancreatic" enzymes in the spleen.

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
Carboxypeptidase 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

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. 8A). 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. 8B) 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. 9). Furthermore we show that these same pancreatic enzymes found in murine DC are also found in human monocyte-derived DC (Fig. 10).

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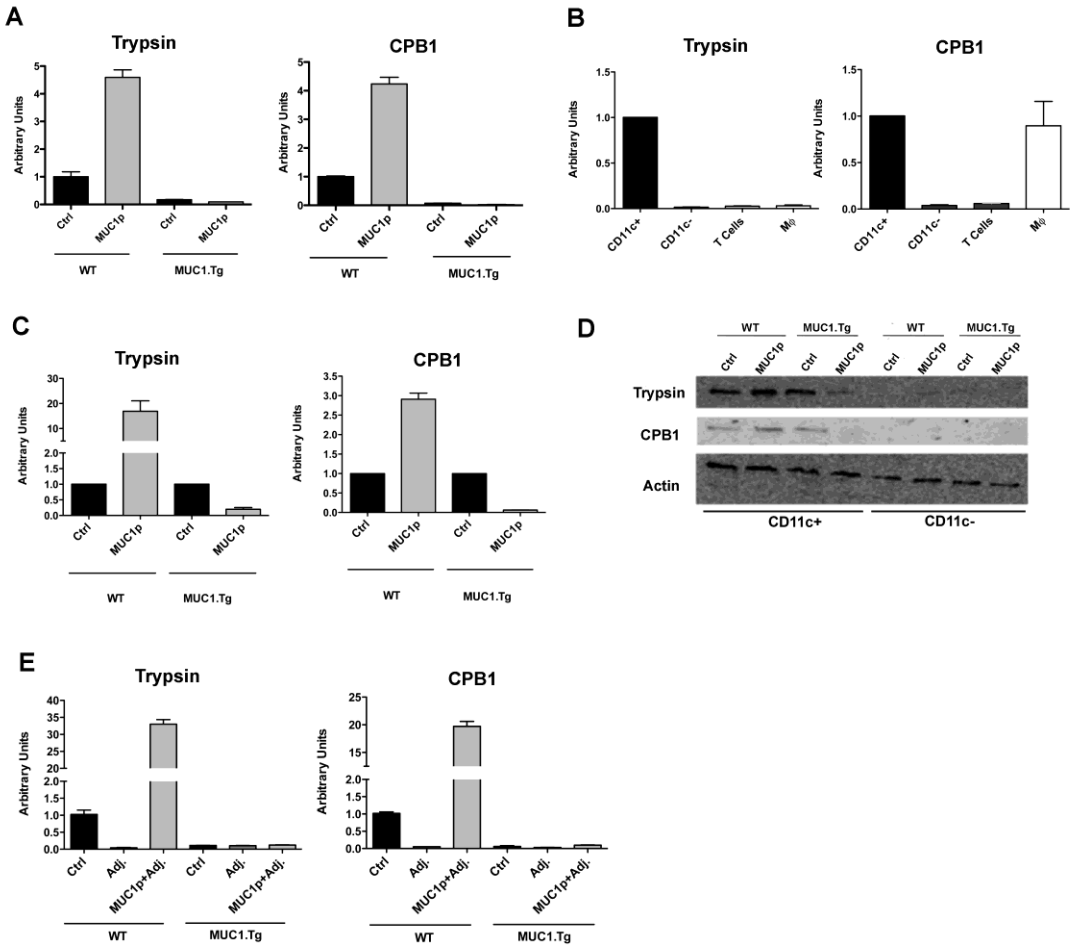


Figure 8 Immunization of WT but not MUC1.Tg mice with MUC1p results in up-regulation of pancreatic enzymes in splenic DC.

(A) WT and MUC1.Tg mice (n=3/group) were injected i.v with unloaded BMDC (ctrl) or BMDC loaded with MUC1 peptide (MUC1p). 24h 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 days 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 (n=3/group) were immunized as in (A). At 24h, 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). 24h later spleens were harvested for qRT-PCR analysis. Bars represent mean ± SEM normalized to PBS control and are representative of four independent experiments.

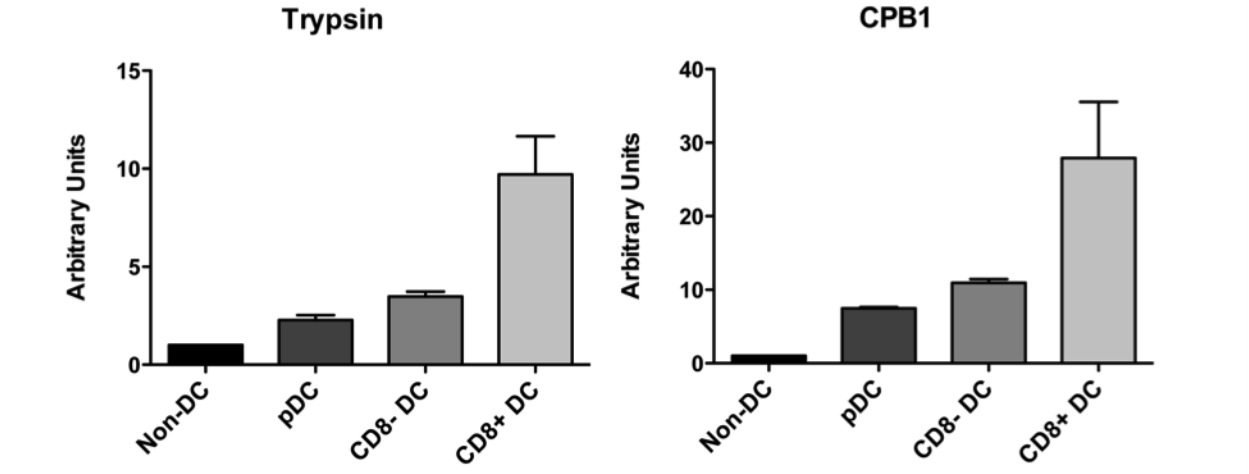


Figure 9 All major resident splenic DC subpopulations express higher levels of trypsin and CPB1 than CD11c$^-$ splenocytes.

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.

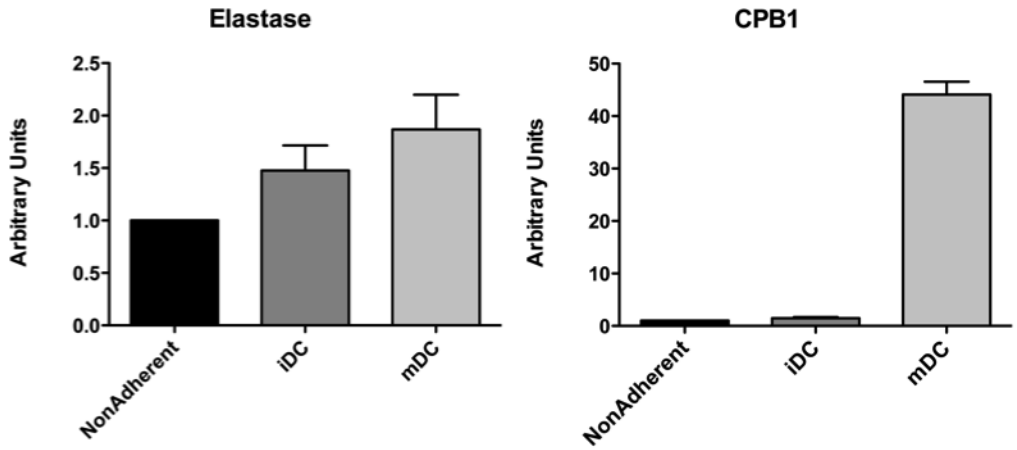


Figure 10 Human monocyte-derived DC upregulate elastase and CPB1 expression upon TLR3 stimulation.

Peripheral blood mononuclear cells (PBMC) obtained from the leukopaks of healthy donors were cultured for 5 days in the presence of 100IU/mL of GM-CSF and 200IU/mL IL-4. Before culture, non-adherent lymphocytes (NonAdherent) were collected after failure to adhere to culture plastic after 1h and frozen for later analysis. On d5, immature DC were harvested, RNA extracted and qPCR conducted for elastase and CPB1. Remaining DC were matured overnight with 30μg/mL of Poly-ICLC and trypsin and CPB1 levels examined the following day to query differences in mDC. Data are representative of 2 independent experiments from 2 separate donors.

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. 8E). 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 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. 11A and 11B).

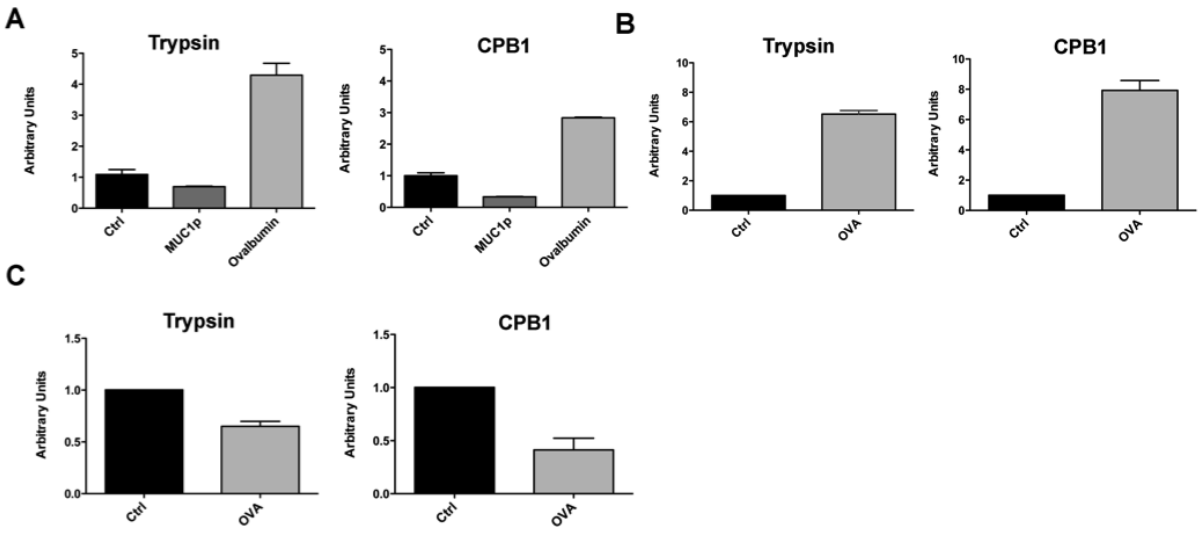


Figure 11 Failure of DC to upregulate trypsin and CPB1 is recapitulated in the OVA model of self-tolerance.

(A) MUC1.Tg mice (n=3/group) were immunized i.v. with PBS (ctrl), soluble MUC1p or ovalbumin (OVA) admixed with Poly:ICLC. Spleens were harvested at 24h post immunization 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 (n=3/group) were immunized i.v. with unloaded DC (ctrl) or DC loaded with OVA (OVA). 24h post-immunization 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 (n=3/group) were immunized and processed as in (B). Bars represent mean ± SEM normalized to ctrl vaccination. Data are representative of two independent experiments.

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 (197), with DC loaded with OVA. The DC recovered from these mice also failed to up-regulate trypsin and CPB1 (Fig. 11C).

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 (48, 198), 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. 12A)

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. 12B).

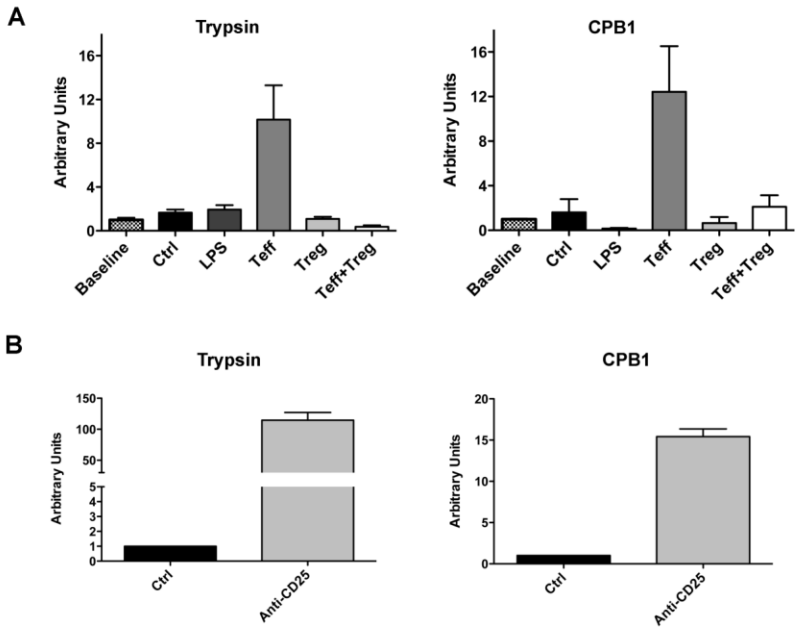


Figure 12 Interactions between DC and CD4$^+$ T cells control expression levels of pancreatic enzymes in DC.

(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 24h of co-culture DC were separated and mRNA was extracted for qRT-PCR analysis. Units were standardized against levels pre-culture (baseline). Bars represent mean ± SEM. Data are representative of two independent experiments. (B) MUC1.Tg mice were treated with an antibody against CD25 to deplete regulatory CD4 T cells (Anti-CD25) or with an isotype control (ctrl). 2 days following depletion, mice were vaccinated with soluble MUC1p plus Poly-ICLC i.v. Splenic RNA was extracted 24h post vaccination for qRT-PCR analysis. Units were standardized against isotype control treated mice. Bars represent mean ± SEM respectively. Data are representative of 3 independent experiments.

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 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. 13A). Given the known ability of IL-10 to modulate DC phenotype and function in the direction of tolerance versus immunogenicity (199), 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. 13B). 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. In support of the suppressive effect of IL-10 on DC enzyme expression, we immunized IL-$10^{-/-}$ mice crossed onto the MUC1.Tg background (MUC1/IL$10^{-/-}$) with MUC1p. Immunization in the absence of IL-10 also resulted in the reconstitution of trypsin, CPB1, and elastase expression in DC (Fig. 13C).

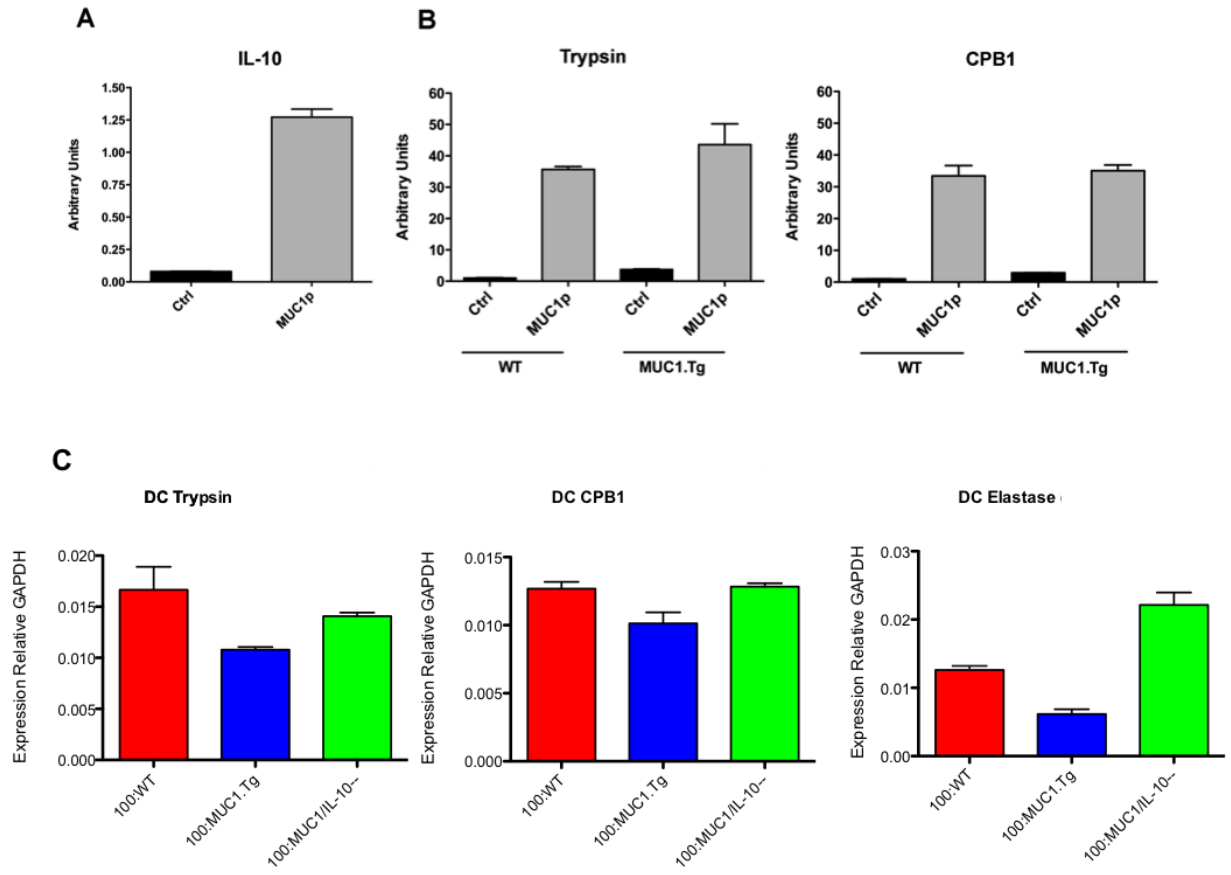


Figure 13 IL-10 is required *in vivo* for control of trypsin, CPB1, and elastase expression in DC

(A) MUC1.Tg (n=3) mice were immunized with PBS (ctrl) or soluble MUC1p admixed with Poly-ICLC (MUC1p). IL-10 expression was measured by qRT-PCR on total splenic mRNA 24h post vaccination. Bars represent mean ± SEM. Data are representative of at least 4 independent experiments. (B) WT and MUC1.Tg mice were treated with an IL-10R blocking antibody followed by i.v. immunization with PBS (ctrl) or MUC1p as in (A). 24h post vaccination 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. (C) WT, MUC1.Tg and MUC1$^{+/+}$IL-10-/- (n=3/group) mice were immunized with DC:MUC1p (100:WT, 100:MUC1.Tg, and 100:MUC1/IL-10$^{-/-}$). After 24h, splenic DC were isolated and the relative amounts of trypsin, CPB1, and elastase were measured by qRT-PCR. Bars represent mean ± SEM. Data are representative of 3 independent experiments.

2.4 DISCUSSION

Our data reveal the presence of a new pancreatic enzyme signature in DC that may be predictive very early post -vaccination (24-72h) 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 endogenous splenic DC compartment. A number of other peptidases utilized by DC, especially in the context of antigen processing and presentation, have been characterized (89) and an expanding repertoire of enzymes involved in generating MHCI-restricted peptides is beginning to be elucidated (86). 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 in 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., MUC1p in WT mice) and suppressed following exposure to a self-antigen (e.g., MUC1p in MUC1.Tg mice). Importantly, the signature of pancreatic enzyme expression by DC is not dependent on whether the antigen is also a tumor antigen. Our data show that immunization of RIP.OVA mice with OVA results in a similar failure to up-regulate DC enzymes while vaccination of MUC1.Tg mice induces up-regulation, suggesting that this is a general marker corresponding to the maintenance of self-tolerance rather than a unique characteristic of MUC1-specific immunity. 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 illustrates the fact 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 (28), 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 (200-202)

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 in addition to diffusion of IL-10 and/or other cytokines 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 (203, 204).

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 (205). Tolerogenic DC have also been implicated because of their ability to promote the generation of iTreg (206, 207). 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 (135). 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 cognate 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 they are involved in antigen processing or other DC-intrinsic functions. 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.

