HUMAN TUMOR ANTIGEN MUC1 AS AN INDUCER OF DENDRITIC CELL MIGRATION AND DISTORTED MATURATION

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The immunostimulatory outcome of the interactions of many pathogens with dendritic cells (DC) has been well characterized. There are many fewer examples of similar interactions between DC and self-molecules, especially the abnormal self-proteins such as many tumor antigens, and their effects on DC function and the immune response. We show that human epithelial cell antigen MUC1 mucin, is recognized in its aberrantly glycosylated form on tumor cells by immature human myeloid DC as both a chemoattractant (through its polypeptide core) and a maturation and activation signal (through its carbohydrate moieties). Interestingly, MUC1 is chemotactic for immature DC but not for other cells of the immune system. Not only is this the first example of a tumor antigen that has chemotactic abilities but there are also no known receptors that are expressed uniquely on immature DC. However, we have determined that the MUC1 chemotactic receptor is a member of the chemokine receptor family based on its sensitivity to pertussis toxin, the formation of pseudopods upon ligand binding, and the Ca^{2+} flux in response to MUC1. Upon encounter with MUC1, similar to the encounter with LPS, immature DC increase cell surface expression of CD80, CD86, CD40 and CD83 molecules and the production of IL-6 and TNF-α cytokines, but fail to make IL-12. When these DC are co-cultured with allogeneic $CD4^+$ T cells, they induce production of IL-13 and IL-5 and lower levels of IL-2, thus failing to induce a type 1 response. Our data suggest that in cancer patients *in vivo*, MUC1 attracts immature DC to

the tumor through chemotaxis and then subverts their function by negatively affecting their ability to stimulate type 1 helper T cell responses important for tumor rejection.

Included with this dissertation are 3 movies (.avi files) on CD-ROM.

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I must have something to engross my thoughts, some object in life which will fill this vacuum, and prevent this sad wearing away of the heart.

Dr. Elizabeth Blackwell

I have a great deal of work, what with the housekeeping, the children, the teaching and the laboratory, and I don't know how I shall manage it all.

Dr. Marie Curie, letter to her brother, Jozef

I wanted to start with these quotes from two women who inspired me to go into medicine and science when I was young. The two desires they express, the need to occupy your mind and the need to live your life, are often pitted against one another. However, both of these scientists must have managed a sort of balance and the world is better off because of it. Of all the things that I learned from my mentor Dr. Olja Finn, I think the ability to strike a balance between these two forces is foremost not only because of its importance but also because of its difficulty. Olja, I would like to thank you for your instruction, your optimism, and your love. These have been the most intellectually invigorating years of my life because of your guidance and passion for the scientific process.

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1. Introduction

1.1. Immunosurveillance for tumor cells

1.1.1. Interaction between tumor cells and dendritic cells

Since Coley's observations in the early part of the 20th century, that an immune response to a fulminant bacterial infection can also eliminate an established tumor (1), medicine has been interested in the ability of the immune system to combat cancer. Cells of the innate and the adaptive immune system are capable of not only protecting an organism from external threats, but also controlling the internal health of the body by maintaining normal body homeostasis. Tumors are a double challenge to the immune system. They are both a "foreign" threat presented by the transformed phenotype of the tumor cells, as well as a "self" that has lost normal homeostasis. Although there is extensive evidence for tumor immunity as well as for success of immunotherapy of cancer, there are also many examples where the immune system can contribute to tumor growth. Furthermore, the cancer cells growing in the presence of immune cells can develop ways to circumvent normal immune functions. Dendritic cells, as coordinators of the immune response, are very important in ensuring tumor recognition by the immune system, but they can also be hijacked by the tumor for subversion of the anti-tumor responses. In this section, we will discuss the important role for DC in priming effective immune responses to tumors, the changes in tumor cells that can be recognized by DC leading them to alert the immune system to the presence of the tumor, and finally, how the tumor and the tumor microenvironment can divert DC function from what is good for the immune system to what is beneficial for the tumor.

1.1.2. Basic DC biology

DC are sentinels of the immune system. They are bone marrow derived cells that migrate and reside in virtually every organ of the body. In these peripheral sites they can take up antigens from the extracellular space as well as from dying and dead cells. In the steady state, these DC sample self antigens and after a certain period of time migrate to the draining lymphatics. In the lymph node, DC present antigens acquired in the periphery to cells of the adaptive immune system. The DC presenting self antigens generally do not stimulate an inflammatory response due to thymic negative selection of T cells specific for self antigens with the added protection that the DC lack high expression of co-stimulatory molecules. Due to the low expression of co-stimulatory molecules, self-reactive T cells that do escape negative selection would not be stimulated by these DC to proliferate and respond. Instead, the DC must have been activated in the periphery to express high levels of the appropriate co-stimulatory molecules and cytokines to induce antigen specific T cell activation and proliferation.

DC take up antigens through pinocytosis, phagocytosis and receptor mediated endocytosis. However, on immature DC, the majority of the MHC Class II molecules are not found on the cell surface. Instead they are found in intracellular vesicles in association with the molecules HLA-DM and H2-M that stabilize the Class II molecule in preparation for activation (2). Upon activation, peptide is loaded into the MHC Class II molecules and these complexes traffic to the cell membrane thus greatly increasing the surface concentration and preparing the DC to activate $CD4^+$ T cells. Before activation, there is still some MHC Class II on the surface but it most likely contains self peptides and is presented without concomitant increase in co-stimulatory molecules. There is very little MHC Class I on immature DC and the levels also increase on activated DC but largely through new protein synthesis (3).

There are distinct DC subtypes in both mouse and human. In humans, the main two subtypes found in circulation are the myeloid ($CD11c^{+}CD1c^{+}$) and the plasmacytoid DC ($CD123^{high}$, BDCA-2^{+}, BDCA-4^{+}). There are also DC that are derived *in vitro* from monocytes in humans and from bone marrow cells in mice. In general, *in vitro* derived DC are similar to *in vivo* DC but there are distinct differences in the expression of certain receptors and magnitude of cytokine production (4). In mice, the DC subsets are mainly grouped by their expression of CD11c, CD4, and CD8. There are differences between these subtypes in terms of their ability to induce T helper cell (Th) commitment, produce type I interferons and cross-present antigens (5). However, there is also strong evidence that there is a degree of plasticity in these DC so that they do not always simply induce one type of immune response. DC can respond to endogenous signals such as cytokines but also to signals from molecules from pathogens to tailor the type of stimulation to elicit.

1.1.3. DC cytokine production and T cell priming

The activation and polarization of Th cells into Th1 or Th2 cells is determined by three signals. First, the T cell must be stimulated through its T cell receptor (TCR) binding to the correct peptide in the MHC Class II molecule on the DC surface. This is termed signal one. Second, the T cell must receive co-stimulation through its CD28 molecule binding to CD80 and CD86 on the DC. This is termed signal two. Third, the DC gives a polarizing signal to the Th cell by becoming polarized itself in a reaction to the environment in which the DC was activated (6). This is termed signal three. DC that induce Th1 and Th2 polarization are termed DC1 and DC2, respectively. DC1 polarizing molecules include IL-12, IL-23, IL-27, type I interferons, and surface expression of ICAM-1 (7-9). DC2 polarizing signals include CCL2 and OX40 ligand (10, 11). Toll like receptors (TLRs) have also been implicated in determining the polarization of Th cells. DC that have been stimulated with LPS through TLR4 induce a Th1

response, while DC that have been stimulated with Pam3cys, a bacterial lipoprotein antigen, through TLR2 induce a Th2 response. Although both receptors use similar signaling machinery, they differentially modulate members of the MAP kinase family and hence result in different DC cytokine profiles (12). Activated NK cells have also been identified as strong inducers of DC1 phenotype (13). Conversely, DC that have bound immune complexes through their FcγR do not produce IL-12p70, an important Th1 cytokine, and induced T cells to polarize towards a Th2 phenotype (14). These two examples, NK cells and immune complexes, illustrate that the skewing of Th1 and Th2 subsets towards cell mediated and antibody based immune responses, respectively, gives positive feedback to one pathway and negatively regulates the other pathway. Even without the presence of MHC Class II on the tumor, $CD4^+$ T cells, especially in their production of IFN-γ, are important for activating other cells that can kill the tumors (15). Interestingly, evidence exists that DC instruct $CD8^+$ T cells how to home back to the original site of antigen capture (16). There are numerous reports that indicate that Th2 type T cell responses predominate over Th1 responses in cancer (17-22). In conclusion, the DC are critical in the formation of a strong helper response.

1.1.4. Need for effective DC in cancer

As discussed above, DC are uniquely able to prime naïve T cells (23). It is known that tumors express a variety of proteins either de novo or in aberrant forms that can be recognized by the adaptive immune system (24). To achieve immune stimulation DC must take up tumor antigens, migrate to the lymph nodes and present these antigens to T cells in an immunostimulatory context. Tumor-derived proteins can also be brought into the lymph node through the afferent lymphatics, where they can be taken up by lymph node resident DC. DC

sample antigens in their environment through receptor-mediated endocytosis or receptor independent pinocytosis. These antigens are either soluble products from tumor cells, or apoptotic or necrotic tumor cells. As will be discussed below, the origin of the antigen is critically important. For example, apoptotic and necrotic tumor cells can suppress or enhance DC maturation, respectively (25, 26). Both $CD8^+$ and $CD4^+$ T cells are required for an effective anti-tumor response (23, 27). Most of the experiments to date suggest that to achieve effective destruction of tumors, DC priming of naïve T cells should result in the activation of $CD4^+$ helper T cells of the Th1 type and expansion of $CD8^+$ T cells capable of direct tumor cell lysis (CTL) (27). DC elicit this type of immunity best in their fully mature state when they produce inflammatory cytokines such IL-12 and express high levels of co-stimulatory molecules.

Tumor antigen specific T cells are found in many cancer patients providing evidence that a certain level of T cell priming does take place during the growth of the tumor (28). However, since the frequency of such cells is usually low and the cancer progresses in spite of their presence, the whole process of T cell priming is considered to be sub-optimal in cancer patients. A whole new field of research has developed toward understanding the reasons for the lack of sufficient T cell help and how this may be improved by ensuring an optimal DC function. These studies have lead to the development of approaches that use *in vitro* manipulated DC as components of vaccines for cancer (29, 30).

DC are also important in the activation of innate immunity. This activation occurs at the tumor site and can be enhanced by DC maturation or activation in response to endogenous danger signals released by the tumor, molecules such as high mobility group box 1 protein (HMGB-1) or uric acid, which will be discussed in more detail below (31-33). Most notably, DC have been shown to activate NK cells that are central to anti-tumor responses. With many

tumors downregulating MHC class I molecule expression as a means of escape from adaptive immunity, it is important to be able to activate NK cells that do not require class I molecules to exert anti-tumor effects (34). DC-derived IL-12 activates both NK and NKT cells to proliferate, produce IFN-γ, and become cytotoxic effector cells (35, 36). DC can deliver indirectly the "help" message to T cells by inducing NK cell cytokine production (13, 37, 38). Furthermore, DC can enhance and direct the migration of other cells of the innate immune system into the body of the tumor by producing chemokines such as CXCL8, CCL4, CCL17, and CCL22 (39).

1.1.5. DC defects in cancer patients

1.1.5.1. Inhibition of maturation and differentiation

There are a variety of mechanisms underlying tumor escape from immune recognition and destruction (40) and many of these involve subverting DC function. Although cancer is associated with chronic inflammation (25, 41-43), once the tumor is established, patients have widespread immunosuppression with fewer circulating DC (44-47). The loss of DC seems to be more pronounced in the myeloid than in the plasmacytoid DC compartment (46, 47). Removal of the tumor can sometimes restore the number of circulating myeloid DC (44, 46, 47). DC can be found in rapidly growing tumors where they usually do not express co-stimulatory molecules CD80 and CD86 and have reduced T cell stimulatory activity (48-51). Tumors can thwart DC development and function by 1) blocking the development of mature and functional DC; 2) increasing the number of immature DC that cannot upregulate MHC class II, co-stimulatory molecules, and inflammatory cytokine production; and 3) inducing production of Gr-1^+ immature myeloid cells (myeloid suppressor cells) (40, 52). Tumors make TGF-β, IL-10, PGE_2, VEGF and other molecules that exert their effects on DC and other immune cells (53). For example, PGE_2 through its receptor EP2 inhibits DC differentiation and function (54, 55).

The recent observation of an abundant population of cells in cancer patients, termed immature myeloid cells (ImC), has helped explain in part the low number and impaired function of DC in some cancer patients. ImC represent an early stage of myeloid and hence DC differentiation. These cells are also found in mouse models of tumors (40, 56). Tumors alter hematopoiesis by the production of factors that induce ImC (57, 58). ImC express Gr-1, CD11b, and low levels of MHC class II and low to no co-stimulatory molecules (59, 60). The induction of Stat3 in myeloid cells is one of the ways that tumors induce this cell population (61). The presence of ImC is bad for anti-tumor immunity not only because it represents the failed development of DC but, in addition, these cells are capable of suppressing IFN-γ production by CD8+ T cells in an antigen specific manner (44).

Another newly discovered reason for insufficient DC in cancer patients is the VEGF-induced recruitment of immature DC to become endothelial cells (62). This is also of interest for basic DC biology because DC have been considered terminally differentiated cells, which is not supported by this observation. This, along with another recent observation that immature DC can become osteoclasts (63), suggests that under certain conditions, such as those present in cancer patients, immature DC can transdifferentiate into other cell types.

The production of VEGF is another way that cancer cells prevent maturation of DC. VEGF causes dysfunctional maturation of DC (*in vitro* and *in vivo*) through impaired NF-κB activation (64-66), which can be corrected with antibodies against VEGF (67). Furthermore, activation of Stat3 in tumor cells is implicated in upregulation of IL-10 and VEGF, and impaired maturation of DC resulting in T cell tolerance (68). Tumors also make NO, PGE_2, IL-10, and IFN-α, IL-12 p40 homodimers and TGF-β, all of which can lead to the generation of tolerogenic or regulatory T cells (69, 70). Lastly, tumors can induce the expression of molecules on the DC

that are negative regulators of co-stimulation, such as B7-H1 (71) that selectively induces IL-10 production in resting T cells. It can also induce apoptosis of activated T cells (72).

1.1.5.2. Influence on migration

Because the composition and number of DC at the tumor site is often different from that in normal tissue, it is clear that tumors also alter the ability of DC to migrate to and from the tumor environment. Overall, there is an increase in immature DC within the tumor mass as opposed to mature DC that are rarely found within the tumor. Instead, mature DC are found in the peri-tumoral area (73). There are a number of chemokines produced by tumor cells that can attract immature DC, such as CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL20 and CXCL12. Many of these chemokines are not specific for DC and can attract a variety of immune cells to sites of inflammation (74).

Some tumors are able to selectively attract certain DC subsets. This is clear in the case of ovarian cancer, where there is an increase in plasmacytoid DC at the tumor site. This is thought to occur as a result of the production of CXCL12 (SDF-1) chemokine acting on the CXCR4 receptor on plasmacytoid DC (75). Plasmacytoid DC, in the absence of strong activation through type I interferons or toll receptor ligands, promote development of regulatory $CD8^+$ T cells (76). These $CD8^+$ T cells display poor secondary proliferation and cytolytic function, due in large part to the production of IL-10. They also inhibit activation of bystander naïve $CD8^+$ T cells (76).

1.1.5.3. Suppression of function

Another recently documented mechanism by which DC from cancer patients induce weak T cell activation is altered tryptophan catabolism through IDO (indoleamine 2,3-dioxygenase)

production. IDO is an enzyme that catabolizes tryptophan in an oxidative reaction. Since tryptophan is an essential amino acid, depletion of tryptophan from the local environment by IDO produced by tumor cells and/or tumor resident DC (77) limits proliferation of immune cells in the tumor area. It has also been postulated that the toxic metabolites of tryptophan catabolism, such as quinolinic acid, kynurenine, and 3-hydroxy-anthranilic acid, may play a role in immunosuppression of T cell proliferation (78-80). Interestingly, tumors have hijacked production of IDO as an immune response inhibitor from normal cells involved in immune response regulation. IDO is expressed in subsets of normal myeloid cells, including mouse $CD11c^{+}CD8a^{+}$ DC and plasmacytoid DC, and a significant portion of *in vitro* monocyte-derived human DC (81-83). IDO expression by DC correlates with weak T cell proliferation in mixed leukocyte reactions, enhanced apoptosis, and weak responses by TCR transgenic $CD8^{+}$ T cells (82, 84, 85). This effect can be overcome by the addition of 1-methyl-tryptophan or excess tryptophan *in vitro* (86).

Treatment of DC with IL-10, which is produced by a majority of cancer cells, induces T cell anergy (87, 88). T cell anergy is a state where the T cells become unresponsive to secondary stimulation through the TCR even in the presence of co-stimulatory molecule engagement. Anergic T cells can be generated through the lack of co-stimulation or the presence of regulatory cytokines, such as IL-10, during primary stimulation (89). The preponderance of immature DC in cancer would be expected to block T cell stimulation since they have been shown to induce T cell anergy *in vitro* and *in vivo* (90-92). Finally, the tumor specific T cells interacting with DC at the tumor site may be prevented from forming an effective synapse because of the lack of a proper environment (93). Whereas in the lymph node, the architecture is supportive of cell-cell interactions, the large number of stromal cells and high concentration of collagen and

extracellular matrix molecules at the tumor site can prevent the close interactions needed between immune cells. In addition to these specific mechanisms identified to date, there is also a frequently observed general downregulation of the various components of the antigen processing and presentation machinery in DC under the influence of tumor cells (94-97).

Understanding how tumors drive DC dysfunction is vital since preventing or overcoming the DC dysfunction has the potential to lead to tumor rejection. The goal is to understand what mechanisms that the tumor uses to suppress the endogenous immune response and how these can be manipulated to improve the DC function. Another goal is to understand what are the limits of function for DC that have differentiated in the immunosuppressive environment of the tumor. These are the DC that are called upon to respond to cancer vaccines administered to patients with cancer. Effective use of therapeutic vaccines will involve measures that can overcome tumor-induced DC dysfunction.

1.1.6. Do DC recognize cancer cells as abnormal?

The immune system must discriminate between many different stimuli and determine whether to respond by activation of effector cells or establish a state of tolerance and/or anergy. DC appear to play a pivotal role in these decisions by being able to assess, through a variety of cell surface receptors, different stimuli from the tissue environment, and to process other contextual information such as the molecular characteristics and relative concentration of the stimuli (23, 98). Multiple changes in tumor cell protein profiles occur during carcinogenesis. How these altered proteins and other molecules affect DC and the subsequent tumor specific response is a new and growing area of investigation.

There are several examples of proteins released from tumor cells that are recognized by DC. Stress proteins such as heat shock proteins are released during periods of high cell turnover, as seen during tumor growth. It is known, for example, that DC express CD91 which is ligated by the heat shock protein gp96 released by necrotic cells (99-101). By binding to CD91 and activating DC, gp96 sends a danger signal to the immune system. Similarly, HMGB1 and uric acid are released by necrotic cells and represent internal danger signals. The receptors for HMGB1 on the DC are toll-like receptor 2 (TLR2) and RAGE (receptor for advanced glycated end-products) (31, 102). HMGB-1 function is associated with DNA "bending" and when released from necrotic cells it induces maturation of DC (31). Uric acid, which exists in the extracellular environment in the form of monosodium urate, is also perceived as a danger signal by DC that can then transmit that activation signal to the immune system (103).

Proteins can also be differentially posttranslationally modified in tumor cells compared to normal cells and this difference can alert the DC. For example, due to changes in the levels of various glycotransferases, tumor cells can add fewer and/or different sugars to glycoproteins or oversialylate the sugars that have been successfully added. Lower levels of glycosylation expose peptide epitopes that on normal molecules are hidden by large carbohydrate side chains. Tumor specific carbohydrate moieties, such as the Thomsen-Friedenreich (β-Gal-[1->3]-α-GalNAc-O-serine) antigen (TF), also known as the T antigen, and the Tn antigen (α-GalNAc-O-serine/threonine), are recognized by antibodies as tumor markers. Furthermore, these tumor-specific carbohydrates can bind to and crosslink receptors on the surface of immune cells, especially DC and other cells of the innate immune system.

Recognition by DC of new carbohydrate structures on transformed cells can serve as a danger signal to the immune system. One example is recognition by DC of the MUC1 mucin

that is overexpressed on tumor cells and aberrantly glycosylated with tumor specific sugars such as T and Tn described above. It has been shown that tumor forms of MUC1 bind to receptors on the surface of cells of the innate immune system, such as the mannose receptor, sialoadhesin (Siglec-1), and Gal/GalNAc-specific calcium dependent lectins (104-106). Tumor MUC1 can alter the differentiation of monocytes into DC *in vitro* (107). Monocytes co-cultured with MUC1+ tumors or their supernatants in the presence of GM-CSF and IL-4 become DC that express low levels of co-stimulatory molecules and increased IL-10 production. These DC do not make high levels of IL-12 and induce greater number of IL-4 producing $CD4^+$ T cells (107).

Gangliosides are another family of molecules aberrantly expressed by cancer cells. Gangliosides are sialic acid-containing glycosphingolipids that are components of the plasma membrane. Gangliosides produced by tumor cells differ from those on normal cells. For example, GM3 is the only ganglioside made by normal melanocytes but melanoma cells produce a wide variety of gangliosides (108, 109). These tumor gangliosides have been shown to bind to siglec receptors (110) and impair DC development and function (111). DC matured in the presence of the ganglioside G(D1a) showed decreased levels of co-stimulatory molecules CD40 and CD80 and impaired production of inflammatory cytokines IL-6, IL-12 and TNF-α and were also poor stimulators of T cell proliferation (112).

1.1.7. Summary

Optimal maturation and function of DC are critically important for the development of strong and long-lasting tumor immunity. It is clear from the examples we discussed above, that certain changes that occur during malignant transformation can serve as danger signals picked up by DC and that the DC respond to these changes. However, a stronger immune response is not always the final outcome. The weakened overall immune response in cancer patients appears to

be mostly a result of mechanisms that a tumor develops to divert the DC away from their proper function. Tumors block DC development and suppress DC maturation. Tumors express chemokines that draw immature DC into the suppressive tumor environment. They can even recruit DC to trans-differentiate into endothelial cells as the tumor promotes angiogenesis. They can also block the expression of MHC molecules and co-stimulatory molecules that are important for the induction of a T cell response. By inducing DC to produces cytokines and molecules that block the type 1 immune response, cancer cells prevent the few tumor-specific T cells that do get stimulated from being effective against the tumor. With such a large arsenal of DC subverting mechanisms, it is clear that the stimulation of the immune system against cancers, through such strategies as those discussed, must occur before the tumor overwhelms the body's ability to protect itself.

1.2. Danger signals and pattern recognition receptors

1.2.1. Recognition of molecular motifs as abnormal

Pattern recognition receptors (PRRs) are germline encoded receptors that bind to specific conserved patterns on molecules (113). Originally, Janeway proposed that these patterns recognized by PRR were specifically from pathogens and that these patterns were not found in endogenous molecules (114). However, a second hypothesis was put forward by Matzinger that these PRRs had evolved to recognize "danger signals" from normal cells that were undergoing stress or abnormal death (115). There is strong evidence that both of these hypotheses are correct and yet not mutually exclusive as several of the PRRs can bind to both pathogen derived molecules and host derived molecules. In either case, DC and cells of the innate immune

system play dual roles as protectors against invaders and normal host homeostasis and hence must be able to recognize the differences and similarities between these two groups.

1.2.2. Families of PRRs and their functions

PRRs are categorized into families based on similarities in their structure. The three major families of PRRS are siglecs, C-type lectins, and Toll-like receptors (TLRs). These receptors are expressed on a variety of cells. Cells of hematopoietic origin express very high levels of PRRs as well as a large diversity of types of receptors. There are certain profiles of receptor expression that are characteristic of cells and can depend upon the state of the cell. For example, DC express higher levels of PRRs than T cells and the different DC subsets express a different group of receptors. Furthermore, most PRRs are exclusively found on either mature or immature DC. The three major families of PRRs will be discussed below.

Siglecs are members of the immunoglobin superfamily (I-type lectins) that bind to sialic acid residues with high specificity. There are at least 11 family members in humans that are expressed on a variety of cells including monocytes, macrophages, NK cells, dendritic cells, B cells, and T cells (116). Siglecs all possess a NH_2 terminal Ig domain which contains the sialic acid carbohydrate recognition domain (CRD) (117). Siglec-1, or sialoadhesin, lacks a cytoplasmic signaling region. However the other members of this family have signaling capabilities and contain immunoreceptor tyrosine-based inhibitory motifs (ITIM) (118). ITIM motifs are mainly found in the families of I-type and C-type lectins and block cell activation by promoting dephosphorylatioin reactions through the inhibitory phosphatases SHP-1 and SHP-2 (119). CD83, which is also known as a marker of mature DC, is also a sialic acid binding receptor, however it is an atypical member of this family (120). The ability of Siglecs to bind

sialic acid is sometimes prevented by masking of the sialic acid binding site by interactions with sialic acid containing proteins on the same cell surface as the siglec (117).