We expect that immune hypo-responsiveness reported to vaccines based on many other tumor associated antigens (208, 209) could also be explained by how similar or different they are from those same antigens expressed on normal cells. The ability of immunization with self-antigens other than MUC1p to tolerize endogenous splenic DC remains to be tested. However, the conservation of enzyme expression patterns between immunizations using different self-antigens leads us to envision a similar conservation in the resultant DC phenotype. Therefore,

pancreatic enzyme expression in DC represents a new finding and suggests an easily accessible signature that can be used to assess almost immediately the suitability of a particular antigen and 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 efficacy early after vaccination, rather than waiting for the results of a tumor challenge in an animal or tumor recurrence in a patient. Previous work has shown that various MUC1p-based vaccines can fail to eradicate or slow the growth of $MUC1^+$ tumors in MUC1.Tg mice while remaining effective in WT mice where MUC1p is a foreign antigen (175, 192). Vaccine-induced control of tumor growth is dependent on $CD4^+$ T cells which are not fully functional in immunized MUC1.Tg mice compared to WT (191) (4). This study provides evidence that the defect in anti-tumor immunity in MUC1.Tg mice is attributable in part to splenic DC preferentially priming $CD4^+$ T cells into $Foxp3^+$ Treg versus $IFN\gamma^+$ cells likely via low costimulatory molecule expression and impaired motility.

Our previous studies have emphasized the importance of antigen selection, especially in the case of non-viral tumor associated antigens (151). 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 (210), 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 TnMUC1 results in strong immunogenicity rather than tolerance in immunized MUC1.Tg mice (211). 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 (212). Depletion of Treg with anti-CD25 antibodies or diphtheria toxin have shown a good deal of promise in preclinical models of cancer immunotherapy (213-217). 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 (155, 218). We propose that these treatments work because they prevent DC-propagated infectious tolerance.

2.5 MATERIALS AND METHODS

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) (219) 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.

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 (4) by the University of Pittsburgh Genomics and Proteomics Core Laboratories. $OVA_{323-339}$ peptide and ovalbumin protein were purchased from Sigma.

Mouse DC culture and vaccines

BMDC were generated according to established protocol (5). Briefly, female C57BL/6 mice (Jackson) were sacrificed and their femurs and tibiae removed. Marrow was 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 $1.5\text{-}2\times10^6$/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 30μg/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 $.5\text{-}1\times10^6$ DC. Soluble peptide immunizations consisted 100μg of MUC1 100mer peptide or ovalbumin and 50μg of Poly-ICLC in 100μL of PBS.

Microarray

Whole spleen from WT and MUC1.Tg mice (n=3/group) was harvested 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 (220). Microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) with the accession number GSE43503 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43503).

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 (221).

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.

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 6, 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

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 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.

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).

***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).

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).

Human DC Culture

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 trypsin and CPB1. Remaining DC were matured overnight with 30μg/mL of Poly-I:C, and trypsin and CPB1 levels examined the following day to query differences in mDC.

3.0 NOVEL MECHANISMS UNDERLYING THE IMMEDIATE AND TRANSIENT GLOBAL TOLERIZATION OF SPLENIC DC FOLLOWING VACCINATION WITH A SELF-ANTIGEN

3.1 ABSTRACT

Dendritic cells (DC) are potent directors of the immune response. As such, vaccines designed to elicit immunity against pathogens and tumors rely on DC directly or indirectly. However, the role of endogenous DC in mediating vaccine-induced tolerance and immunity is unclear. Using the tumor antigen MUC1 as a relevant self-antigen, we previously showed that i.v. immunization of MUC1.Tg mice, but not WT, with MUC1 resulted in a tolerized splenic DC compartment marked by low costimulatory molecule expression, induction of regulatory T cells (Treg), and suppressed expression of trypsin and Carboxypeptidase B1 (CPB1). Here we examined the functional phenotype underlying these tolerized DC. We found that immunization with self-antigen results in an endogenous, splenic DC population that shares some characteristics with immature DC such as a less inflammatory cytokine/chemokine profile, deficient activation of NFκB, and sustained expression of *zDC* and CCR2. However, these tolerized splenic DC concurrently acquire novel attributes in response to exposure to self antigen, including the inducible expression of Aldh1/2 and phospho-STAT3. We also found that suppressed expression of trypsin impedes the ability of DC to degrade extracellular matrix protein, while

metallopeptidases including CPB1 are required by DC to induce optimal, antigen-specific $CD4^+$ T cell proliferation. DC were not refractory to maturation after stimulation with a TLR3 agonist, illustrating that this tolerized state is not terminal, and these DC are capable of inducing immunity to foreign antigen after exposure to self antigen as been cleared.

3.2 INTRODUCTION

Dendritic cells (DC) can induce potent immunity by presenting foreign antigens (Ag) derived from infectious agents or abnormal self antigens derived from tumors (222). However, DC can also maintain tolerance to self Ag derived from healthy tissues, environmental agents, and commensal microbiota (76, 100). Ablation of DC during homeostasis results in a breakdown of $CD4^+$ T cell tolerance with ensuing fatal autoimmunity (119, 179). Multiple mechanisms assist DC in maintaining peripheral tolerance to self Ag, such as low expression of costimulatory molecules such as MHC II, CD40, CD80, and CD86, production of anti-inflammatory molecules such as IL-10, IDO, and retinoic acid (RA), exposure to low Ag dose, and expression of cell surface molecules such as DEC-205 and CD103 that mark DC that preferentially prime peripheral regulatory T cells (Treg) (75, 76, 100, 103, 105). Vaccines designed to induce immunity against tumors often utilize antigens that are closely related to self-antigens, or are a combination of normal (self) and abnormal (foreign) epitopes, resulting in a less robust immune response (223). Immune responses to vaccines are controlled by many different mechanisms in the periphery. Better understanding of these mechanisms is important for designing vaccines that elicit the desired outcome: strong, protective immunity against pathogens and cancer, or tolerance for self antigens to avoid autoimmunity.

We have been studying immune responses to cancer vaccines based on peptides derived from the human tumor antigen MUC1 that range from those closest to self (i.e. peptides with no sugar moieties), to those representing "abnormal self" (i.e. peptides decorated with tumor-specific, O-linked GalNAc adducts) (5, 224). We recently published that i.v. immunization of human MUC1.Tg mice (MUC1.Tg) with an unglycosylated MUC1 peptide (MUC1p) resulted in the transient tolerization (between 24h-72h) of the entire splenic DC population that failed to

upregulate costimulatory molecules, lacked motility, and preferentially primed Foxp3$^+$ Treg. These vaccine-induced, tolerized DC also differed in their gene expression profile from immunogenic DC by suppressed expression of a group of "pancreatic" enzymes, specifically trypsin and carboxypeptidase B1 (CPB1) (6), which we proposed as being predictive biomarkers of vaccine outcome . These results were consistent with the previously observed low level of vaccine-induced immunity against MUC1p in MUC1.Tg mice (4, 5, 173).

In the current study we elucidated several molecular and cellular pathways that characterize DC tolerized by vaccination with a self antigen . We show that, in contrast to endogenous splenic DC recovered from mice vaccinated with a foreign antigen, splenic DC from mice vaccinated with self-antigen inhibit NFκB while simultaneously increasing signaling through phospho-STAT3. As a result, they produce fewer inflammatory cytokines and chemokines. These DC also induce expression of aldehyde dehydrogenase 1 (Aldh1), the enzyme catalyzing the last step in the biosynthesis of immunosuppressive retinoic acid (RA), suggesting a novel role for RA in promoting this vaccine-mediated DC tolerance.

We also examined the functional consequence of the previously reported suppressed expression of "pancreatic" proteases in tolerized DC that are highly upregulated in immunogenic DC. We show that increased expression by DC of metallopeptidases, such as CBP1, is required for optimal proliferation of MUC1p-specific CD4$^+$ T cells, suggesting their role in increasing efficiency of antigen processing and presentation. We found that trypsin expression, on the other hand, is required for degradation of the extracellular matrix (ECM), facilitating DC motility. Suppressed expression of these enzymes in tolerized DC contributes to their low T cell stimulatory capacity and low motility, resulting in low or no response to a self- antigen vaccine.

3.3 RESULTS

Immunization with a self-antigen promotes non-inflammatory DC with decreased chemotactic potential and a novel transcriptional signature

As we previously reported, splenic DC recovered from MUC1.Tg mice immunized i.v. with MUC1p express lower levels of MHC II, CD40, and CD86, compared to DC from WT mice immunized with the same peptide. They also preferentially prime naïve $CD4^+$ T cells into Treg versus IFNγ-producing effector T cells. Additionally, there are fewer DC 24h post-immunization in the spleens of MUC1.Tg mice compared to WT (6). Low costimulatory molecule expression and priming of Treg are hallmarks of an immature DC (iDC) (100, 225, 226). We now show, using a cytokine blot array, that DC recovered from MUC1p-immunized MUC1.Tg mice produce lower levels of pro-inflammatory cytokines (i.e., IL-1a, TNFa, IL-6) relative to WT **(**Figs. 14A and 14B**)**. These tolerized DC also produced less lymphotactic chemokines such as MIP-1b, RANTES, and IL-16 as well as less CXCL1, MIP-2, and CCL2. Tolerized DC failed to down-regulate expression of CCR2, the receptor for CCL2, and a phenotypic marker of iDC (227, 228) **(**Fig. 14C). This CCR2 was functional as evidenced by the ability of more tolerized DC to directionally migrate towards a linear CCL2 gradient compared to DC from immunized WT mice (Fig. 15).

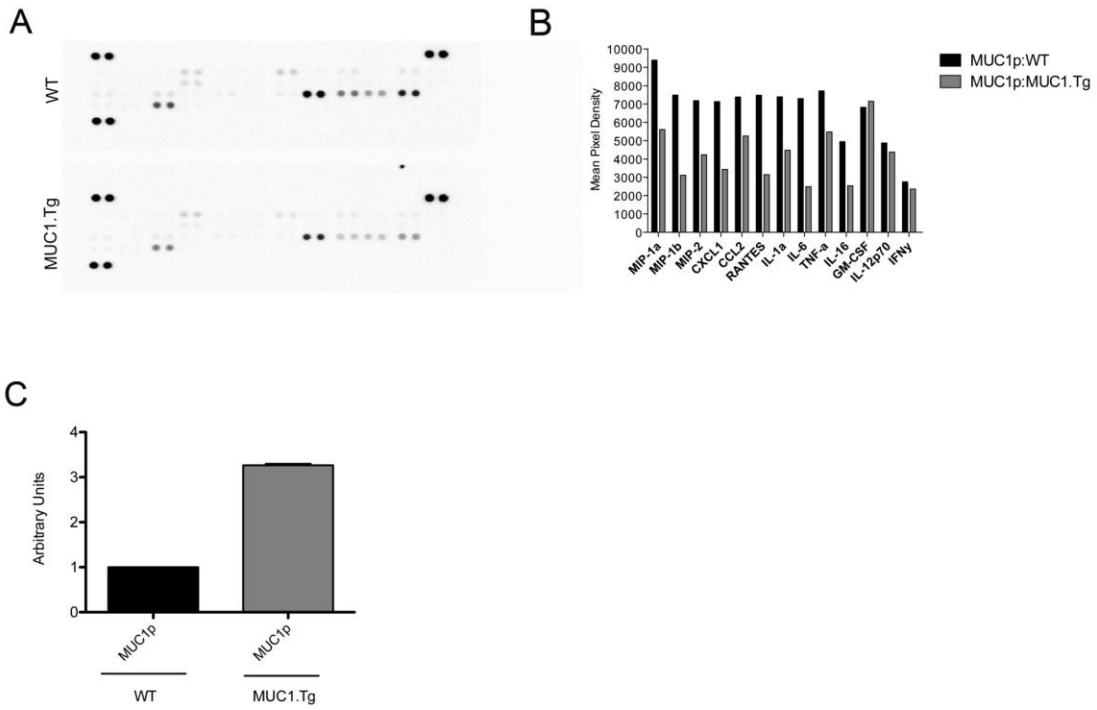


Figure 14 DC recovered from MUC1p-immunized MUC1.Tg mice are less inflammatory than DC from immunized WT mice.

(A) WT and MUC1.Tg mice (n=3/group) were immunized (i.v.) with DC:MUC1p (MUC1p) (1×10^6 cells). 1d later isolated splenic DC were cultured overnight in the presence of 500ng/mL LPS and Brefeldin A. DC lysates were then incubated on a mouse cytokine blot array and developed according to the manufacturer instructions. Blots are shown in (A) and are representative of 2 independent experiments. (B) Densitometry of the blots from (A) quantified with Image J. (C) Mice were immunized with MUC1p as in (A) with splenic DC isolated at 24h and qPCR performed for CCR2. Data are normalized to MUC1p-immunized WT mice, with bars representing the mean± SEM. Data are representative of 3 independent experiments.

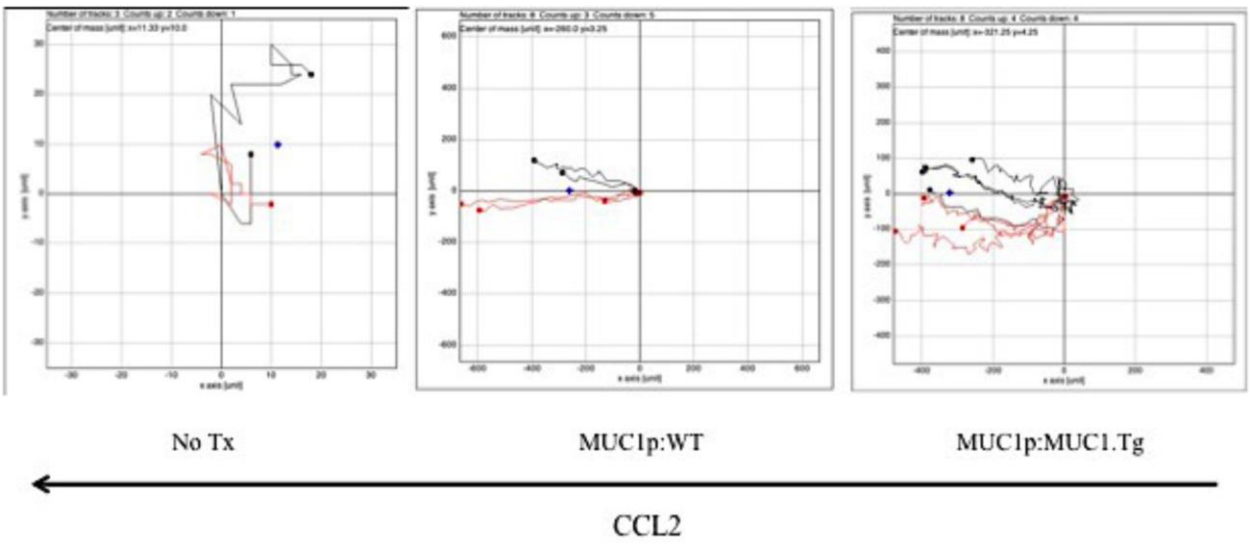


Figure 15 Splenic DC isolated from immunized MUC1.Tg mice express functional CCR2

Mice (n=3/group) were immunized as in Fig. 14 and DC isolated at 24h and plated onto chemotaxis slides ± 50ng/mL linear gradient of recombinant CCL2. Cells were imaged for 4h and the cells with directional migration quantified from 5 fields/group. Data are representative of 2 independent experiments.

Suppressor of cytokine signaling 3 (SOCS3) and histone deacetylase 11 (HDAC11) have been implicated in suppressing pro-inflammatory cytokine production and promoting IL-10 expression respectively in DC (122, 182). While SOCS3 expression was decreased by approximately 50% in DC recovered from MUC1p vaccinated WT mice, as would be expected of a DC developing an immunostimulatory phenotype, its expression was unchanged in DC from immunized MUC1.Tg mice (Fig. 16A). HDAC11 levels in immunized WT or MUC1.Tg mice were not significantly different (Fig. 16B). This suggested that these two transcription factors, which play a role in DC that are actively immunosuppressive, do not have the same role in either inducing or maintaining the transient tolerized state of DC from vaccinated MUC1.Tg mice. We also examined the transcription factor *Zbtb46* (zDC) that has recently been identified as a conventional DC-lineage marker whose expression is inversely correlated with DC maturational

status (119, 120, 229). Expression of zDC was approximately 2.5-fold higher in tolerized DC recovered from immunized MUC1.Tg mice compared to WT (Fig. 16C). Expression of zDC and CCR2 decreased after stimulation of DC with Poly:ICLC *ex vivo* demonstrating that these DC are not refractory to maturation. (Figs. 16C and 16D).

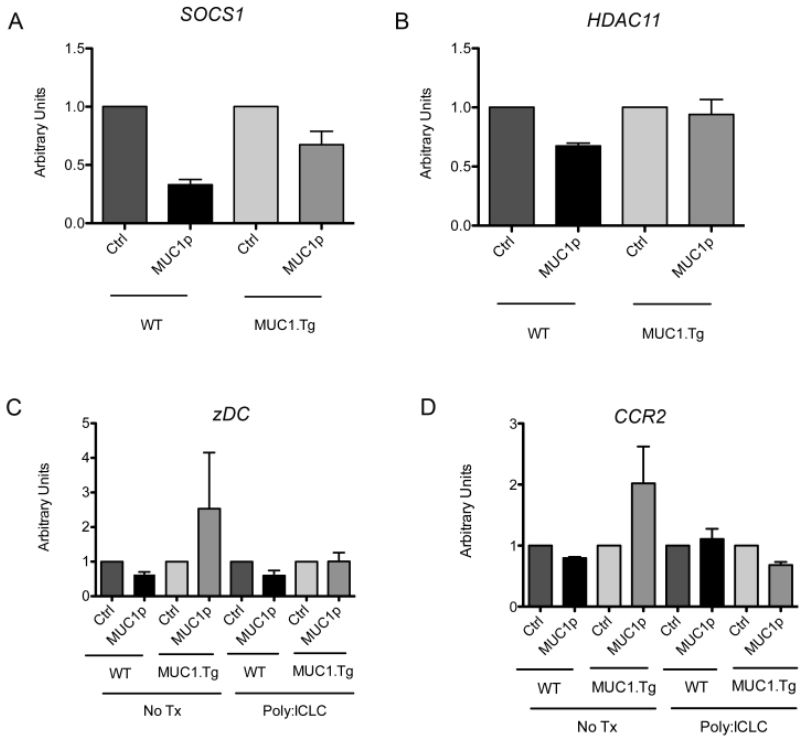


Figure 16 SOCS3 and HDAC11 do not contribute to vaccine-tolerized DC while sustained expression of *Zbtb46* and *CCR2* maintain an immature DC phenotype

WT and MUC1.Tg mice (n=3/group) were immunized (i.v.) with DC:MUC1p (MUC1p) (1×10^6 cells) or unloaded DC (Ctrl). 1d later, isolated DC were pooled for qRT-PCR for *SOCS3* (A) or *HDAC11* (B). (C and D) Mice were immunized with MUC1p as in (A) with splenic DC isolated at 24h and qRT-PCR for *zDC* (C) or *CCR2* (D) conducted with (Poly:ICLC) or without (No Tx) stimulation with 30ug/mL of Poly:ICLC for 4h. Bars represent mean± SEM after normalization to respective control vaccinations and are representative of 3 independent experiments

Increase in phospho-STAT3 and decrease in NFκB p65 in tolerized DC

Activation of STAT3 characterizes DC that are unable to prime efficient Th1 responses, and is considered a negative regulator of DC function (123, 230). Splenic DC isolated from MUC1p immunized MUC1.Tg mice upregulated phospho-STAT3 24h post-immunization (Fig. 17A). Conversely, NFκB pathway activation resulting in degradation of IκBα and phosphorylation of p65, is critical for DC phenotypic maturation and immunogenic function (118, 231). As shown in Figs. 17B and 17C, tolerized DC from immunized MUC1.Tg mice express less phospho-p65 with a concurrent increase in total IκBα. Therefore, the low immunogenicity of tolerized DC is due, in part, to deficient activation of NFκβ signaling and self-antigen-induced STAT3 signaling.

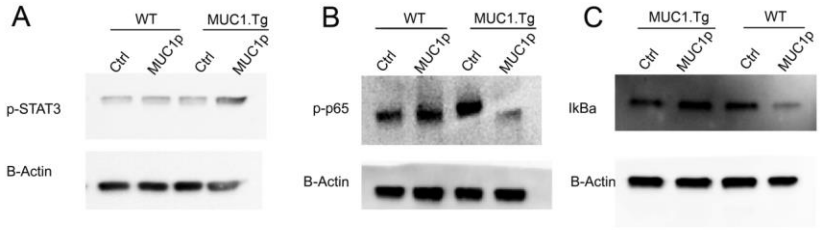


Figure 17 Deficient NFκB activation and enhanced STAT3 signaling in vaccine-tolerized DC.

WT and MUC1.Tg mice (n=3/group) were immunized (i.v.) with DC:MUC1p (MUC1p) (1×10^6 cells) or unloaded DC (Ctrl). 1d later, isolated DC were pooled and whole cell lysates were Western blotted for phospho-STAT3 (A), phospho-p65, (B) or total IκBα (C). B-Actin is shown as a loading control for each blot. Data are representative of 2-3 independent experiments for each molecule.

Aldh1/2 expression as a new hallmark of splenic DC tolerized by immunization with self-antigen

Retinoic acid is a well-characterized dietary metabolite and mediator of tolerance in the gut, and is involved in the induction of $CD4^+$ $Foxp3^+$ T cells by $CD103^+$ DC presenting oral and commensally derived Ag (76, 116). The last step in RA biosynthesis is the oxidation of retinaldehyde to RA by Aldh1/2. Recent data indicate that $CD103^-$ DC in the skin and lung also express Aldh1/2 (232) and that RA production is not restricted to the intestine. Splenic DC from immunized MUC1.Tg mice expressed both *Aldh1* and Aldh1/2 (Figs. 19A-C), while those recovered from all other vaccinations did not. There was no difference in the number of $CD103^+$ DC after immunization of either WT or MUC1.Tg mice (Fig. 18). This suggests that RA is expressed by vaccine-tolerized DC independent of the CD103 marker and may be responsible for their observed capacity to preferentially prime Treg (6).

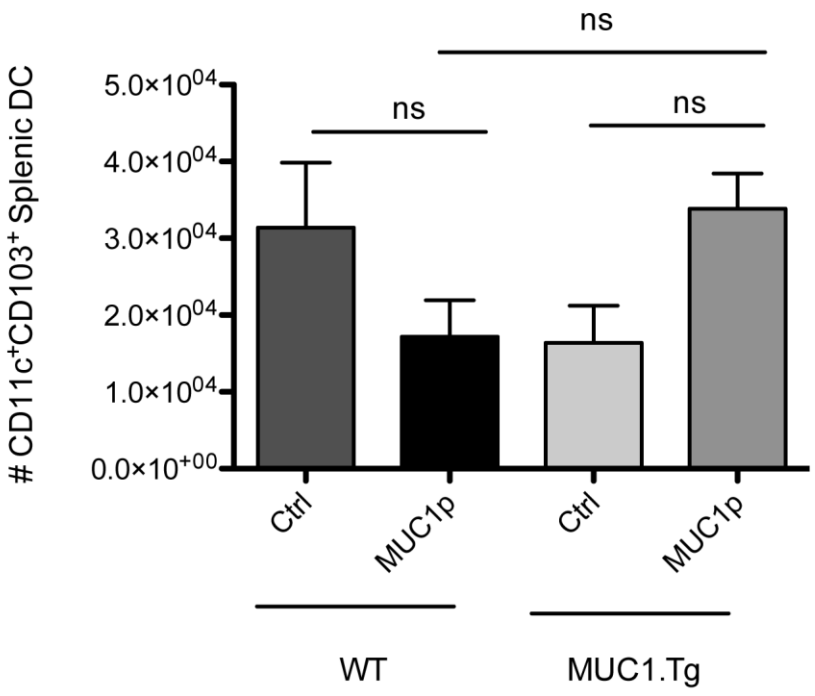


Figure 18 No difference in CD103 expression between DC that produce Raldh1/2 and those that do not.

24h after immunization of WT and MUC1.Tg mice (n=3/group) spleens were harvested for FACS. Bars represent mean ± SEM and data are representative of 2 independent experiments.

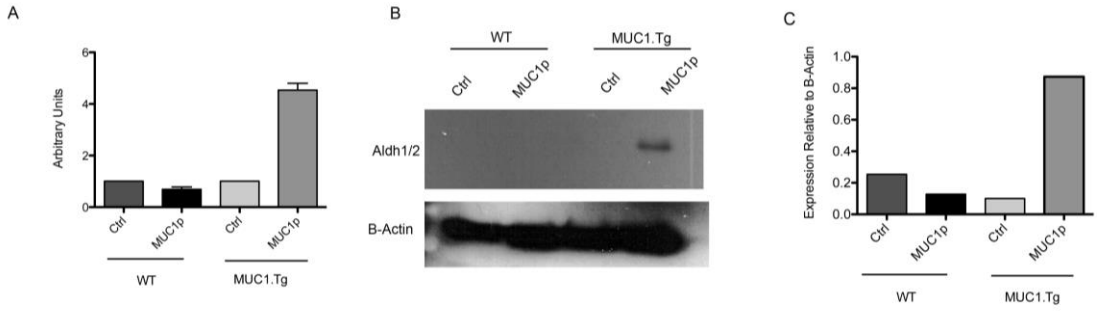


Figure 19 Inducible production of aldehyde dehydrogenase by tolerized DC.

WT and MUC1.Tg mice (n=3/group) were immunized (i.v.) with DC:MUC1p (MUC1p) (1×10^6 cells) or unloaded DC (Ctrl). 1d later, isolated DC were pooled and qRT-PCR conducted for Aldh1 (A). Concurrently, whole cell lysates from recovered DC were Western blotted for Aldh1/2 (B) and bands quantified via densitometry (C). Bars represent mean± SEM after normalization to respective control vaccinations (A) and are representative of 3 independent experiments.

F**unction of pancreatic enzymes in DC**

Expression of "pancreatic" proteases by splenic DC is coordinately regulated such that immunization of WT mice with MUC1p results in a 10-40 fold increase in their expression by 24h, while the same immunization in MUC1.Tg mice results in their profound suppression. We found that this expression profile is a predictive biomarker of DC immunogenicity and the ensuing T effector or Treg responses (5, 6). However, the actual function of these newly discovered enzymes in DC remained unknown.

We explored the potential function of two of these enzymes, CBP1 and trypsin, as representatives of two broad families, metallopeptidases (CBP1) and serine proteases (trypsin).

We hypothesized that both might be involved in the processing and/or presentation of MHC II-restricted peptides derived from MUC1p, since a diverse repertoire of enzymes, including serine, aspartyl, and cysteine peptidases, as well as aspraginyl endopeptidase are involved in this pathway (89). To test the contribution of trypsin and CBP1 to the ability of DC to prime an antigen-specific $CD4^+$ T cell response, we cultured bone marrow derived DC (BMDC) overnight in the presence of MUC1p and n-orthophenanthroline, an inhibitor of all metallopeptidases including CPB1, or a trypsin-specific inhibitor derived from chicken ovalbumin. Neither inhibitor had a direct effect on DC viability as determined by Annexin-V/PI staining (Fig. 20). Antigen-loaded DC were then co-cultured for three days with MUC1p-specific TCR.Tg $CD4^+$ T cells labeled with CFSE to quantify proliferation. Figs. 21A and 21B show that inhibition of metallopeptidases, as measured by inhibition of CPB1, resulted in low $CD4^+$ T cell proliferation suggesting lower antigen processing and/or presentation. Inhibition of trypsin did not have any effect.

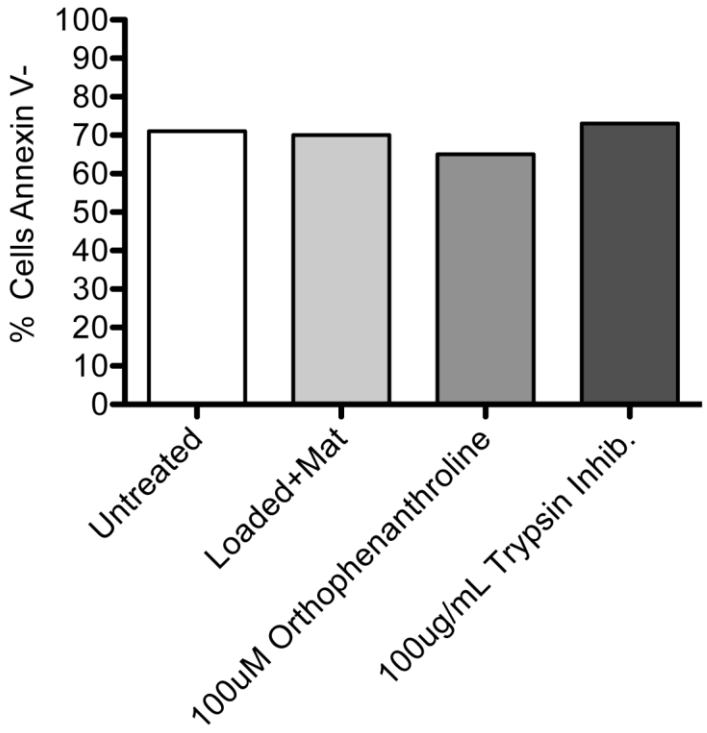


Figure 20 Treatment of BMDC with n-orthophenanthroline and trypsin inhibitor from chicken egg white does not affect DC viability.

BMDC were treated overnight with the indicated concentrations of n-orthphenanthroline or trypsin inhibitor from chicken egg white. The following day, cells were stained with PI and Annexin V to determine the frequency of apoptosis and necrosis. Data are representative of 2 independent experiments.

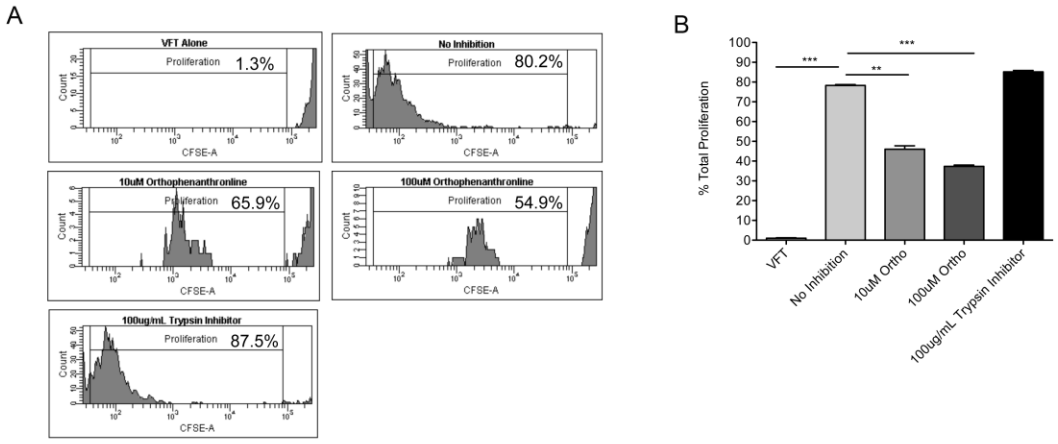


Figure 21 DC metallopeptidases, including CPB1, are required for optimal proliferation of MUC1p-specific CD4$^+$ T cells.

(A) BMDC were cultured overnight in the presence of 30 μg/mL Poly:ICLC and 30 μg/mL MUC1p with or without added protease inhibitors (10μM or 100μM n-orthophenanthroline or 100μg/mL trypsin inhibitor). The following day, naïve, CFSE labeled, MUC1p-specific CD4+ T cells were added to cultures at a 1:5, DC:T cell ratio, and cultured for 3d. Cells were then harvested and percent proliferation determined by CFSE dilution. Histograms are representative of 2 independent experiments. (B) Quantification of (A). Bars represent mean± SEM with data representative of 2 independent experiments.

We found, on the other hand, that suppression of trypsin expression was likely responsible for our previously reported observation that DC recovered from MUC1.Tg mice migrated shorter distances *ex vivo* compared to those recovered from immunized WT mice (6). *In vivo*, DC in lymphoid and non-lymphoid tissues must remodel ECM proteins such as collagen, elastin and fibronectin by using secreted or membrane bound proteases (233). Because the optimal pH of both trypsin and CPB1 activity are within physiologic range (7.5, and 7-9 respectively) (234, 235), we cultured DC and assayed the supernatants for secreted trypsin and

CPB1 by Western blotting. Both proteins were secreted by 40h with the majority of CPB1 being the 45kDa pro-enzyme form. There were multiple trypsin bands detected with at least one of them corresponding to an active, 23kDa enzyme (Fig. 22A). To determine if there was a difference in the ability to degrade ECM between DC recovered from MUC1p-immunized WT mice that upregulate these enzymes, and DC from MUC1p-immunized MUC1.Tg mice that do not, we plated both DC populations on FITC-Gelatin coated slides and examined them 4h later. The regions of the slide devoid of FITC fluorescence correspond to the amount of gelatin degraded by the DC. DC from immunized WT mice were able to degrade more FITC-gelatin compared to those from immunized MUC1.Tg mice (Figs. 22B and 22C). When we then cultured DC on FITC-gelatin in the presence of a specific trypsin inhibitor derived from chicken ovalbumin, the ability of DC from immunized WT mice to degrade the matrix was impeded (Figs. 22B and 22C).

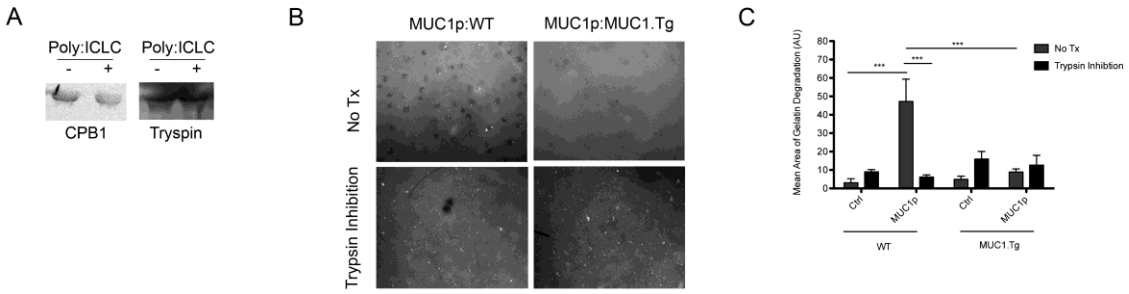


Figure 22 Extracellular trypsin enhances degradation of the extracellular matrix by DC.

(A) Splenic DC were isolated from naïve, WT mice and cultured for 40h in the presence or absence of 30μg/mL Poly:ICLC. Cell-free supernatants were collected and Western blotted for the presence of trypsin and CPB1. Data are representative of 3 independent experiments. (B) WT and MUC1.Tg mice (n=2/group) were immunized (i.v.) with DC:MUC1p (MUC1p) (1×10^6 cells) or unloaded DC (Ctrl). 2d later, splenic DC were isolated, pooled, and plated onto chamber slides coated with a FITC-Gelatin matrix in the presence or absence of 100μg/mL of trypsin inhibitor. Cells were fixed after 4h and images collected on an epifluorescent microscope and quantified with Image J (C). Bars represent mean± SEM. Data are representative of 2 independent experiments.

3.4 DISCUSSION

We previously showed that, immunization with a self antigen almost immediately (i.e. within 24h) precludes the maturation and immunogenicity of all splenic DC *in vivo*, and not just the relatively few DC that present the antigen (6). This rapid tolerization of all splenic DC in immunized MUC1.Tg mice but not in WT mice is likely due to the increased frequency of pre-existing MUC1p-specific Treg in MUC1.Tg mice (6, 236). Therefore, the earliest effects of vaccination occur not just at the stage of T cell priming, which takes several days to realize, but

upstream, at the level of Ag-specific interactions between thymically-derived Treg and DC, which take only hours to realize. Here, we show that DC tolerized after immunization with a self peptide share some characteristics with iDC while concurrently acquiring a novel, transient phenotype based on Aldh1/2, trypsin and CPB1 expression. Like iDC, tolerized DC have a less inflammatory cytokine/chemokine profile that likely results in impaired DC-mediated inflammation and chemotaxis. Specifically, tolerized DC produced less CCL2, suggesting an inability to recruit additional $CD11c^{+}CCR2^{+}$ cells. CCR2+ DC ameliorate progression of diabetes in NOD mice expressing CCL2 in the pancreas (228). Conversely, maintenance of CCR2 expression by DC serves as another indicator of a cell that is refractory to vaccine-induced maturation *in vivo*. (227, 237).

Tolerized DC rely on β-catenin, SOCS1/3, NFκB, RAR/RXR, HDAC11, zDC and STAT3 to induce and sustain various anti-inflammatory programs (60, 100, 119, 121, 182, 238) We found that DC isolated from MUC1p-immunized WT mice downregulated SOCS3 as expected, but those from immunized MUC1.Tg mice did not increase expression, suggesting that SOCS signaling is not involved in our system. Similarly, expression of HDAC11 is inversely correlated with the degree of IL-10 transcription.(122). However, tolerized splenic DC did not show the expected decrease in HDAC11 and. by extension, did not produce increased levels of IL-10 relative to WT (Fig. 23).

The transcription factor *Zbtb46* (*zDC)*, a repressor of DC maturation, was expressed approximately 2.5-fold higher in DC from immunized MUC1.Tg mice relative to those from WT illustrating another similarity between iDC and vaccine-tolerized DC. Activation of the NFκB pathway has been shown to be critical for DC maturation (118). Tolerized DC from MUC1.Tg mice were deficient in NFκB signaling compared to WT, demonstrating a shared transcriptional

signature with iDC. A possible link between zDC and NFkB, other than their shared responsiveness to TLR ligands, has not been described. Importantly, the expression of both zDC and CCR2, another marker of iDC, decreased when DC were stimulated with a TLR3 agonist directly *ex vivo*, indicating that these cells maintain the potential for maturation/immunogenicity when removed from *in vivo* suppressive signals, and are thus only transiently tolerized. This would allow for the host to control a potentially autoimmune response against self Ag while preserving the ability to prime a T cell response against foreign Ag, once self Ag was removed

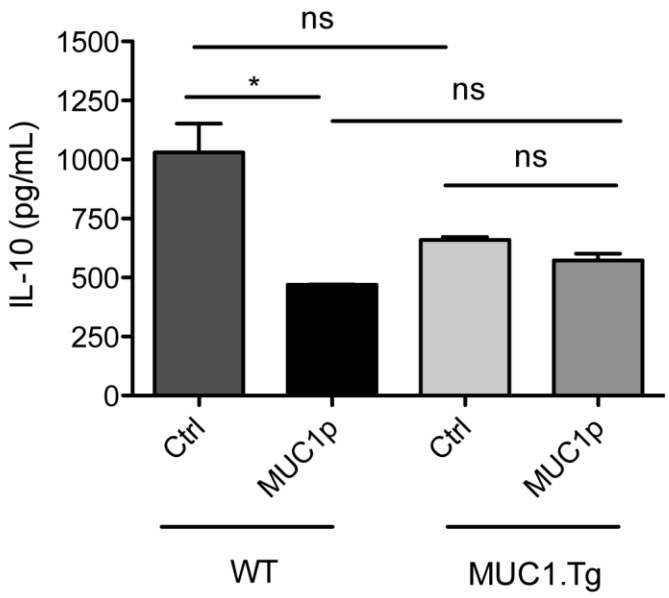


Figure 23 No difference in the amount of IL-10 produced by DC recovered from MUC1p-immunized WT vs. MUC1.Tg mice.

WT and MUC1.Tg mice (n=3/group) were immunized (i.v.) with DC:MUC1p (MUC1p) (1×10^6 cells). 1d later isolated splenic DC were cultured with 500ng/mL LPS for 24h with brefeldin A added during the last 6h of stimulation. Culture supernatants were then assayed for IL-10 by ELISA. Bars represent mean± SEM. Data are representative of 2 independent experiments.