The C-type lectin family is made up of 4 subfamilies: the macrophage mannose receptor (MMR) family, the NK receptor family, the Type II receptor family, and the selectins (121). Ligand binding by C-type lectins is Ca^{2+} dependent. These receptors bind to carbohydrates through their carbohydrate recognition domains (CRDs). Each receptor and each CRD within their extracellular domain has a specificity of binding to certain types and arrangements of sugars. For example, although the C-type lectins mannose receptor and DC-SIGN can both bind mannose, mannose receptor preferentially binds to single mannose residues while DC-SIGN binds to more complexly configured mannose residues. There is also some evidence that the CRD domains might be able to bind lipids and proteins in addition to carbohydrates (122).

Several members of these families have been shown to function as recognition and endocytic receptors on cells of the innate immune system. Mannose receptor and DEC-205 (CD205) are members of the MMR family and function as endocytic receptors. DC-SIGN and BDCA-2, members of the Type II receptor family are also endocytic. Mannose receptor has been shown to deliver antigens to early endosomes and recycle to the surface whereas the other three receptors deliver proteins to late endosomes. C-type lectins can form oligomers on the cell surface and thereby increase and change the avidity of binding to ligands (123, 124). Mannose receptor can be shed from the cell surface of human monocyte-derived DC, especially immature DC (125). As discussed above, there is a difference in C-type lectin expression between subgroups of DC. For example, there are many more C-type lectins expressed by monocyte derived DC than by circulating DC (126).

PRRs can recognize pathogen derived molecules such as β-glucan from fungus and HIV but they can also recognize self molecules (127-131). Mannose receptor can recognize lysosome hydrolases and thyroglobulin (132, 133) and DC-SIGN can bind both ICAM-2 and ICAM-3 (134, 135). Mannose receptor engagement has been implicated in the upregulation of cytokines, lysosomal enzymes, reactive oxygen species, and arachidonic acid metabolites (136). The use of antibodies to these receptors that bind but do not induce signaling can increase the processing and presentation of antigens bound to the antibody and increase the number of antigen specific T cells (137).

Members of the lectin family can also synergize or antagonize another family of pattern recognition receptors, the TLRs (138-142). Toll family members mainly bind to motifs expressed by pathogens and induce strong inflammatory signals to the cells. Lectins and TLRs can bind the same molecules although they might deliver differing signals to the cell. There is a hypothesis that binding to lectins alone induces immunosuppression but that binding to both lectins and TLRs induces immune activation (139, 143). There is also some evidence in macrophages that the combination of the uptake of apoptotic bodies in combination with TLR ligands can actually suppress the upregulation of IL-12 and TNF-α seen with TLR ligand alone (144, 145).

In human DC the myeloid and the plasmacytoid DC have mutually exclusive expression of several TLRs. Myeloid DC express TLR2, TLR3, TLR4, TLR5, TLR6, and TLR8 while plasmacytoid DC express TLR7 and TLR9 (146). TLR7 and TLR9 are expressed by plasmacytoid DC but are generally not expressed by myeloid DC. Plasmacytoid DC, but not myeloid DC, lack TLR4 and hence do not respond to LPS through that pathway. Most TLRs are expressed on the cell surface, but TLR7 and TLR9 are expressed mainly in endosomes. The

intracellular localization of these receptors may have evolved to have better access to virus that replicate within the cell. Signaling through TLRs 3, 7, and 9 can induce cross-presentation in DC but TLR4 and TLR2 do not (147). TLRs signal through two basic pathways. One pathway is via the MyD88 adaptor protein and the second pathway is through the Toll-IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) molecule and the IFN regulatory factor-3 (IRF-3) transcription factor (148). Both pathways lead to the activation of NF-κB and its translocation to the nucleus.

1.2.3. PRRs can suppress APC function

Many of these receptors, when appropriately triggered, can induce either pro-inflammatory or anti-inflammatory signals. One example of this is the mannose receptor, CD206. Mannose receptor has been traditionally known as an important endocytic receptor that greatly enhances the presentation of proteins that it binds by DC. However, mannose receptor can function as a receptor for distorted maturation. When bound by the anti-mannose receptor antibody PAM-1, DC display markers of maturation such as expression of CD83 and migration towards CCL19, a CCR7 ligand. However they make an array of cytokines that render the DC unable to polarize $CD4^+$ T cells towards a Th1 response (149, 150). Also, mannose receptor, when bound by *Mycobacteria tuberculosis*, can inhibit LPS induced IL-12 production (151).

Other PRRs have also been implicated in blocking activation of DC through various pathways. The C-type lectin DEC-205 has been used to target antigens for immunization to DC *in vivo*. However, targeting an antigen to DEC-205 on DC resulted in the induction of tolerance to that antigen (152, 153). Also, activation of $CD4^+$ T cells with anti-CD3 and anti-CD46, a pattern recognition receptor, induced the differentiation of T cells into Tr1 IL-10 producing T cells (154). Engagement of CD33 (Siglec-3) with anti-CD33 antibody on monocytes and myeloid precursors blocks the generation of DC (155). The addition of antibodies for siglec-8 to

eosinophils results in increased apoptosis (156). All of these examples show that simply binding to a PRR does not result in DC activation and a strong inflammatory response. On the contrary, it seems that PRR signal along a continuum of levels that determine whether the DC will induce Th1, Th2, or T regulatory cells once they reach the lymph node.

1.2.4. Motifs on tumor cells that can be recognized by PRRs

As mentioned above, many of the molecules expressed by tumors are different from normal cells. Also, PRRs can bind to and respond to self proteins. So the question becomes, are there abnormal tumor markers that can bind to PRRs and alert the immune response to tumors. With many of these molecules, the consequences of PRR engagement are still unknown. For example, the tumor antigen MUC1 binds to the mannose receptor and although it is known that this subverts its endocytic processing, it is unknown how this binding changes the phenotype of the DC. However, it is clear that these molecules can either positively or negatively regulate DC activation and that the sum of these tumor molecules will determine the overall response.

Tumor derived molecules can suppress the immune response to tumors as exemplified by gangliosides. Gangliosides are recognized by cells of the innate immune system and function in suppression of the anti-tumor response. Gangliosides are tumor antigens that are sphingolipids and are present on tumors that arise from the neuroectoderm. The pattern of gangliosides shed from normal cells and malignant cells differs significantly(157). Gangliosides can bind to members of the siglec family through their sialic acids (110). Gangliosides have been shown to inhibit the development of human monocytes into DC *in vitro* (112, 158). DC differentiated in the presence of gangliosides have a decreased ability to uptake and present antigen and to stimulate T cells (112, 158). Monocyte activation is also inhibited as well as their ability to stimulate T cells (112). When produced by renal cell carcinomas, gangliosides can induce the degradation of NF-κB in T cells (159). The ganglioside GD3, can inhibit NK cell cytotoxicity

through its interaction with siglec-7 (160). Interestingly, although many gangliosides were capable of inhibiting the function of PBMCs (161, 162), not all gangliosides inhibited DC differentiation (157). The inhibition of PBMC function was largely dependent upon the carbohydrate side chains and the length of the underlying ceramide structure (161, 162).

Other tumor molecules can activate the immune system. These include heat shock proteins and uric acid. The function of heat shock proteins (HSPs) is to ensure the proper folding of proteins intracellularly. However, when a cell dies, the HSPs are released along with the proteins that are bound to them. HSPs can bind to endocytic receptors, such as CD91, that can activate DC and also increase the uptake of proteins bound to the HSPs. Uric acid is released from cells undergoing necrotic cell death but not apoptosis. Maturation is enhanced by uric acid and in turn enhances the DC's ability to stimulate T cells (32, 163, 164). Further work has shown that tumor cells undergoing rejection have elevated levels of uric acid removing uric acid slowed the rejection process (33).

1.2.5. Summary

DC are poised to sense and respond to their environment through pattern recognition receptors. These receptors bind to a variety of ligands present on pathogens and endogenous molecules. Although there is some controversy as to the evolutionary drive behind these receptors, it is clear that they have the capability to direct the immune response. The three main families of PRRs are siglecs, C-type lectins and TLRs. Their expression is determined by the cell type and cell's state of activation. Sometimes their signaling can synergize and sometimes they antagonize each other. Tumors express molecules that bind to PRRs, however it is not yet known what signal many of these ligand-receptor interaction give to the immune system.

1.3. Chemotaxis

1.3.1. Normal DC chemotaxis

Normal trafficking of DC involves the movement of DC into peripheral tissue and upon antigen uptake, the migration of DC to secondary lymphoid tissue to stimulate naïve T cells. DC migration is controlled by chemokines and their receptors as well as adhesion molecules. The array of chemokine receptors expressed by DC changes in response to their maturation state. No one chemokine has been implicated in the movement of DC from the bone marrow to the peripheral tissues and/or how extravasation occurs for DC. However, in mice, it has been shown that immature but not mature DC migrate to a site of inflammation beginning at 6 hours and that this migration is dependent upon ligands for endothelial selectins (165, 166). Chemokine receptors that are on immature DC are down-regulated upon maturation by reduction of receptors at the membrane and by reductions in mRNA expression (167-170). CCR7 is critical for the migration of DC to secondary lymphoid tissue, which produce the CCR7 ligands CCL21 and CCL19, and is only present upon mature DC (168, 169). Another example of a chemokine receptor that is differentially expressed on immature and mature DC is the fMLP receptor, which is only expressed on immature DC. Some receptors are expressed on both immature and mature DC, such as C5aR (171).

Chemoattractants include formyl peptides (172, 173), proteins from the complement pathway(173), lipid metabolites (174), and the chemokine superfamily. Within the chemokine superfamily there are subgroups based on the presence of cysteine motifs: the CXC, CC, C, and CX_3C chemokine subgroups (also known as the α, β, γ, and δ subfamilies, respectively) (175-177). The CXC family is subdivided into ELR (glutamic acid/leucine/arginine) and non-ELR containing groups (178). In general, ELR containing chemokines attract neutrophils while non-ELR CXC chemokines attract lymphocytes (175, 179). Two chemokines have been identified that have mucin-like domains, CXCL16 and CX_3CL1/fractalkine, although the mucin-like

domains are not thought to contribute to their chemotactive properties (178). It is interesting to note that these two chemokine can also act as adhesion molecules in their membrane bound form and when cleaved from the cell surface, they act as chemokines. Finally, the enzyme, TACE, which is known to cleave MUC1 from the cell surface, also acts to cleave CX_3CL1 from the cell surface (180, 181).

Chemokines can generally be thought of as being of two families in terms of function. Homeostatic chemokines are important for the normal homing of DC from the bone marrow to the tissue and then from the tissue to the secondary lymphoid organs. Inflammatory chemokines are induced by microbes or cellular damage and draw cells from the surrounding areas and circulation to sites of damage. Some chemokines belong to both groups and are called "dual function" chemokines (182). Chemokines can bind to glucosaminoglycans, part of the extracellular matrix, that stabilize them for function *in vivo*. Without this binding site, many chemokines fail to function. Chemokine function is also regulated by extracellular proteases such as matrix metalloproteases and elastases (182). Cleavage of chemokines by proteases can lead to antagonist formation, block of function, or enhancement of function. Chemokines can also act as antagonist of other chemokines. For example, ligands for CXCR3 are antagonists for CCR3 (183).

DC constitutively express CCL22 (MDC), CCL15 (MIP-1γ), and DC chemokine 1 (DC-CK1) (184-186). In response to TLR activation and other inflammatory signals, DC can make CXCL8, CCL3, CCL4, CCL5, and CXCL10 and there seems to be some specificity in which of these are induced depending upon the TLR that is stimulated (170, 187, 188). Mature myeloid DC, but not plasmacytoid DC, can also make high levels of CCL22 and CCL17 (TARC) (39, 189). The production of CCL17, CCL18, CCL19 and CCL22 by mature DC functions to attract

T and B cells (190-192). DC, in general, are excellent chemokine producers and can produce log-fold greater amounts of chemokines than many other cells (189).

1.3.2. Chemokine receptors

Chemokine receptors are G protein-coupled seven-transmembrane receptors. The N-terminus is extracellular and, in addition to the seven transmembrane domains, there are three intracellular loops and three extracellular loops. The C-terminus is intracellular and contains serine and threonine phosphorylation sites. The chemokine receptors can also function in other capacities besides chemotaxis. For example, CCR4 and CCR5 serve as co-receptors for HIV (177). These receptors are usually sensitive to inhibition by pertussis toxin. Amongst chemokine receptors, there is a phenomenon known as heterologous desensitization. This basically means that stimulation through one chemokine receptor can desensitize the cell to stimulation through another chemokine receptor for a period of time (193). Chemokine receptors also undergo homologous desensitization when stimulated. There is also evidence for other receptors affecting chemokine function. For example, Partida-Sanchez *et al.* showed that CD38 on DC is critical to the function and Ca^{2+} flux of many chemokine receptors (194). There is also a great deal of promiscuity in terms of ligand binding as many of the chemokine receptors have multiple binding partners.

The reception of a chemotactic signal results in the formation of a pseudopod at the leading edge of the cell and a uropod at the retracting edge of the cell (195). While the pseudopod is being formed at the leading edge of the cell, the formation of pseudopods at the lateral edges is repressed. Unstimulated DC lack polarity and although there is a constant movement of the cell membrane termed “membrane ruffling,” there are no true pseudopods present. The majority of

studies in eukaryotic cell chemotaxis have been studied in neutrophils. The description below is based on neutrophil chemotaxis but, in general, should be representative of signaling in the DC as well. The leading edge pseudopod is enriched in actin filaments while the uropod is enriched in myosin filaments. Even when actin polymerization is blocked, the chemokine gradient is still recognized as shown by the localization of phosphatidlyinositol-3,4,5-triphosphate and its regulating enzymes at different membrane regions relative to the gradient (196). Although most chemokine receptors are G protein-coupled, the chemokine-chemokine receptor interaction can also activate pathways independent of G proteins such as Ca^{2+} flux, receptor phosphorylation, MAPK pathway activation and STAT pathway activation (197-199). Once a chemotactic signal is received, several proteins that contain a pleckstrin-homology (PH) domain are recruited from the cytosol to the plasma membrane at the leading edge (196, 200, 201). The binding of PH domain containing proteins, such as protein kinase B/Akt, results in the local accumulation of phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-triphosphate. Rho-family GTPases, especially Rac and Cdc42, play a role in inducing actin polymerization. Myosin is regulated by a kinase, myosin regulatory light chain kinase (MLCK), which is in turn activated by Ca^{2+} and Rho-stimulated kinase (ROCK) (195).

Human myeloid DC express a variety of chemokine receptors summarized in Table 1. The expression of these receptors changes with maturation. Even though some receptors are expressed on both mature and immature DC, this does not always mean that they are functional on both. For example, CXCR4 is on immature and mature myeloid DC but was recently found to only function on immature DC. There are few chemokine receptors that are known to be present and functional on plasmacytoid DC. They respond to a distinct set of cytokines from myeloid DC as they are not typically found in peripheral tissue like myeloid DC. The

plasmacytoid DC do not respond to inflammatory chemokines but do respond to the lymph node homing chemokines. One plasmacytoid DC chemokine receptor is ChemR23. ChemR23 is also expressed on human myeloid DC but not on Langerhans cells. ChemR23 is bound by the chemokine chemerin and can induce the trans-endothelial migration of plasmacytoid and myeloid DC (202). Another chemokine receptor on the plasmacytoid DC is CXCR4 and plasmacytoid DC respond to its ligand CXCL12.

Table 1: Chemokine receptor expression on human dendritic cells

Chemokine Receptors on Immature DC	Chemokine Receptors on Mature DC
CXCR1 (170, 203)	CXCR4 (170, 205)
CXCR2 (203)	CXCR5 (190)
CXCR4 (170, 203-206)	CCR2 (213)
CCR1 (170, 203, 204, 206)	CCR4 (184)
CCR2 (170, 203, 204)	CCR7 (168-170, 214, 215)
CCR3 (204, 206)	Platelet-activating factor receptor (PAFR) (174)
CCR4 (170, 204)	C5aR (171)
CCR5 (170, 203-206)	
CCR6 (169, 207-212)	
Platelet-activating factor receptor (PAFR) (174)	
Formyl peptide receptor (171)	
C5aR (171)	

1.3.3. Modulation of DC chemotaxis by tumors

The effects of tumors on DC migration was briefly discussed in section 1. In addition to the chemokines discussed there, chemerin that attracts plasmacytoid DC, was initially discovered in tumor cells. It is not known if chemerin acts *in vivo* at the tumor site but this is likely to be the case (216).

DC chemotaxis could also be downregulated at tumor sites. Several cytokines and prostaglandins can inhibit DC migration. DC exposed to IL-10, IL-4 and the prostaglandin $PG(D)_2$ did not upregulate CCR7 and failed to respond to inflammatory chemokines (217-219). Furthermore, having more DC at a tumor site might seem to be helpful to the anti-tumor immune response since they could ingest and present tumor-derived antigens, but this is not always true. In fact, when the potent DC chemokine CCL20 (MIP-3α) was transfected into a tumor cells that were transplanted into rats, the tumors actually grew faster despite a huge influx in immature DC (220). Without a strong activating signal, these DC remained immature and failed to induce a strong anti-tumor response. CCL2 and CCL5 have also been implicated in drawing immune cells towards tumors and contributing to tumor growth (221-223).

1.3.4. Self proteins as chemotactic factors

Although all of the classical chemokines are in reality "self" proteins, they are distinctly different from the chemotactic factors discussed here. Classical chemokines are small (~ 8kDa), dependent upon cysteine residues, and bind to traditional chemokine receptors. However, self protein chemoattractants have a variety of structures and bind to receptors that may not be be considered part of the chemokine receptor family or may serve another well known function. An example of this is norepinephrine and its receptor α_{1b}-adrenergic receptor. Norepinephrine and its receptor are traditionally thought of as neurotransmitters, however, α_{1b}-adrenergic receptor is also expressed on immature DC and norepinephrine can induce both chemokinesis and

chemotaxis (224). Many of these self protein chemokines have been identified recently and to illustrate the variety amongst this group, several interesting examples are discussed below.

β-defensins are anti-microbial peptides that can also act as chemokines. They are recognized by CCR6. CCR6 is present on immature DC, resting memory $CD4^+$ T cells and some B cells. At concentrations approximately 10-100 fold lower than their effective microbicidal concentratioin (μM), β-defensins can induce chemotaxis of CCR6+ cells (210). Tyrosyl tRNA synthetase has also been reported to be chemotactic for neutrophils (225, 226) Other aminoacyl tRNA sythetases have also been found to be chemotactic for immature DC, activated monocytes, and T cells (227, 228). Interestingly these aminoacyl tRNA synthetases can also be targets of antibodies in autoimmune diseases. CCR5 has been identified as the receptor for histidyl tRNA synthetase (227, 228) and CCR3 has been identified as the receptor for asparaginyl tRNA synthetase (228). Two other autoantigens, the retinal autoantigen S-Antigen, and the interphotoreceptor retinoid binding protein, involved in uveitis were demonstrated to be chemotactic for immature DC and lymphocytes. These antigens work through CXCR5 and CXCR3 or CXCR3, respectively (229). FRPL1 (formyl peptide receptor-like 1) has been identified as a receptor for serum amyloid A, which is a chemoattractant for leukocytes (230-233).

1.3.5. Summary

DC chemotaxis is critical to DC function. DC normally circulate from the bone marrow, through the blood stream to peripheral tissue and, upon activation or steady state maturation, to the secondary lymphoid tissues. DC make large amounts of chemokines, including inflammatory chemokines, when stimulated with pro-inflammatory triggers. They also express a variety of canonical chemokine receptors, although the expression profile of these receptors

changes drastically upon DC maturation. Self proteins, which do not fit into one of the classical chemokine families, have been shown capable of attracting cells. Tumors can also alter DC chemotaxis although it is unclear yet whether the chemotactic factors that they release differ from chemokines found under normal conditions.

1.4. MUC1

1.4.1. MUC1 biochemistry and cell biology

1.4.1.1. MUC1 genetics

The human mucin 1, MUC1, is located on the long arm of chromosome 1 (1q21-24) and is 4-7 kb in length. It is also known as polymorphic epithelial mucin (PEM), peanut reactive urinary mucin (PUM), and episialin. The gene is polymorphic mainly in the region of variable number of tandem repeats (VNTR) in the extracellular portion of MUC1 within exon 2. Amongst the human alleles, the length of this repeat region ranges from approximately 25 to 125 repeats of 60 bp (234, 235). There is also some variation in the actual repeat sequence within individual repeat domains (236). MUC1 contains 7 exons. The major form of MUC1 uses all 7 exons although splice variants do exist (237). There is a large amount of variation in the tandem repeat region in mammals, however there is great conservation in the transmembrane domain and cytoplasmic tail. This conservation suggests that these regions are critical to the normal function of MUC1.

There is some instability in chromosome 1 in cancer patients. At 1q21 there is a significant increase in chromosomal alterations in cancer cells. These alterations include alterations at 1q42-43 and 1q 33-35 in the region of the MUC1 gene (238). There are also alterations in the introns of MUC1 (239) as well as the exons (240, 241). These alterations in exons can lead to different splice variants of MUC1 (240, 241).

The control of MUC1 gene expression is not clearly defined although several critical regions have been identified within the promoter region. Two DNase I hypersensitivity sites (DHS) were identified in the 5' region in both humans and MUC1 transgenic mice (242). In the 5' region there are binding sites for general transcription factors such as SP1 and AP-1 as well as an E box that function in the regulation of MUC1 (243, 244). Indole-3- carbinol, a chemical known to have a chemopreventive effect for cancer, down-regulates MUC1 gene and protein expression (245). Erb-B2, as a homodimer, has been implicated as an inhibitor of MUC1 expression (246, 247). This is especially interesting because the activation of the proto-oncogene Erb-B2 occurs in c-Neu tumors and many breast cancers. Erb-B2 is the target of antibody based therapy in humans although there is no data at present to suggest what the effects of this therapy has on MUC1 expression.

There are also possible consensus sequences for hormone receptor binding. In fact, in human endometrium there is regulation of MUC1 expression during different phases of endometrial growth and during embryo implantation (248). Similarly, in the rabbit, the levels of the rabbit homologue of MUC1 were increased by progesterone in the endometrium although progesterone did not affect the level of expression of rabbit MUC1 in the cervix (249). There are conflicting reports on whether tamoxifen, a selective estrogen receptor modulator, can inhibit MUC1 expression in cancer cells (250-252) Estradiol was found to increase MUC1 expression as measured by [^{3}H] glucosamine incorporation into MUC1 (251, 252). Phorbol myristate acetate (PMA), a phorbol ester and protein kinase C activator, was also able to induce MUC1 expression which was inhibitable by bisindolylmaleimide, a protein kinase C inhibitor (251).

Several other inducers of MUC1 expression in the cytokine families have been defined. Tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) have both been shown to induce

MUC1 expression through a κB binding site in the MUC1 promoter (253-255). IFN-α could also increase MUC1 expression although not to the level that IFN-γ could induce (255). There is also a STAT-binding element in the promoter (253, 256). IFN-γ and IL-6, by activating STATs, are able to increase MUC1 expression (256). Furthermore, in many cancer cells there is constitutive activation of members of the STAT family. In particular, STAT3 is often constitutively activated and has been shown to up-regulate MUC1 expression (257). IL-7 and IL-15 have recently been observed to upregulate MUC1 expression in primary human T cells (258).

1.4.1.2. MUC1 structure

MUC1 is transcribed as a single messenger RNA and further translated as a single protein chain. Upon protein synthesis, the large single chain is cleaved near the C-terminus of the protein at a Gly-Ser protein bond (259). Mutagenesis of this site does not affect overall MUC1 expression at the cell surface but it does block the cleavage of the initial MUC1 protein chain in the endoplasmic reticulum (259). The two proteins remain associated despite the lack of covalent linkage between the two sub-units. The tandem repeat region is contained within the larger N-terminal fragment that is also the majority of the protein in the extracellular region. The C-terminal fragment is significantly smaller and contains the transmembrane and cytoplasmic tail regions. The extracellular portion of the C-terminal fragment is only 65 a.a. in length. The overall structure of MUC1 as it is expressed on the cell surface is shown in Figure 1.

There are several MUC1 splice variants. The splice variant MUC1/Y, which contains the transmembrane and cytoplasmic portions of MUC1 but very little of the extracellular domain, can bind to two other splice variants of MUC1, MUC1/SEC and MUC1/REP. MUC1/REP and MUC1/SEC are made up of the extracellular domain of MUC1 without the transmembrane and

cytoplasmic domains (260). The interaction between MUC1/Y and MUC1/SEC leads to phosphorylation of the cytoplasmic tail of MUC1/Y and changes in cell morphology (260). Splice variants of MUC1 are also overexpressed on cancer cells and can affect tumorigenesis. MUC1/Y overexpression is correlated with increased tumorigenic potential of DA3 mouse mammary epithelial cells (261).

The majority of the tandem repeats of MUC1 are comprised of the sequence PDTRPAPGSTAPPAHGVTSA. The secondary structure of the tandem repeat region is a polyproline β-turn helix-like formation. Within each repeat there is a hydrophilic region (PDTRPAP) with 2 β-helices and a hydrophobic region (262, 263). The structure of the tandem repeat region is very rigid and not affected by the number of repeats present (264).

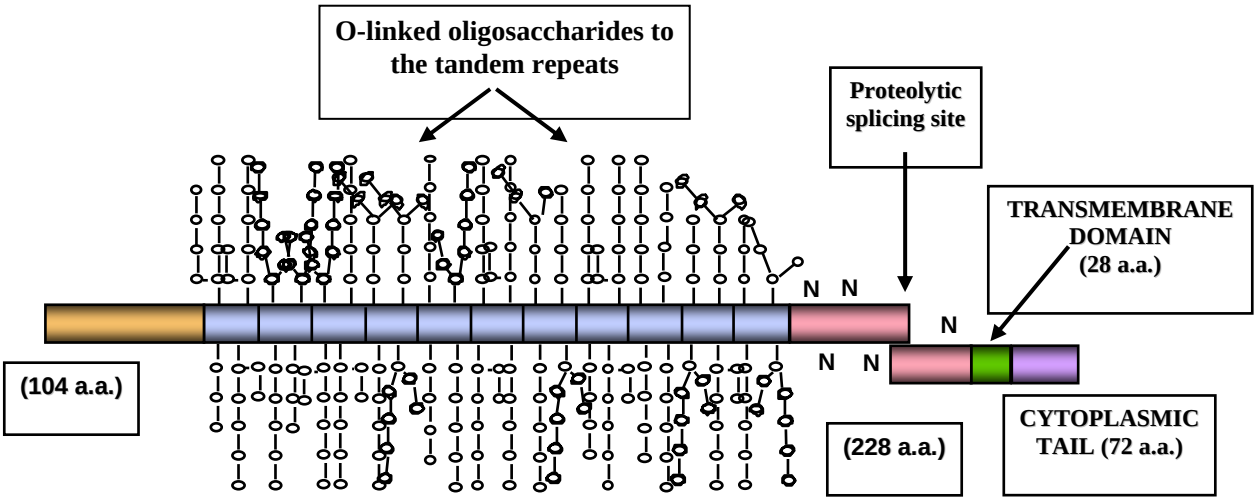

Figure 1: The structure of normal MUC1.

The cytoplasmic tail is colored purple followed by the transmembrane domain colored green. The extracellular non-VNTR region (pink) contains sites for possible *N*-linked glycosylation (N). The VNTR region (blue) has a high concentration of *O*-linked carbohydrates represented by the circles and lines. The N-terminal domain is colored tan.

The allelic variation in the number of repeats within the VNTR region has been associated with several diseases. Shorter alleles have been associated with gastric cancer and its premalignant condition incomplete intestinal metaplasia, and *H. pylori* infection (265-267).

Longer alleles have been associated with susceptibility to acne and with complete intestinal metaplasia of the stomach (266, 268). Individuals heterozygous in the MUC1 VNTR region have a lower incidence of chronic atrophic gastritis and intestinal metaplasia, which are premalignant conditions for gastric adenocarcinoma (266).

Shedding of the extracellular portion of MUC1 occurs in cancer cells and in uterine cells during the receptive phase of implantation. Several enzymes have been implicated as the MUC1 "sheddase". In rabbit endometrium, a transmembrane metalloprotease, a disintegrin, and metalloprotease (ADAM) 9 may be responsible for localized MUC1 loss during implantation (269). In a human uterine cell line, TNF-α induced MUC1 shedding. This shedding was partially mediated through protein kinase C (PKC) and was inhibited by a metalloprotease inhibitor, a TNF-α protease inhibitor (TAPI) and tissue inhibitor of metalloprotease (TIMP)-3 but not by TIMP-1 or a variety of serine, threonine or aspartyl protease inhibitors (254). Phorbol 12-mystirate 13-acetate also induced MUC1 shedding which was inhibited by TAPI as well as by TIMP-3 (270). The enzyme responsible for these effects was identified as tumor necrosis factor-α converting enzyme (TACE)/ADAM 17 (270). The tyrosine phosphatase inhibitor vanadate was also found to increase MUC1 shedding even in TACE deficient cells. This activity was blocked by TIMP-2 and TIMP-3 but not by TIMP-1. The enzyme responsible for this MUC1 sheddase activity is the membrane-type matrix metalloprotease 1 (MT1-MMP) (271).

1.4.1.3. MUC1 signaling

The intracellular cytoplasmic tail of MUC1 contains several tyrosines that can be phosphorylated and contribute to intracellular signaling (272-275). Of the seven intracellular tyrosines, Y(20), Y(46), Y(60) and Y(29) were activated by the binding of anti-CD8 antibody to

a fusion protein of the CD8 extracellular region with the MUC1 cytoplasmic tail (276). This same fusion protein was used to show that phosphorylation of MUC1 results in the activation of ERK1/2, but not SAPK/JNK, or ERK5 (277). Y(20) and Y(60) are required for efficient endocytosis of MUC1 from the cell surface (278).

When the tyrosines in the MUC1 tail are phosphorylated they are potential binding sites for the SRC homology 2 (SH2) domains of many kinases including c-Src and Grb2/SOS (279, 280). Src phosphorylates Y(46) and the Y(20) site can be phosphorylated by ζ-associated protein 70 (ZAP-70) (280, 281). The association of Src with MUC1 blocks the binding of another kinase, GSK3β. Once Src phosphorylates MUC1, MUC1 has increased binding to β-catenin (280). The cytosolic tail of MUC1 can associate with glycogen synthase kinase 3β (GSK3β) and β-catenin (282, 283). GSK3β phosphorylates a serine (S(44)) near the β-catenin binding site which decreases β-catenin binding to MUC1 (283).

MUC1 expressed in Jurkat and primary T cells becomes phosphorylated upon TCR engagement (281). Upon phosphorylation, MUC1's cytoplasmic tail associates with lck tyrosine kinase. Knocking down MUC1 expression in T cells with small interfering RNA (siRNA) leads to inhibition of downstream signaling upon TCR activation including lower level of Ca^{2+} flux and ERK1/2 phosphorylation (281). This leads to lower levels of expression of CD69, less proliferation and decreased IL-2 production by the T cell (281).

In other cell types, MUC1 has been shown to act through other signaling pathways as well. Transfection of MUC1 into fibroblasts resulted in an increase in activated Akt and Bad which was mediated through the activation of phophoinositide-3-kinase upstream. Furthermore, in these cells, MUC1 prevented apoptosis and increased expression of the anti-apoptotic protein Bcl-XL (284). MUC1 can associate with all members of the erbB family of receptors and the

addition of erbB ligands induce MUC1 cytoplasmic tail phosphorylation (285). This pathway results in the activation of ERK1/2. MUC1 can associate and co-immunoprecipitates with adenomatous polylposis coli (APC) (286). APC downregulates β-catenin signaling and is decreased in colon and breast carcinomas. The interaction between APC and MUC1 is increased by the addition of epidermal growth factor and is increased in cancerous and metastatic cells compared to normal breast tissue (286). $MUC1^{+}$ cells may also be more sensitive to FasL induced apoptosis by an upregulation of Fas expression on their cell surface in response to FasL (287). Finally, upon binding intracellular adhesion molecule-1 (ICAM-1), signaling through MUC1 causes Ca^{2+} flux in the cell. This MUC1-ICAM-1 signal involves a Src family kinase, lipid rafts, and phospholipase C but not MAP kinase (288).

1.4.1.4. Function of MUC1

The normal role of MUC1 is not clear although the role of mucins in general has been hypothesized to be to protect the epithelial cells from damage or pathogens. However, many studies have been done to look at the function of eukaryotic homologues of MUC1. MUC1 homologues in eukaryotes such as *Leishmania major* and *Trypanosoma cruzi* show that these mucins are involved with attachment and invasion (289). In *Saccharomyces cerevisiae*, the MUC1 homologue is critical for pseudohyphal differentiation and invasive growth (290).

A study of the expression of mouse Muc1, which shares homology with human MUC1 in the cytoplasmic tail and transmembrane domain but very little with the extracellular domain, in embryos showed that Muc1 expression was correlated with the epithelial differentiation of many organs such as the lung, pancreas, and salivary glands (291). Muc1 synthesis also increased with time indicating that mouse Muc1 expression is controlled both temporally and spatially. In the hamster, the MUC1 homologue was downregulated by squamous cell differentiation (292).

There are pathologies present in the muc1 null mice that lack the murine homologue of human MUC1. These mice, when kept in non-barrier animal facilities, have significant fetal loss during the post-implantation period. This is accompanied by a large number of necrotic decidual cells in the uterus (293). There is controversial evidence that Muc1 null mice, also in non-barrier facilities, had a propensity to develop blepharitis and conjunctivitis (294, 295). Muc1 null mice had a slower rate of cell growth in tumors induced by the polyoma middle T antigen (296). In a mouse model of cystic fibrosis, Muc1 was shown to play an important role in intestinal blockage caused by mucus. In the Muc1 null/cystic fibrosis mouse, there was less obstruction and these animals showed better survival on solid foods (297). Furthermore, muc1 knockout mice show decreased uptake and absorption of cholesterol from the intestine and a decrease in the formation of gall stones (298, 299). The lower reproductive tract of Muc1 null mice was more susceptible to infections, mainly caused by opportunistic pathogens, when kept in normal animal husbandry. However, the endometrium of muc1 null mice was better at attaching to blastocyst than wild type endometrium (300).

In humans MUC1 is expressed by a vast number of tissues, usually in the ductal epithelial cells. A few examples are listed below. MUC1 is expressed normally in the distal convoluted tubules and in the collecting ducts of the kidney (301). It is expressed by the parotid and submandibular and minor salivary (302, 303). MUC1 is expressed throughout the entire tracheobronchial epithelium and the collecting ducts in the lung but not in the submucosal glands or bronchioles (304, 305). MUC1 is expressed by the mucous cells in the epithelial layer of the stomach and neck of the antrum and in the body of the stomach in the pyloric and oxynthic glands (306, 307). MUC1 is expressed by the stratified squamous epithelia of the conjuctiva and cornea of the eye (308).

There are some data that provide clues to the role of MUC1 in humans. MUC1 is expressed around areas of ulceration in the gut suggesting that it may play a role in either wound healing or ulceration (309). MUC1 has also been associated with epithelial cell differentiation in humans in organs such as the lungs (310). MUC1 may also be selectively expressed during erythropoeisis in humans indicating that it participates not only in the formation of epithelial cells but also hematopoetic cells (311) MUC1 transfection into MUC1 negative cell lines was associated with reduced cell cohesion and enhanced migration on type I collagen coated surfaces as well as altered cell morphology when grown in three-dimensional type I collagen gels (312, 313). MUC1 has also been implicated in regulating the stress response to reactive oxygen species by upregulating anti-oxidant enzymes (314).

1.4.1.5. MUC1 glycosylation

MUC1 is heavily glycosylated in normal tissue. The VNTR region contains five possible sites of *O*-linked glycosylation. In normal MUC1, these sites are glycosylated as MUC1 passes through the Golgi compartment and further glycosylated upon recycling of MUC1 form the cell surface, back through the Golgi compartment. There are also sites of *N*-linked glycosylation in the extracellular portion of MUC1 near the transmembrane domain. The level of glycosylation of MUC1 in normal cells is so extensive that the peptide backbone of the VNTR region is hidden.

Glycotransferases and glycosidases add or remove carbohydrate residues, respectively, and the levels of these enzymes within a cell varies during differentiation and malignant transformation. The presence of carbohydrates on a molecule not only serves as a possible ligand for receptors but it also changes the overall physical properties, such as charge and mass, of the protein. Normal MUC1 has a larger proportion of core-2 based *O*-glycans (Galβ1,3-

GalNAc-β1,6GlcNAc) and tumor MUC1 has a greater portion of core-1 based *O*-glycans(Galβ1,3GalNAc) (315).

The addition and structure of oligosaccharides changes drastically in malignant cells (316). There is a general increase in certain residues such as the Tn antigen (GalNAc), the T antigen, (Galβ1-3GalNAc), sialyl-Tn, and the Lewis blood group antigens (316). The alterations occur because of alterations in the profile of glycotransferases and sialyltransferases within the Golgi compartment of the cancer cells. These changes in the pattern of carbohydrate residues occurs in diseases that put patients at high risk for cancer as well. Colonic and intestincal epithelia from patients with inflammatory bowel disease show increased levels of the Tn and T antigens, a decrease in sulfation, and an overall increase in the sialylation of surface proteins (317). The addition of carbohydrates to MUC1 induces a more rigid and extended structure to the peptide backbone (263, 318). Furthermore, there is some evidence that the carbohydrate residues and the peptide backbone can form hydrogen bonds that can also alter the conformation of the molecule (318). The addition of glycosylation to MUC1 can protect the peptide backbone amine groups from solvent exchange.

To study the complex process of glycosylation, several fusion proteins and truncated forms of the MUC1 VNTR region have been used. Using a probe of six tandem repeats containing a secretory signal and a tag for purification, Hanisch *et al.* were able to study the carbohydrate side chains added to MUC1 by a variety of tumor cells (319). In general, no glycans containing more than two sialic acids or modified with sulfate or phosphate were found with any of the tumor cell lines tested (319). Also, there was a tendency to a higher density of glycans, although of shorter length, on tumor cell expressed MUC1 than lactating milk MUC1 (319). However, each cell line had a unique pattern of glycans expressed. Using enzymes from

the lysates of pancreatic tumor cells, only two out of the five sites were glycosylated *in vitro* (GVTSA and GSTAP) although this does not rule out that other sites are glycosylated *in vivo* (320). The addition of GalNAc to these sites was very dependent upon the primary amino acid sequence and the relative position of the glycosylated amino acid within the repeat. The addition of monosaccharides or disaccharides to serines/threonines in the repeat can enhance or block the addition of sugars to the other possible sites of glycosylation (321). In MUC1 derived from human milk, the use of all 5 potential sites of glycosylation per repeat can be found (322).

MUC1 glycosylation affects its presentation to the adaptive immune system by DC. Truncation of *O*-glycans on MUC1 inhibits the cell surface expression of MUC1 and upregulates its recycling through clathrin-mediated endocytosis (323). When comparing synthetic unglycosylated MUC1 to forms glycosylated by tumor cells, DC, in both mouse and human, were only capable of degrading the unglycosylated MUC1 (324). The main sites of cleavage, likely by the protease cathepsin L, for the synthetic 100mer unglycosylated form were at the His-Gly (PAHGVT) and at the Thr-Ser (GVTSAP) residues within the repeat. If short sugars such as Tn (GalNAc) or T antigens (Galβ1-3GalNAc) are present, they are not removed although they do block the cleavage sites if they are joined to residues adjacent to the cleavage sites. This last point explains why the tumor forms are not degraded by DC and is in accordance with several other papers (105, 325-327) looking at MUC1 processing by DC. Several groups have attempted to immunize against these carbohydrate antigens alone and are successful at generating antibody responses but no T cell responses (328, 329). In general, the addition of these tumor specific glycans to the MUC1 backbone proved to be a better vaccine than the glycans alone or glycans bound to a non-specific peptide backbone (330). To generate a T cell response, the carbohydrate antigen needs to be bound to a peptide capable of binding within the MHC clefts.

Complexly tumor glycosylated MUC1 is not processed for presentation on MHC Class II and fails to stimulate $CD4^+$ T cells (325). Unglycosylated MUC1 and MUC1 with short sugars, such as Tn, can elicit anti-MUC1 $CD4^+$ T cell responses (325, 327). As MUC1 becomes more glycosylated, the ability of DC to stimulate MUC1 specific CTL decline (326). However, unlike for MHC Class II presentation, highly glycosylated MUC1 was able to be processed and presented on MHC Class I, albeit at low levels (325, 326). Natural antibodies to MUC1 from patients with breast cancer were more reactive to MUC1 with short glycans than to the completely unglycosylated MUC1. However, upon vaccination with the unglycosylated form, the predominant MUC1 specific antibodies were to the unglycosylated form (331).

These carbohydrate antigens on MUC1 have been shown to play a role in carcinogenesis. The loss of sialyl Lewis X epitopes on MUC1 has been implicated in allowing tumor emboli to pass through the vasculature without binding to endothelial cells (332) The use of the inhibitor benzyl-N-acetyl-alpha-D-galactosamine, an inhibitor of O-linked glycosylation, in a temporal manner causes the loss of sialic acid residues followed by T antigen residues from newly produced MUC1. This loss increases the $MUC1^+$ cells adherence to Type I collagen (333). Blocking extension of carbohydrate branching on mucins has been shown to increase the binding of cancer cells to a macrophage cell line and peritoneal murine macrophages (106).

Sialic acid residues can be added to both *O*-linked and *N*-linked glycans. They add a negative charge to the protein and hence can alter the binding capabilities of the protein. Sialic acids can also be recognized as ligands for receptors such as the siglec and selectin families of receptors. Sialylation can terminate the ability of a receptor to bind a protein such as with the galectin family of receptors. The addition of sialic acid terminates the ability to add further sugars to the carbohydrate chain and thus it is classified as a terminal sugar. An increase in the

cell surface levels of α2,6-sialylation increased binding of cancer cells to collagen, decreased cell-cell adhesion, and increased invasion capacity (334). Increased sialylation on colon cancer mucins was associated with a more metastatic potential (335, 336). The use of disaccharides as decoys for carbohydrate forming reactions in tumor cells lowered the level of sialic acid on surface proteins and made the tumor cells more susceptible to attack by immune cells (337). The sialyl-Tn antigen, when overexpressed by transfection of cells with sialyltransferase-6-GalNAc I, decreased cell growth and adhesion and increased cell migration (338). Sialylated core-1 based glycans on MUC1, but not core-2 based glycans, increased tumor cell growth in MUC1 transgenic mice but interestingly, not in wild type mice (339).

Mucin binding to receptors is dependent upon the sugars present as well as the peptide back bone (340). MUC1 binds to ICAM-1, sialoadhesin, and mannose receptor through the combination of the peptide backbone and carbohydrate side chains (341). Glycosylation can also alter the presentation of MUC1 on MHC molecules. This alteration can affect presentation in two ways. One, the combined carbohydrate-peptide epitope can be recognized by glycoepitope specific T cells (327). Secondly, the addition of a glycans can alter the conformation of the peptide and how it sits in the binding cleft of the MHC molecule. Glycosylation of MUC1 also affects antibody binding to MUC1 (342).

1.4.1.6. Summary

MUC1 is a large transmembrane protein expressed by a wide variety of human tissues. The expression of MUC1 is regulated by a number of transcriptional pathways. It is translated as a single protein that is cleaved into two pieces that remain in association after cleavage. Once in the membrane, MUC1 signals through tyrosines found in its cytoplasmic tail. It associates with kinases that contain SH domains, especially of the Src family of kinases. MUC1 can also be

shed from the cell surface through the actions of enzymes such as TACE. Little is known about how MUC1 functions normally in humans but studies with the MUC1 homologues in other species suggest that it plays a role in epithelial and hematopoeitic differentiation, reproduction, protection from pathogens and stress responses. Normal MUC1 is heavily *O*- and *N*-glycosylated in its extracellular region and the pattern of glycosylation changes between cell types and during differentiation or malignant transformation. The aberrantly glycosylated MUC1 protein expressed by tumor cells is recognized by receptors and can serve as additional targets for the anti-tumor response. However, it is not known what the effects of MUC1 binding to these receptors are and how they affect the body.

1.4.2. MUC1 as a tumor antigen

MUC1 was first identified as a tumor associated antigen through the isolation of MUC1 specific T cells from the draining lymph nodes of pancreatic cancer patients (343). Tumor associated antigens are proteins that are expressed in normal tissues but are normally not exposed to the immune system either because they are expressed in a sequestered location, have extremely low levels of expression, or are expressed differently in tumor cells. Because MUC1 is expressed on so many human tumors, it is a good target for cancer immunotherapy. Through the attempts to target the immune response to MUC1, it has also become clear that MUC1 plays a function in promoting cancer cell growth and adhesion. Although many groups have tried to immunize against MUC1, it is becoming clear that the abnormal expression of MUC1 and many other tumor antigens occurs very early in carcinogenesis and that effective therapies to target tumor antigens will need to occur prior to symptomatic disease.

1.4.2.1. MUC1 expression by cancers

Even though immune responses to MUC1 are considered to be beneficial for cancer patients, the expression of MUC1 on tumors is usually correlated with a worse prognosis. The

level of expression of MUC1 on the surface of cells is increased in virtually every adenocoarcinoma. For example, increased expression of MUC1 in colon cancer, invasive ductal carcinomas of the pancreas, invasive cholangiocarcinomas of the liver, renal cell carcinoma, gastric cancer, adenocarcinomas of the esophagus, and gall bladder cancer correlates with a worse prognosis (344, 345), (346, 347). These mucins often have an increase in the carbohydrate antigens Tn, T and sialyl-Tn antigens with a decrease in Type 3 core *O*-glycans and *O*-acetyl-sialic acid. Furthermore, MUC1 expression is correlated with increased invasive and metastatic behavior. This is demonstrated by invasive ductal carcinomas of the pancreas and invasive cholangiocarcinomas of the liver which both show invasive growth and a poor prognosis and high levels of MUC1 expression. However, MUC1 and its sialylated epitopes are usually not expressed on the less pathogenic intraductal papillary mucinous tumors of the pancreas or bile duct cystadenocarcinomas of the liver (344). In addition, only invasive cases of mucinous cystic neoplasm of the pancreas express MUC1. Their non-invasive counterparts generally lack MUC1 expression (348, 349). The loss of polarity of MUC1 is also correlated with a more metastatic phenotype and poorer prognosis (348, 350, 351). Another characteristic of MUC1 expression is its association with a less differentiated phenotype when expressed by lymphoid malignancies such as plasmacytomas (352). Finally, there are high levels of MUC1 found circulating in pancreatic cancer patients as well as many patients with other types of cancers (353). When the circulating MUC1 was found in conjugation with anti-MUC1 antibodies in patients with breast cancer, thus indicating an anti-MUC1 response had been at least partially generated, these patients had a better rate of survival (354). MUC1 expression in colon cancer was associated with lymphocytic infiltrate (355). These studies show that MUC1

plays a role in tumor growth and malignancy and hints to a possible role in immune modulation and tumor invasion.

In some premalignant states there appears to be changes in MUC1 expression. In Crohn's disease, an inflammatory disorder of the gut, there is little change in MUC1 expression between affected and unaffected areas of the small intestine (356). However, there is an increase in MUC1 in the serum of patients with interstitial lung disease, especially when the disease is active and MUC1 is expressed by premalignant lesions in the lung (304, 357). Another inflammatory bowel disease with an increased risk of cancer is ulcerative colitis. Some patients with ulcerative colitis develop antibodies to MUC1 (358, 359). MUC1 is upregulated in Barrett's esophagus, a premalignant condition of the esophagus, as well as in dysplastic tissue in the esophagus (360). MUC1 expression is correlated with high-grade dysplasia in colorectal cancer (361, 362). Since MUC1 is available to the immune system in these early lesions, it is clear that an immune response to MUC1 is being shaped at these early time points. To effectively alter this response, therapies would have to be targeted at these early lesions.

1.4.2.2. Targeting MUC1 through immunization

Immune responses to MUC1 can be generated in other conditions, especially during lactation and pregnancy. MUC1 specific MHC Class I restricted CTLs and $CD4^+$ T cells can be generated *in vitro* from the T cells of multiparous women but not from normal donors suggesting that pregnancy results in a natural immunization against MUC1 (363, 364). This is particularly relevant because pregnancy and lactation have been associated with a lower risk for breast cancer. MUC1 antibody levels are increased in non-pregnant women compared to pregnant women although MUC1 serum levels were increased in the pregnant women compared to the non-pregnant women indicating that some of the anti-MUC1 antibodies in pregnant women may

be bound to MUC1 in circulation (365). Lactating women also have significantly higher levels of the anti-MUC1 IgG isotype compared to non-lactating women (365). An interesting case report has been noted where a woman with breast cancer had high levels of B- and T-cell epitopes and she survived this cancer. Subsequently, she became pregnant and developed fulminant lymphocytic mastitis, an inflammation of the breast tissue. This patient's breast tissue expressed similar epitopes to the tumor in terms of MUC1 and had circulating anti-MUC1 antibodies and MUC1 specific CTL. This suggests that in the right circumstance, a cancer can prime a strong anti-MUC1 response and that this can result in survival as well as a long lasting memory response (366). Together these data indicate that anti-MUC1 immune responses can be generated in healthy individuals and points towards the hypothesis that a strong anti-MUC1 immune response can prevent tumor growth.

Several epitopes recognized by cancer patients have been identified within the MUC1 protein. One HLA-A2 restricted epitope is from the leader sequence of MUC1 (LLLLTVLTV) and stimulates $CD8^+$ T cells in patients with multiple myeloma (367, 368). Some other HLA restricted epitopes are from the VNTR region (STAPPVHNV and STAPPHGV) (368-370). Epitopes have been identified in the degenerate repeats present in the terminal regions of the VNTR domain including the HLA-A2 epitope SAPDNRPAL which is very similar to the VNTR epitope SAPTRPAP (371). When HLA-A2+ DC were transfected with total RNA from a breast cancer cell line and used to stimulate T cells, four immunodominant peptides were found. Two were from the tumor antigen Her-2/neu and two were from MUC1 including the epitope STAPPVHNV from the tandem repeat region and the epitope LLLLTVLTV from the leader sequence (372). The identification of these epitopes not only points towards good epitopes for

vaccination but also indicates that there are several target regions within the MUC1 molecule for vaccination.

Several formulations of vaccines have been developed against MUC1. These vaccines have been tested in normal mice, MUC1 transgenic mice and humans. Different forms of MUC1 used in trials include MUC1 fused to the carbohydrate mannan (373-376), MUC1 KLH (377), DNA vaccines (378) and vaccinia virus expressing MUC1 (379). Tumor cells transfected with different adjuvants (380) as well as tumor cells fused with dendritic cells have also been tested (381, 382). Consistently in MUC1 transgenic mice, the administration of MUC1 without a strong inflammatory signal such as with a weak adjuvant or as irradiated tumor cells results in weak antibody responses and little to no detectable T cell responses (383, 384). This is true in the immunization of human cancer patients as well (377).