DC from immunized MUC1.Tg mice did not produce more IL-10 than those from WT (Fig. 23), yet demonstrated increased phosphorylation of STAT3, suggesting that a paracrine source of IL-10 was contributing to their tolerized state. We previously showed that both Treg and IL-10 actively suppress expression of trypsin and CPB1 in splenic DC, and by extension, contribute to tolerizing those DC. Because MUC1.Tg mice have a higher frequency of MUC1p-specific Treg (28), Treg and paracrine IL-10 may act on DC to induce STAT3 phosphorylation. IL-6 also signals through STAT3, however, tolerized DC produce less IL-6 compared to immunogenic DC and there is no increase in IL-6 transcript in the spleens of WT or MUC1.Tg mice post-immunization (data not shown). The cellular source of this IL-10 remains to be identified. STAT3 can also directly inhibit NFκB signaling as well as the production of IL-6 and TNFα as observed in DC recovered from immunized MUC1.Tg mice (239). Thus, STAT3 phosphorylation induced by immunization with self antigen is a novel characteristic of tolerized splenic DC not shared by iDC.

Tolerized DC express Aldh1/2, the enzyme responsible for oxidizing retinaldehyde to immunosuppressive RA. $CD11c^{+}CD103^{+}$ cells from the gut have been shown to use RA in the maintenance of tolerance to commensal flora, and RA also plays a role in oral tolerance to food Ag (116). RA is also produced by $CD11c^{+}CD103^{-}$ in the lung and dermis (232) demonstrating that this molecule is not restricted to the gut, and that CD103 may not be a universal marker of RA-producing DC. However, RA production by splenic DC in response to vaccination with a self Ag has not, to our knowledge, been demonstrated. This suggests a new mechanism of peripheral tolerance in MUC1.Tg mice in which DC inducibly express Aldh1/2 after immunization with self Ag, and also represents a characteristic of tolerized DC not shared by iDC.

Metallopeptidases including CPB1 are necessary for DC to prime an optimal, antigen-specific $CD4^+$ T cell response, suggesting that CPB1 may be involved in the generation of MHC II-restricted MUC1 peptides. The majority of endolysosomal peptidases involved in generating peptides to be loaded onto MHC II are the cathepsins, consisting of cysteine, serine, and aspartyl peptidases. It is possible that the metallopeptidase inhibitor we used, n-orthophenanthroline, also affected other susceptible enzymes involved in the MHC II pathway.. However, there are currently no known metallopeptidases reported to be involved in MHC II processing and presentation, and only two carboxypeptidases, suggesting that in this particular pathway, CPB1 can be implicated as playing a role directly or indirectly in increasing the efficiency of MHC II-restricted MUC1p presentation (240, 241).

Our previous data showed that DC recovered from MUC1p-immunized MUC1.Tg mice are less motile *ex vivo* compared to those from WT. We wanted to determine if this was due to a defect in the ability of DC to migrate through the ECM. Because ECM degradation is dependent on extracellular protease expression, we examined DC and found that they were able to secrete trypsin and CPB1, making them candidates for remodeling ECM proteins. Inhibition of trypsin decreased the ability of DC recovered from immunized WT mice (immunogenic DC) to degrade gelatin, suggesting that it is necessary for remodeling of the ECM. *In vivo* it is likely that suppressed expression of trypsin in tolerized DC from immunized MUC1.Tg mice retards their ability to migrate to T cell areas within the spleen, as the majority of DC are initially localized in the red pulp and marginal zone (65). We also observed that the 45kDa proenzyme form of CPB1 was secreted into DC supernatant while an active 23kDa trypsin isoform was secreted. Because trypsin activates the CPB1 zymogen (242) it is likely that both enzymes are functional in the extracellular space. The active suppression of trypsin and CPB1 expression, and resulting effects

on DC gelatinolysis and T cell priming also constitute part of the novel phenotype of splenic DC tolerized by immunization.

Together our data demonstrate that vaccination with self-antigen has an early and global tolerizing effect on DC within the draining lymphoid tissue. Once tolerized, these DC possess phenotypic hallmarks of iDC as well as novel features precluding their immunogenicity such as expression of Aldh1/2, and suppressed expression of trypsin and CPB1 An understanding of early events after immunization will ultimately allow for better rational design of vaccines.

3.5 MATERIALS AND METHODS

Mice

C57BL/6 (WT) mice were purchased from The Jackson Laboratory. MUC1.Tg mice were originally purchased from Dr. Sandra Gendler (Mayo Clinic) (219) and subsequently bred at the University of Pittsburgh. All colonies were housed under specific pathogen free-conditions. Experiments were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Peptides

A 100-mer MUC1p represents five repeats of the 20-aa sequence HGVTSAPDTRPAPGSTAPPA from the MUC1 VNTR region. It was synthesized as described previously by the University of Pittsburgh Genomics and Proteomics Core Laboratories (4).

DC Culture and Vaccines

Bone marrow–derived DC (BMDC) were generated according to a previously reported protocol (5). Briefly, female C57BL/6 mice (Jackson) were sacrificed and their femurs and tibiae harvested. Marrow was flushed with RPMI-1640 and cells were passed through a 70-mM strainer and pelleted before RBC lysis using ACK buffer. Cells were resuspended in AIM-V (Life Technologies), counted, and plated at 1.25* 10^6/mL in AIM-V containing 20 ng/ml GM-CSF (Miltenyi Biotec). On days 3 and 5, half of the media was replaced with fresh AIM-V and GM-CSF. On day 6 of culture, DC were harvested with 2 mM EDTA, counted, and (when indicated) loaded with 30 μg/ml MUC1 100-mer and matured with 30 μg/ml polyinosinic-polycytidylic acid and poly-L-lysine (Poly-ICLC; Hiltonol), a generous gift from Oncovir, overnight. On day 7, cells were harvested as above. For immunizations, day 7 DC were washed and resuspended in sterile PBS. Mice were immunized i.v. via the lateral tail vein with 1*10^6 DC.

DC Protease Inhibition

BMDC were cultured overnight in the presence of 10μM or 100μM N-orthophenanthroline (Sigma) or 100μg/mL of trypsin inhibitor from chicken ovalbumin (Sigma), and 30μg/mL MUC1p 100mer, and 10ng/mL GM-CSF (Miltenyi). The following day, MUC1p-specific TCR.Tg CD4+ T cells (VFT) labeled with 5μM CFSE (Invitrogen) were added to culture at a 5:1, CD4:DC ratio. After 3d of coculture, cells were harvested and T cell proliferation determined by CFSE dilution on an LSR II cytometer (BD).

Quantitative RT-PCR

RNA was extracted from bead-isolated CD11c+ splenic DC (Miltenyi) using an RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed using Oligo(dT) primes and SuperScript III reverse transcriptase (Invitrogen). cDNA was amplified using the following primers: CCR2 (forward: 5'-CTGCAAAGACCAGAAGAGGGC-3', reverse: 5'-CCACCACCCAAGTGACTACA-3'), SOCS3 (forward: 5'-CCAGCCTGCGCCTCAAGACC-3', reverse: 5'-GCGTGCTTCGGGGGTCACTC-3'), Aldh1 (forward: 5'-CCATGCCGGGCGAGGTGAAG-3', reverse: 5'-TCCGGGTGGAAAGCCAGCCT-3'), and zDC (forward: 5'-AGAGAGCACATGAAGCGACA-3', reverse: 5'-CTGGCTGCAGACATGAACAC-3'). Quantitative PCR was conducted using a QuantiTect SYBR Green Kit (Qiagen). Reactions were run on a StepOne Plus cycler (Applied Biosystems) with data generated using ΔC_T methodology (221).

Western Blotting

Cells were lysed in RIPA buffer and proteins run on 10% Tris-HCL Mini-PROTEAN TGX gels (Bio-Rad) followed by transfer onto a polyvinylidene difluoride membrane. Membranes were blocked in 5% milk followed by overnight incubation at 4° with one of the following Abs: Rb X-trypsin (M-60), Rb X-CPB1 (M-134), Ms X-Aldh1/2 (H-8), Rb X-IκB-α (C-21) or Rb X-p-STAT3 (Ser 727) (Santa Cruz), and Rb X-p-p65 (93H1) (Cell Signaling).

Cytokine Array

DC were MACS (Miltenyi) isolated from spleens 24h post-MUC1p immunization of WT and

MUC1.Tg mice. DC were then cultured overnight in the presence of 500ng/mL LPS (Sigma) and GolgiPlug (BD). The following day, cells were lysed and total DC protein queried for cytokine/chemokine expression using a Proteome Profiler Array (R&D Systems). Densitometry was conducted on blots using Image J (NIH).

Gelatin Degradation Assay

DC were MACS isolated (Miltenyi) from spleens 24h post-immunization of WT and MUC1.Tg mice. Cells (1×10^5) were immediately plated into 8-well chamber slides coated with FITC-Gelatin using the QCM Gelatin Invadopodia Kit (Millipore). Images were collected on an Olympus Provis fluorescent microscope with a 40X objective. Areas of FITC-Gelatin degradation were quantified at 4h and 24h using binary thresholding in Image (NIH). Where indicated, DC were cultured for indicated timepoints in the presence of 10μM N-orthophenanthroline and/or 100ug/mL trypsin inhibitor from chicken ovalbumin (Sigma).

CCL2 Chemotaxis Assay

WT and MUC1.Tg mice were immunized with DC:MUC1p and splenic DC MACS isolated (Miltenyi) 24h later. DC were labeled with 5μM CFSE (Invitrogen) and $1.8*10^4$ DC were plated into one chamber of a μ-chemotaxis slide (Ibidi) while the opposite chamber was filled with media containing 2μg/mL of recombinant CCL2 (Peprotech). After 1h at 37° during which a stable linear gradient of CCL2 was established, cells were imaged at 1 frame/5min on a Nikon live cell system for 6h. Data was quantified and plotted using WinTaxis (Wimasis) tracking only DC that showed directional chemotaxis towards the CCL2 gradient.

4.0 TRANSCRIPTIONAL PROFILE OF WT AND MUC1.TG MICE IMMUNIZED WITH TN.MUC1, A HYPOGLYCOSYLATED ANTIGEN RESTRICTED TO MALIGNANT AND INFLAMED EPITHELIA

4.1 ABSTRACT

While MUC1.Tg mice do not mount a robust immune response after vaccination with MUC1p due to peripheral tolerance, the addition of disease-specific sugar moieties render it significantly more immunogenic. This is a result of cryptic peptide epitopes that are revealed to T cells by disease-restricted glycosylation, and the ability of those glycoepitopes to be processed and presented by DC. We were interested in determining the early (24h) events in the spleen that contribute to the immunogenicity of MUC1.Tn-based vaccines in both WT and MUC1.Tg mice. Accordingly, we immunized both strains with DC loaded with MUC1.Tn (DC:Tn100mer) and analyzed differentially expressed transcripts in total splenic RNA by microarray. We found that in spite of the equivalent immunogenicity of DC:Tn100mer in both WT and MUC1.Tg mice, there were signatures of two different types of immune response to the same vaccine. Immunized WT mice induced transcripts associated with DC, DC recruitment, and DC cross-presentation, while MUC1.Tg mice expressed a plethora of genes associated with the Type I IFN response. These results demonstrate that seemingly divergent early responses to vaccine-delivered antigen can ultimately result in the same degree of immunogenicity days and weeks later.

4.2 INTRODUCTION

Malignant transformation and inflammation of ductal and glandular epithelial cells results in an altered profile of MUC1 expression that includes loss of polarity, overexpression, and hypoglycosylation (164, 243, 244). These biochemical differences between "normal" and "tumor" MUC1 make it an attractive target for both immunoprevention and immunotherapeutic vaccine strategies due to better accessibility to the immune system, and the disease-restricted revelation of cryptic peptide epitopes. In mouse models, TCR.Tg $CD4^+$ T cells that recognize an epitope derived from a hypoglycosylated MUC1 peptide (i.e. RFT cells recognizing the TnMUC1 peptide) proliferate equivalently after vaccination in both WT and MUC1.Tg mice, demonstrating a lack of peripheral tolerance against this antigen (5, 176). Conversely, $CD4^+$ T cells that recognize unglycosylated MUC1 (i.e. VFT cells recognizing the MUC1p peptide) proliferate less after immunization in MUC1.Tg mice compared to WT due to peripheral tolerance against MUC1p as a self-antigen (4, 5). Importantly, these tumor-associated epitopes are recognized by cytotoxic T lymphocytes (CTL) in patients with multiple malignancies, including breast and pancreatic cancer (245, 246). Therapeutic immunization of prostate cancer patients with a polyvalent vaccine consisting, in part, of a TnMUC1 32mer stimulated a high titer, specific IgG response, and a current Phase I/II trial examining the efficacy of a DC vaccine loaded with the Tn antigen in prostate cancer is currently underway (247) (Trial NCT00852007, clinicaltrials.gov).

Hypoglycosylation of MUC1 does not result in decoration of its peptide backbone with a random assortment of sugar moieties. Rather, short, conserved glycoantigens such as the Tf, Tn, and sialyl-Tn antigens are generated due to premature carbohydrate chain terminations by glycosyltransferases (159, 162). The Tn antigen has been particularly well characterized and

consists of an O-linked N-acetylgalactosamine (GalNAcα-*O*-S/T) added in the Golgi by a polypeptide-N-acetylgalactosaminyltransferase (248). This glycoepitope is not removed during antigen processing by DC, and is recognized by cognate, glycopeptide-specific $CD4^+$ T cells (224).

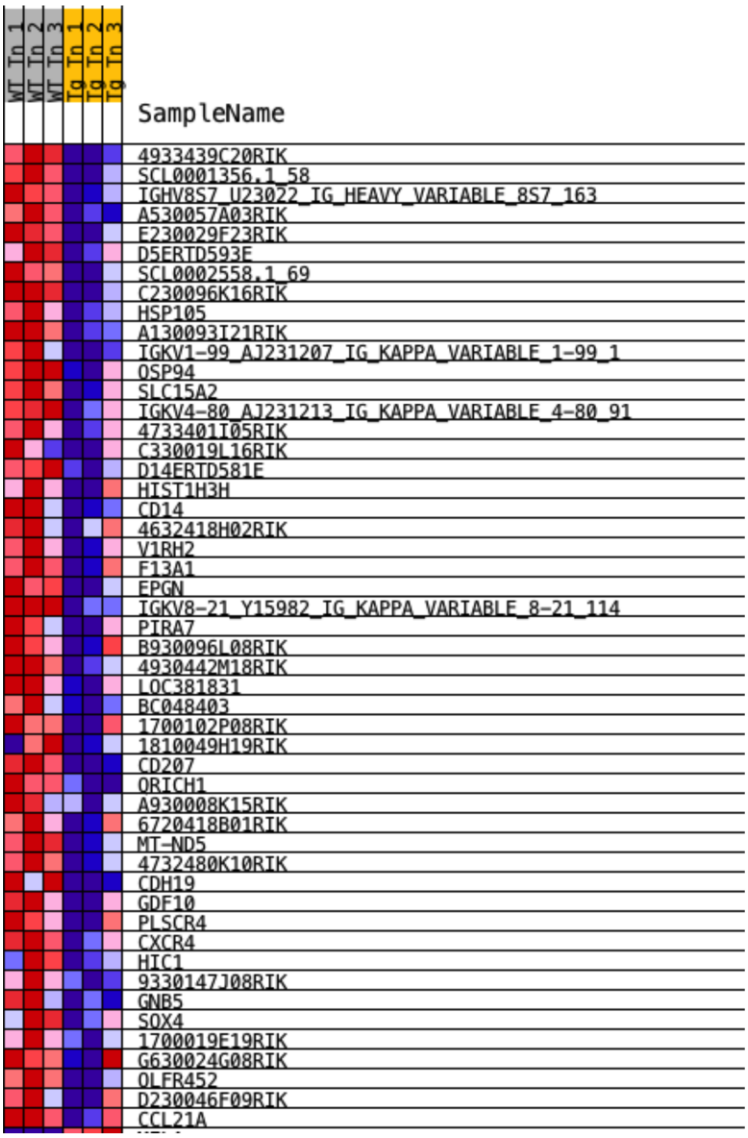
Because of the universal immunogenicity of the MUC1.Tn antigen in WT and MUC1.Tg mice, we were interested in understanding early, global events in the spleen after i.v. immunization. Accordingly, mice were immunized with DC:Tn100mer and total splenic RNA was extracted at 24h and 72h post-immunization followed by whole transcriptome analysis. We found overexpression of CXCR4, CCL21, CD207, HSP105, and CD14 in immunized WT mice and overexpression of CTLA-4 and IRF7 in immunized MUC1.Tg mice. Data from the 24h timepoint are presented here as there were no changes in the relative expression of these transcripts, nor any new transcripts expressed at 72h.

4.3 RESULTS

Expression differences between WT (n=3) and MUC1.Tg (n=3) mice immunized with DC:Tn100mer were determined by microarray analysis of pooled, total splenic RNA at 24h post-immunization. Data were analyzed using the Gene Set Enrichment Analysis program (GSEA). GSEA determines changes in the expression of single genes as well as allowing for the comparison of cohorts of genes that belong to related signaling pathways (249). Examination of the 50 most overexpressed and underexpressed transcripts in immunized MUC1.Tg mice relative to immunized WT revealed differential expression of multiple immune and non-immune related genes (Fig. 24)

The most statistically significant, differentially expressed, immune-related genes were HSP105, CD14, CD207, CXCR4, and CCL21A and were preferentially induced in WT mice, while IRF7 was induced in MUC1.Tg mice. Both sets of genes can mediate immunogenic functions via different mechanisms, as might be expected for a vaccine that induces strong immunity in both mouse strains. HSP105 is a positive regulator of NKT cell activation as well as MHC I biosynthesis (250). Interestingly, CD207 (Langerin), considered a marker of skin-resident Langerhans cells, is also expressed on marginal zone, $CD8\alpha\alpha^{+}$ DC (251). CD14 is a co-receptor, along with MD2 and TLR4, for LPS and is expressed by myeloid cells including DC, while CCL21 is a potent chemoattractant for naïve T cell and iDC homing to and within secondary lymphoid tissue. CXCR4 has well-characterized functions as an HIV-1 co-receptor on T cells, however it is has also been implicated, along with its ligand, CXCL12 in DC survival and chemotaxis (252). These molecules may constitute the early phase of a DC-centric immune response to Tn100mer in WT mice. Conversely, IRF-7, along with IRF-3, are master regulators of the Type I interferon response in virally infected cells as well as pDC suggesting a possible early, innate-type inflammatory response underlying Tn100mer immunogenicity in MUC1.Tg mice dependent on Type I IFN signaling (253).