The level of tolerance to MUC1 in the MUC1 transgenic mouse is elevated compared to wild-type mice (384). This tolerance is largely in the $CD4^+$ T cell compartment as shown by the administration of wild-type $CD4^+$ cells primed *in vitro* and then administered to MUC1 transgenic mice. These recipient mice showed increased survival when challenged with a $MUC1^+$ tumor (385). Still, with a strong enough vaccine in the MUC1 transgenic mice, both antibody class switching and CTLs can be generated (379, 384). The key elements for tumor rejection of the B16.MUC1 tumor (a melanoma transfected with MUC1) were $CD4^+$ T cells, FasL, lymphotoxin (LT) α, CD40, CD40L, and CD28 (381, 386). Perforin, $\delta\gamma$ T cells, IL-4, IL-10, IL-12 or TNFR-I were not absolutely required although NK cells seemed to play a role in rejection (386). Two subsets of NKT cells have recently been shown to recognize MUC1 and to lyse $MUC1^+$ targets after expansion in the presence of MUC1 (387). However, even with a good vaccine there are elements within the tumor site that can suppress an active immune response. In

a MUC1 transgenic model of spontaneous pancreatic cancer, infiltrating $CD8^+$ T cells become cytolytically anergic and are tolerized to MUC1 (388). These studies show that an effective immune response to MUC1 requires several arms of the immune system to be activated and that even with activation, the tumor can develop mechanisms to suppress the immune response.

1.4.2.3. MUC1 in tumorigenesis

As briefly mentioned above, MUC1 has also been suggested to function in tumorigenesis in humans. To study this role and the possible tolerance to MUC1 in humans, a mouse model of MUC1 expression was developed. The MUC1 transgenic mouse expresses human MUC1 under the control of the human MUC1 promoter. MUC1 expression in the MUC1 transgenic mouse is very similar to the expression pattern in humans. MUC1 also undergoes similar changes in polarity and glycosylation during tumorigenesis. Experiments in these mice, injecting $MUC1^+$ tumors into MUC1 transgenic and wild type mice, show that $MUC1^+$ tumors grew in the transgenic mice but not in wild type mice. This indicates that there is a level of tolerance that exists in these mice (389).

Another way MUC1 can affect tumorigenesis is through altering intracellular signaling, conferring resistance to apoptosis, and modulating adhesion or migration. A model of spontaneous breast cancer formation in MUC1 transgenic mice showed that MUC1 can potentiate the signaling through MAP kinases of epidermal growth factor (EGF) in lactating mammary glands (390). Tumors that arise in multiparous mice from this study show a tumor specific co-immunoprecipitation between MUC1 and β-catenin. Also, in uniparous mice, MUC1 decreased postlactational apoptosis, increased whey acidic protein expression (a protein normally downregulated with postlactational apoptosis) and resulted in aberrant pErk2 activation (390). Overexpression of MUC1 in fibroblasts and epithelial cells induces anchorage independent

growth and confers tumorigenicity (391, 392) Furthermore, the cytoplasmic tail of MUC1 complexed with β-catenin and γ-catenin can be found in the nucleus (391, 393-395). MUC1 can activate anti-apoptotic pathways and the loss of MUC1 expression in cancer cell lines through siRNA knock down results in increased sensitivity to genotoxic drugs (396). MUC1 is localized to the tips of filipodial protrusions of MCF-7 breast cancer cells in association with the protein ezrin which may indicate that it plays a role in migration (397). Tumor MUC1 enhances binding of cells to extracellular matrix proteins and normal human lung tissue in vitro most likely through the unglycosylated VNTR region (398). MUC1 localization to the generalized or basal surfaces of cells indicates a more invasive phenotype of cell (399).

1.4.2.4. Summary

MUC1 is overexpressed by over 80% of human tumors in a non-polarized fashion. The changes in the level, localization, and glycosylation of MUC1 enable the immune system to discern normal MUC1 expression from abnormal MUC1 expression. Human cancer patients have immune responses to MUC1 but they are weak and ineffective. However, immune responses to MUC1 can be generated in non-cancer patients and there is some evidence that conditions associated with decreased risk of cancer, such as pregnancy and lactation, immunize against MUC1 naturally (400). To increase the immune response to MUC1 in cancer patients, several vaccines have been developed. However, eliciting an effective immune response against tumors is difficult and further complicated by the fact that the immune system of cancer patients is suppressed. Furthermore, MUC1 contributes to tumorigenesis and since it is present on pre-malignant lesions, the immune response to MUC1 has been shaped by the tumor long before the patient has symptomatic disease. It is still not known if MUC1 actually contributes to the immune suppression of the anti-MUC1 response or to the general anti-tumor response.

1.4.3. MUC1 and the immune system

MUC1 is a very complex molecule that can function on the cells expressing it both by signaling itself and perhaps through binding to receptors. With the large amount of effort that has gone into creating a MUC1 cancer vaccine, especially ones using DC, it will be important to know if MUC1 can bind and alter the function of cells in the immune system. MUC1 has also been found on cells on the immune system and may function in normal as well as malignant settings.

1.4.3.1. MUC1 and human pathogens

MUC1 has been shown to interact with human pathogens. *Helicobacter pylori* has been shown to colonize areas in the stomach with incomplete intestinal metaplasia that express MUC1 but not MUC2 (401). *H. pylori* is capable of binding to both MUC1 and MUC5AC (402). Furthermore, MUC1 from bovine milk inhibits a neuraminidase sensitive strain of rotavirus (403). Human milk fat mucin binds rotavirus and inhibits its growth both *in vitro* and *in vivo* and this inhibition is dependent upon sialic acid (404) Similarly, the human milk fat mucin can bind S-fimbriated *Escherichia coli* and prevent *E.coli* adhesion to epithelial through its sialic acid residues (405). These examples illustrate that MUC1 can bind to pathogens and alter the growth of these bacteria. Thus, MUC1 contributes to host defense by preventing bacterial growth and invasion.

1.4.3.2. MUC1 and T cells

MUC1 is expressed on T cells that are activated *in vivo* or *in vitro* although it is not expressed at all by resting T cells (275). The level of MUC1 protein expressed by these T cells is much lower than the amount of MUC1 expressed by either normal epithelial cells or cancer cells. The glycosylation of T cell MUC1 has a greater abundance of core-2 based structures

compared to core-1 based structures, making T cell MUC1 more similar to normal epithelial MUC1 than MUC1 expressed by cancer cells (275). This expression has recently been shown to be induced by both IL-7 and IL-15. (258). MUC1 can also be upregulated on T cells by IL-12 but is unaffected by IL-2, IL-4, IL-5, IL-10, IL-13 or TNF-α (406). When DC make IL-7, they can induce MUC1 expression on T cells and the MUC1 is expressed in a polarized fashion opposed to the DC-T cell interface (258). The crosslinking of MUC1 on the T cell surface may inhibit T cell proliferation (406). When T cells are stimulated by the chemokine RANTES (CCL5) they polarize and MUC1 localizes to the leading edge of the T cell unlike other mucin-like molecules that localize to the uropod (275).

Although it seems that MUC1 expression is a marker of activated T cells, it can also block T cell function when soluble. These studies have used tumor-like forms of MUC1 and hence the reason activated T cell expression does not result in suppression may be because T cell MUC1 is more highly glycosylated. At the same time, MUC1 from cancer patients' ascites fluid or synthetic unglycosylated MUC1 repeats can inhibit the proliferation of T cells in vitro. This inhibition was overcome by the addition of IL-2 or anti-CD28 antibody (407). MUC1 from tumor supernatants has been shown to block T cell proliferation and activation in response to PHA stimulation (408). These T cells maintained low levels of IL-2R and the suppression could not be overcome by exogenous IL-2 or IFN-γ (408). MUC1 expressed by tumor cells can also inhibit NK cell lysis of K562 cells and is able to bind both cell types (409). Purified $CD4^+$ T cells treated with colon cancer mucin (mainly composed of MUC1 and MUC2) and stimulated with PMA or anti-CD3 antibody produced lower levels of IL-2, bound less anti-CD28 antibody, and produced less IFN-γ (410).

1.4.3.3. MUC1 and antigen presenting cells

Since the antigen presenting cells in the body are crucial to the development of the anti-MUC1 immune response, it is critical to understand the effects MUC1 may have on these cells. It has been shown that MUC1 can bind to receptors expressed on these cells (104, 105). MUC1 can bind to sialoadhesin (Siglec-1) on macrophages that are infiltrating breast cancers (104). Sialoadhesin lacks a signaling domain, but mannose receptor, the second receptor on APC that can bind MUC1 does have diverse signaling capabilities as described above. Hiltbold *et al.* has shown that tumor MUC1 from ascites fluid can bind to mannose receptor on human DC. This interaction results in the uptake of MUC1 into early endocytic vesicles. However, the strong avidity of MUC1 binding to mannose receptor results in a block in processing. MUC1 fails to be transported to the late endosomes to be proteolyzed and instead recycles back out to the cell surface. This mechanism prevents MUC1 presentation in MHC Class II molecules. Despite blocking its own processing, MUC1 did not block the processing of other proteins (105). Blocking the mannose receptor greatly reduced the uptake of MUC1 but did not totally eliminate it suggesting that MUC1 can bind to DC through one or more other receptors. It is not known what effect MUC1 has on these cells.

There is controversial evidence that MUC1 is expressed by human dendritic cells. One group has shown that MUC1, as recognized by the BC2 antibody (directed against the VNTR region) and the CT1 antibody (directed against the cytoplasmic tail), is expressed on “activated” human blood DC, monocytes, and monocyte derived DC (273). This group has also shown that the mouse homologue of MUC1 is expressed by murine splenic DC (273). Another group agrees that MUC1 is expressed on monocyte and monocyte-derived DC but not on blood DC, thymic DC or tonsillar DC (411). Cloosen *et al.* also found MUC1 on DC in the synovial fluid of patients with rheumatoid arthritis, which is especially interesting because this is also a site of *in*

vivo T cell MUC1 expression (275, 411). MUC1 may also be expressed on follicular DC in the MALT (412). However, work done in our lab has shown that MUC1 as recognized by the HMFG-1 antibody is not expressed by either blood DC nor monocyte derived DC (data not shown). All groups are in agreement that MUC1 is expressed by bone marrow derived DC generated from the human MUC1 transgenic mice. MUC1 has also been reported to be expressed by neutrophils but only in the cytoplasm (413).

Work done by D. Lopez *et al.* examined the possible immunomodulatory effects of MUC1/SEC, a secreted form of MUC1 that contains the extracellular portion of MUC1 with a unique sequence in the C-terminal region, VSIGLSFPMLP. They have identified some tumor preventative effects of this peptide sequence although it is unclear how this peptide functions (414). However, it does not function in immunocompromised mice, thus suggesting a role for the immune system in the anti-tumor effects. Further work done by this lab has suggested that MUC1/SEC recruits three to four times as many macrophages to the tumor site and may be able to induce the expression of the chemokine CCL2 by tumor cells (415).

Many tumor-derived molecules have recently been shown to affect the differentiation of monocytes into dendritic cells. Monti *et al.* have shown that MUC1 has this affect as well. When monocytes are co-cultured with $MUC1^{+}$ tumor cells, the DC that are formed make IL-10 but no IL-12 and do not reach full activation even with CD40 ligation (107). They implicate the sialylated glycoepitopes on MUC1 as playing a role in this deviation. Despite displaying a regulatory DC phenotype, these DC do upregulate the chemokine receptor CCR7, which indicates that these DC could migrate to the lymph node and interact with naïve T cells. Accordingly, when cultured with T cells, these DC show diminished ability to induce T cell proliferation and induce T cells to produce greater amounts of IL-4 and lesser amounts of IFN-γ

(107). This T cell cytokine profile indicates that DC that have differentiated in a MUC1 rich environment drive naïve $CD4^+$ T cells away from a Th1 phenotype and towards a Th2 phenotype. As mentioned above, this is a phenotype that is often displayed by cancer patients.

1.4.3.4. Summary

MUC1 is expressed by some cells of the immune system in activated states. It is not clear what role MUC1 plays on these cells. In the case of T cells, the form of MUC1 expressed is similar to normal MUC1. The addition of the secreted form of MUC1 to a cancer cell line induced influx of immune cells through a mechanism that induces the production of CCL2. Most importantly, MUC1 can alter the development of DC from monocytes. It is unclear how MUC1 accomplishes this alteration but its effects on the DC skew their ability to produce cytokines towards IL-10 and away from IL-12. These effects block the development of Th1 cells and presumably would have this effect *in vivo* in cancer patients. These studies suggest that MUC1 has distinct effects on the immune system but the mechanisms and breadth of these effects are not fully characterized.

1.5. Statement of the problem

MUC1 binds to members of the PRR family that are expressed by cells of the immune system, especially APCs. This interaction is known to prevent processing of MUC1 and to prevent the differentiation of DC from monocytes (105, 107). Furthermore, the stroma surrounding $MUC1^+$ tumors displays an interesting pattern of DC migration with larger numbers of immature DC within the tumor site and more mature DC in the peri-tumoral area. At this point in time, it was not known whether MUC1 might attract DC to the tumor site, like it is predicted to do for fibroblasts (416), and whether the interaction of MUC1 with receptors on the DC might affect their function and stimulatory capacity. These issues are important to

understand because MUC1 is expressed on early pre-malignant lesions. Therefore, if MUC1 is affecting DC function and migration, the $MUC1^{+}$ tumor would be capable of directing and controlling the anti-tumor immune response long before the disease becomes symptomatic.

We hypothesized that this might be happening, based on what had been learned about anti-MUC1 immunity in cancer patients and designed studies in this dissertation to test this hypothesis. Our results show that MUC1, as we hypothesized, induces the migration of DC and suppresses their function. This work suggests that the tumor MUC1 interacts with DC in the body and alters their function. It has strong implications that MUC1 present on early premalignant lesions can contribute to tumor-induced immunosuppression and argues for early targeting of tumor antigens through prophylactic vaccination. It also suggests that chemotactic forms of MUC1, when co-administered with a strong adjuvant, will be a superior vaccine compared to the use of non-chemotactic forms.

2. Human Tumor Antigen MUC1 is Chemotactic for DC

Some of the contents of section 2 have been modified from article "Human tumor antigen MUC1 is chemotactic for immature dendritic cells and elicits maturation, but does not promote Th1 type immunity." Copyright (2005), with permission from "The American Association of Immunology". Copyright permission is on file with Casey A. Carlos. Some of the work shown in this chapter was done in collaboration with the laboratory of Dr. Joost J. Oppenheim at the National Cancer Institute.

2.1. Introduction

Tumor stroma is critical for the growth of cancer cells and, in fact, the stromal tissue can often make up a larger proportion of the tumor mass than the tumor cells. The cells that comprise the stroma are thought to be recruited by factors derived from the cancer cells. Tumor stroma cells include fibroblasts, immune cells, and endothelial cells. There is a level of selection amongst cells. For example, not all immune cells or subtypes of fibroblasts are found at the

tumor. This variety indicates that the tumor-derived chemoattractive factors are selectively produced (417). Some of the chemokines produced by tumor cells are CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL20 and CXCL12 (418). What is interesting and different about the immune cells at the tumor site is that there is not a common distribution among cells. For example, macrophages are not distributed to the same areas as dendritic cells and T cells are not found in the same region as NK cells.

DC are part of the tumor stroma and are also found within many tumors (74). A functional DC would be advantageous to the development of an anti-tumor response if the DC could take up and present tumor antigens. However, despite the close proximity of the DC to tumors cells and tumor antigens, these DC fail to elicit strong anti-tumor responses in most patients. The lack of response comes from a tumor induced defect in DC function or from a failure of the DC to migrate to the lymph node. Both of these issues probably play a role in late tumor growth. Also, there is a preponderance of immature DC within the tumor mass with the more mature DC located in the peritumoral area. This differential distribution is not unexpected as immature and mature DC express different chemokine receptors (170). Yet, it is not known which chemokines determine these differences.

MUC1, a human tumor antigen expressed by over 80% of human tumors, has been shown to be chemotactic and chemokinetic for human lung and skin fibroblasts (416). Platelet-derived growth factor, fibroblast growth factor, and fibronectin were also chemotactic for these cells and the migration towards MUC1 was comparable to migration to these proteins (416). Fibroblasts are part of the tumor stroma and hence $MUC1^+$ tumors could attract fibroblasts to the tumor site through MUC1. To date, the effects of MUC1 on the migration of other cells that make up the tumor stroma has not been examined. Here we show that the tumor antigen MUC1 is

chemotactic for immature human DC. MUC1 was not chemotactic for other cells of the immune system including monocytes, lymphocytes, NK cells and mature DC. The circulating form of MUC1 found in cancer patients and a synthetic form containing only the peptide backbone of the VNTR region were both chemotactic. The addition of sugars to the serines and threonines of MUC1 through *O*-glycosylation blocked DC chemotaxis indicating that only regions with low to no sugars added, i.e. tumor-like forms, of MUC1 attract DC. The chemotactic effect could also be blocked by an antibody to MUC1. These results show that the expression of tumor MUC1 by pre-malignant and malignant cells attracts immature DC. The presence of DC at the tumor site could be a good thing if the DC function and are activated by the danger signals at the tumor site such as uric acid from necrotic cells. However, if the DC fail to activate, this could lead to the induction of tolerance and/or anergy to the tumor antigens. This scenario would lead to tumor progression and a failure of tumor immunosurveilance.

2.2. Materials & Methods

2.2.1. Reagents

All chemokines and cytokines were obtained from the NIH cytokine repository or Peprotech (Rocky Hill, NJ) unless otherwise noted. Reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Anti-MUC1 antibody MF11 used in the chemotaxis blocking studies was obtained from ISOBM TD-4 International Workshop on Monoclonal Antibodies against MUC1 (419). It is specific for the PPAH sequence in the 20 amino acid-long MUC1 tandem repeat sequence GVTSAPDTRPAPGSTAPPAH.

2.2.2. Chemotaxis assay

Cells were resuspended in chemotaxis medium (RPMI 1640 media containing 1% bovine serum albumin, 25 mM HEPES, pH 8.0) at 1-5 x10^6 cells/ml. Chemokines diluted in chemotaxis medium were placed in the lower wells of a microBoyden chemotaxis chamber (Neuroprobe,

Cabin John, MD). When primary leukocytes were analyzed, five-micrometer polycarbonate membranes were placed over the chemokines. Lymphocytes required that the membranes be precoated with 50 μg/ml of fibronectin. After the micro-chemotaxis chamber was assembled, 50 μl of cells were placed in the upper wells. The filled chemotaxis chambers were incubated in a humidified CO_2 incubator for 90 minutes (monocytes, immature human DC) or 3 hours (lymphocytes, NK cells). After incubation the membranes were removed from the chemotaxis chamber assembly followed by gently removing cells from the upper side of the membrane. The cells on the lower side of the membrane were stained using Rapid Stain (Richard Allen, Kalamazoo, MI). The number of migrated cells in three high-powered fields (x200) was counted by light microscopy after coding the samples. In many cases counting was computer assisted using the BIOQUANT program (R & M Biometrics, Nashville TN). Additional chemotaxis experiments done with $CD1c^+$ DC were conducted similarly but with chemotaxis plates (5.7 mm diameter, 30 ml, 5 μm pores; Neuroprobe, Gaithersburg, MD.) Results are expressed as the mean value of the migration of triplicate samples with the standard deviation shown by bars.

2.2.3. MUC1 antigen preparations.

MUC1 100mer synthetic peptide (GVTSAPDTRPAPGSTAPPAH) x 5 represents five unglycosylated tandem repeats from the variable number of tandem repeats (VNTR) region comprising most of the extracellular domain of MUC1. MUC1 100mer was synthesized on a Chemtech 200 automated peptide synthesizer (Advanced ChemTech, Inc., Louisville, KY) with N-(9-fluorenyl)methoxycarbonyl chemistry and purified by high-pressure liquid chromatography (HPLC) at the University of Pittsburgh Cancer Institute Peptide Synthesis Facility. MUC1 Tn-100mer is a glycosylated 100mer peptide that was prepared as described previously (321, 327). It contains 15 GalNAc residues bound to the threonines in VTSA regions and serines and threonines in GSTA regions of the tandem repeat sequence GVTSAPDTRPAPGSTAPPAH

(glycosylated residues indicated in bold), consistent with the site-specificity of the recombinant glycosyltransferase rGalNAc-Ts used to glycosylate the peptide. Ascites MUC1 was purified from ascitic fluid obtained from cancer patients and characterized as previously described (420). Generation and purification of recombinant tumor forms MCF-7 MUC1 and HEK MUC1 was described previously (319). In brief, a mammalian episomal expression vector pCEP-PU (421) encoding six tandem repeats of MUC1 (120mer peptide) under the control of the cytomegalovirus promoter was used to transfect the breast cancer cell line MCF-7 and the EBV-transformed human embryonic kidney cell line 293/EBNA (obtained from the American Type Culture Collection.) This vector contains the secretory signal peptide of BM40, an extracellular matrix protein also known as SPARC or osteonectin, followed by a hexa-histidine sequence and a Myc tag used for affinity purification of the MUC1 protein produced by the transfected cells. HEK MUC1 and MCF-7 MUC1 were purified from the conditioned supernatant of confluent cell layers by passage through a Ni^{2+}-nitrilotriacetic acid Superflow column (Quiagen, Hilden, Germany). They were further purified by HPLC on a C8 silica column (Vydac 208TP1015; MZ Analysentechnik, Mainz, Germany). Quality of purified recombinant MCF-7 MUC1 and HEK MUC1 were checked by SDS-PAGE followed by blotting onto a nitrocellulose membrane. Peptides were detected with an anti-myc monoclonal antibody (319) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA.) Specific O-glycosylation profiles were determined by hydrazinolysis and normal phase chromatography of 2-aminobenzamide-labeled glycans as previously described (319). All MUC1 peptides were tested for LPS with the limulus amebocyte lysate assay and found to have <0.05 EU/ml by Cambrex (Cambrex Bio Science Walkersville, Inc., Walkersville, MD.)

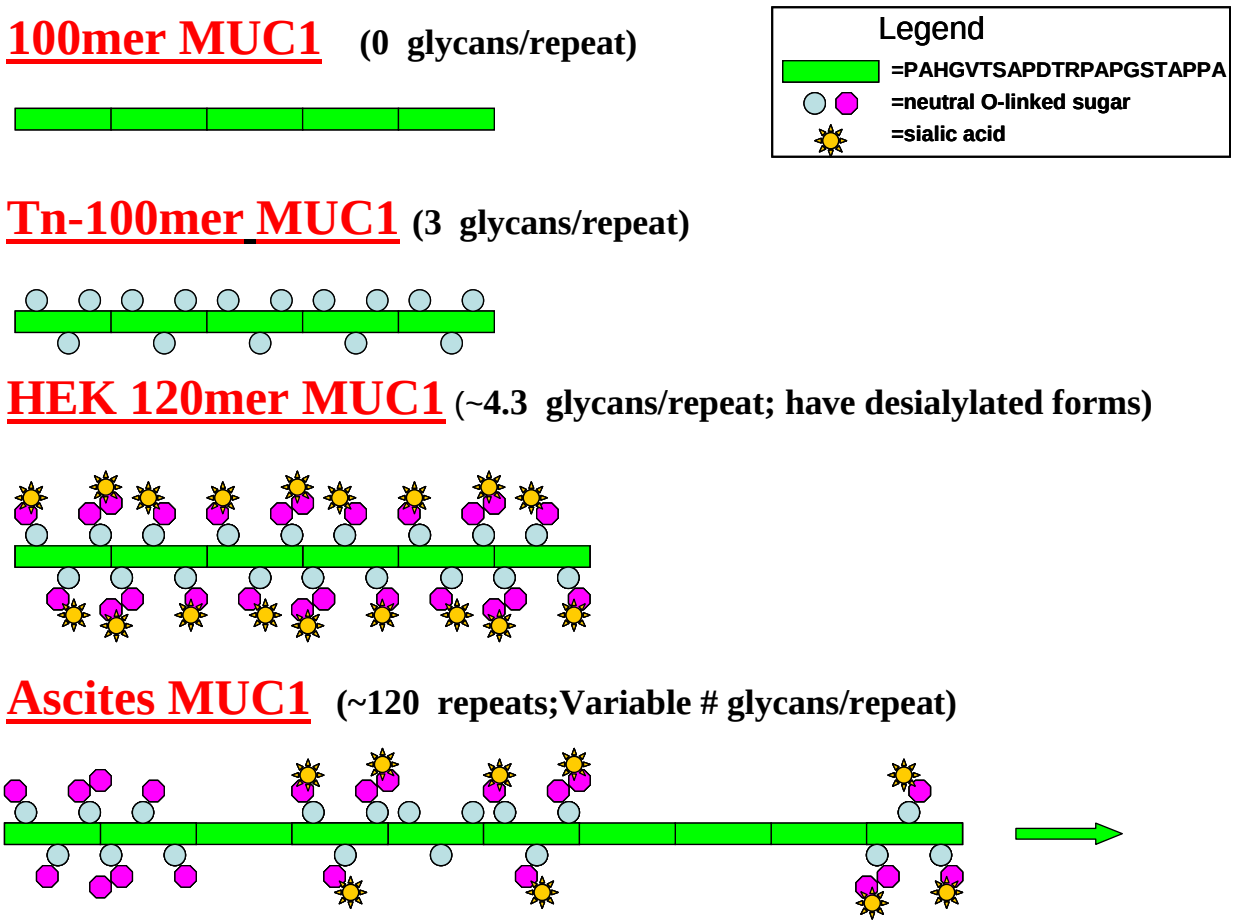

Figure 2: MUC1 forms used in these studies.

100mer MUC1 has 5 repeats and lacks *O*-linked glycosylation. Tn-100mer MUC1 has 5 repeats and a single sugar, GalNAc, added to 3 out of 5 possible sites of *O*-linked glycosylation/repeat. HEK MUC1, and similarly MCF-7 MUC1 (not shown), contains 6 repeats. HEK MUC1 has an average of 4 out of 5 possible glycosylation sites used and has a high density of terminal sialic acid residues. Ascites MUC1 is approximately 120 repeats in length and contains regions similar to each of the 3 forms shown above because of heterogeneity in the addition of carbohydrate side chains *in vivo*.

2.2.4. DC and T cell isolation and purification.

Primary human leukocytes were isolated from fresh normal donor leukapheresis packs under an approved human subjects protocol as previously reported (422). For NK cell purification, nylon wool column separation was used. In some studies, percoll purified lymphocytes, monocytes, or NK cells were cultured at 1 x 10^6 cell/ml in RPMI 1640 (Bio Whittaker, MD) containing 10% fetal bovine serum (HyClone, Logan, UT) and 2 mM glutamine, 100 U/ml penicillin and streptomycin (Quality Biologicals, Gaithersburg, MD), with 100 U/ml of

recombinant human IL-2 for 16 hours or 7 days in a 5% CO_2 humidified tissue culture incubator. Human imDC were generated from purified human peripheral blood monocytes (>95%) as previously described (209) and their phenotype confirmed by flow cytometry. Immature DC were $CD1a^{+}$(Ab clone H1149), $CD14^{-}$ (Ab clone M5E2), $CD40^{low}$ (Ab clone 5C3), $CD83^{-}$ (Ab clone HB15ε), $CD86^{low}$ (Ab clone 2331(FUN-1)), HLA-DR^{medium} (Ab clone G46-6 (L243)). Mature DC were generated by culturing imDC with 1 μg/ml of LPS (Sigma L-9764) for 48 hours. The phenotype of mDC was $CD83^{high}$, $CD86^{high}$, HLA-DR^{high}. Human $CD1c^{+}$ DC were immunomagnetically purified with BDCA-1/CD1c microbeads (Miltenyi Biotec, Auburn, CA) from primary human leukocytes as previously described (423). In brief, percoll purified mononuclear cells were washed and resuspended in 2mM EDTA containing 0.5% heat inactivated fetal bovine serum (Invitrogen, Grand Island, NY). Cells were incubated with anti-CD19 microbeads and anti-CD1c-biotin antibody. Cells were then washed and passed through a column to remove $CD19^{+}$ B cells. The remaining cells were then incubated with strepavidin-microbeads for 15 minutes. Cells were washed and passed through a second magnetic column to capture $CD1c^{+}$ cells. This purification resulted in >90% $CD11c^{+}$ cells that were also uniformly phenotypically immature being HLA-DR^{low}, $CD83^{-}$, and $CD86^{low}$.

2.3. Results

2.3.1. Tumor antigen MUC1 is chemotactic for immature DC.

Using a highly purified form of tumor MUC1 obtained from the ascitic fluid of cancer patients (ascites MUC1) we set up chemotaxis assays with immature myeloid DC. We found that ascites MUC1 induced migration of imDC (Fig 3A). Ascites MUC1 is large in size (over 100 tandem repeats on average) and presents to the DC a wide variety of unglycosylated, as well as complex glycosylated epitopes in the tandem repeat and the N-terminal region (420). To

determine what may be the nature of the epitope responsible for the chemotactic activity, we examined two well-characterized recombinant MUC1 molecules made by the cell lines MCF-7 (breast cancer cell line) and HEK293/EBNA (EBV-transformed human embryonic kidney cell line). MCF-7 MUC1 has a lower overall level of glycosylation compared to other breast cancer cell lines, with a large number of polylactosamine-type *O*-glycans with neutral and fucosylated sugars. HEK MUC1 has a greater density of carbohydrates with a high number of glycans terminating in sialic acid residues (319). Both fucosylation and sialylation can drastically change the binding affinity of ligands to receptors and influence the masking or exposure of epitopes (424). As shown in Figure 3B, the less glycosylated MCF-7 MUC1 was chemotactic for imDC while the more glycosylated and sialylated HEK MUC1 was not. Because unlike ascites MUC1, the recombinant molecules are composed of only tandem repeats and lack the N-terminal segment, we concluded that the chemotactic activity seen in the native and the recombinant forms resides in the tandem repeat region of MUC1.

2.3.2. Tumor MUC1 is not chemotactic for other cells of the immune system.

Since most chemokines attract a variety of cells, we also tested to see whether MUC1 was chemotactic for other cells of the immune system. Most importantly, we tested mature DC. Mature DC did not migrate to either MUC1 100mer or Tn-100mer (Fig. 4A). This fits with most chemotactic responses for the DC because the majority of chemokine receptors expressed on immature DC are downregulated or fail to function once the DC is mature. Lymphocytes and monocytes were also tested and neither cell type migrated in response to either ascites MUC1 or 100mer MUC1 (Fig 4B & 4C).

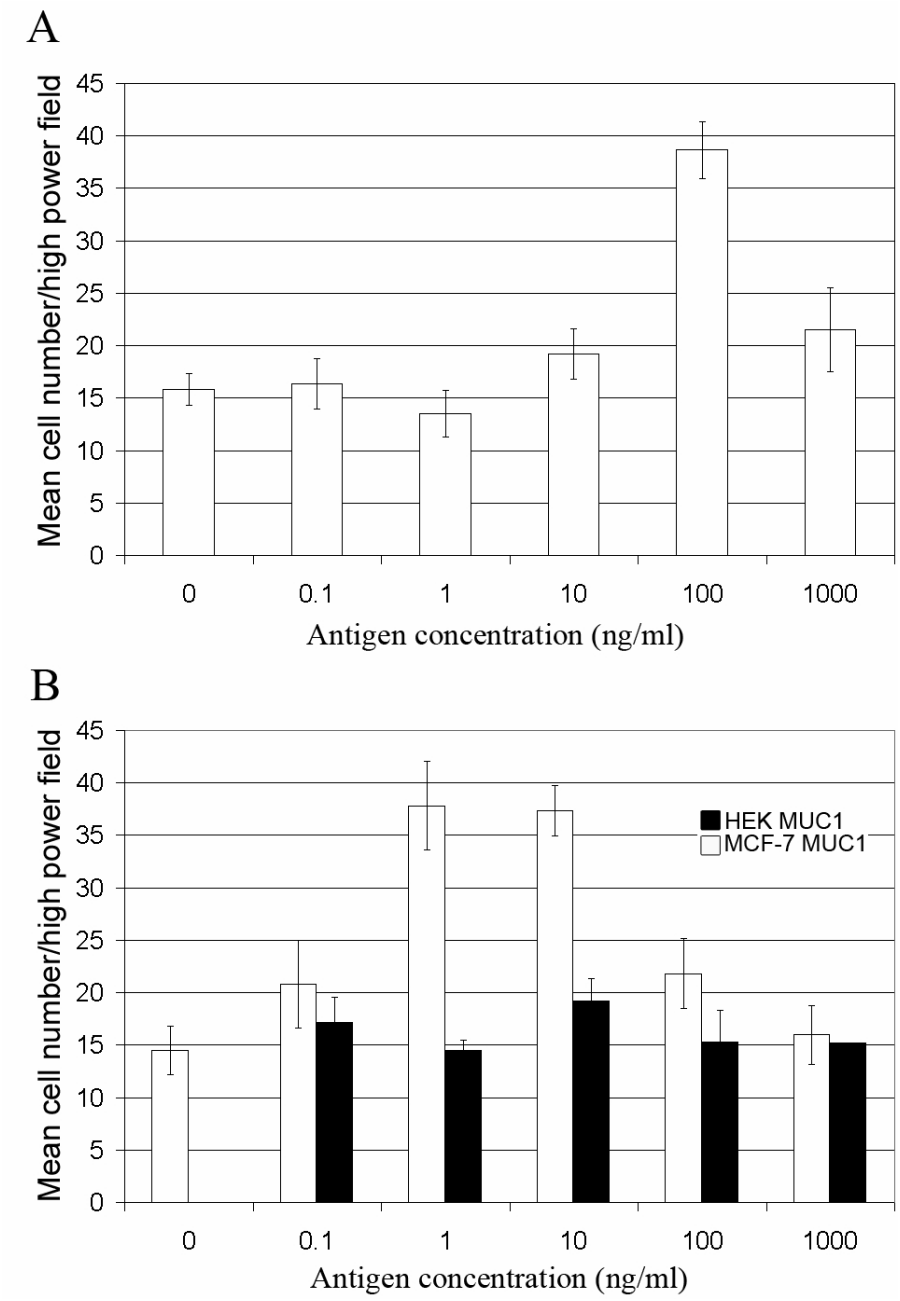

Figure 3: Tumor forms of MUC1 are chemotactic for immature myeloid DC.

For A and B, concentrations of MUC1 in the lower chamber are shown on the x-axis. Increasing concentrations of A) ascites MUC1 and B) MCF-7 MUC1 and HEK MUC1 were added to the lower chamber. Immature human monocyte-derived DC were then added to the upper wells. After 90 minutes, the numbers of DC in the bottom chambers were counted and triplicate wells were averaged. Data shown are representative of three independent experiments.

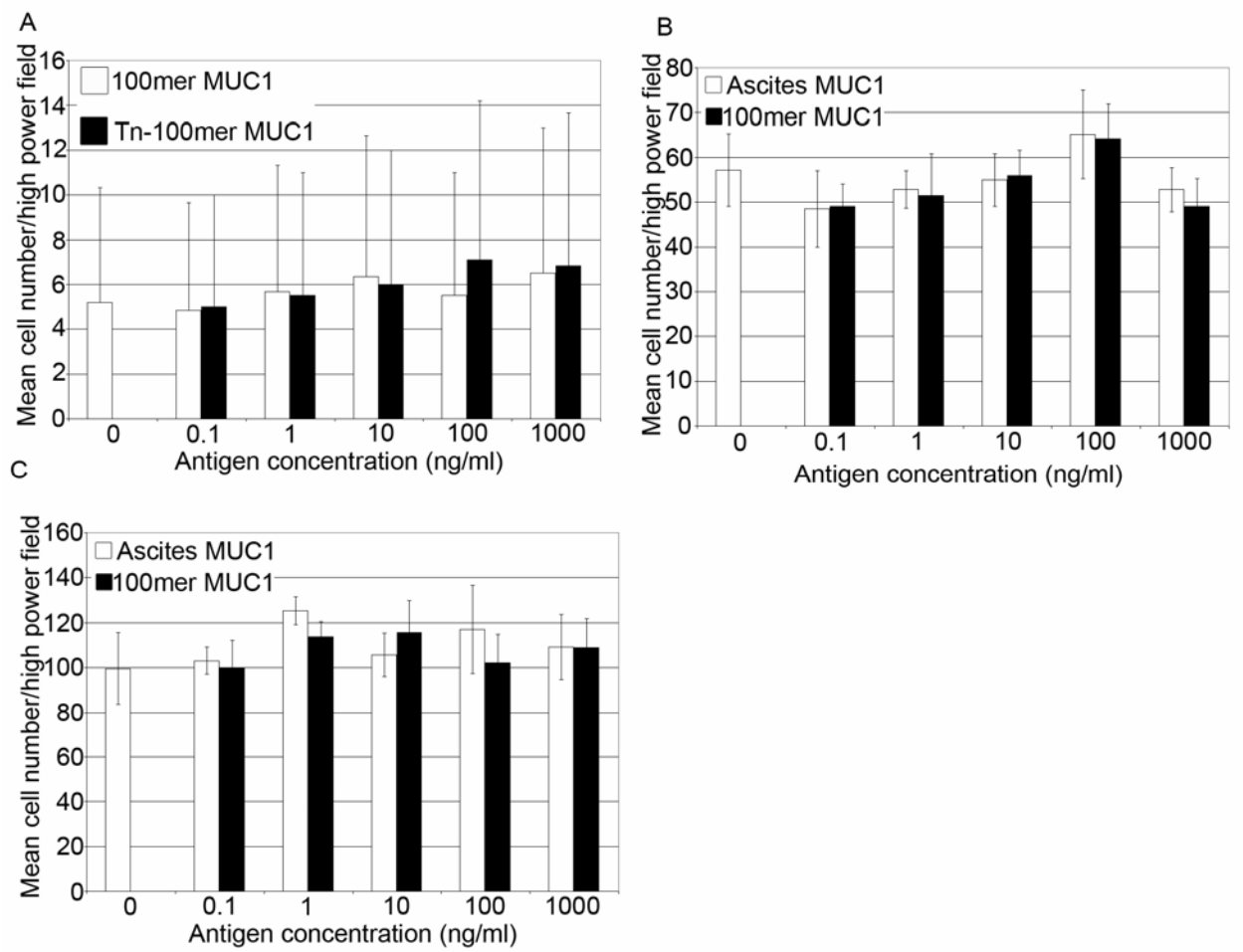

Figure 4: MUC1 does not induce chemotaxis of mature DC, lymphocytes, or monocytes.

Chemotaxis experimtents were conducted as described in Material & Methods section. A, 100mer and Tn-100mer MUC1 do not induce chemotaxis of mature human DC. B, Ascites MUC1 and 100mer MUC1 do not induce chemotaxis of human lymphocytes. C, Ascites MUC1 and 100mer MUC1 do not induce chemotaxis of human monocytes.

We also tested NK cell chemotaxis. Similar to lymphocytes and monoctyes, NK cells were not attracted to MUC1 (Figure 5). The data clearly show that MUC1 is only chemotactic for immature DC. This result is unusual since the known chemokine receptors are expressed by multiple cell types within this group and hence MUC1 should induce migration of more than one of these cell types if it is binding to a characterized chemokine receptor.

2.3.3. **The MUC1 chemotactic function is carried out by its peptide backbone**

Because our data show that MUC1 with fewer sugars had better chemotactic activity (ascites MUC1 and MCF-7 MUC1), we repeated the chemotactic assays with two synthetic MUC1 peptides. Although the purity of ascites MUC1 and MCF-7 MUC1 preparations was high and documented by several analytical methods (319, 420) there remained a small possibility that some chemokine might have contaminated the preparations and is responsible for the chemotactic activity. Therefore we used the synthetic unglycosylated 100mer MUC1 peptide and the same 100mer peptide containing a single GalNAc (Tn) attached to fifteen of the possible twenty-five serines and threonines (Tn-100mer) on the five repeats of the MUC1 tandem repeat backbone. The unglycosylated 100mer MUC1 induced chemotaxis but the Tn-100mer MUC1 did not (Figure 6). This indicates that the unglycosylated peptide backbone is responsible for the chemotactic effect. Furthermore, it shows that this chemotactic activity can be blocked by glycosylation. Some of the other glycosylated MUC1 forms were chemotactic such as ascites MUC1 and MCF-7 MUC1. The discrepancy is likely due to the greater heterogeneity in the glycosylation of these forms compared to Tn-100mer. Tn-100mer is glycosylated *in vitro* using recombinant glycosyltransferases.

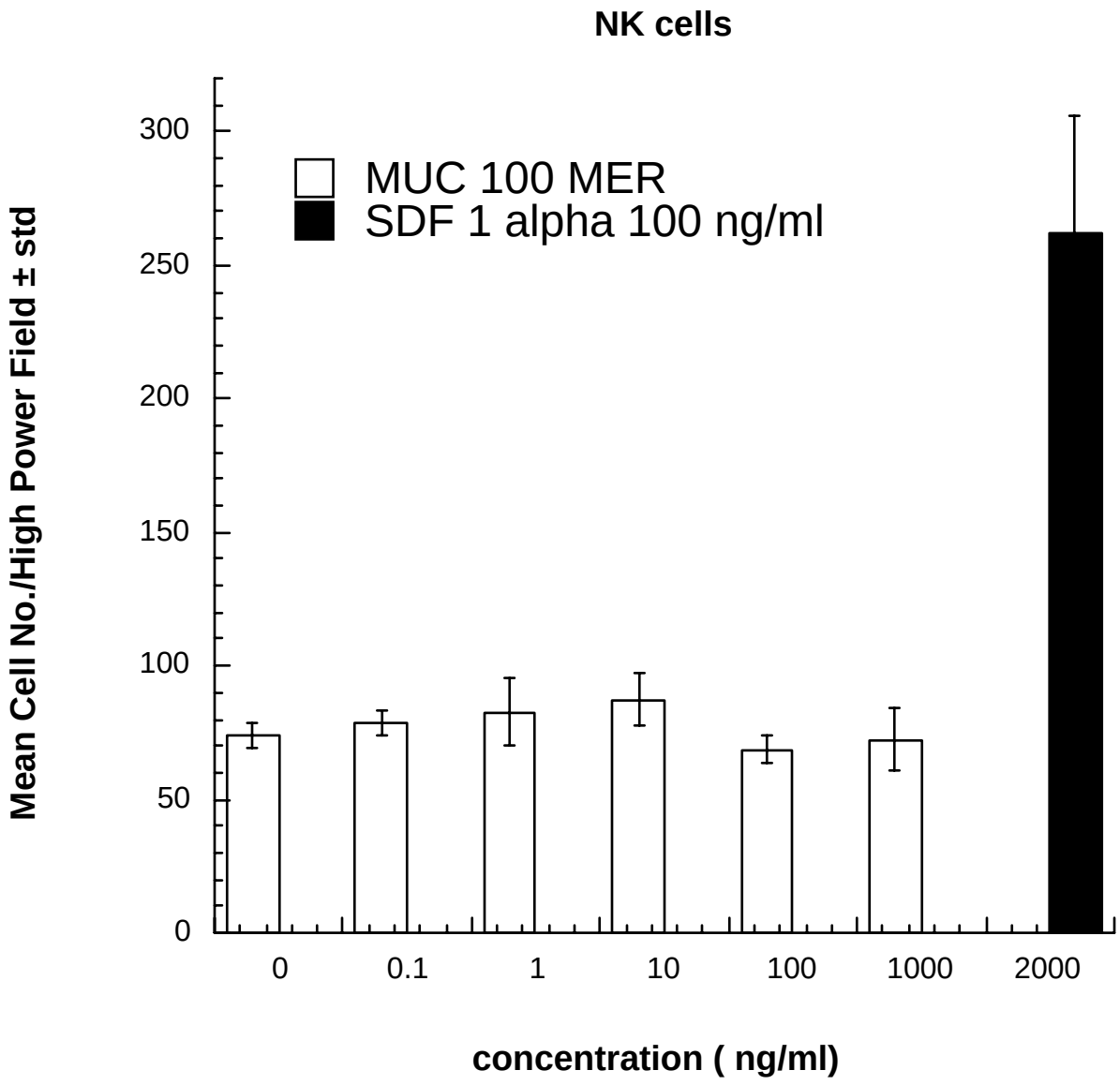

Figure 5: MUC1 100mer is not chemotactic for human NK cells.

NK cells were purified from PBMCs and used in chemotaxis studies as described in Materials and Methods. Concentration of 100mer MUC1 or SDF-1α are shown on the *x*-axis. SDF-1α was used as a positive control for NK cell migration. Data shown are average of 3 chambers with standard deviations and is representative of three independent experiments.

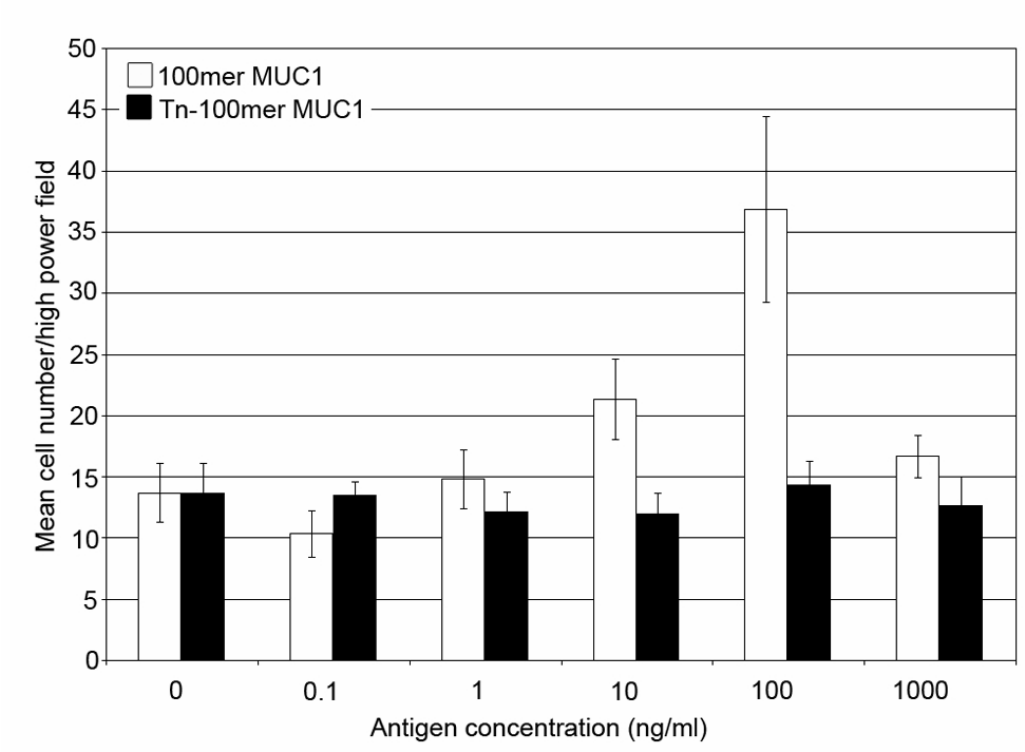

Figure 6: The unglycosylated tandem repeats of tumor MUC1 are the chemoattractive regions.

Chemotaxis experiments were conducted as described in Materials and Methods. Unglycosylated 100mer MUC1 and glycosylated Tn-100mer MUC1 were added in increasing concentrations in the lower chamber of the chemotaxis apparati. Data shown are representative of 3 separate experiments.

This provides great uniformity in the glycosylation of the tandem repeats, such that all three potential glycosylation sites on each repeat are occupied by sugar leaving very few unglycosylated peptide segments. MCF-7 MUC1, on the other hand, retains chemotactic activity due to less uniform *in vivo* glycosylation from one repeat to another. This leaves repeats that are either unglycosylated or under glycosylated and exposes larger unglycosylated peptide segments.

In Figure 7 we show that the chemotactic effect was specific for the MUC1 peptide sequence and could be blocked with an antibody against MUC1, MF11, but not by a control antibody. MF11 antibody is specific for the peptide epitope PPAH in the MUC1 tandem repeat sequence GVTSAPDTRPAPGSTAPPAH. This further confirms that the VNTR peptide

backbone is responsible for the chemotactic effects of MUC1 and suggests that the PPAH region is part of or close to the chemotactic region of MUC1.

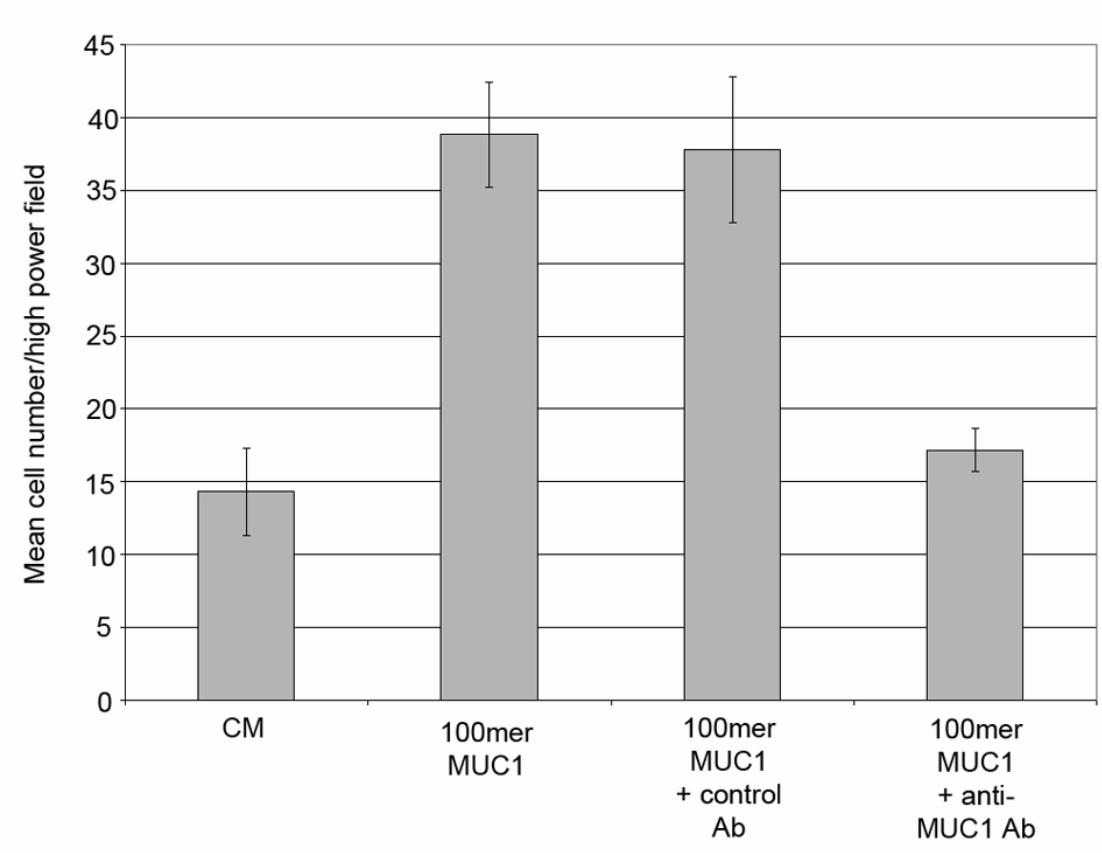

Figure 7: Chemotaxis to MUC1 100mer can be blocked by the addition of anti-MUC1 antibody.