While not all statistically significant, examination of DC:Tn100mer-induced transcriptional changes by gene sets rather than individual genes provides some insight into how this vaccine affects several important pathways. Figure 25 shows heat maps comprised of genes associated with the CCR5 and STAT3 pathways, along with genes involved in the general inflammatory response. In the inflammatory cluster, the only consistent changes amongst the technical replicates were the overexpression in MUC1.Tg spleens of Cathepsins C and S. Both enzymes are involved in antigen processing and presentation in APC, with cathepsin S cleaving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WT Tn 2
WT Tn 3
Tg Tn 1
Tg Tn 2
Tg Tn 3
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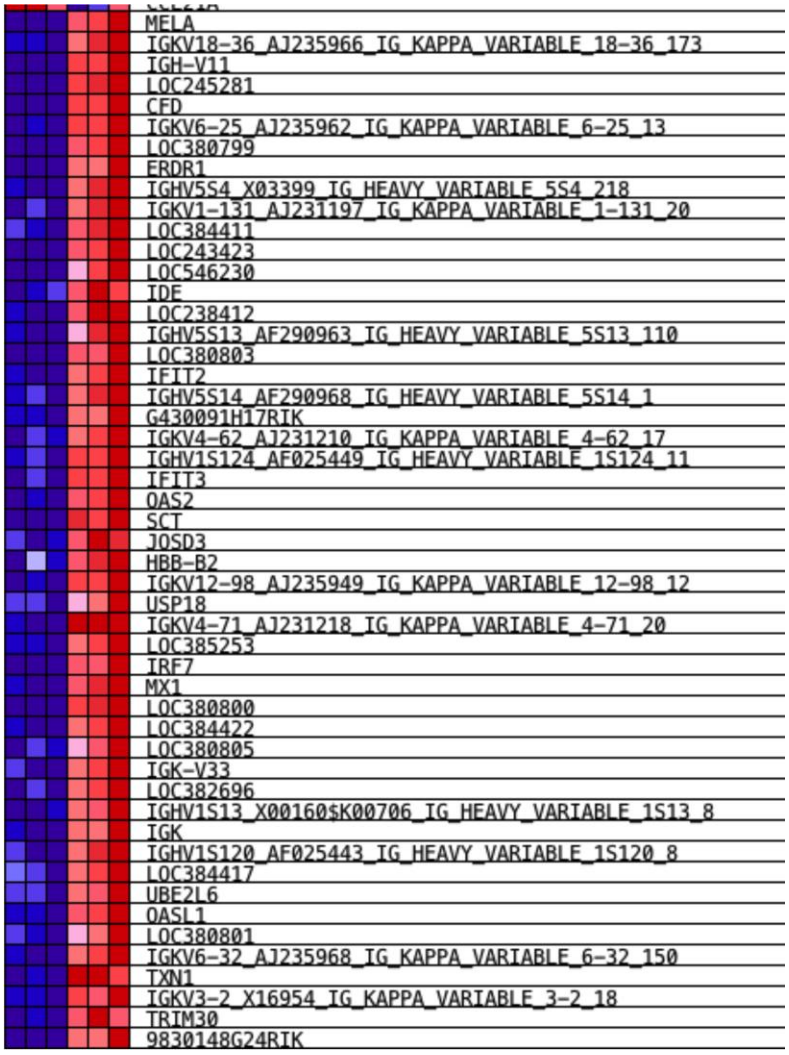

Figure 24 Differentially expressed genes in WT and MUC1.Tg mice immunized with DC:Tn100mer.

WT and MUC1.Tg mice (n=3/group) were immunized i.v. with BMDC loaded with 34ug/mL of Tn:100mer. 24h later, total splenic RNA was pooled and whole transcriptome analysis conducted. Technical triplicates were run on arrays (Samples WT Tn 1-3 and Tg Tn 1-3). The heat map represents fold changes in MUC1.Tg mice relative to WT. Red and pink signify overexpression while blue and light blue represent underexpression. Data were analyzed using GSEA.

MHC II invariant chain into CLIP before H2-M catalyzed loading of peptide (89). In the STAT3 cluster IFIT3 was upregulated in MUC1.Tg mice. This protein plays a downstream role in the Type I interferon response, fitting in with the induction of IRF7 in the same animals. In the CCR5 cluster there was induced expression in WT mice of CXCL12, (SDF-1), the ligand for CXCR4.

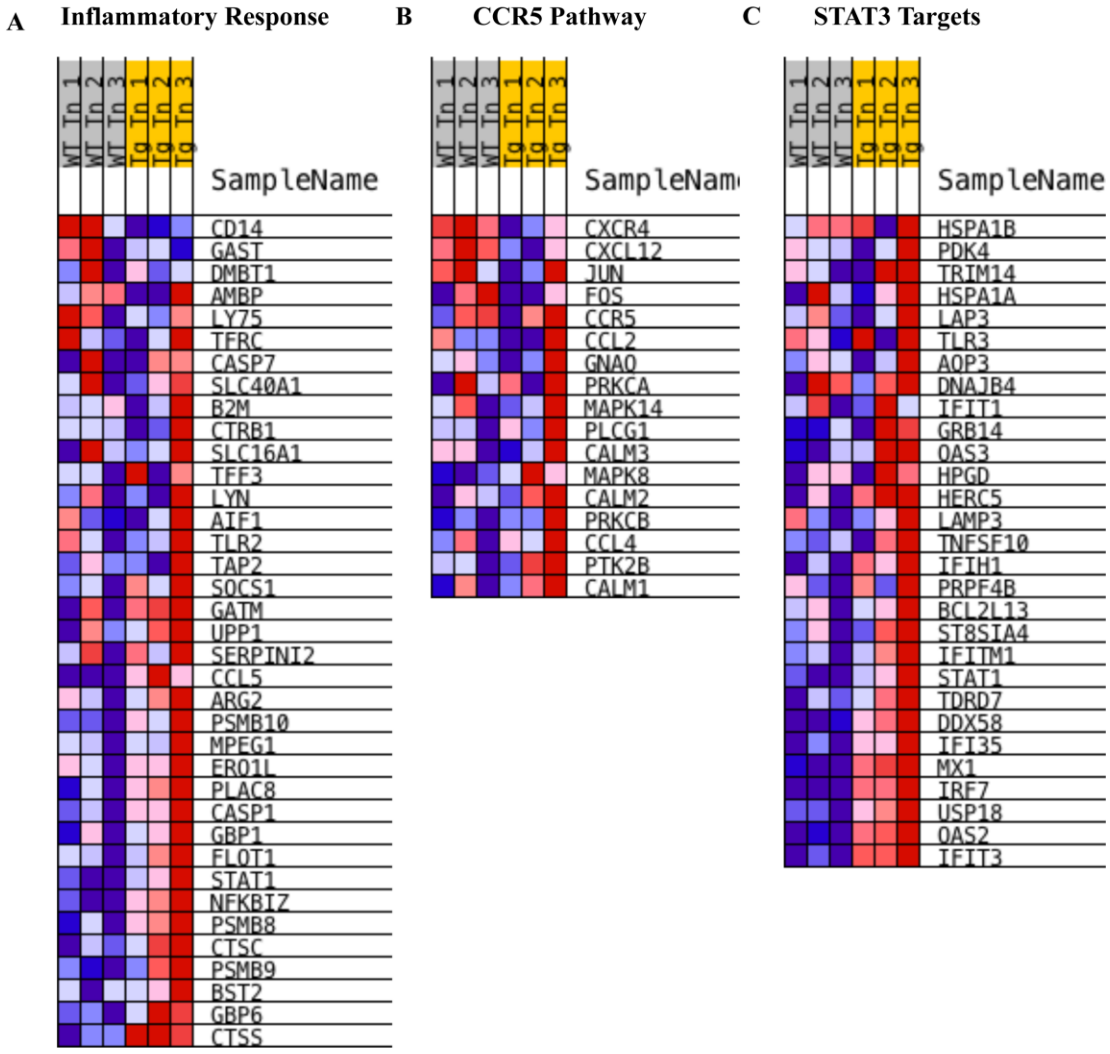

Figure 25 Changes in CCR5, STAT3, and inflammatory pathway genes in WT and MUC1.Tg mice 24h post-vaccination with DC:Tn100mer.

Single transcript fold changes were grouped into gene clusters using curated gene sets from GSEA.

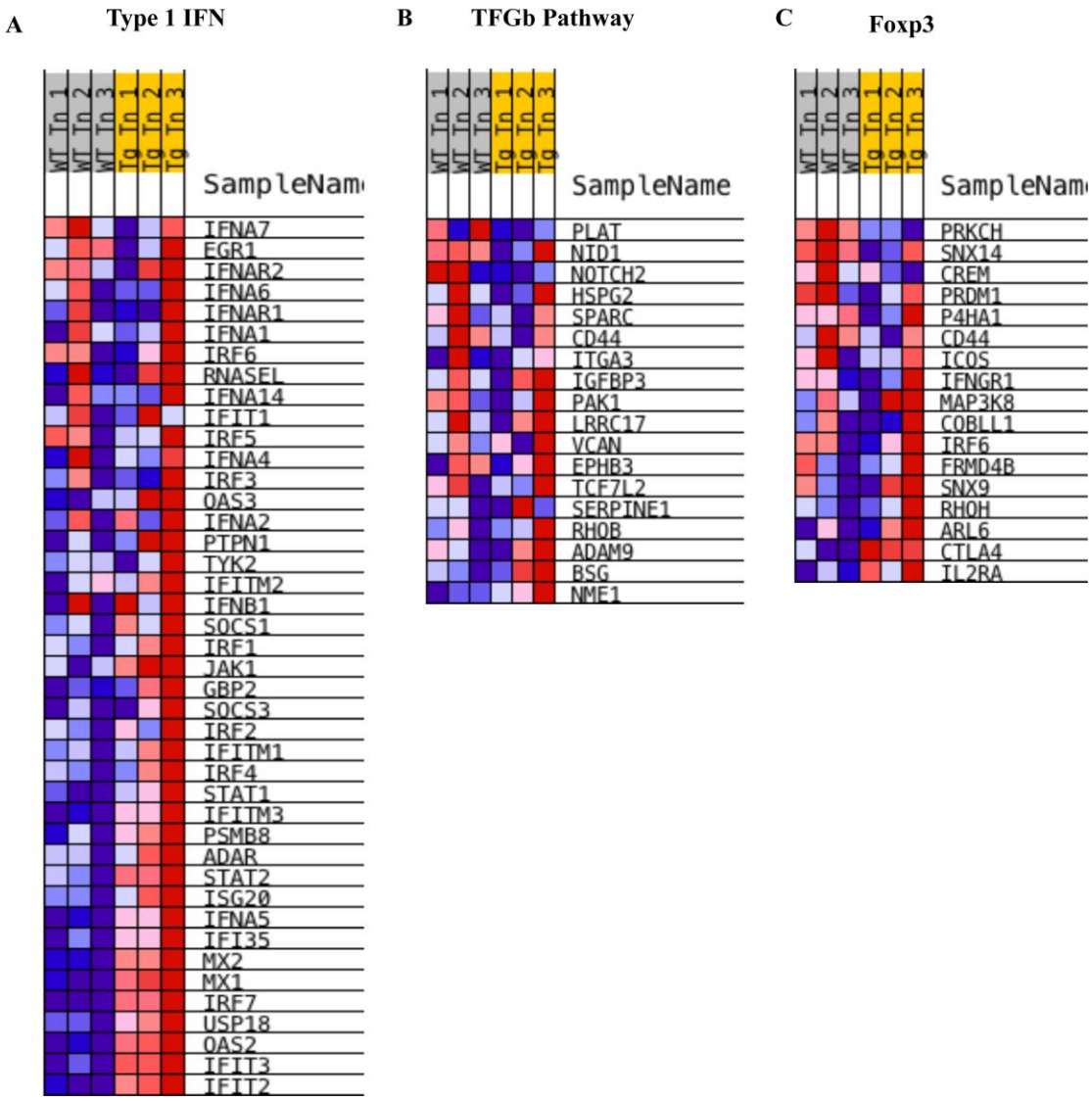

Figure 26 Changes in Type I IFN, TGF-β, and Foxp3 pathway genes in WT and MUC1.Tg mice 24h post-vaccination with DC:Tn100mer.

Single transcript fold changes were grouped into gene clusters using curated gene sets from GSEA.

Examination of the Type I IFN pathway cluster showed an overall enhanced induction of Type I IFN signaling in immunized MUC1.Tg mice relative to WT as would be expected to coincide with the relative overexpression of IRF7 and IFIT3. Interestingly, there was decreased SOCS1/3 transcript in WT mice. The suppressor of cytokine signaling proteins (SOCS) act to

preclude production of pro-inflammatory cytokines, particularly in DC. In the Foxp3 cluster, there is a trend towards induction of CTLA-4 and IL-2Ra in immunized MUC1.Tg mice. These mice have more MUC1-specific, thymically-derived Treg compared to WT, and these transcripts may reflects an early, direct, or MUC1p-cross reactive regulatory response to Tn100mer. There were no consistent changes in genes associated with the TGF-β cluster.

4.4 DISCUSSION

The addition of tumor-specific carbohydrates to the MUC1 100mer allows for recognition by a T cell repertoire that is not subject to the same mechanisms of peripheral tolerance that those specific for unglycosylated peptide are. As a result, the Tn100mer is equivalently immunogenic in both WT and MUC1.Tg mice (5, 211). Microarray analysis of early, global changes in gene expression in the spleen suggests that WT and MUC1.Tg mice initially engage different programs, that ultimately result in a similar degree of immunogenicity (as measured by equivalent RFT proliferation) in both mouse strains. Vaccination of WT mice with DC:Tn100mer induces expression of a group of DC-related genes, particularly those that may be involved in initiating cross-priming of a TnMUC1-specific $CD8^+$ T cell response. CD207 expression in the spleen coincides with $CD8\alpha\alpha^+$ DC, a population considered to have enhanced cross-priming capability (68, 251, 254). In combination with higher expression of HSP105, which acts as a positive regulator of MHC I biogenesis (250), enhanced cross-presentation of Tn100mer is a possibility. Immunized WT mice also show additional hallmarks of DC involvement and immunogenicity within the first 24h. Induction of CD14, whose expression is restricted mainly to myeloid cells, particularly DC and macrophages, suggests enhanced

recruitment or retention of those populations after immunization. CCL21 expression, likely by $CD45^-$ stromal cells, is a potent chemotactic molecule for naïve T cells and DC and may underlie the relative surfeit of DC-related transcripts in WT spleens, as well as acting to recruit circulating T cells. Interestingly, expression of SOCS1 and SOCS3 were not expressed in WT mice to the degree they were in MUC1.Tg. SOCS proteins are critical inhibitors of proinflammatory cytokine production by DC (238), again supporting an immunogenic role for DC in initiating the immune response against Tn100mer. Finally, immunized WT mice had enhanced expression of CXCR4. CXCR4 has been shown to decrease the expression of the anti-apoptotic protein Bcl-2 in DC thus enhancing DC survival (252). If the CXCR4 expression is confined to DC, this would further support an early, and sustained immunogenic response to MUC1.Tn in WT mice that is likely DC-mediated.

In contrast to the transcriptional program initiated in WT mice, the response to immunization with Tn100mer in MUC1.Tg mice appears to rely more on the initiation of Type I IFN signaling. The higher relative expression of IRF7, IFITM3 and numerous other genes up and downstream of IFNα/β signaling supports an early, biased response that may be mediated by pDC (Fig. 26). Somewhat counter-intuitively, there is preferential induction, albeit not within the cohort of genes with the highest fold changes, of the suppressive Treg-associated genes CTLA-4 and IL-2Rα. Because Tn100mer induces immunity in MUC1.Tg mice, these suppressive transcripts represent either an early, and ultimately overcome, regulatory response, or the presence of Tn100mer-specific Treg that are present in the MUC1.Tg spleen, but not WT. The medullary thymic epithelial cells of both humans and MUC1.Tg mice express glycosylated and unglycosylated MUC1, while thymi from WT do not (28). Therefore, the enrichment in CTLA-4 and IL-2R are most likely derived from thymic Treg that are resident in the spleen. Interestingly,

there was no differential expression of either trypsin or CPB1 in splenic RNA from WT or MUC1.Tg mice. We would hypothesize that the immunogenicity of MUC1.Tn would result in induction of these enzymes in DC from both mouse strains, therefore precluding the detection of differences in expression that were determined using GSEA.

This analysis represents the first step towards understanding the mechanisms behind the immunity induced by an effective vaccine in WT and MUC1.Tg mice. While it will be important to determine in which splenic compartment each gene is changing expression in, it is tempting to speculate that the early role endogenous DC play in mediating tolerance after immunization with a self-antigen may also be true after immunization with an "abnormal-self" or "foreign" antigen. A comprehensive understanding of the early response to effective and ineffective MUC1 vaccines is important to improvements in future iterations.

4.5 MATERIALS AND METHODS

Mice

C57BL/6 (WT) were purchased from the Jackson Laboratory. MUC1.Tg mice were purchased from Dr. Sandra Gendler (Mayo Clinic) (219) and bred in the University of Pittsburgh animal facility. All colonies were 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.

Peptides

A 100mer MUC1 peptide (MUC1p) represents 5 repeats of the 20- amino-acid sequence

HGVTSAPDTRPAPGSTAPPA from the extracellular, MUC1 VNTR region. It was synthesized as described previously (4) by the University of Pittsburgh Genomics and Proteomics Core Laboratories. The Tn100mer peptide was synthesized by enzymatic addition of GalNAc moieties to the 100mer via recombinant human UDP-GalNAc:polypep- tide N-acetyl-galactosaminyltransferase rGalNAc-T1 (5)

DC culture and vaccines

BMDC were generated according to established protocol (5). Briefly, female C57BL/6 mice (Jackson) were sacrificed and their femurs and tibiae removed. Marrow was 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 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 loaded with 34μg/mL TnMUC1 100mer and matured with 30μg/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 1×10^6 DC.

Microarray

Whole spleen from WT and MUC1.Tg mice (n=3/group) was harvested at 24h and 72h post-immunization with DC loaded with TnMUC1 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 using GSEA (http://www.broadinstitute.org/gsea) (249).

5.0 CONCLUSIONS AND SIGNIFICANCE

5.1 GLOBAL CONCLUSIONS

In this study we show that the relative expression of a group of "pancreatic" enzymes by splenic DC after vaccination is an early marker of the ensuing immune response, distinguishing future effective immunity from tolerance. Intravenous vaccination of MUC1.Tg mice with MUC1p, or RIP.OVA mice with OVA (as representative self-antigens) results in the suppressed expression of trypsin and CPB1 by splenic DC, while vaccination of WT mice with MUC1p or OVA (as representative foreign antigens) results in their upregulation. Therefore, expression of trypsin and CPB1, after immunization with a foreign peptide, is induced in all DC in the draining lymphoid organ and suppressed after immunization with self-peptide. We show that both IL-10 and Treg actively prevent expression of these enzymes, as pre-immunization depletion of either restores trypsin and CPB1 to WT levels. Importantly, DC with suppressed enzymes post-vaccination are phenotypically and functionally tolerized, as determined by reduced expression of CD40, CD86 and MHC II, a less inflammatory cytokine/chemokine profile, and ability to prime naïve $CD4^+$ T cells into $Foxp3^+$ Treg versus $IFN\gamma^+$ cells *ex vivo* (Fig. 27). We suggest that our data provides *in vivo* support for a two-step model of infectious tolerance: the transfer of tolerance from one population of cells to another (205). The first transfer of tolerance occurs when a MUC1p-specific Treg recognizes a DC presenting MUC1p and signals it to suppress expression of

pancreatic enzymes (i.e. trypsin and CPB1). The higher frequency of MUC1p-specific Treg in MUC1.Tg mice compared to WT means that the DC from immunized MUC1.Tg mice are induced to suppress enzyme expression while those from WT are not. This enzymatic profile is a marker of a DC that has become tolerized, and these DC subsequently fail to recruit and prime cognate $CD4^+$ effector T cells, resulting in a second transfer of antigen-specific tolerance to naïve $CD4^+$ T cells (Fig. 27). Vaccine-mediated effects on DC occur rapidly, but with each transfer of tolerance characterized by different kinetics. In MUC1.Tg mice, Treg suppress DC trypsin and CPB1 within hours, while the downstream effects of tolerized DC on T cell proliferation and phenotype occur within days. Combined with a decrease in motility and corresponding ability to degrade the ECM, these tolerized DC constitute an additional layer of peripheral tolerance against the generation of T cell responses directed against MUC1 as previously reported (4, 5, 173, 175, 191, 211).