MUC1 100mer (100 ng/ml) was added to the lower chamber with an antibody specific for the unglycosylated form of MUC1, MF11, or an isotype control antibody. CM, control media. Data shown are representative of three independent experiments.

2.3.4. MUC1 is chemotactic for human peripheral blood DC

There are a number of differences between *in vitro* derived DC and DC *in vivo*. DC directly purified from the blood have a lower level of expression of PRRs, a less robust production of cytokines, and a lower sensitivity to activating signals compared to DC generated *in vitro*. Since the circulating DC are the cells that will be predicted to move from the blood to the $MUC1^+$ tumors, we tested the ability of human peripheral blood DC to migrate towards MUC1.

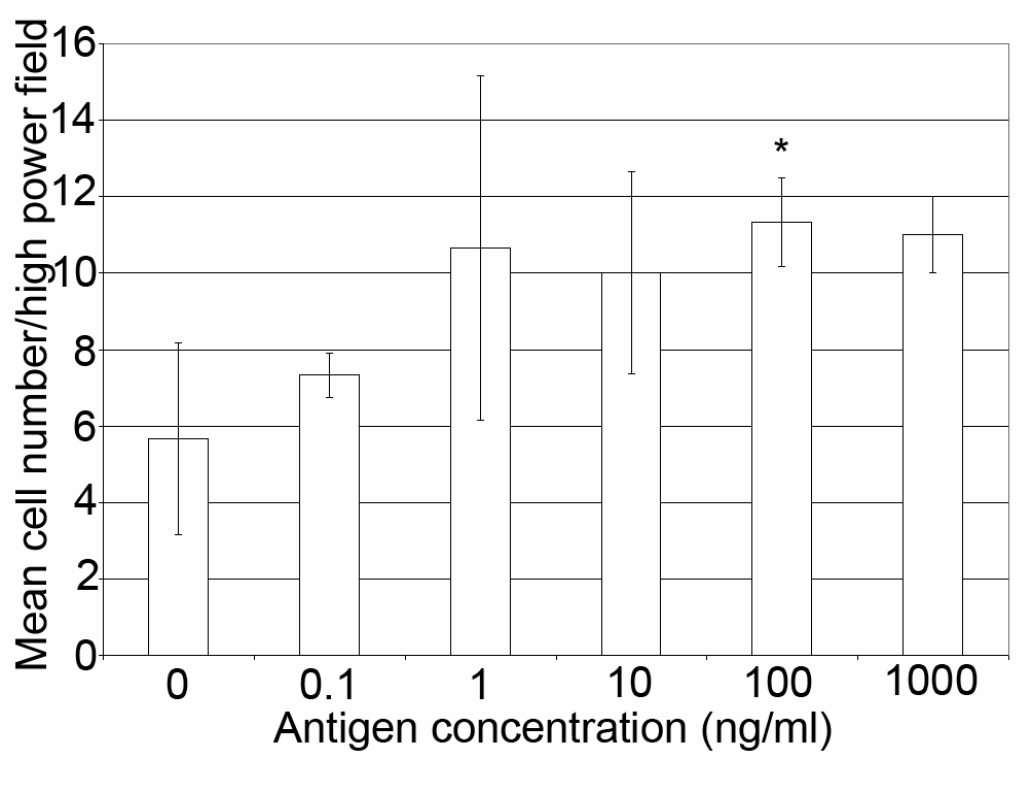

Figure 8: 100mer MUC1 is chemotactic for human CD1c$^+$ peripheral blood DC.

CD1c$^+$ DC were purified from PBMCs of normal donors as described in Materials & Methods. 100mer MUC1 was added to the bottom chamber in the concentrations shown. Cells in lower chamber were counted after 90 minutes and triplicate wells were averaged. CM: control media. *, $p < 0.028$ compared to control media. Results shown are representative of five independent experiments with different normal donors.

As shown in Figure 8, CD1c$^+$ DC from the peripheral blood of normal donors did migrate to 100mer MUC1. However, similar to the reduced ability of these cells to respond to activating signals, these DC did not migrate in large numbers. There was a statistically significant level of migration compared to control media and 100 ng/ml, which is the same concentration of 100mer MUC1 that attracts monocyte-derived DC. From these data, it appears that circulating DC need the same concentration of MUC1 to react but upon receiving this signal, they do not migrate as readily.

2.3.5. MUC1 is chemotactic not chemokinetic for immature human DC

Some molecules are able to increase the random movement of cells. This induced random movement is termed chemokinesis and, unlike chemotaxis, it is not dependent upon a concentration gradient. However, in *in vitro* chemotaxis assays, increased chemokinesis can result in apparent chemotaxis because of an increased random movement across the transwell membrane. To differentiate chemokinesis from chemotaxis, a checkerboard analysis is used. In this experimental set-up increasing concentrations of the chemokine in question are added to both the bottom and top chambers of the chemotaxis apparatus. If the chemokine is truly chemotactic, cells will migrate to the bottom chamber only in the presence of a concentration gradient. This migration shows that the chemotactic effect observed is not due to a simple increase in chemokinetic activity of the DC. As shown in Table 2, the use of a checkerboard analysis confirms that MUC1 is chemotactic and not simply chemokinetic, and hence, a chemokine to immature human DC.

Table 2: Checkerboard Analysis of MUC1 Induced Chemotaxis.

Lower chamber (ng/ml)	Upper chamber (ng/ml)			
	0	1	10	100
0	34.5 (2.6)[a]	36.7 (3.5)	36.7 (3.8)	40.7 (4)
1	52.3 (10)	42.2 (6.3)	45 (2.8)	51.2 (5.1)
10	80.7 (5.5)	61.7 (3.3)	43 (5.2)	44.8 (4.3)
100	105.7 (15)	64.8 (7.4)	49 (9.2)	45.8 (9)

[a]Data shown are average (standard deviation) and representative of three separate experiments

2.3.6. Discussion

The consequence of MUC1 hypoglycosylation and overexpression on tumor cells has been explored in a variety of disease related situations (425). This includes alteration of the

adhesive capabilities of tumor cells, the suppression of T cell function, and alterations in the *in vitro* generation of DC (107, 407, 426-428). This is the first time that MUC1 has been shown to be chemotactic for immature DC. Although certain chemokines have been identified that contain mucin domains (429, 430), no chemotactic regions have previously been described within those domains. Our experiments showing that chemotaxis is induced by peptide epitopes that are only exposed on the tumor-derived molecule suggest that MUC1, which is a secreted as well as a transmembrane molecule, may be acting as a tumor specific chemokine.

Prior studies have shown that there is a large number of DC at sites of $MUC1^+$ tumors (431, 432). Our results suggest that the overexpression of the tumor form of MUC1 may be the signal that brings those imDC from the circulation to the tumor site. Unlike the structure of normal MUC1, the unglycosylated peptide backbone of tumor MUC1 has a characteristic shape and highly stable tandem repeat structure that may be highly effective at binding receptors on DC (262, 433, 434). Efforts are underway to identify this receptor. However, because MUC1 is a complex molecule with many epitopes that could potentially interact with not one but several receptors on DC, the identity of the receptor(s) may be difficult to determine.

DC migration induced by the chemotactic stimulus from MUC1 has similarities to DC migration during inflammation rather than the steady state migration. It is the pro-inflammatory chemokines that characteristically induce migration in only the immature DC population. Immature DC express chemokine receptors such as CCR1, CCR5, and CXCR2 and are primed to respond to pro-inflammatory chemokines such as CCL2 (MCP-1), CCL5 (RANTES), and CXCL8 (IL-8). Upon maturation, they down-regulate these receptors and instead up-regulate other receptors, such as CCR7, which are required for migration toward the secondary lymphoid tissues (178, 435). Underglycosylated MUC1 is produced only by abnormal cells, and hence it

should not be encountered during normal DC migration in healthy tissues. More likely, when $MUC1^+$ pre-malignant cells or small tumors are growing and have not yet gained the mutations to make large amounts of other chemokines, such as CXCL8 (IL-8) and CXCL1-3, MUC1 can attract immature DC (436).

The stroma that surrounds the tumor cells is critical to the survival of malignant cells. That stroma includes cells fibroblasts, cells of the immune system, angiogenic cells and extracellular matrix proteins (437). Tumor derived molecules such as basic fibroblast growth factor, VEGF, PDGF, epidermal growth factor receptor (EGFR) ligands, and various cytokines play a role in stromal formation (437). The data shown here and previous work showing that MUC1 can be chemotactic for fibroblasts (416) indicate that MUC1 can be one of the players in the formation of this desmoplastic tissue that promotes tumor growth and survival.

3. Human Tumor Antigen MUC1 induces maturation of DC and prevents effective induction of Th1 type cells

3.1. Introduction

Unique molecular patterns expressed by pathogens are recognized by antigen presenting cells through pattern recognition receptors (PRRs) such as Toll-like receptors (438), C-type lectins (121), and I-type lectins (117). Immature dendritic cells (imDC) respond to signals from the PRRs by undergoing maturation and turning on the production of specific sets of cytokines typical for individual receptors and the pathogens they recognize. While it has been assumed

that these receptors evolved to signal the presence of invaders from the outside, i.e. pathogens, there is evidence that PRRs also recognize some self molecules suggesting that they may have evolved to alert the immune system to changes in the body that threaten the integrity of the organism that might require its involvement (439).

A tumor is an example of an invader from within and although derived from the host, tumor cells display molecular patterns that distinguish them from normal cells. The presence of tumor specific immune responses in cancer patients are clear evidence that the immune system has been alerted to the presence of the tumor, but the progressive growth of the tumor suggests that the process of immune activation is not usually carried out successfully.

We have studied the ability of dendritic cells to signal to the adaptive immune system the presence of malignant epithelial cells expressing abnormal forms of an epithelial cell glycoprotein MUC1. This molecule is normally expressed on the apical surfaces of ductal epithelial cells and in the lumen of the ducts (thus, outside of the body) but gains access to the inside of the body during epithelial cell transformation and the growth of epithelial adenocarcinomas (425). The extracellular portion of MUC1 is largely composed of a region of multiple tandem repeats of a 20 amino acid sequence. On normal epithelia, MUC1 is expressed at low levels and is complexly *O*-glycosylated, but on tumor cells it is greatly overexpressed and markedly hypoglycosylated with more simple and shorter chains. Reduced glycosylation exposes the peptide backbone resulting in novel peptide epitopes as well as novel truncated carbohydrate epitopes characteristic of tumor cells, such as T (Gal-GalNAc-*O*-Ser/Thr), Tn (GalNAc-*O*-Ser/Thr), and sialyl-Tn (NeuAc-GalNAc-*O*-Ser/Thr) (440). We have published previously that MUC1, either purified from tumor cells or synthesized as a long synthetic tandem repeat peptide with and without *O*-linked carbohydrates, can bind to immature DC and be

internalized through an active endocytic process (105). In a subsequently published study we addressed what type of epitopes, peptides and/or glycopeptides, are generated and could be presented by the DC that bind these different forms of MUC1 and we showed that both peptides and glycopeptides can be found bound to MHC-Class II (327). It is unclear what effects MUC1 binding, through its glycans, has on the DC and whether MUC1 gives an activating signal similar to *Escherichia coli* or a suppressive signal like apoptotic bodies (Figure 9).

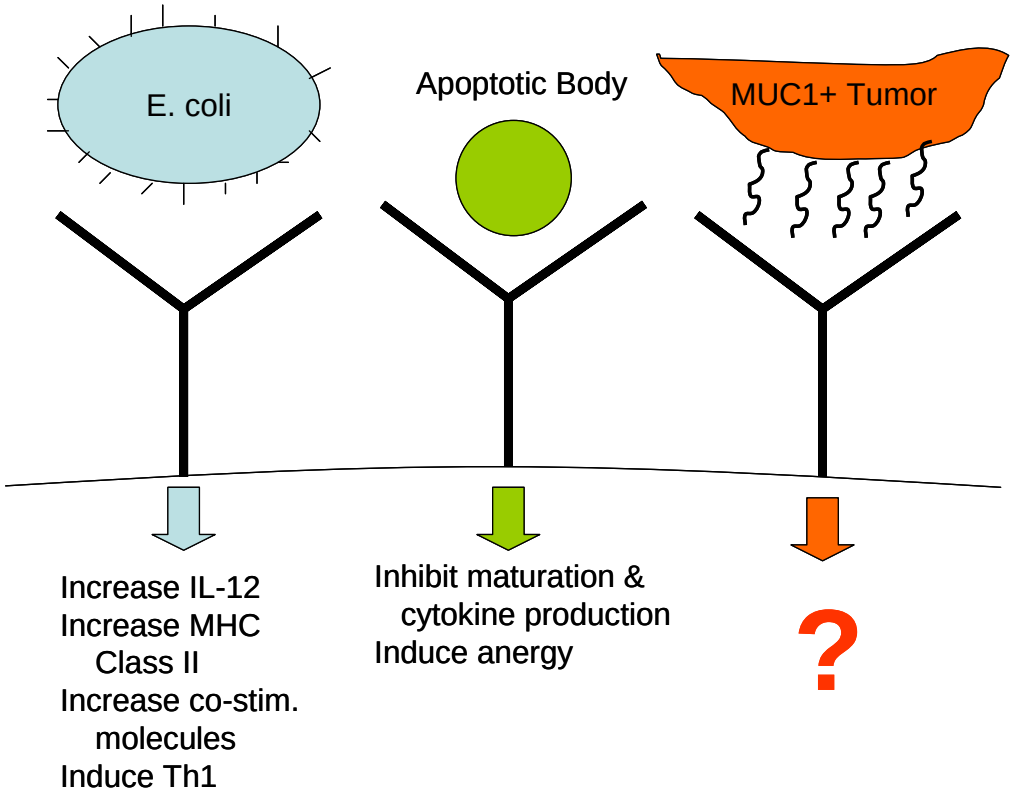

Figure 9: Understanding the effects of MUC1 binding to PRRs on DC function.

In this chapter, we describe an unexpected finding that MUC1 bearing short sialylated carbohydrates, which we previously showed binds and is internalized by DC (327), induces DC activation and maturation phenotypically similar to that induced by LPS. Both molecules induce an increased expression of CD40, CD80, CD86 and CD83. Importantly, whereas LPS treated immature DC (imDC) turn on production of proinflammatory cytokines, DC exposed to tumor

forms of MUC1 produce IL-6, TNF-α, and IL-10, but extremely low levels of IL-12. When naïve T cells are stimulated with allogeneic DC that were exposed to MUC1, they produce higher levels of IL-13 and IL-5 compared to T cells activated with LPS treated DC, which make low levels of these cytokines and high levels of IFN-γ.

Th1 type immunity is considered to be important in tumor rejection (441). DC that have interacted with MUC1 would not be expected to induce a strongly polarized Th1 response either against MUC1 itself or other antigens simultaneously expressed on MUC1+ tumors. High levels of aberrantly glycosylated MUC1 are expressed on all human adenocarcinomas as well as on the known pre-malignant lesions, precursors to some of these tumors (442). Our data show a remarkable ability of dendritic cells to be alerted to the existence of tumors by the ability of some antigens, like MUC1, to subvert their function and prevent development of efficient Th1 type anti-tumor immunity.

3.2. Materials and Methods

3.2.1. Reagents.

Human GM-CSF and IL-4 were obtained from Schering-Plough, Kenilworth, NJ. All other chemokines and cytokines were obtained from the NIH cytokine repository or Peprotech (Rocky Hill, NJ). Reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Anti-MUC1 antibody MF11 used in the chemotaxis blocking studies was obtained from ISOBM TD-4 International Workshop on Monoclonal Antibodies against MUC1 (419). It is specific for the PPAH sequence in the 20 amino acid-long MUC1 tandem repeat sequence GVTSAPDTRPAPGSTAPPAH. Monoclonal antibodies used to phenotype DC were purchased from BD Biosciences (San Diego, CA), unless otherwise indicated.

3.2.2. MUC1 antigen preparations.

MUC1 100mer synthetic peptide (GVTSAPDTRPAPGSTAPPAH) x 5 represents five unglycosylated tandem repeats from the variable number of tandem repeats (VNTR) region comprising most of the extracellular domain of MUC1. MUC1 100mer was synthesized on a Chemtech 200 automated peptide synthesizer (Advanced ChemTech, Inc., Louisville, KY) with N-(9-fluorenyl)methoxycarbonyl chemistry and purified by high-pressure liquid chromatography (HPLC) at the University of Pittsburgh Cancer Institute Peptide Synthesis Facility. MUC1 Tn-100mer is a glycosylated 100mer peptide that was prepared as described previously (321, 327). It contains 15 GalNAc residues bound to the threonines in VTSA regions and serines and threonines in GSTA regions of the tandem repeat sequence GVTSAPDTRPAPGSTAPPAH (glycosylated residues indicated in bold), consistent with the site-specificity of the recombinant glycosyltransferase rGalNAc-Ts used to glycosylate the peptide. Ascites MUC1 was purified from ascitic fluid obtained from cancer patients and characterized as previously described (420). Human milk fat globule (HMFG) MUC1 was purified from human milk as previously described (322). Generation and purification of recombinant tumor forms MCF-7 MUC1 and HEK MUC1 was described previously (319). In brief, a mammalian episomal expression vector pCEP-PU (421) encoding six tandem repeats of MUC1 (120mer peptide) under the control of the cytomegalovirus promoter was used to transfect the breast cancer cell line MCF-7 and the EBV-transformed human embryonic kidney cell line 293/EBNA (obtained from the American Type Culture Collection.) This vector contains the secretory signal peptide of BM40, an extracellular matrix protein also known as SPARC or osteonectin, followed by a hexa-histidine sequence and a Myc tag used for affinity purification of the MUC1 protein produced by the transfected cells. HEK MUC1 and MCF-7 MUC1 were purified from the conditioned supernatant of confluent cell layers by passage through a Ni^{2+}-nitrilotriacetic acid Superflow column (Quiagen, Hilden,

Germany). They were further purified by HPLC on a C8 silica column (Vydac 208TP1015; MZ Analysentechnik, Mainz, Germany). Quality of purified recombinant MCF-7 MUC1 and HEK MUC1 were checked by SDS-PAGE followed by blotting onto a nitrocellulose membrane. Peptides were detected with an anti-myc monoclonal antibody (319) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA.) Specific O-glycosylation profiles were determined by hydrazinolysis and normal phase chromatography of 2-aminobenzamide-labeled glycans as previously described (319). The major sialylated glycans on HEK MUC1 were chemically desialylated by treatment of the fusion protein with 0.1M aqueous trifluoroacetic acid for 1 hour at 80^{o}C followed by drying in a vacuum centrifuge (unpublished observations.) Sialidase treatment of HEK MUC1 was accomplished with *Clostridium perfringens* sialidase (Biolabs) using 5 units in a total volume of 50 μl of reaction buffer (0.1 M sodium acetate, pH 5.5) for 18 hours at 37^{o}C. The effectiveness of desialylation was checked by gel electrophoresis and desalted on a NAP-5 column. All MUC1 peptides, with the exception of HMFG MUC1, were tested for LPS with the limulus amebocyte lysate assay and found to have <0.05 EU/ml by Cambrex (Cambrex Bio Science Walkersville, Inc., Walkersville, MD.)

3.2.3. DC and T cell isolation and purification.

Primary human leukocytes were isolated from fresh normal donor leukapheresis packs under an approved human subjects protocol as previously reported (422). In some studies, percoll purified lymphocytes or monocytes were cultured at 1 x 10^{6} cell/ml in RPMI 1640 (Bio Whittaker, MD) containing 10% fetal bovine serum (HyClone, Logan, UT) and 2 mM glutamine, 100 U/ml penicillin and streptomycin (Quality Biologicals, Gaithersburg, MD), with 100 U/ml of recombinant human IL-2 for 16 hours or 7 days in a 5% CO_2 humidified tissue culture incubator. Human imDC were generated from purified human peripheral blood monocytes (>95%) as previously described (209) and their phenotype confirmed by flow cytometry. Immature DC

were $CD1a^{+}$(Ab clone H1149), $CD14^{-}$ (Ab clone M5E2), $CD40^{low}$ (Ab clone 5C3), $CD83^{-}$ (Ab clone HB15ε), $CD86^{low}$ (Ab clone 2331(FUN-1)), HLA-DR^{medium} (Ab clone G46-6 (L243)). Mature DC were generated by culturing imDC with 1 μg/ml of LPS (Sigma L-9764) for 48 hours. The phenotype of mDC was $CD83^{high}$, $CD86^{high}$, HLA-DR^{high}. Human $CD1c^{+}$ DC were immunomagnetically purified with BDCA-1/CD1c microbeads (Miltenyi Biotec, Auburn, CA) from primary human leukocytes as previously described (423). In brief, percoll purified mononuclear cells were washed and resuspended in 2mM EDTA containing 0.5% heat inactivated fetal bovine serum (Invitrogen, Grand Island, NY). Cells were incubated with anti-CD19 microbeads and anti-CD1c-biotin antibody. Cells were then washed and passed through a column to remove $CD19^{+}$ B cells. The remaining cells were then incubated with strepavidin-microbeads for 15 minutes. Cells were washed and passed through a second magnetic column to capture $CD1c^{+}$ cells. This purification resulted in >90% $CD11c^{+}$ cells that were also uniformly phenotypically immature being HLA-DR^{low}, $CD83^{-}$, and $CD86^{low}$.

$CD4^{+}$ T cells were purified from peripheral blood mononuclear cells using the $CD4^{+}$ T cells isolation kit (Miltenyi Biotec.) In brief, non-$CD4^{+}$ T cells were removed by labeling cells with a cocktail of biotinylated monoclonal antibodies to CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and glycophorin A and then mixing labeled cells with anti-biotin microbeads. Labeled cells were removed by passing cells through a magnetic separation column were the labeled cells became trapped in a magnetic field. The negative cells contained in the column effluent were > 90% $CD4^{+}CD3^{+}$ by flow cytometry. The $CD4^{+}$ positive fraction was then labeled with CD45RO microbeads (Miltenyi Biotec) and depleted of $CD45RO^{+}$ cells by magnetic separation.

3.2.4. Assessment of DC phenotype and cytokine production.

DC phenotype was assessed by flow cytometry. In brief, cells were washed and counted prior to incubation with normal mouse serum (Jackson Laboratory, Bar Harbor, ME) for 15 minutes at 4 ^{o}C to block non-specific Fc receptor binding. Cells were then stained with specific antibodies for 30 minutes at 4 ^{o}C in the dark and washed extensively prior to fixation in 1% paraformaldehyde solution. Cytometry was performed with either a Becton Dickenson FACScaliber cytometer or LSR II cytometer. Flow data was analyzed using CellQuest software.

DC supernatants were collected and stored at -80^{o}C until testing. Cytokines were measured with the Luminex multiplex system (Luminex Corp., Austin, TX) (443). Luminex DC and T cell isolation and purification. Luminex combines the principle of a sandwich immunoassay with the fluorescent-bead-based technology allowing multiplex analysis of several different analytes in a single microtiter well (444). Microspheres were obtained from BioSource (Camarillo, CA) to measure a panel of DC cytokines (IL-12p70, IL-10, IL-6, IL-1β, and TNF-α). Assays were performed in 96-well microplate format according to the protocol by BioSource International. A filter-bottom, 96-well microplate (Millipore, Billerica, MA) was blocked for 10 minutes with PBS/BSA. To generate a standard curve, five-fold dilutions of appropriate standards were prepared in media diluent. Standards and supernatants were pipetted at 50 µl/well in duplicate and mixed with 50 µl of the mixture. The microplate was incubated for 1 h at room temperature on a microplate shaker. Wells were then washed three times with washing buffer using a vacuum manifold. Phycoerythrin (PE)-conjugated secondary antibody was added to the appropriate wells and the wells were incubated for 45 minutes in the dark with constant shaking. Wells were washed twice, assay buffer was added to each well, and samples were analyzed using the Bio-Plex suspension array system, (Bio-Rad Laboratories, Hercules, CA).

3.2.5. MLR and assessment of T cell cytokine production.

1 x 10^5 allogeneic $CD4^+CD45RO^-$ T cells were co-cultured with $1x10^4$ DC per well in complete RPMI in triplicate in a 96-well plate. On day 6, the plates were spun down and supernatants collected. Cytokines in the supernatants were measured by Luminex as described above for assessment of DC cytokine production, with a panel of microspheres specific for T cell cytokines IL-5, IL-4, IL-13, IL-10 and IFN-γ, (BioSource).

3.3. Results

3.3.1. Tumor forms of MUC1 induce cell surface maturation markers on DC.

The observation that tumor MUC1 is chemotactic for immature DC raised the question of the immunological consequences of the interaction of MUC1 with the DC once they are recruited from peripheral blood to the tumor site. To model this *in vitro*, we purified myeloid DC from PBMCs of normal donors and exposed them to various forms of MUC1. LPS treatment served as the positive control for DC activation and maturation. DC were immature at the time of purification and responded strongly to LPS by upregulation of surface markers CD83, CD80, CD86, CD40, and MHC Class II (Figure 10A and data not shown). No change in the expression of these markers was seen upon interaction with the chemotactic forms, the unglycosylated 100mer MUC1, Tn-100mer MUC1, or MCF-7 MUC1 (data not shown). However, HEK MUC1, the form without chemotactic activity, but with high levels of sugars and terminal sialic acid residues, induced increased expression of all the maturation markers (Figure 10A).

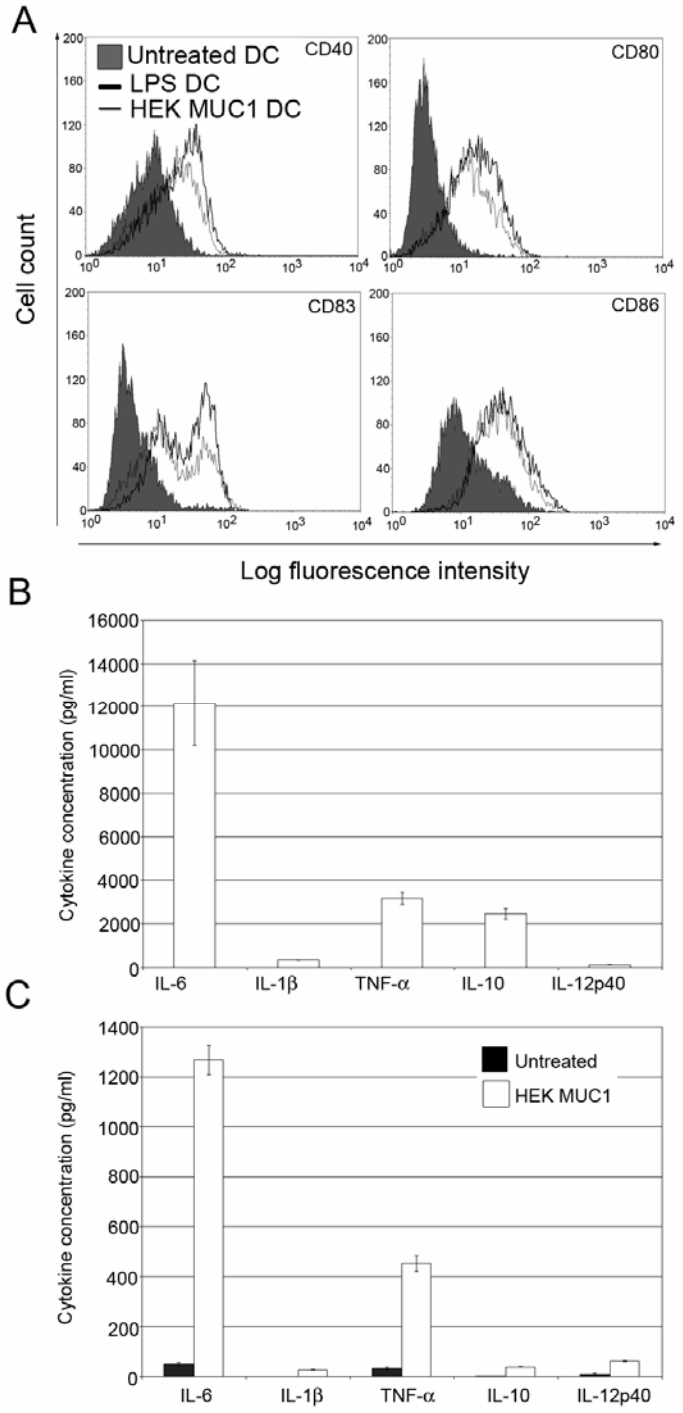

Figure 10: Tumor MUC1 induces the maturation and cytokine production of human peripheral blood DC.

CD1c$^+$ human myeloid DC were purified from the peripheral blood mononuclear cells of normal donors. A) Expression of maturation and co-stimulatory makers CD40, CD80, CD83 and CD86 on peripheral blood DC. DC were untreated or treated with 1 μg/ml of LPS or 50 μg/ml of HEK MUC1 for 18 hours prior to staining. B) Cytokine production by LPS DC at 18 hours as measured by multiplex analysis. C) Cytokine production by untreated DC and HEK MUC1 treated DC at 18 hours as measured by multiplex analysis. For B & C, triplicate wells were averaged and standard deviations were calculated. Results are representative of 3 independent experiments with different donors.

Our observation that tumor MUC1 can activate and mature DC, thus presumably making them more potent antigen presenting cells, appeared to be in contrast to several reports that terminal sialic acid residues on other tumor derived molecules and on mucins from other organisms have immunosuppressive effects on DC and T cells (111, 160, 445). Since upregulation of maturation markers does not always guarantee an immunostimulatory DC (446), we also examined the production of cytokines by DC that have interacted with various forms of MUC1 and compared them to the DC activated and matured by LPS. We were especially interested in the production of cytokines that would make the DC effective in priming naïve $CD4^+$ T cells to become Th1 cells. Culture supernatants were collected from DC left untreated or treated with LPS or tumor MUC1. The presence of various cytokines was assessed by multiplex analysis with Luminex. LPS treated DC produced high levels of TNF-α, IL-6, IL-10, and IL-1β and a low but detectable level of IL-12p40 that correlated with increasing concentrations of LPS (Figure 10B). We found that human $CD1c^+$ DC isolated directly from peripheral blood produced approximately 100 fold lower amounts of IL-12p40 then monocyte-derived DC, even when treated with large amounts of LPS with and without other DC maturation signals, such as CD40L (Figure 10B and unpublished data). Tumor MUC1 treated DC produced lower overall levels of cytokines than LPS treated DC but significantly greater amounts of IL-6, TNF-α, and IL-10 than untreated DC (Figure 10C). IL-12p40 expression by tumor MUC1 treated DC was extremely low. Different donors differed in the overall magnitude of response but not in the profile of cytokines produced.

3.3.2. HMFG MUC1 from human milk activates DC

MUC1 is shed into human milk from lactating mammary glands. It is likely that the MUC1 shed from lactating breast tissue is more similar to tumor MUC1 than normal MUC1 because these epithelial cells are rapidly growing and shedding MUC1. This form of MUC1 also

has high levels of sialylation on its glycans. Therefore we tested whether this highly sialylated form of MUC1, this time directly derived from a human source instead of a tumor cell, could activate human DC.

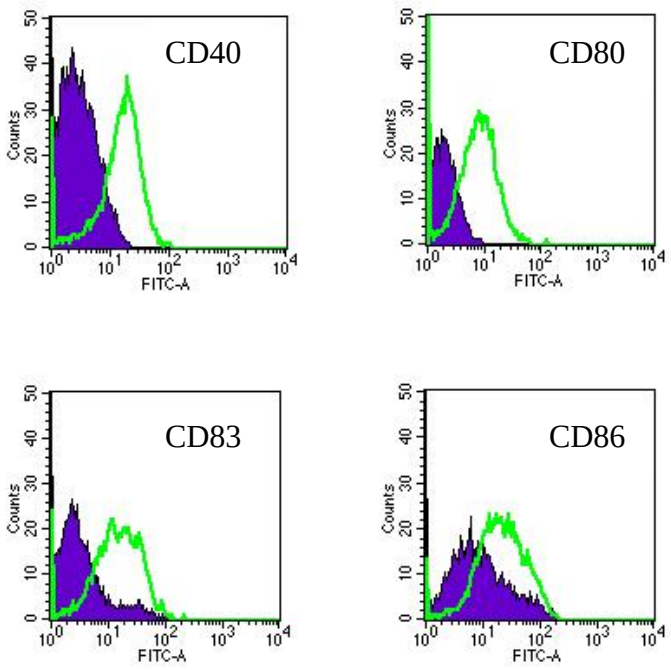

Figure 11: HMFG MUC1 activates human DC.

CD1c$^+$ DC were purified as previously described and left untreated or treated with HMFG MUC1 (50 μg/ml) or LPS (1 μg/ml, data not shown) for 18 hours. Purple histogram, untreated DC; Green histogram, HMFG MUC1 treated DC. Results shown are representative of three separate experiments.

As shown in Figure 11, HMFG MUC1 clearly activated the immature DC. Although we can not completely rule out that there is not LPS contamination of this protein, because we were not able to test the protein for LPS due to the low amount of available protein, is is likely that the response we see is due to MUC1 and its sialic acid residues. The cytokine production (Figure

12) and DC activation elicited by this form was even stronger than HEK MUC1. This indicates that a larger molecule of MUC1 with more repeats may be able to induce even stronger signaling to the DC, perhaps by crosslinking a greater number of receptors. It also raises the interesting point that MUC1 may play a role both in the immune response to MUC1 generated by lactation as well as in the newborns that ingest forms of MUC1 like HMFG MUC1.

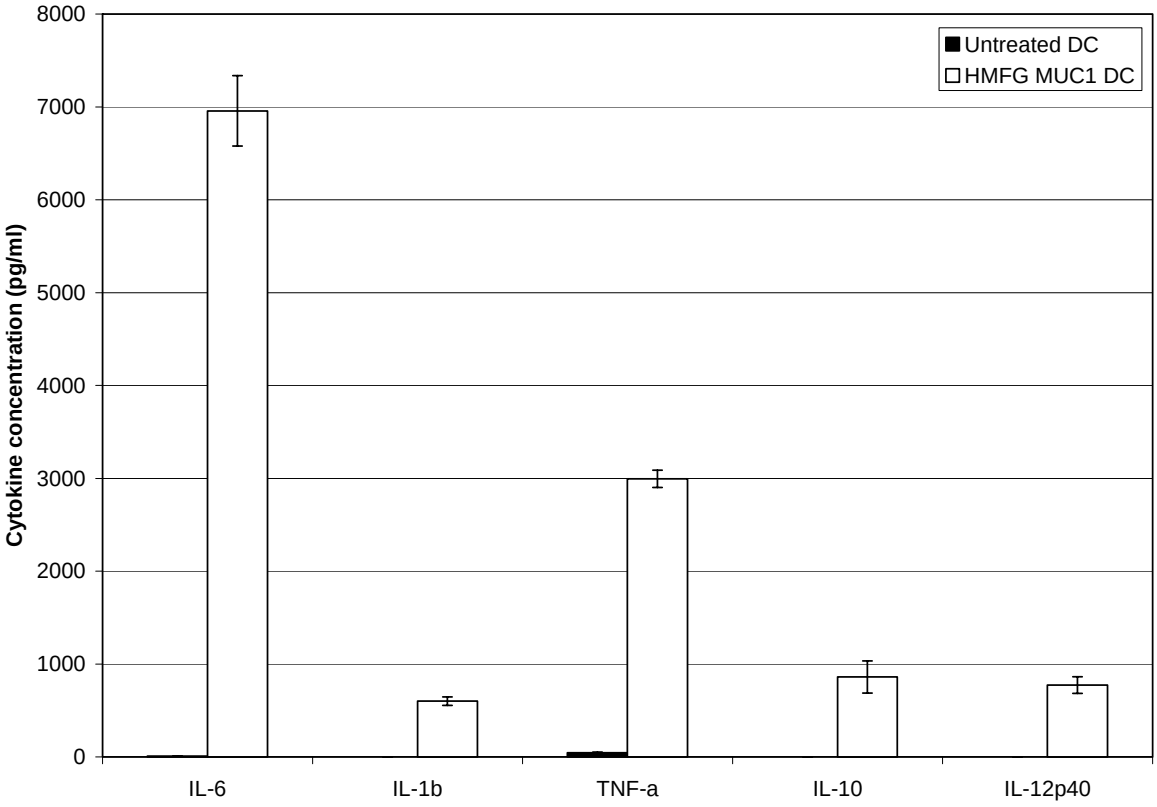

Figure 12: HMFG MUC1 induces high levels of cytokine production.

Supernatants from DC treated as in Figure 11 were collected and cytokine concentrations were measured by Luminex. Results shown are representative of three independent experiments.

3.3.3. Terminal sialic acid residues on MUC1 induce altered DC maturation

Sialylation has been shown to be important in many receptor-ligand interactions (117, 424) and thus we tested its involvement in MUC1 induced DC maturation and cytokine production. We show in Figure 13A that desialylation of HEK MUC1 [(S-) HEK MUC1]

through chemical removal of terminal sialic acids, abolished its ability to increase expression of maturation markers. Removal of sialic acid residues with bacterial sialidase, although not as complete as chemical removal, also inhibited the ability of HEK MUC1 to induce up-regulation of maturation markers (Figure 13A). Combined with the data above, these data suggested that *in vivo*, imDC could be attracted to the tumor site through the chemotactic activity of the unglycosylated peptide epitopes on the tumor MUC1 tandem repeats and then through the interaction with the sialylated carbohydrate epitopes, acquire a more mature phenotype, i.e. up-regulate their cell surface levels of antigen presenting and co-stimulatory molecules. Similar to the results that we saw for upregulation of maturation markers, the induction of cytokine production was dependent upon the terminal sialic acids and with the more efficient desialylation, with TFA treatment, the better the prevention of cytokine induction (Figures 13B&C).

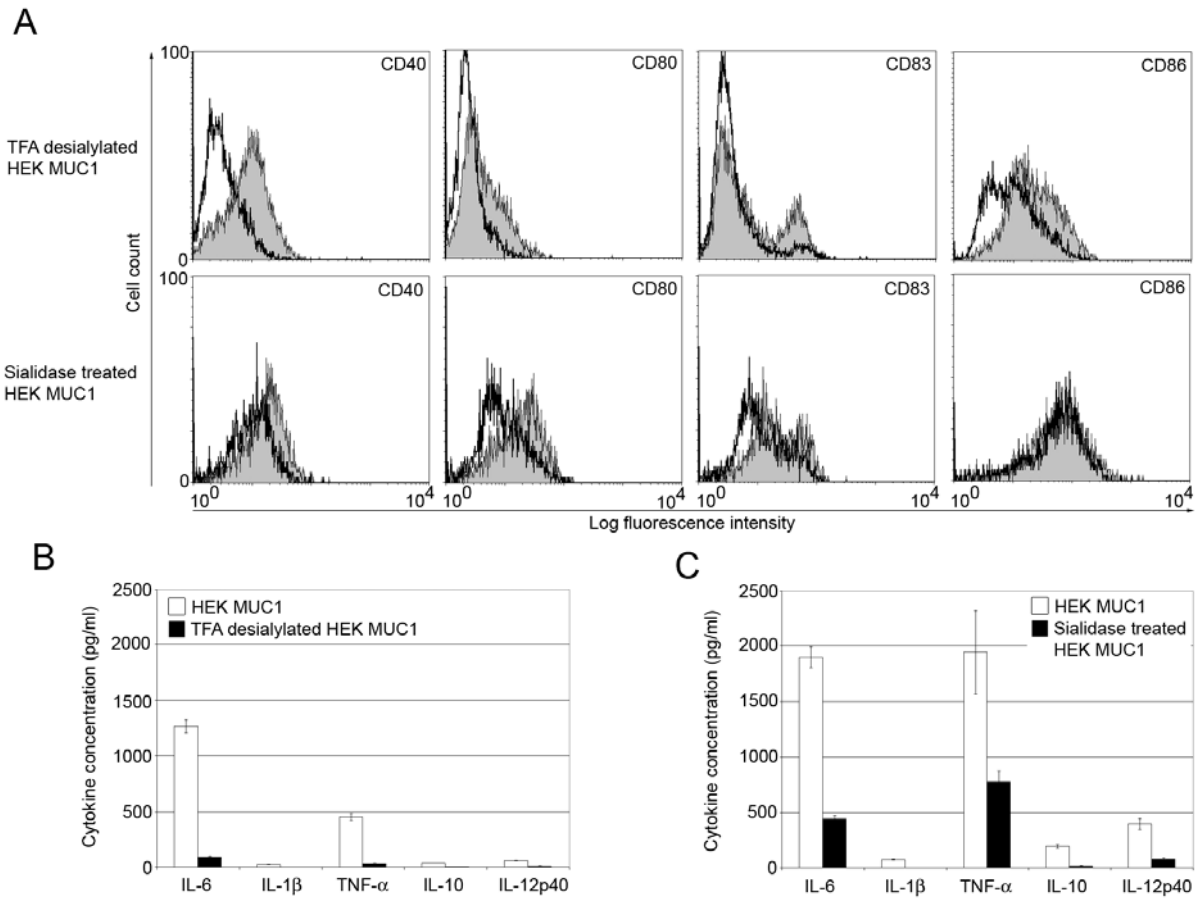

Figure 13: The removal of terminal sialic acids from tumor MUC1 prevents the induction of maturation and cytokine production by DC.

$CD1c^+$ human myeloid DC were purified from the peripheral blood mononuclear cells of normal donors. DC were treated with 50 μg/ml of HEK MUC1, TFA chemically desialylated HEK MUC1, or sialidase treated HEK MUC1 for 18 hours prior to flow analysis. Filled histogram, HEK MUC1 DC, thick line upper panels, TFA treated HEK MUC1; thick line lower panels, sialidase treated HEK MUC1. B) Cytokine production by HEK MUC1 DC and TFA desialylated HEK MUC1 DC at 18 hours as measured by multiplex analysis. C) Cytokine production by HEK MUC1 DC and sialidase treated HEK MUC1 DC at 18 hours as measured by multiplex analysis. Results shown are representative of 3 independent experiments with different donors.

3.3.4. MUC1-"matured" DC fail to promote T cell commitment to Th1

Since DC showed increased levels of cytokine production and co-stimulatory molecules upon interaction with tumor MUC1, we examined how effective these DC would be in stimulating and polarizing naive $CD4^+$ T cells. Interestingly, as shown in Figure 14, the proliferation induced by untreated, LPS treated and HEK MUC1 treated DC was very similar despite the distinct differences between the levels of co-stimulatory molecules and cytokines expressed by these groups of DC. It is unclear why there is no difference.

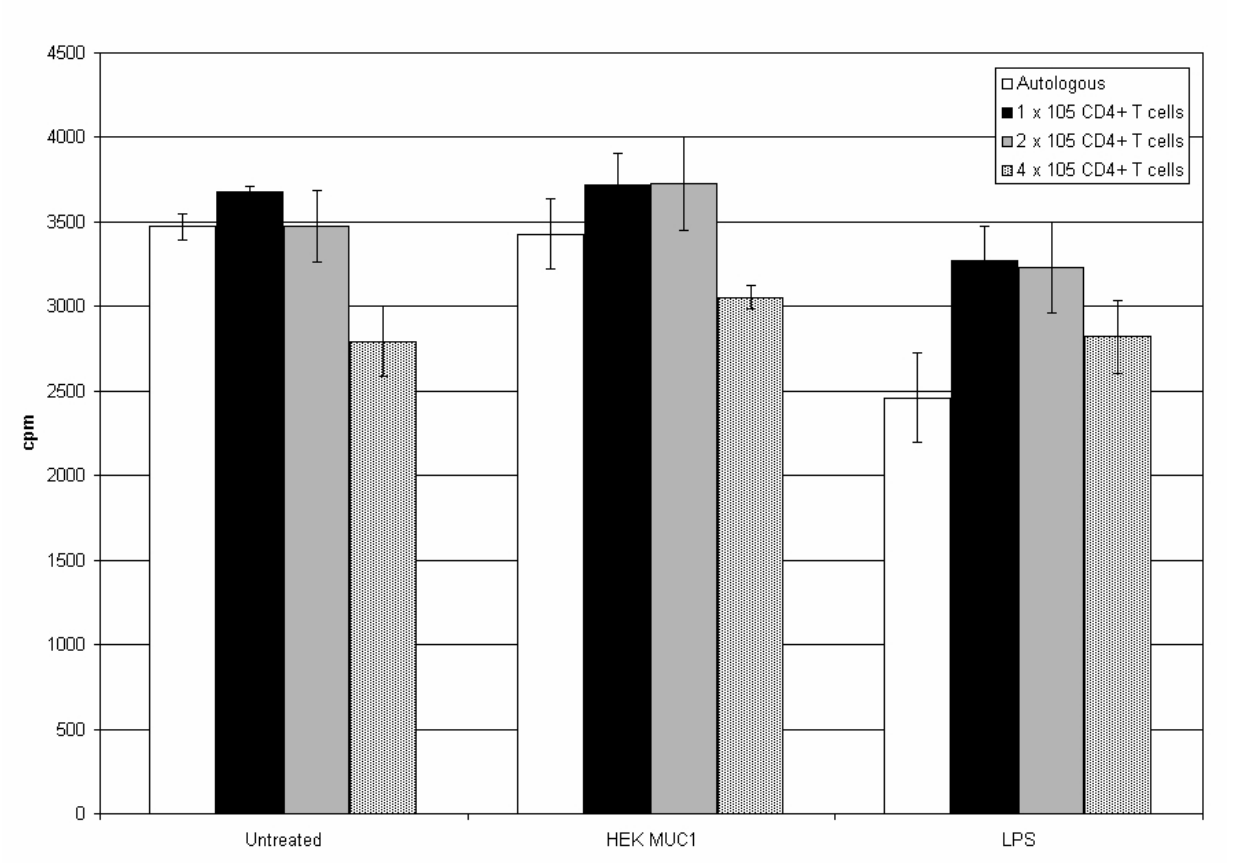

Figure 14: $CD4^+$ T cell proliferation in response to HEK MUC1 treated DC.

Human $CD1c^+$ DC were treated with nothing, LPS (1μg/ml) or HEK MUC1 (50 μg/ml) for 12 hours prior to washing and co-culture with T cells. 1 x 10^4 DC were added per well and the number of cells added per well are indicated in the legend. On day 4 of mixed leukocyte reaction, H^3-thymidine was added for 18 hours to measure proliferation. At end of incubation, cells were lysed and incorporated thymidine was measured by β-emission. Results shown are averages of triplicate wells with standard deviations and are representative of 3 independent experiments.

However, there are sharp differences in the cytokine production of these T cells despite the lack of difference in proliferation. As shown in Figure 15, T cells stimulated with untreated immature allogeneic DC produced low levels of IL-5, IL-13 and IFN-γ, indicating that they did not have the capacity to skew the development of $CD4^+$ T cells towards either Th1 or Th2 phenotype. LPS treated DC were fully capable of inducing Th1 T cells that produce IFN-γ and repressing IL-5 and IL-13 producing T cells (data not shown). HEK MUC1 treated DC did not skew CD4+ T cells towards either Th1 or Th2 type but instead induced IL-5, IL-13 as well as IFN-γ producing T cells and suppressed IL-2 production. IL-4 and IL-10 were not significantly induced in any of these experiments (unpublished observations). As in the previous experiments, desialylation of tumor MUC1 blocked the induction of T cell cytokine production compared to the sialylated form of MUC1 (Figure 15).

3.3.5. Discussion

Overexpression of MUC1 and its glycosylation with sialylated core-type glycans are seen on a large number of human cancers (447, 448). Our data, shown in chapter 2, suggest that the hypoglycosylated MUC1 draws immature DC to the tumor site where they could pick up tumor antigens for presentation to T cells in the lymph node. In this chapter we show that through interactions with the highly sialylated MUC1, these DC acquire a surface phenotype of activated and matured DC that are fully expected to promote T cell activation and to skew the response to the Th1 type important for tumor rejection. On a closer examination, however, these DC show instead a state of semi-maturation, produce IL-6 and TNF-α that have been implicated in tumor metastasis and progression, and in the lymph node do not support T cell commitment to Th1. In

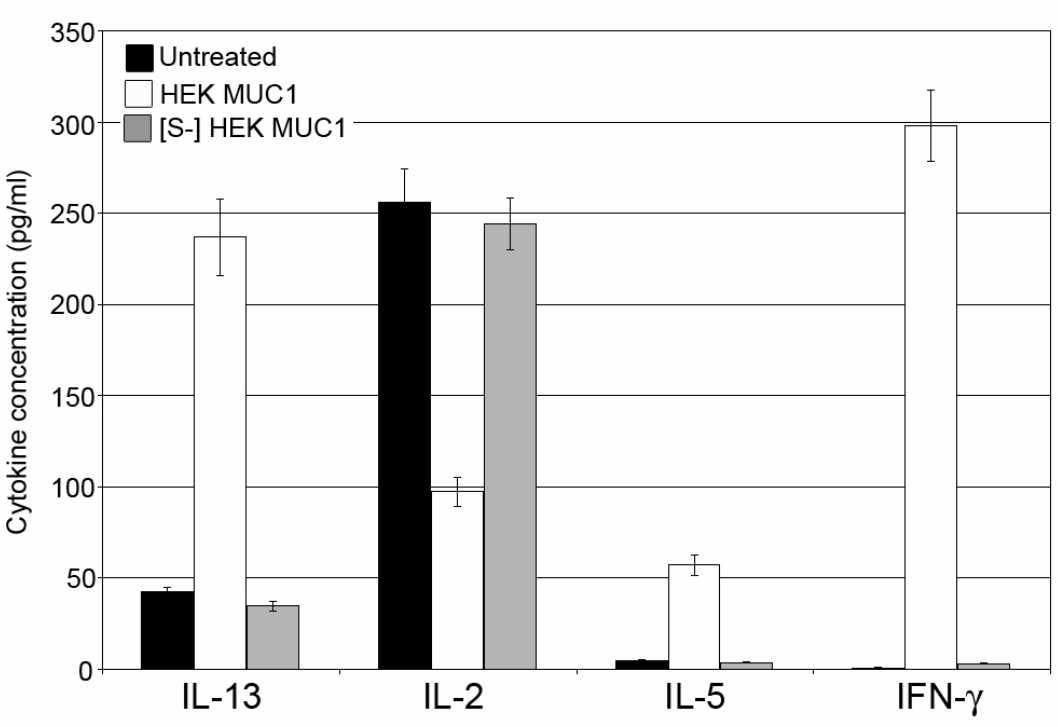

Figure 15: DC that have interacted with MUC1 fail to induce strong Th1 responses in T cells despite expressing co-stimulatory molecules.