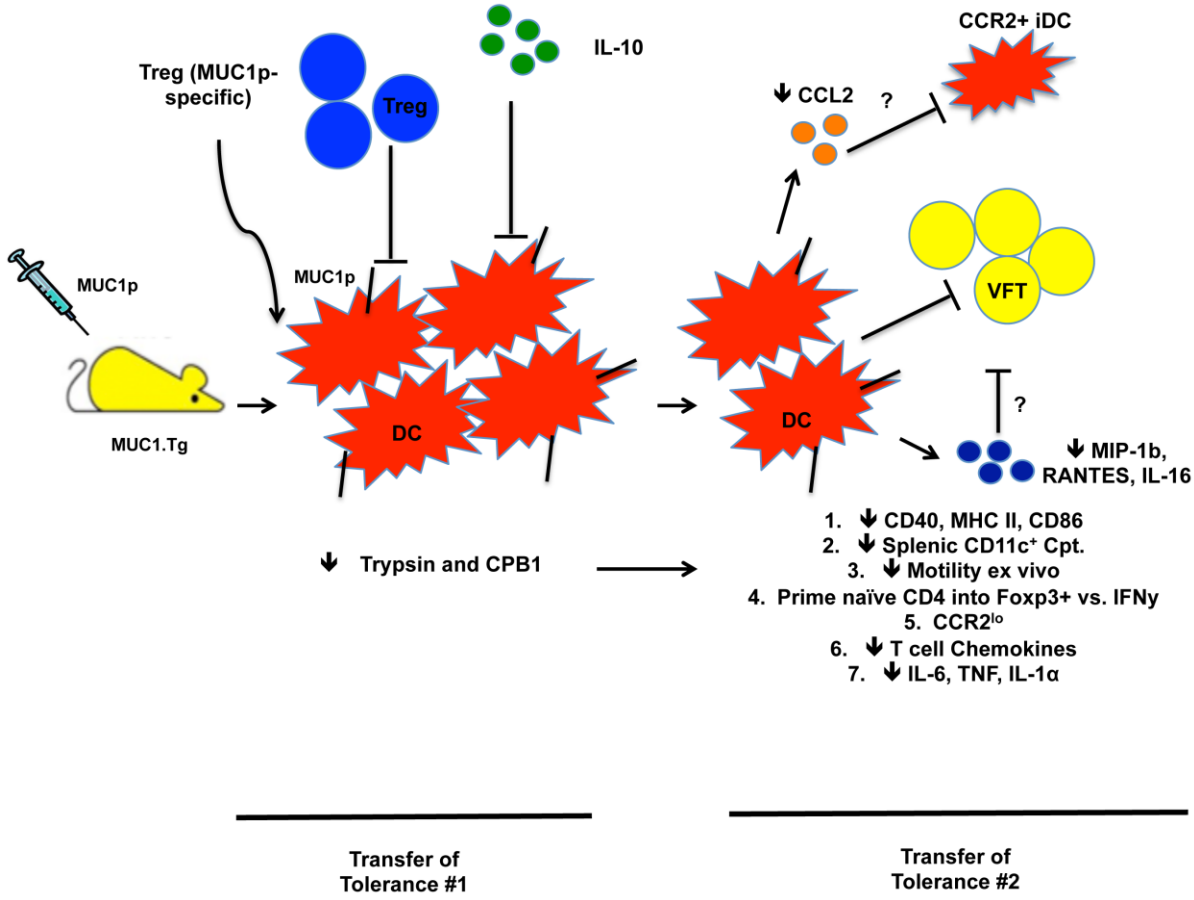

Figure 27 Propagation of tolerance to the self-antigen MUC1p in vivo.

Immunization of MUC1.Tg mice with MUC1p results in the rapid tolerization of Ag-presenting DC by cognate thymic Treg. These DC are prevented from upregulating expression of trypsin, CPB1 and other "pancreatic" enzymes. This profile of suppressed enzyme expression is a marker of a DC that is tolerized as determined by low costimulatory molecule expression, decreased motility, and decreased chemotactic and inflammatory cytokine/chemokine production. These DC subsequently prime Treg rather than effector CD4+ T cells. The role of CCL2 in recruiting additional DC to the spleen as well as the role for MIP-1b, RANTES, and IL-16 in precluding productive contacts between DC and $CD4^+$ T cells is currently unknown.

Some of the mechanisms underlying DC tolerization after vaccination with MUC1p are shared with iDC. There are, however, additional novel mechanisms we identified in DC that contribute to, and perpetuate tolerance against self-antigen. Like iDC, tolerized DC have

decreased activation of the NFκβ pathway, and sustained expression of *zDC* and *CCR2* compared to the immunogenic DC from MUC1p-immunized WT mice. Unlike iDC however, these tolerized cells have enhanced phospho-STAT3 expression, mediated upstream by increased sensitivity to IL-10, as well as inducible expression of Aldh1/2, and likely RA (Fig. 28). This tolerized state is transient as DC from MUC1p-immunized MUC1.Tg mice are not refractory to maturation with TLR ligands *ex vivo*. This demonstrates that the suppressive factors required *in vivo* to tolerize DC in a MUC1p-specific fashion (e.g. Treg and IL-10) do not do so terminally. After the threat of self-antigen passes, these splenic DC are capable of maturing and initiating immunity to appropriate foreign antigens. Therefore, DC tolerized after immunization with self-antigen do not simply fail to mature, but rather share characteristics of iDC in addition acquiring more active mechanisms to transiently prevent induction of immunity to self.

In addition to showing that DC express the enzymes trypsin and CPB1 amongst others (Table I), we present data demonstrating their involvement in DC immunogenicity. Accordingly, an additional, novel hallmark of tolerized DC that distinguish them from iDC is the functional consequence of actively suppressed trypsin and CPB1 expression. Trypsin, secreted by DC, is required for optimal degradation of the ECM. As a result, tolerized DC with suppressed trypsin expression may not be able to traffic efficiently to T cell areas within secondary lymphoid tissue as a variety of enzymes, including matrix metalloproteinases, are required for DC migration within tissues (255, 256). Metallopeptidases, including CPB1, are required for optimal presentation of MUC1p to cognate $CD4^+$ T cells. This constitutes another mechanism by which tolerized DC can also be described as "tolerogenic", in that the ineffective processing and presentation of MUC1p due to suppressed CPB1 expression precludes priming of an optimal T cell response. It is possible that the priming of naïve $CD4^+$ T cells by tolerized DC into Treg is

mediated in part by the suboptimal presentation of MUC1p:MHC II complexes, as lower pMHC density on DC can favor Treg rather than effector T cell development (80, 257).

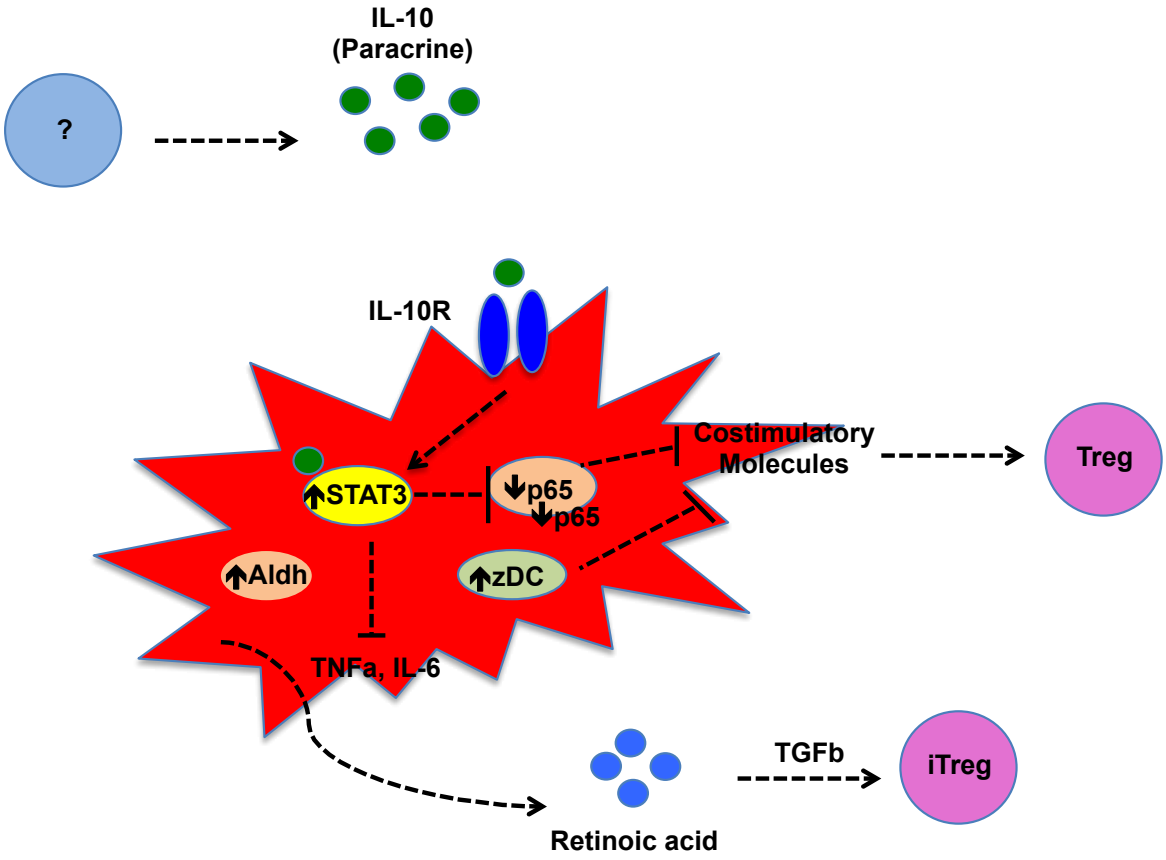

Figure 28 Signaling pathways underlying tolerized DC.

DC tolerized after immunization with self-antigen share some of the signaling pathways that underlie iDC such as sustained expression of zDC and suboptimal NFkB activation. However, these DC also show increased STAT3 phosphorylation and inducible expression of Aldh1/2 revealing the simultaneous acquisition of active mechanisms of tolerance. The cellular source of IL-10 and the contribution of the combination of RA and TGF-β to iTreg generation has not been determined.

5.2 SIGNIFICANCE

Our results identify the relative expression of "pancreatic" enzymes in splenic DC as a very early biomarker predictive of the ensuing immune response to a given vaccine. Trypsin and CPB1 expression measured as early as 24h post-vaccination is highly correlated with the relative immunogenicity of the DC in which it occurs (6). Furthermore, we show that the relative immunogenicity of splenic DC after i.v. immunization determines the quality of the $CD4^+$ T cell response against MUC1 (5). Current correlates of vaccine protection often rely on measuring antibody titers or T cell responses that take days or weeks to develop, so the ability to predict vaccine efficacy within hours represents a potentially useful advance (258). Similarly, the assays used to quantify the response to vaccination, with the exception of flow cytometry, (e.g. ELISA, ELISPOT, *ex vivo* cytotoxicity) are multiday procedures. While our results represent an important proof of concept, challenges remain before DC-based biomarkers are translated. Firstly, current correlates of vaccine protection are largely based on Ig levels and T cell responses derived from sera and peripheral blood mononuclear cells (PBMC). While the degree to which a tissue-based immune response is mirrored in the peripheral circulation is questionable, the convenience of monitoring serial blood draws from a patient to track vaccine response is not to be overlooked. However, in both mice and humans DC only constitute around 1% and .5% of PBMC respectively, with the majority localized in lymphoid and parenchymal tissues (259, 260). This presents potential obstacles in isolating and purifying enough DC from the blood to quantify enzyme expression (limited mostly by the requirement for enough RNA to perform qPCR), as well as the possibility that peripheral DC will not show changes in trypsin and CPB1. An alternative would be to examine these markers in monocyte-derived DC from PBMC as established protocols exist to differentiate peripheral mononuclear cells (261), and

these cells express CPB1 and elastase (6). However, changes in expression after vaccination have not yet been examined in these cells.

Secondly, we examined endogenous DC in the draining lymphoid tissue, the spleen, after intravenous immunization. Most cancer vaccines, with the exception of some DC-based vaccines, are administered either intramuscularly, intradermally, or subcutaneously (262). As a result, collecting DC from the relevant draining LN would require biopsy followed by isolation. While this is technically feasible it represents a more invasive procedure, and the benefit to risk ratio would have to be acceptable for it to be a viable option.

Our results also contribute to a previously underappreciated role for endogenous DC in influencing the direction of the ensuing immune response to vaccination. It is generally thought that DC-based vaccines act by directly presenting antigen to cognate T cells, with the subsequent response contingent on both the immunizing antigen as well as the phenotype of the vaccine DC. Recently it has been demonstrated that the viability and MHC II expression of the DC used for vaccination are dispensable, and it is DC-derived antigen processed by endogenous DC that directly primes the T cell response (263). Our data show that post-vaccination changes in the phenotype of the splenic DC compartment are global, suggesting a mechanism by which DC tolerance initiated by Treg is transmitted to the entire DC population, tissue-wide. This data underscores the importance of vaccine antigen choice by highlighting a system in which vaccine signals are quickly and efficiently dispersed and amplified.

We also identify inducible expression of Aldh1/2 in $CD103^-$ splenic DC in response to immunization with self-antigen. While the immunosuppressive metabolite of Aldh1/2 activity, RA, is a well-described mediator of tolerance to oral, respiratory and commensal antigens in the gut and lungs, its function has not been well characterized either in the spleen, nor in response to

vaccine-delivered antigens (76, 116). Similarly, the phenotype assumed by tolerized DC is a unique mosaic of both canonical features of DC-mediated tolerance (e.g. low costimulatory molecule expression and anti-inflammatory cytokine production), combined with the novel, immunomodulatory functions of trypsin and CPB1 that we describe. Our identification of trypsin and CPB1 expression and function in splenic DC adds to the growing list of potential molecules and pathways that can be targeted to modulate DC function, before and after vaccination.

5.3 REMAINING QUESTIONS

Like all new information, our results open as many questions as they answer. Our data showing changes in endogenous DC phenotype were all measured using total, splenic DC. Therefore, either a small subset of DC dramatically changes phenotype (e.g., enzyme expression, cytokine production etc.) such that the observed differences are apparent in the population as a whole, or the majority of cells within that population undergo more biologically feasible changes in phenotype. We propose that the latter is the more likely scenario and that there are at least two major mechanisms by which the introduction of relatively few antigen-bearing, vaccine DC might propagate either a tolerogenic or immunogenic signal to all other DC in the draining lymphoid tissue. First, antigen derived from vaccine DC could be distributed to all other endogenous DC in the tissue. This could occur through the shedding and subsequent internalization of antigen-containing exosomes or via the transfer of antigen through physical, tubular connections between DC termed tunneling nanotubules (204, 264). Second, cognate Treg:DC interactions could be relatively few, with the resulting signaling mediators (e.g.,

calcium flux) being transmitted to all tissue DC rather than antigen. As discussed in Appendix C, a variety of microscopic approaches need to be utilized to address the precise mechanism by which vaccine DC integrate into the resident DC network.

We show that Aldh1/2 is made by tolerized DC. However, a direct role for RA, in conjunction with TGF-β, as being involved in the priming of $Foxp3^+$ inducible Treg by tolerized DC *ex vivo* remains to be determined. Similarly, the relative contributions of low costimulation and fewer MUC1p:MHC II complexes (due to suppressed CPB1 expression) to the ability of tolerized DC to prime Treg is unknown. That is, would RA, low costimulation, or suppressed CPB1 expression alone maintain the tolerogenicity of these DC, or do all three mechanisms act in concert to produce the observed function.

Our observations demonstrating impaired *ex vivo* motility of tolerized DC as well as their relative inefficiency in degrading ECM raise important questions regarding how effectively these DC can interact with cognate effector T cells *in vivo*. During homeostasis, the majority of DC are localized in the splenic marginal zone, or scattered throughout the white pulp, however, they must respond to a CLL19/CCL21 gradient to migrate to T cell areas during their maturation (65). While there are equivalent numbers of VFT $CD4^+$ T cells in the spleen after adoptive transfer, it remains to be determined whether there are an equivalent number of productive DC:VFT interactions in immunized WT and MUC1.Tg mice. The decrease in tolerized DC motility and gelatinolysis in concert with their decreased production of T cell chemokines may act as an additional safeguard against autoimmunity by precluding the ability of DC to physically traffic to T cell areas in the spleen.

Finally, we show that the endogenous DC compartment is only transiently tolerized after exposure to self-antigen. DC stimulated *ex vivo* can be induced to mature with TLR ligands, and

levels of trypsin and CPB1 return to baseline several days after immunization. The signaling networks responsible for a return to the steady-state, be they decreased IL-10, the egress or apoptosis of cognate Treg, or others, remain to be determined. Additionally, at the biochemical level, an understanding of what molecules control transcription of trypsin and CPB1 would be of utility to identify potential overlaps with the IL-10, NFκβ, STAT3 and zDC pathways. Similarly, identifying the enzymatic cascade upstream of bioactive trypsin and CPB1 formation remains to be determined. In the duodenum, secretion of enterokinase cleaves trypsinogen into active trypsin which then acts both in an autocatalytic loop, as well as activating pro-CPB1 to CPB1 (265). While our array data did not show any differential expression in enterokinase between immunized MUC1.Tg and WT mice, its relevance in potentially activating DC-expressed trypsin would still be useful to examine given the limits of microarray sensitivity. A more nuanced understanding of the regulatory networks controlling DC tolerance versus immunity will allow for interventional modulation so as to drive the immune system towards the desired outcome.

APPENDIX A

SUBCELLULAR LOCALIZATION OF TRYPSIN AND CPB1

Our data suggests a role for CPB1, and likely additional metallopeptidases, in mediating optimal $CD4^+$ T cell priming by DC. When CPB1 activity was inhibited using the broad metallopeptidase inhibitor n-orthophenanthroline, DC were unable to induce efficient proliferation of cognate, MUC1p-specific $CD4^+$ T cells compared to control. The generation of MHC II-restricted peptides depends on processing by various proteolytic enzymes such as the cathepsins in progressively more acidic endolysosomal compartments. Ultimately, peptides derived from the original protein are loaded onto empty MHC II molecules by H2-M before transport via endolysosomal tubules to the plasma membrane (83, 93, 266, 267). Accordingly, we were interested in determining the sub-cellular localization of CPB1 with the hypothesis being that it would be expressed in compartments associated with Class II processing and presentation. Additionally, since DC maturation is accompanied by enhanced lysosomal acidification and peptide processing (62), we wanted to assess any differences in CPB1 quantity and/or distribution after a variety of maturational stimuli. To this end, bone marrow-derived DC (BMDC) were permeabilized and stained for trypsin, CPB1 and MHC II after no treatment, or overnight culture with LPS, Poly:ICLC, or X-CD40. Cells were then fixed and imaged with

confocal microscopy. As shown in Figure 29, both enzymes are predominantly cytosolic in the steady state with a large degree of overlap with internal MHC II, perhaps in MHC II compartments (MIIC) (268).

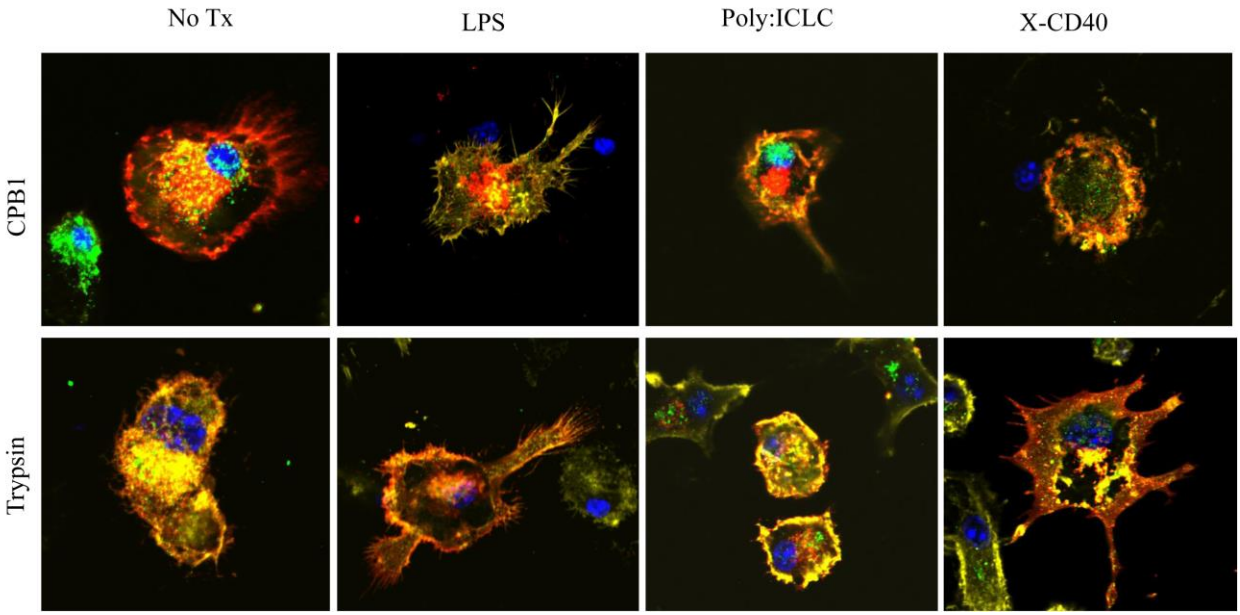

Figure 29 Trypsin and CPB1 move towards the periphery of BMDC after maturation.

BMDC were left untreated overnight, or stimulated with 1μg/mL LPS, 25μg/mL Poly:ICLC, or 10μg/mL anti-CD40. Cells were then fixed, permeabilized, and stained with antibodies to trypsin or CPB1 (Green) and an antibody to I-Ab (Red) followed by counterstaining with DAPI (Blue). Slides were imaged on an upright Olympus Fluoview 1000 at 60X. Data are representative of 2 independent experiments.

After maturation of DC with LPS, there is a peripheralization of both proteases, with CPB1 redistributing away from the nucleus and trypsin moving near the cell membrane. This pattern is recapitulated after CD40 and Poly:ICLC induced maturation as well, particularly for the membrane distribution of trypsin. Maturation also enhanced co-localization between CPB1 and intracellular MHC II^+ compartments and trypsin and MHC II on or near the membrane. These data suggest that CPB1 may be present in late endosomal compartments where peptides

are being loaded onto MHC II, while trypsin, which we identified as being secreted by DC and important for gelatinolysis, is localized on or near the membrane for remodeling of the ECM.

LAMP-1 (CD107a) is a marker of acidic, lysosomal and late endosomal compartments where chaperoned MHC II molecules are loaded with peptide (269). To further examine which endocytic compartments CPB1 may be part of, we performed immunofluorescence as above to examine co-localization between the enzymes of interest and LAMP-1^{+} lysosomes and/or endolysosomes. As shown in Figure 30, there is minimal co-localization between CPB1 and LAMP-1^{+} lysosomes, and no overlap was observed for trypsin (data not shown). This implicates CPB1 as playing a role in antigen processing and/or presentation earlier in the MHC II endocytic pathway. Its putative localization in MIIC, but not LAMP-1 vesicles suggests that it catalyzes proteolysis of proteins, or plays some other function earlier in the pathway, before maximal acidification of the phagolysosome. Because the optimal pH for CPB1 activity is between 7-9 (235) it would likely not be optimally functional at a typical lysosomal pH of 4.5-6 but would operate in the early endosomal pH range of 6.1-6.8 (270).

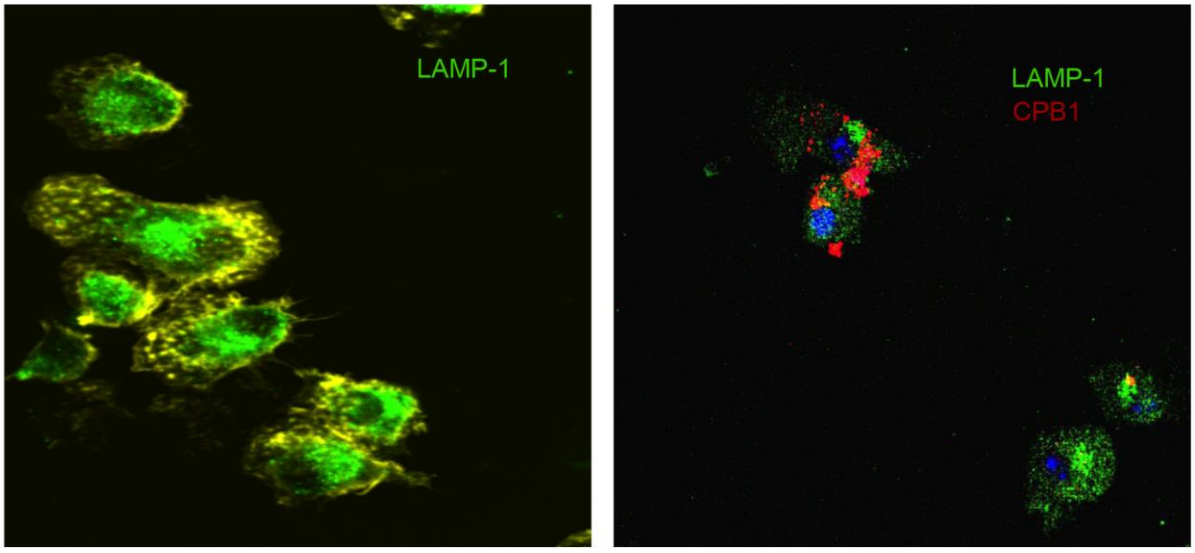

Figure 30 Minimal Colocalization between CPB1 and LAMP-1+ compartments.

BMDC were permeablized and stained with antibodies to LAMP-1 (Green) and CPB1 (Red). Cells were fixed and imaged using confocal microscopy. Slides were imaged on an upright Olympus Fluoview 1000 at 60X. Data are representative of 2 independent experiments.

APPENDIX B

EFFECTS OF TOLERIZED DC ON THE EARLY CD4$^+$ T CELL RESPONSE TO MUC1

Previous work has shown that MUC1.Tg mice are hyporesponsive to immunization with MUC1p as determined by a number of correlates (i.e. cognate CD4$^+$ T cell proliferation, MUC1p-specific IgG, IFNγ production, and specific lysis of MUC1$^+$ tumor cells) (4, 5, 175). As these readouts are all part of the adaptive response, they were necessarily quantified between 5d-14d after immunization of WT and MUC1.Tg mice. Because tolerized DC produce less of the CD4-chemotactic chemokines (i.e. MIP-1b, RANTES, and IL-16) 24h after vaccination (6), we hypothesized that there might exist a defect in the recruitment or retention of cognate CD4$^+$ T cells in the spleen. Similarly, the interactions between DC that have low costimulatory molecule expression, are less inflammatory, and preferentially prime Foxp3$^+$ Treg versus IFNγ$^+$ effector T cells *ex vivo* would likely prime naïve, MUC1p-specific T cells differently *in vivo* than the more immunogenic DC from immunized WT mice. To determine the early effects of a tolerized splenic DC compartment on nascent T cell responses we transferred (i.v.) 5*10^6 CFSE-labeled, MUC1p-specific CD4$^+$ T cells (VFT) into WT and MUC1.Tg recipients. After 24h, mice were immunized with DC:MUC1p and 24h later spleens harvested for FACS. The absolute number of VFT T cells that homed to, and were retained in, the spleens of WT and MUC1.Tg mice was

equivalent as was the number of total $CD4^+$ T cells (Fig. 31). There were slightly more VFT cells that were $CD25^{hi}$ suggesting that the DC from immunized MUC1.Tg mice were beginning to prime a regulatory response. *Ex vivo* data previously demonstrated that these DC prime naïve T cells into $Foxp3^+$ Treg in an antigen-specific manner after 7d in culture (6) so it is likely that after 1d of interaction between MUC1p-loaded DC and VFT T cells the beginning of a Treg response is being primed. As might be predicted, there was a higher frequency of $CD69^+$ VFT T cells in the spleens of immunized WT mice (Fig. 31). Because human MUC1p represents a foreign Ag in WT mice, VFT T cells proliferate more compared to MUC1.Tg (4). This is reflected early on, within 24h of DC:T cell interactions and is likely mediated by splenic DC that are more immunogenic relative to MUC1.Tg.

While there were no numerical differences in the pool of VFT T cells available to interact with tolerogenic or immunogenic DC, these data show that as early as 24h post-vaccination, the MUC1-specific $CD4^+$ T cell response begins to diverge in WT and MUC1.Tg mice. This is supported by recent observations illustrating the maintenance of a higher frequency of $Foxp3^+$ VFT T cells in MUC1.Tg mice compared to WT even after several weeks post-immunization (271).

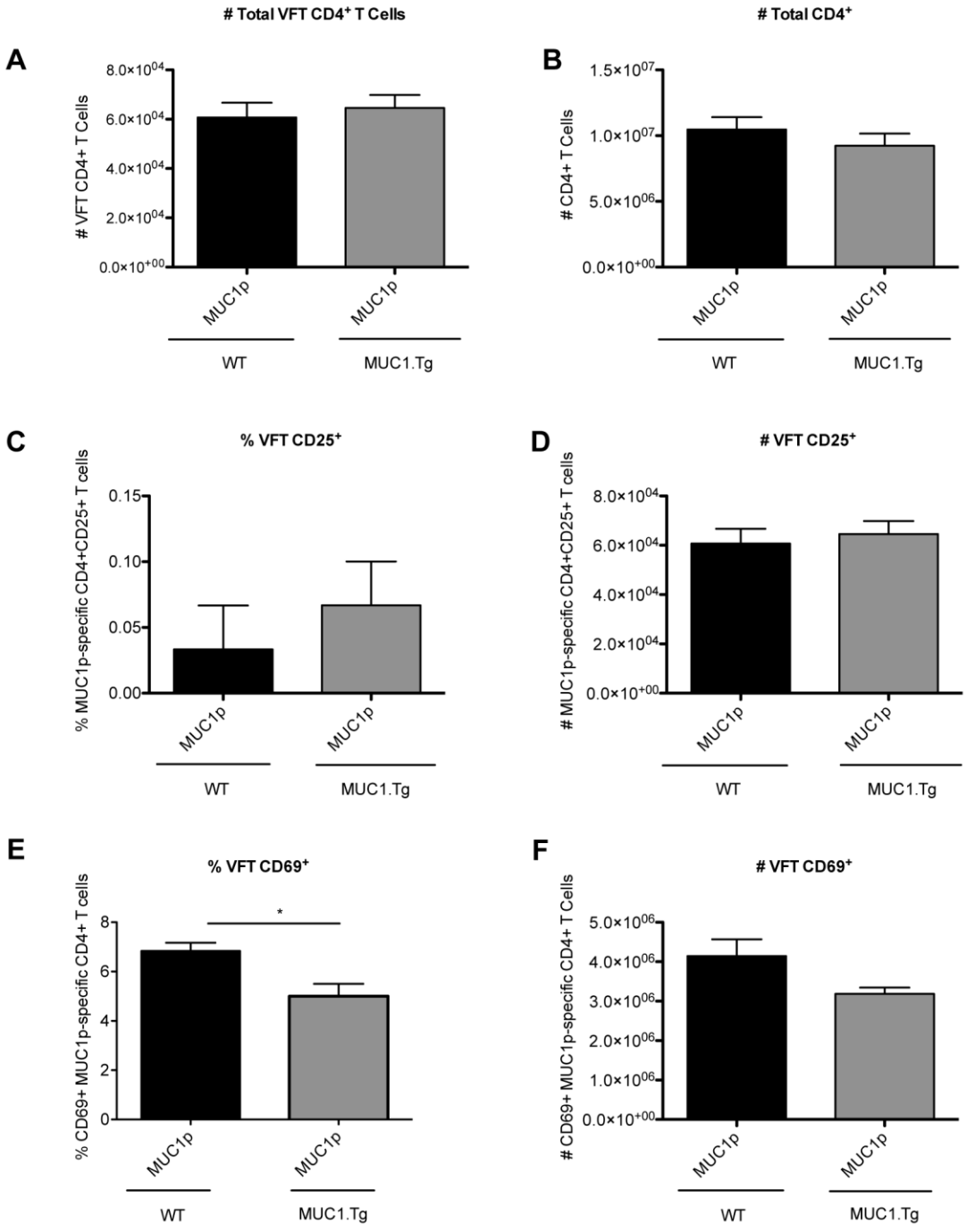

Figure 31 DC from immunized MUC1.Tg mice do not activate MUC1p-specific CD4$^+$ T cells as effectively as those from WT.

$5*10^6$ CFSE-labeled, MUC1p-specific $CD4^+$ T cells (VFT) were transferred (i.v.) into WT and MUC1.Tg recipients. 24h later, mice were immunized with DC:MUC1p and after an additional 24h, spleens were harvested for FACS. (A) Total number of MUC1p-specific $CD4^+$ T cells (VFT). (B) Total number of all $CD4^+$ T cells. (C) Percentage and (D) number of $CD25^{hi}$ VFT cells. (E) Percentage and number (F) of $CD69^+$ VFT T cells. Bars represent Mean ± SEM, data representative of 2 independent experiments.

B.1 DECREASED NUMBER OF SPLENIC DC IN IMMUNIZED MUC1.TG MICE CORRESPONDS TO A CONCURRENT INCREASE IN THE NUMBER OF NK CELLS.

After immunization of MUC1.Tg mice there is a decrease both in the absolute number of $CD11c^+$ splenic DC, as well as the DC used for immunization, compared to immunized WT. Part of this decrease is likely due to a defect in recruitment of $CCR2^+$ DC to the spleen due to decreased DC-produced CCL2 compared to WT (Fig. 14). However, the decreased number of splenic DC could also be explained by cell death, either by apoptosis or direct killing. Specifically, $CD8^+$ T cells and NK cells have been shown to kill iDC both *in vitro* and *in vivo* (272, 273). To examine the possibility that DC were being killed we examined concurrent changes in the number and phenotype of $CD8^+$ T cells and NK cells. While there was no difference in the $CD8^+$ population in the spleen 24h post-immunization, we observed a small but consistent increase in the frequency of $NK1.1^+$ NK cells, and a significant increase in their absolute number in immunized MUC1.Tg mice relative to WT (Figs. 32A and 32C).

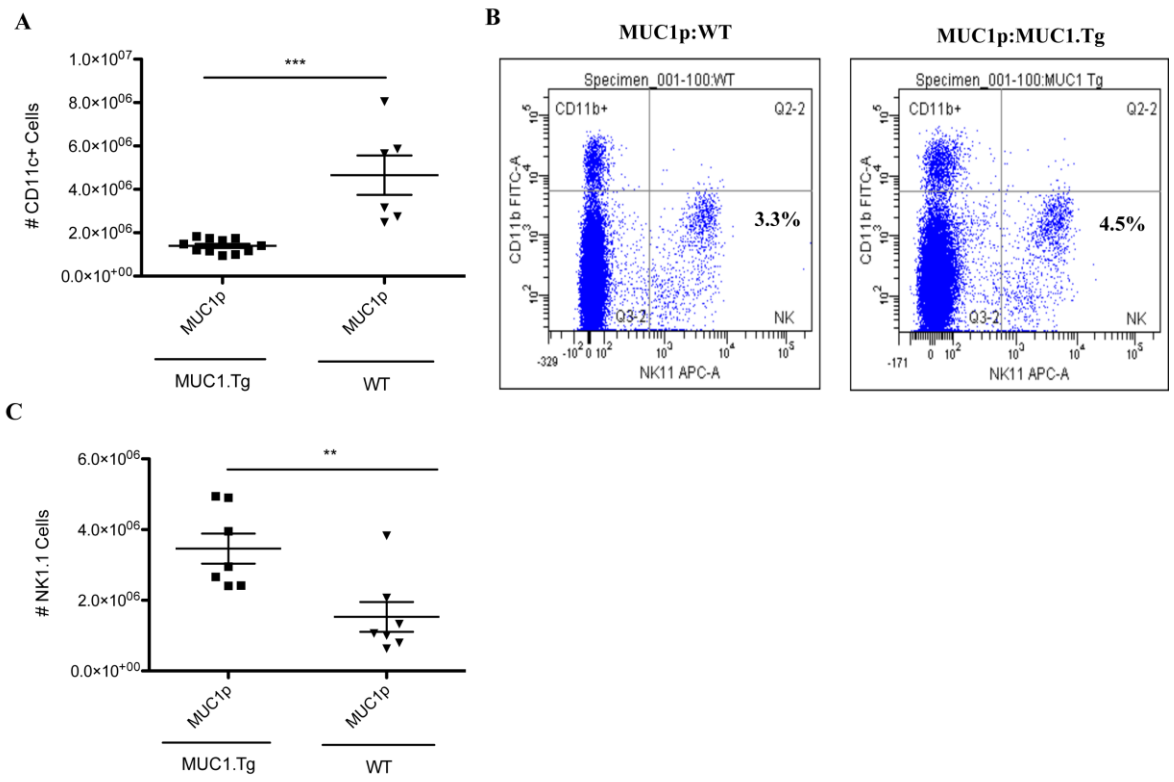

Figure 32 Decrease in DC number after immunization of MUC1.Tg mice corresponds with a concurrent increase in the number of NK cells.

WT and MUC1.Tg mice (n=3) were immunized with MUC1p. 24h later, spleens were harvested for FACS analysis. (A) Number of DC in spleens of immunized mice. (B) Representative dot plot showing small increase in the frequency of NK1.1+ cells after immunization. (C) Number of NK cells in spleens of immunized mice. (A+C) Each point represents an individual mouse. Bars represent mean ± SEM. Data are pooled from 3-5 independent experiments.

The integration of activating and inhibitory signals delivered to NK cells via their receptors determines whether the NK cell will lyse a target cell or not (274). Two of the best characterized interactions between NK cells and their targets are the ligation of the NK activating receptor NKG2D with its ligand Rae-1 (MICA/B in humans), and the killing of target cells with low MHC I expression, (275-277). Activated NK cells can be identified by expression of the early T/NK cell maker CD69 (278). Accordingly, we wanted to determine if the increased

number of NK cells was responsible for direct killing of DC in immunized MUC1.Tg mice. DC from MUC1.Tg mice did not differ in expression of Rae-1 or MHC 1 (data not shown). Phenotypic analysis of the NK compartment revealed a small, but statistically insignificant increase in the number of NK cells that were $NKG2D^{+}CD69^{+}$ in the spleens of immunized MUC1.Tg mice (Fig. 33A). This data suggested that there were no major differences in at least two canonical markers of NK activation between the two mice strains. To determine if there existed functional differences in the ability of NK cells to kill splenic DC we co-cultured DC from immunized WT and MUC1.Tg mice with NK cells isolated from a naïve animal overnight, followed by FACS staining for CD107a (LAMP-1), a marker of T/NK degranulation (279). There were no significant differences in degranulation of NK cells induced by DC from either strain (Fig. 33B). Finally, to determine if there was NK killing of DC through NKG2D-independent pathways, we cultured DC recovered from immunized mice with naïve NK cells and used time lapse microscopy to visualize differences in DC:NK interactions and potential killing. As shown in Figs. 33C and 33D, there was no difference in cytolytic activity between groups, though there was a general trend towards increased duration of DC:NK contact when the DC were from immunized MUC1.Tg mice.

These results suggest that the increased number of NK cells in immunized MUC1.Tg spleens are not killing DC but perhaps act as a compensatory source of IFNγ or play a regulatory role. Another possibility is that the NK are cytolytic but require an activation signal that is missing in *ex vivo* cultures, for example IL-2 and/or IL-15 (280). Similarly, the use of "naïve" NK cells in the assays above may preclude their effector functions, and different results would be obtained if NK cells from immunized mice were co-cultured with DC from the same animal.

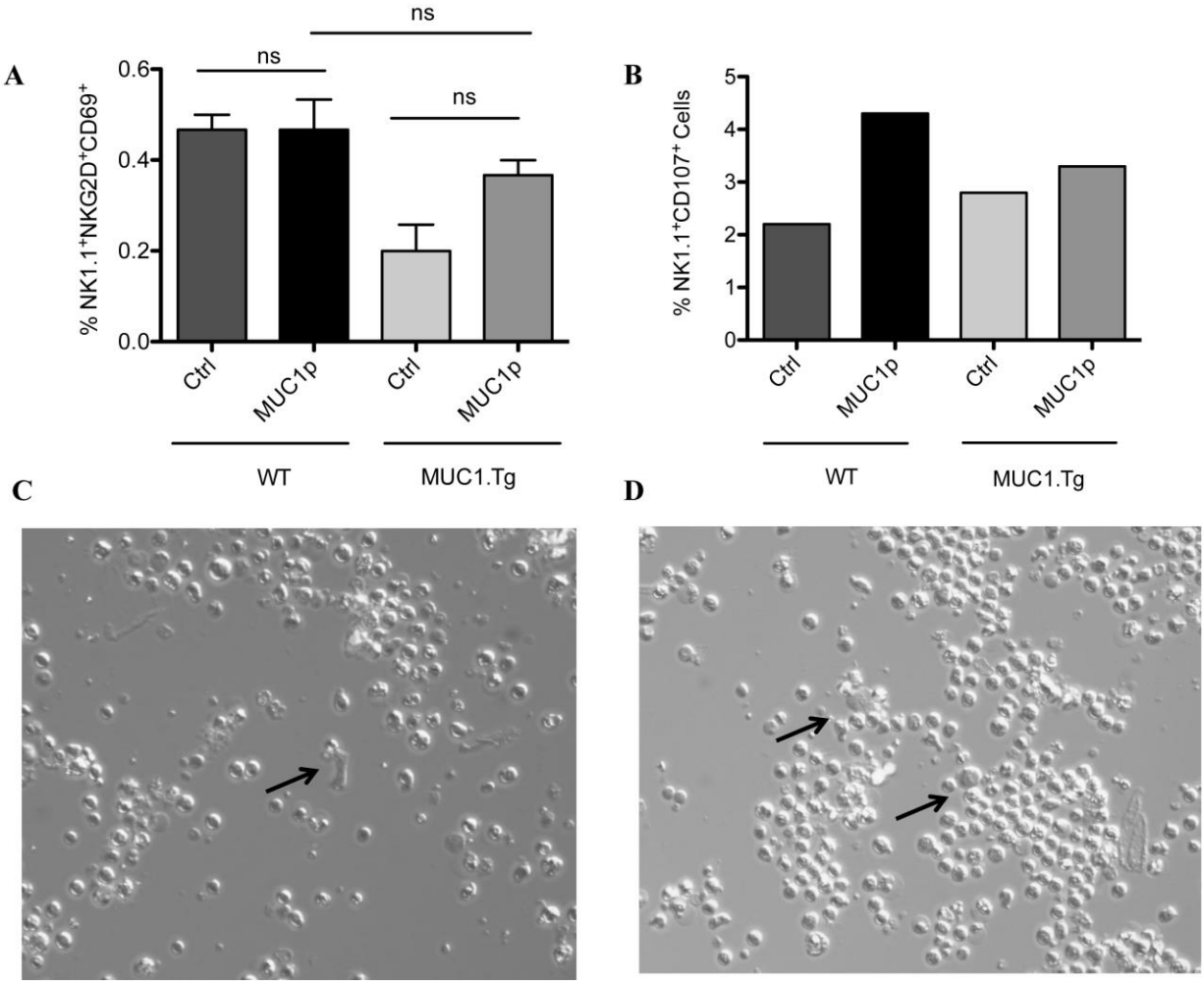

Figure 33 Decrease in splenic DC after immunization of MUC1.Tg mice is not due to enhanced NK killing.

(A) WT and MUC1.Tg (n=2) mice were immunized with soluble MUC1p admixed with Poly:ICLC. 24h later, spleens were harvested and stained for NK1.1, NKG2D and CD69. (B) DC isolated from immunized WT and MUC1.Tg mice were cultured together overnight at a 1:1 ratio with NK cells from a naïve mouse ($5*10^4$ cells/well), 1000U/mL of IL-2, and an anti-CD107a antibody. NK degranulation was quantified with flow cytometry staining for CD107a. (C+D) WT and MUC1.Tg mice were immunized with soluble MUC1p admixed with Poly:ICLC. 24h later DC were isolated and co-cultured overnight with naïve NK cells. Arrows show DC:NK interactions Timelapse DIC images were acquired at 1min intervals at a magnification of 20X. Bars represent mean ± SEM and data are representative of 2 (A), 1 (B), and 1 (C and D) experiments.

B.2 SPLENIC TREG IN IMMUNIZED WT AND MUC1.TG MICE

Treg actively suppress DC expression of trypsin, CPB1, and elastase and by extension, inhibit DC immunogenicity. *In vivo* these effects are realized as early as 24h post-immunization and are therefore mediated by natural, or pre-existing, thymically-derived Treg. We were interested in any changes in the splenic Treg compartment after immunization, as an influx of MUC1p-specific Treg in immunized MUC1.Tg mice might be efficient at rapidly suppressing protease expression by DC presenting MUC1p. To examine this, we immunized WT and MUC1.Tg mice and examined the frequency and phenotype of Treg at 24h. Figure 34 shows that immunization and mouse strain have no effect on the frequency or number of Treg at 24h. There were also no differences in the expression of IL-10, IL-2, and CTLA-4 (data not shown).

Though there were no changes in Treg phenotype or frequency, the increased number of MUC1p-specific thymic Treg in MUCl.Tg mice likely accounts for the recognition of antigen-bearing DC and suppresses enzyme expression in an antigen-specific manner.

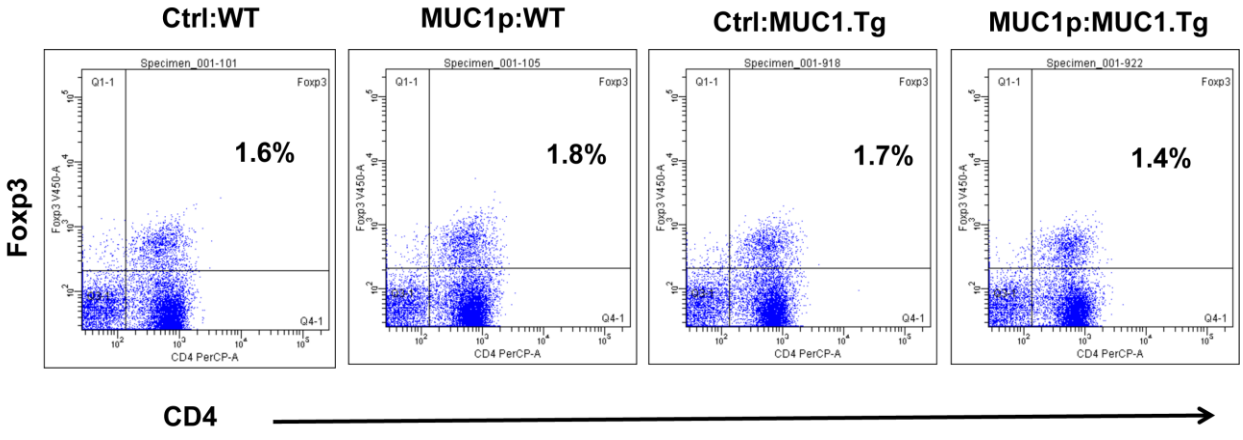

Figure 34 No difference in the frequency of splenic Treg post-immunization of WT and MUC1.Tg mice.

WT and MUC1.Tg mice (n=3) were immunized with MUC1p. 24h later, spleens were harvested for FACS analysis. Dot plots are representative of data from 2 independent experiments.

APPENDIX C

C.1 TRAFFICKING OF DC USED FOR VACCINATION

Given the importance of MUC1-bearing DC in initiating ensuing tolerance or immunity in the spleen, we were interested in determining which tissues they localized to 24h after i.v. immunization. The majority of DC were located in the spleen, as expected, while a small number were found in lung. Almost none of the transferred DC were detected in a non-draining LN (Fig. 35).

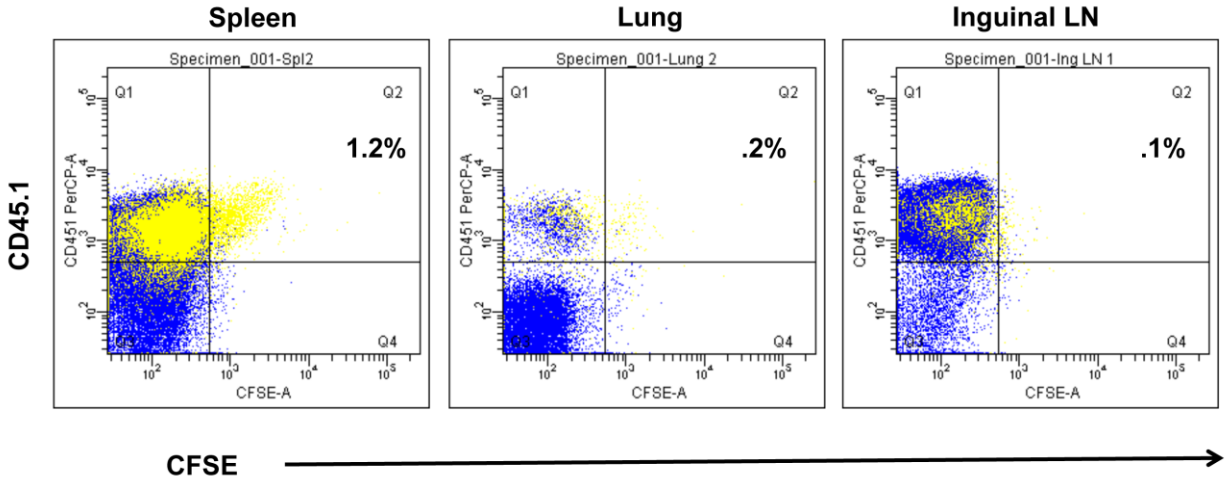

Figure 35 DC predominantly localize to the spleen 24h after intravenous transfer.

$1*10^6$ CFSE-labeled CD45.1 congenic BMDC were injected i.v. into MUC1.Tg (n=3) recipients. After 24h spleen, lung and non-draining (inguinal) LN were harvested and the percentage of DC determined by flow cytometry. Data are representative of 2 independent experiments.

C.2 MECHANISMS UNDERLYING GLOBAL CHANGES IN SPLENIC DC PHENOTYPE POST-IMMUNIZATION

As discussed above (Section 5.2) a small number of the DC used for vaccination induce global, phenotypic changes within the majority of the endogenous DC compartment. Our data suggests that a hallmark of this phenotypic shift is the suppression of trypsin and CPB1 expression in DC that is mediated by Treg, and likely MUC1p-specific Treg. The spleen of a typical 8-10 week old WT or MUC1.Tg mouse yields approximately 1.5-2*10^6 $CD11c^+$ cells after positive selection via MACS (data not shown). Based on Fig. 35, an immunization consisting of 1*10^6 unloaded DC results in the detection of just 1.2*10^4 DC by flow cytometry in the spleen 24h later. Therefore, vaccine DC bearing MUC1p constitute approximately .6% of the total DC compartment in immunized animals. There are several mechanisms by which this paucity of cells might be able to induce changes in the entire endogenous DC compartment. As referenced above, the vaccine DC might be taken up by resident APC resulting in the re-presentation of MUC1p directly to cognate effector and regulatory T cells (263). While this mechanism would represent a partial amplification step as far as increasing the number of DC that are presenting MUC1p, it is unlikely that the phagocytosis/endocytosis of 1.2*10^4 vaccine DC would result in all endogenous APC presenting MUC1p, and thus being tolerized by MUC1-specific Treg. To influence most resident cells, MUC1p must be presented by all splenic DC such that every DC interacts with a cognate Treg. Alternatively, relatively few DC:Treg interactions could occur with the resulting intracellular signals in DC being transmitted to the entire population. The widespread distribution of peptide antigen could occur via physical, tubular connections between DC, or by the shedding of MUC1-containing exosomes and their subsequent uptake, reprocessing, and presentation by recipient DC. Tunneling nanotubules

(TNT) are heterogeneous structures that allow transfer of both second messenger signaling molecules (i.e. Ca^{2+}) as well as small molecular weight compounds (i.e. Lucifer yellow) from an individual DC to others that are distal by as much as several hundred microns (204). Therefore, TNT are candidate structures for disseminating MUC1 peptides as well as intracellular signaling molecules Alternatively, the ability of pMHC-containing exosomes to transfer antigen to distal DC has also been demonstrated (281).

To begin to elucidate the mechanisms underlying global changes in DC phenotype we used multiphoton microscopy to examine the localization and interactions between the DC used for vaccination and endogenous $CD11c^{+}$ cells in live splenic explants. Figure 36 shows the endogenous DC from a CD11c-YFP mouse just beneath the splenic capsule at a depth of approximately 50 microns. The vaccine-derived DC are predominantly located just underneath the (more medullary) endogenous population at a depth of 70-80 microns.

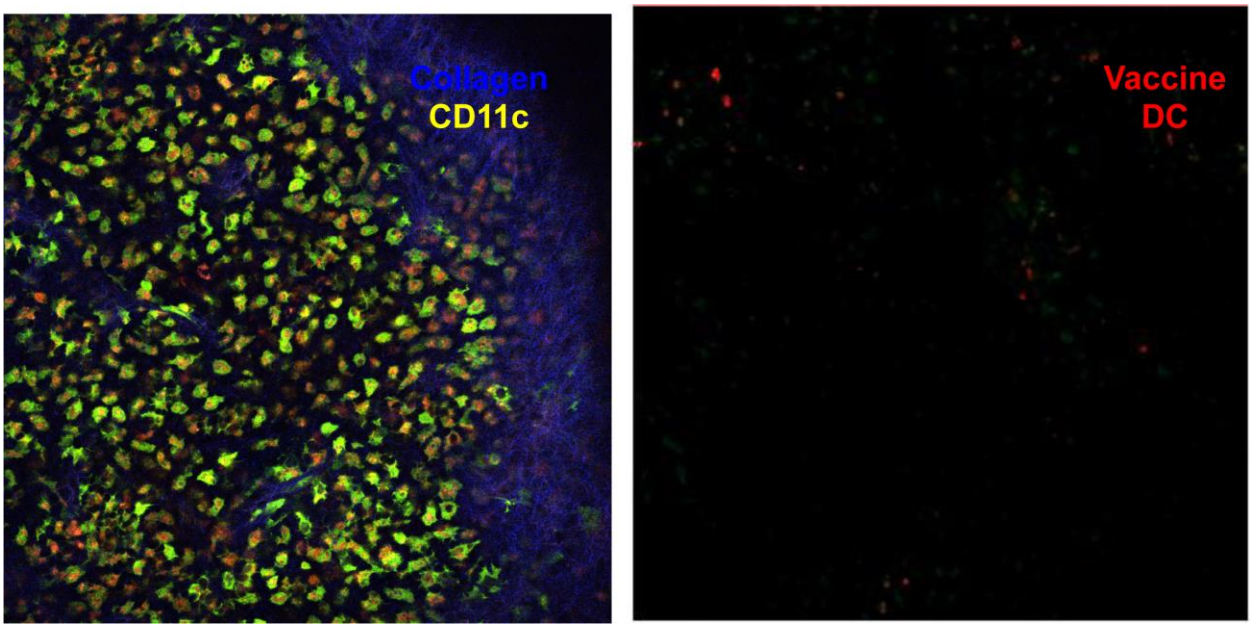

Figure 36 Localization of endogenous and vaccine-derived DC in a live splenic explant.

24h after injection (i.v.) of a CD11c-YFP (n=1) mouse with 2*`10^6 DC:MUC1p labeled with Cy3 , the spleen was explanted and incubated in 37° RPMI and perfused with 95% O2 Images were collected using a Nikon A1R multiphoton system and a 25X, water immersion objective. (A) Image depth approximately 50 microns. (B) Image depth approximately 70 microns. Data are from 1 experiment.

While many injected DC home to a discrete location just beneath the layer of subcapsular resident DC, there are also areas shared by the two populations. Here, there are clear indications that endogenous DC have internalized some, but not all vaccine DC (Fig. 38) supporting the notion that the cells used to immunize are not exclusively the cells that ultimately present antigen to T cells. The morphology of the injected DC is small and rounded in contrast to the larger and more spread shape of resident cells. This may be due to enhanced apoptosis in vaccine DC and/or cells that are in the process of recovery after transfer. It is important to note that immunization of CD11c-YFP mice with MUC1p constitutes vaccination with a foreign antigen rather than a self-antigen. However, the mechanisms by which the endogenous DC network

interact with vaccine DC is likely to be similar regardless of the nature of the antigen introduced. Downstream, however, the types of T cell that DC presenting antigen interact with, the resultant T cell response, and the phenotype of the DC induced by cognate T cells are all intimately linked to the nature of the immunizing antigen.

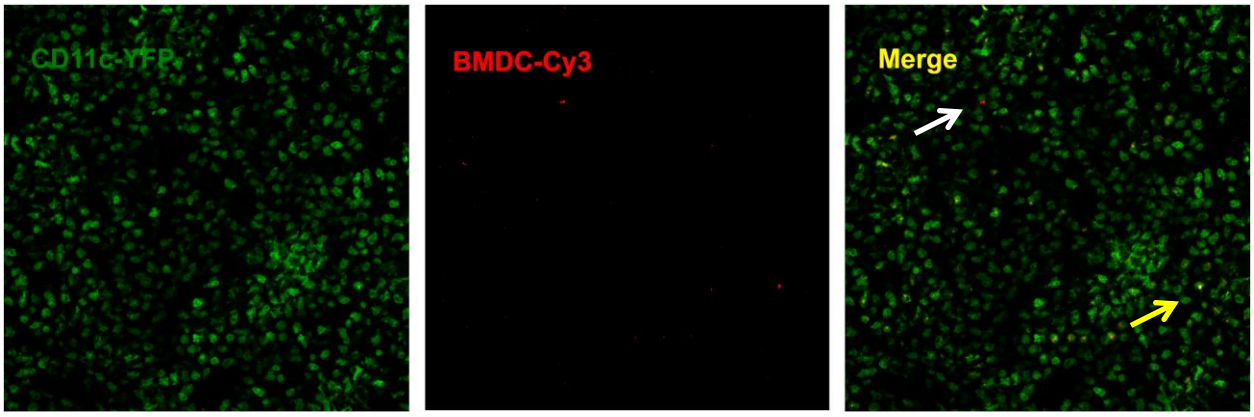

Figure 37 Vaccine-derived DC are internalized by endogenous DC.

24h after injection (i.v.) of a CD11c-YFP mouse (n=1) with $2*10^6$ DC:MUC1p labeled with Cy3 , the spleen was explanted and incubated in 37° RPMI and perfused with 95% O2. The yellow arrow indicates an endogenous DC that has internalized a Cy3-labeled DC used for i.v. immunization. The white arrow indicates a Cy3-labeled DC that has not been internalized. Images were collected using a Nikon A1R multiphoton system and a 25X water immersion objective. Data are from 1 experiment.

While many injected DC home to a discrete location just beneath the layer of subcapsular resident DC, there are also areas shared by the two populations. Here, there are clear indications that endogenous DC have internalized some, but not all vaccine DC (Fig. 37) supporting the notion that the cells used to immunize are not exclusively the cells that ultimately present antigen to T cells. The morphology of the injected DC is small and rounded in contrast to the larger and more spread shape of resident cells. This may be due to enhanced apoptosis in vaccine DC

and/or cells that are in the process of recovery after transfer. It is important to note that immunization of CD11c-YFP mice with MUC1p constitutes vaccination with a foreign antigen rather than a self-antigen. However, the mechanisms by which the endogenous DC network interact with vaccine DC is likely to be similar regardless of the nature of the antigen introduced. Downstream, however, the types of T cell that DC presenting antigen interact with, the resultant T cell response, and the phenotype of the DC induced by cognate T cells are all intimately linked to the nature of the immunizing antigen.

Further experiments are required to determine if MUC1 is physically transported from either population to distal cells via tubules or exosomes, or if the endogenous DC network broadcasts signaling molecules from a relatively few number of DC to the population as a whole. These questions are well suited to a microscopy approach, however their technical execution is not trivial. Multiphoton imaging has the advantage of being able to penetrate deeper into living tissue without the heat damage and photobleaching associated with confocal lasers. However, structural resolution and magnification are limited using the multiphoton objective so a combination of both techniques in living animals, explanted tissue, and fixed tissue sections will be required.

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