$CD1c^+$ DC were treated with nothing, HEK MUC1 (50 μg/ml), or [S-] sialidase treated HEK MUC1 (50 μg/ml) for 12 hours before washing and plating with naïve $CD4^+$ T cells from either the same donor (syngeneic or syn.) or an unrelated donor in triplicate wells. Cell free supernatants from day 6 mixed leukocyte reactions were collected and cytokine levels were measured by Luminex. Supernatants from DC or T cell cultured alone did not produce significant levels of any of the cytokines tested. Graph shown is representative of three independent experiments.

fact, the production of TNF-α and IL-6 have both been linked to cancer and in some cases to a worse clinical prognosis (447-449). Both TNF-α and IL-6 are also keys to the migration of cells through endothelium and hence could ease the escape of metastatic cells from the tumor site. Therefore, imDC that are drawn to the $MUC1^+$ tumor could, through the production of these cytokines, enhance the local immunosuppression and increased tumor invasion.

In addition, these DC promote expansion of T cells that produce large amounts of IL-13, another cytokine that has been implicated in preventing immunosurveillance of tumors and

facilitation of tumor outgrowth by repressing the function of tumor specific CTLs (450, 451). IL-13 has been implicated in facilitating the growth and spread of tumors by direct effects on tumor cells expressing the IL-13 receptor, type II IL-4 R (450). IL-13 also contributes to the stimulation of ImCs to produce TGF-β (452). STAT-6, a downstream mediator of IL-13 signaling, has also been identified as a pivotal player in metastatic disease (453).

Our experiments with sialic acid removal suggest that receptors that signal the DC to mature in response to MUC1 are likely to be sialic acid recognizing receptors of the I-type lectin family known as siglecs (117). MUC1 has been shown to bind to siglec-1/sialoadhesin, but since sialoadhesin lacks a signaling motif, it could not be responsible for the effects that we see (104). The identification of the responsible receptor(s) awaits further data on the expression of various receptors on human circulating DC and reagents for their characterization. HEK MUC1 has tandemly repeated sialic acids in both α2-3 and α2-6 linkages that are predicted to bind to more than one member of the sialic acid binding receptor family confounding their identification (454).

There has been little information to date on factors produced by malignant or pre-malignant cells that can affect fully differentiated DC. Tumor MUC1, along with other tumor derived molecules, has recently been shown to inhibit the differentiation of monocytes into DC *in vitro* (107) and it was suggested that this may contribute to the maintenance of tumor immunosuppression. More importantly, the data presented here show that even normally differentiated DC that encounter abnormal cells expressing MUC1 will be affected and help start the process of tumor-induced DC dysfunction.

Understanding interactions between tumor antigens and the immune system will help in predicting the potential of certain immunotherapeutic approaches. For example, in choosing the

form of MUC1 antigen to use in a cancer vaccine, one can select epitopes that maintain beneficial interactions with DC and eliminate those that negatively affect their function. In the case of $MUC1^+$ tumors, it has to be taken into consideration that cancer patient's T cells are continuously exposed to DC that have interacted with MUC1 in the tumor environment. Therefore, a cancer vaccine administered in the therapeutic setting may not be able to substantially and qualitatively change the ongoing immune response. A more promising scenario would thus be to use the vaccine in a prophylactic setting to elicit a desired immune response and not allow the tumor to determine the fate of tumor specific T cells.

4. Characterization of the MUC1 chemotactic receptor

4.1. Introduction

Classical chemokine receptors are seven transmembrane G protein-linked receptors. They are expressed selectively on certain cell types but are generally each expressed on many cell types. They often respond to more than one ligand although it is not known if different ligands generate different responses. Signaling through these receptors results in the activation of several pathways. Some of these pathways are dependent upon G proteins and some are independent, such as Ca^{2+} flux and activation of the MAP kinase pathway (197-199). Chemokine receptors become inhibited by the addition of pertussis toxin and their function can be modulated by the addition of other chemokines or by prior exposure to its own ligand.

Members of this family include the receptors for the canonical C, CC, CXC, and CX_3C subgroups of chemokines but also for chemokines outside these families such as the C5a receptor.

MUC1 mucin is a human tumor antigen that has been shown to be chemotactic for human fibroblasts and immature DC (416). MUC1 is normally heavily glycosylated and sequestered on the apical surface of ductal epithelial cells. However, when MUC1 is expressed by tumor cells it loses its polarized expression and becomes hypoglycosylated. The lower level of glycosylation exposes the peptide backbone of the tandem repeat region of MUC1, termed the variable number of tandem repeats region or VNTR. The VNTR makes up the majority of the extracellular portion of MUC1, can be shed from the surface of tumor cells, and is found in the circulation of cancer patients. We have identified that the VNTR region is responsible for the chemotactic effects of MUC1. Furthermore, there must be very low to no glycans added to the VNTR to retain the chemotactic effect. From this study, it is clear that tumor MUC1 can attract immature DC and that $MUC1^+$ tumors would also be predicted to induce DC migration. This is one of the first examples of a tumor antigen inducing DC chemotaxis.

Other studies done with tumor MUC1 have shown that MUC1 can interact with immature DC in a second way. Highly sialylated tumor MUC1 induces distorted DC maturation leading to a block in the DC's ability to induce Th1 cell polarization (Chapter 3 of this thesis). Because of this secondary effect, the chemotactic effects of MUC1 could add to the pool of DC that become dysfunctional once they reach the $MUC1^+$ tumor. Therefore, it is important to identify the receptor responsible for MUC1 induced chemotaxis. MUC1 was shown to not have a chemotactic effect on other cells of the immune system. This lack of effect is interesting and puzzling because most known chemokine receptors are expressed on multiple types of immune

cells. The only known chemokine receptor that is uniquely expressed on immature DC is CCR6. However, cells transfected with CCR6 failed to migrate to MUC1 (J.J. Oppenheim, personal communication). This result means that to date there are no receptors that are expressed in the pattern with which we see the effects of MUC1.

To help resolve this issue and because this MUC1 chemotactic receptor may as yet be unknown, we decided to study the basic function of the MUC1 chemotactic receptor. First, the binding of 100mer MUC1 was measured through the use of a fluorescently tagged form of MUC1. Next, since most chemokine receptors are G protein-coupled receptors that are pertussis toxin sensitive, we tested the ability of pertussis toxin to block MUC1 induced chemotaxis. Chemotaxis also requires the formation of pseudopods and can induce Ca^{2+} flux. Live cell microscopy was used to analyze the effects of chemotactic and non-chemotactic forms of MUC1 on DC morphology and intracellular Ca^{2+} levels.

4.2. Materials and Methods

4.2.1. Reagents

All chemokines and cytokines were obtained from the NIH cytokine repository or Peprotech (Rocky Hill, NJ) unless otherwise noted. Reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

4.2.2. Chemotaxis assay

Cells were resuspended in chemotaxis medium (RPMI 1640 media containing 1% bovine serum albumin, 25 mM HEPES, pH 8.0) at 1-5 $x10^6$ cells/ml. Chemokines diluted in chemotaxis medium were placed in the lower wells of a microBoyden chemotaxis chamber (Neuroprobe, Cabin John, MD). When primary leukocytes were analyzed, five-micrometer polycarbonate membranes were placed over the chemokines. After the micro-chemotaxis chamber was assembled, 50 μl of cells were placed in the upper wells. The filled chemotaxis chambers were

incubated in a humidified CO_2 incubator for 90 minutes (immature human DC). After incubation the membranes were removed from the chemotaxis chamber assembly followed by gently removing cells from the upper side of the membrane. The cells on the lower side of the membrane were stained using Rapid Stain (Richard Allen, Kalamazoo, MI). The number of migrated cells in three high-powered fields (x200) was counted by light microscopy after coding the samples. In many cases counting was computer assisted using the BIOQUANT program (R & M Biometrics, Nashville TN). Results are expressed as the mean value of the migration of triplicate sample with the standard deviation shown by bars.

4.2.3. DC isolation and purification.

Primary human leukocytes were isolated from fresh normal donor leukapheresis packs under an approved human subjects protocol as previously reported. Human imDC were generated from purified human peripheral blood monocytes (>95%) as previously described (209) and their phenotype confirmed by flow cytometry. Immature DC were $CD1a^{+}$(Ab clone H1149), $CD14^{-}$ (Ab clone M5E2), $CD40^{low}$ (Ab clone 5C3), $CD83^{-}$ (Ab clone HB15ε), $CD86^{low}$ (Ab clone 2331(FUN-1)), $HLA\text{-}DR^{medium}$ (Ab clone G46-6 (L243)). Mature DC were generated by culturing imDC with 1 μg/ml of LPS (Sigma L-9764) for 48 hours. The phenotype of mDC was $CD83^{high}$, $CD86^{high}$, $HLA\text{-}DR^{high}$. Human $CD1c^{+}$ DC were immunomagnetically purified with BDCA-1/CD1c microbeads (Miltenyi Biotec, Auburn, CA) from primary human leukocytes as previously described (423). In brief, percoll purified mononuclear cells were washed and resuspended in 2mM EDTA containing 0.5% heat inactivated fetal bovine serum (Invitrogen, Grand Island, NY). Cells were incubated with anti-CD19 microbeads and anti-CD1c-biotin antibody. Cells were then washed and passed through a column to remove $CD19^{+}$ B cells. The remaining cells were then incubated with strepavidin-microbeads for 15 minutes. Cells were washed and passed through a second magnetic column to capture $CD1c^{+}$ cells. This purification

resulted in >90% $CD11c^{+}$ cells that were also uniformly phenotypically immature being HLA-DR^{low}, $CD83^{-}$, and $CD86^{low}$.

4.2.4. Assessment of DC phenotype and cytokine production.

DC phenotype was assessed by flow cytometry. In brief, cells were washed and counted prior to incubation with normal mouse serum (Jackson Laboratory, Bar Harbor, ME) for 15 minutes at 4 ^{o}C to block non-specific Fc receptor binding. Cells were then stained with specific antibodies for 30 minutes at 4 ^{o}C in the dark and washed extensively prior to fixation in 1% paraformaldehyde solution. Cytometry was performed with either a Becton Dickenson FACScaliber cytometer or LSR II cytometer. Flow data was analyzed using CellQuest software.

4.2.5. Fluorescent tagging of MUC1 and binding studies

100mer MUC1 was fluorescently tagged with the fluorochrome AlexaFlour® 488 using a protein labeling kit from Molecular Probes (Eugene, OR). In brief, 100mer peptide was reconstituted in water at a concentration of 2 mg/ml. 1 M sodium bicarbonate was added to rasie the pH of the solution and AlexaFluor® 488 was added and mixed with 100mer MUC1 for 1 hour at room temperature. Conjugated 100mer-fluorochrome was purified from unlabeled peptide and unconjugated fluorochrome by separation based on size on a resin column. The first fluorescent dye front representing the conjugated peptide was collected. The concentration of peptide-fluochrome conjugate was measured using the DC protein Assay from Bio-Rad (Hercules, CA).

Binding studies were done with monocyte-derived DC obtained as described above. Using various concentrations of AlexaFluor 488-100mer peptide conjugated and control peptides, cells were incubated on ice for 0, 5, 10 and 15 minutes prior to fixation with 1% paraformaldehyde. Data from fixed cells were collected by flow cytometry using the Becton Dickenson LSR II cytometer and analyzed using CellQuest software.

4.2.6. Live cell microscopy and Ca^{2+} flux

Day 5 human monocyte derived DC were plated on Vitrogen® collagen (Cohesion Technologies, Inc., Palo Alto, CA) coated glass coverslips (Fisher Scientific, Pittsburgh, PA) overnight. The following day, coverslips were placed in a continuous flow chamber system. Images were captured using a Nikon Eclipse 2000E (Nikon Inc., Melville, NY), equipped with a water-cooled Hamamatsu Orca 2 ER camera (Hamamatsu, Tokyo, Japan) and a 40x 1.45NA plan apochromat objective. The software used was MetaMorph (Universal Imaging, Inc., Downingtown, PA). Images were collected at multiple stage positions at 30 second intervals. Peptides were injected as a single bolus into the closed chamber system via an airlock. For Ca2+ flux experiments, cells on coverslip were washed and Fura-2 AM (Molecular Probes, Eugene, OR) in PBS (5 μg/ml) was added to coverslip for 30 minutes. Cells were then washed with fresh media and added to a continuous flow chamber system.

4.3. Results

4.3.1. 100mer MUC1 is capable of binding to immature human DC

Although previous work done in our lab had established that 100mer and ascites MUC1 can bind to the surface of immature monocyte-derived DC (105), we decided to confirm the binding of 100mer MUC1 to the surface of these cells. 100mer MUC1 was fluorescently labeled with the fluorochrome AlexaFluor® 488 and used to label Day 6 monocyte-derived DC. DC were kept on ice during labeling to ensure that no peptide was endocytosed during surface labeling.

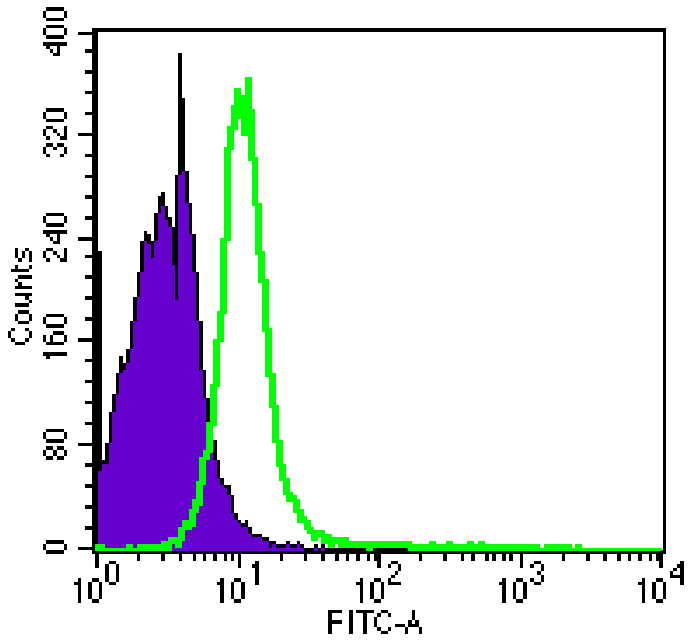

Figure 16: 100mer MUC1 binds to the surface of monocyte derived DC.

Fluorecently labeled 100mer MUC1 was added at a concentration of 1 μg/ml for 10 minutes to Day 6 monocyte DC on ice. At the end of 10 minutes, DC were washed once with FACS buffer and fixed. Purple histogram, unlabeled DC; Green histogram, AlexaFluor® 488 conjugated 100mer MUC1. Results shown are representative of three separate experiments with independent donors

As shown in Figure 16, 100mer MUC1 binds to the surface of the DC. To test for specific binding of 100mer, we attempted to block the binding of fluorescently tagged 100mer MUC1with unlabeled 100mer MUC1. However, as shown in Figure 17, the addition of fifty fold excess unlabeled 100mer MUC1 did not block the binding of labeled 100mer MUC1and, in fact, reproducibly enhanced the binding of 100mer MUC1 to the DC. There is no conclusive reason behind this enhanced binding but we hypothesize, and have some preliminary data, that suggests there could be homotypic binding of 100mer MUC1 to itself. However, this hypothesis has not been fully tested.

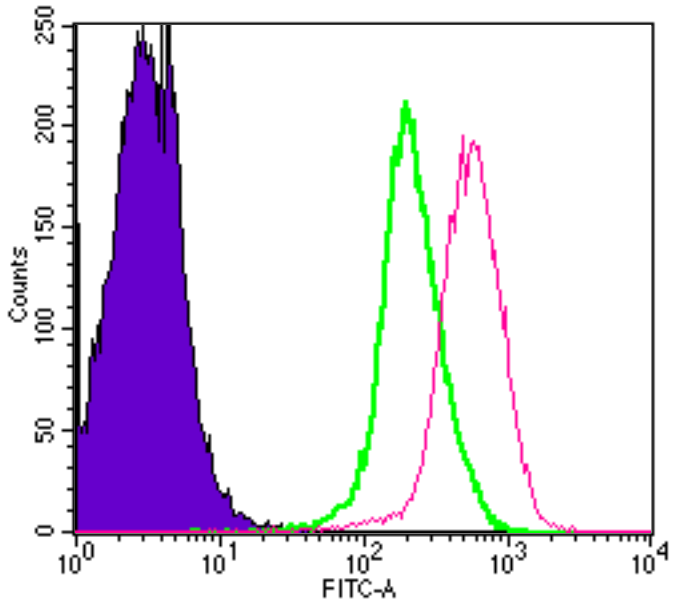

Figure 17: Unlabeled 100mer enhances binding of labeled 100mer to human DC.

Binding study was done as described for Figure 18. AlexaFluor® 488-100mer MUC1 was added at a concentration of 10 μg/ml ± 500 μg/ml of unlabeled 100mer MUC1 for 10 minutes prior to a wash with FACS buffer and fixation. Purple histogram, unlabeled cells; Green histogram, AlezaFluor® 488-100mer MUC1; Pink histogram, AlexaFluor® 488-100mer MUC1 plus 50-fold excess unlabeled 100mer MUC1. Results shown are representative of 3 independent experiments.

4.3.2. The MUC1 chemotactic receptor is a G protein-coupled receptor

Since the majority of chemokine receptors are G protein-linked receptors that are sensitive to inhibition by pertussis toxin, we looked at the effect of pertussis toxin on MUC1-induced chemotaxis. Pertussis toxin functions by catalyzing the ADP-ribosylation of the guanine nucleotide regulatory protein that functions in the receptor-mediated inhibition of adenylate cyclase. The lack of inhibition of adenylate cyclase results in the potentiation of cyclic AMP signaling. As shown in Figure 18, the migration induced by ascites MUC1 was sensitive to pertussis treatment thus implicating one or several G-protein-linked receptors in this process.

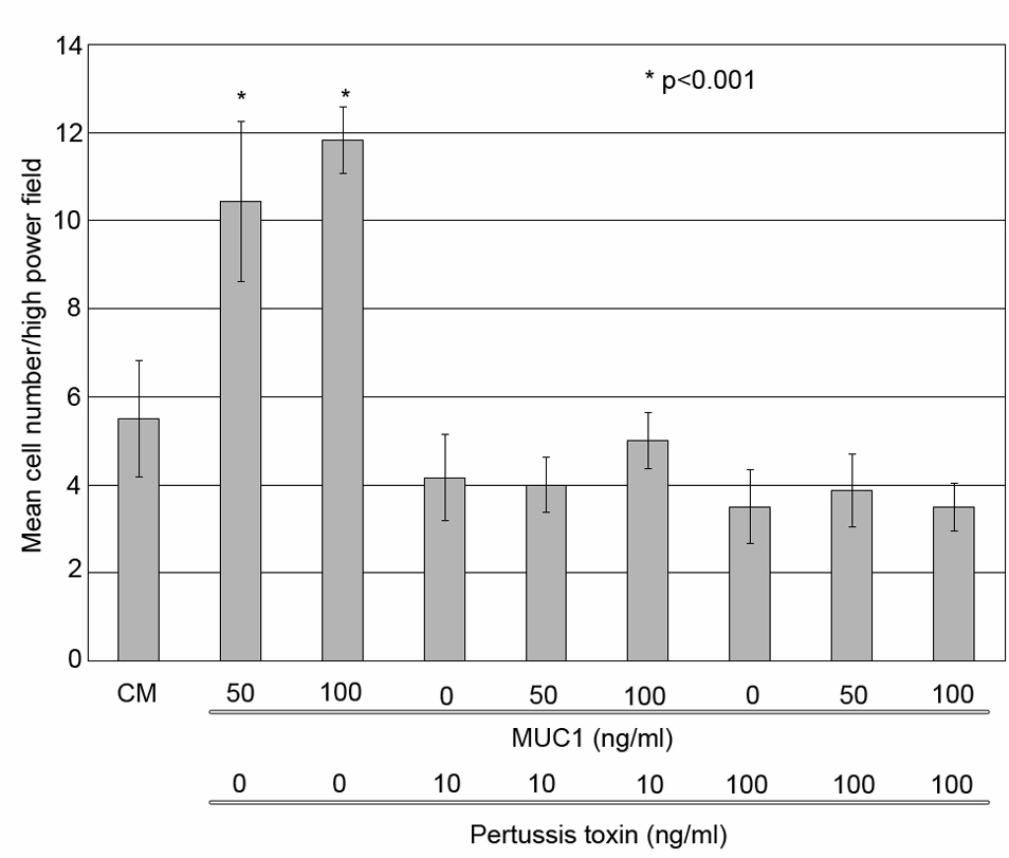

Figure 18: Pertussis toxin inhibits ascites MUC1 induced chemotaxis.

Chemotaxis chambers were set up as described in Material & Methods in the presence or absence of indicated concentrations of pertussis. *, p<0.001. There were no significant differences between control media, CM, and pertussis treated cells.

Chemotaxis mediated by the synthetic 100mer MUC1 was also sensitive to pertussis inhibition (Figure 19). The behavior of these two peptides continues to be the same indicating that the chemotactic receptor for 100mer MUC1 and ascites MUC1 is the same and binds to the unglycosylated VNTR region for each. Thus, similar to other chemokine receptors, the MUC1 chemotactic receptor is a G protein-linked receptor.

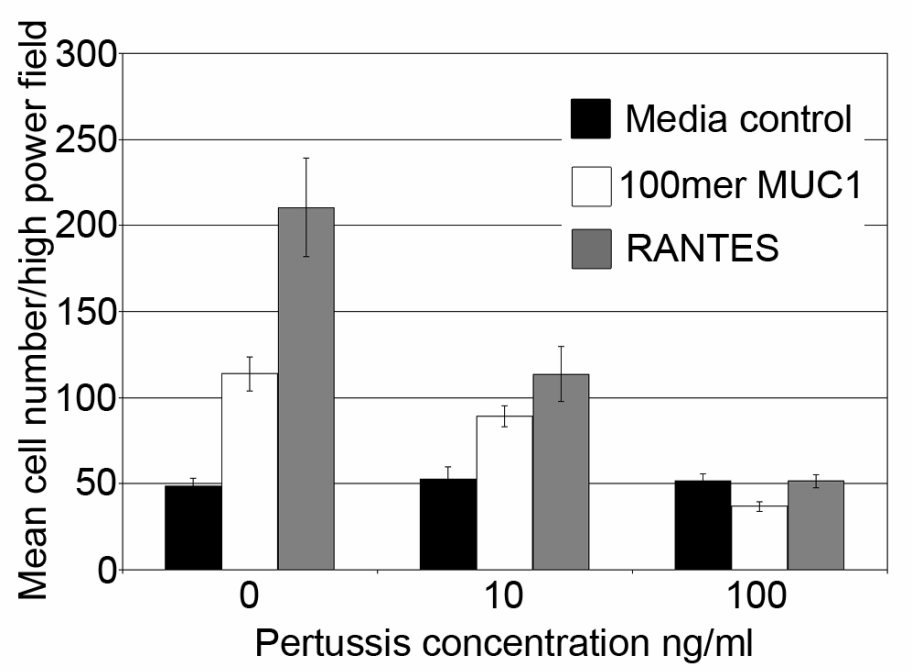

Figure 19: Pertussis toxin inhibits 100mer MUC1 induced chemotaxis.

Chemotaxis chamber were set up as described in Materials & Methods in the presence or absence of pertussis toxin. 100mer MUC1 was used at a concentration of 100 ng/ml and RANTES was used at a concentration of 50 ng/ml.

4.3.3. Chemotactic but not non-chemotactic forms of MUC1 induce pseudopods on human DC

Pseudopods are formed at the leading edge of a cell after it receives a chemotactic signal. Pseudopods contain a large number of actin fibers and concentrate the chemotactic receptor to the pseudopodial portion of the membrane. To examine if pseudopods are formed in response to 100mer MUC1 we conducted microscopy using a live cell apparatus. Cells were plated on coverslips and placed in a closed chamber. Fresh media was washed over the cells during the course of the experiment and the temperature of the cells was maintained at 37^{o}C to keep the cells alive and functional. 100mer MUC1 or HEK MUC1 was added to the chamber and serial photographs were taken of the coverslip over a span of approximately thirty minutes. Images from a single stage position were then combined into a movie. The movies of the DC response to 100mer and HEK MUC1 are included with this dissertation on CD-ROM (100mer MUC1.avi

and HEK MUC1.avi). From Figure 20 one can clearly see that by sixteen minutes, the cells that have been treated with 100mer MUC1 have formed large pseudopodial protusions and have drastically changed shape compared to time zero. In contrast, DC that were treated with HEK MUC1, which is not chemotactic, fail to produce pseudopods and look quite similar at sixteen minutes to their appearance at time zero.

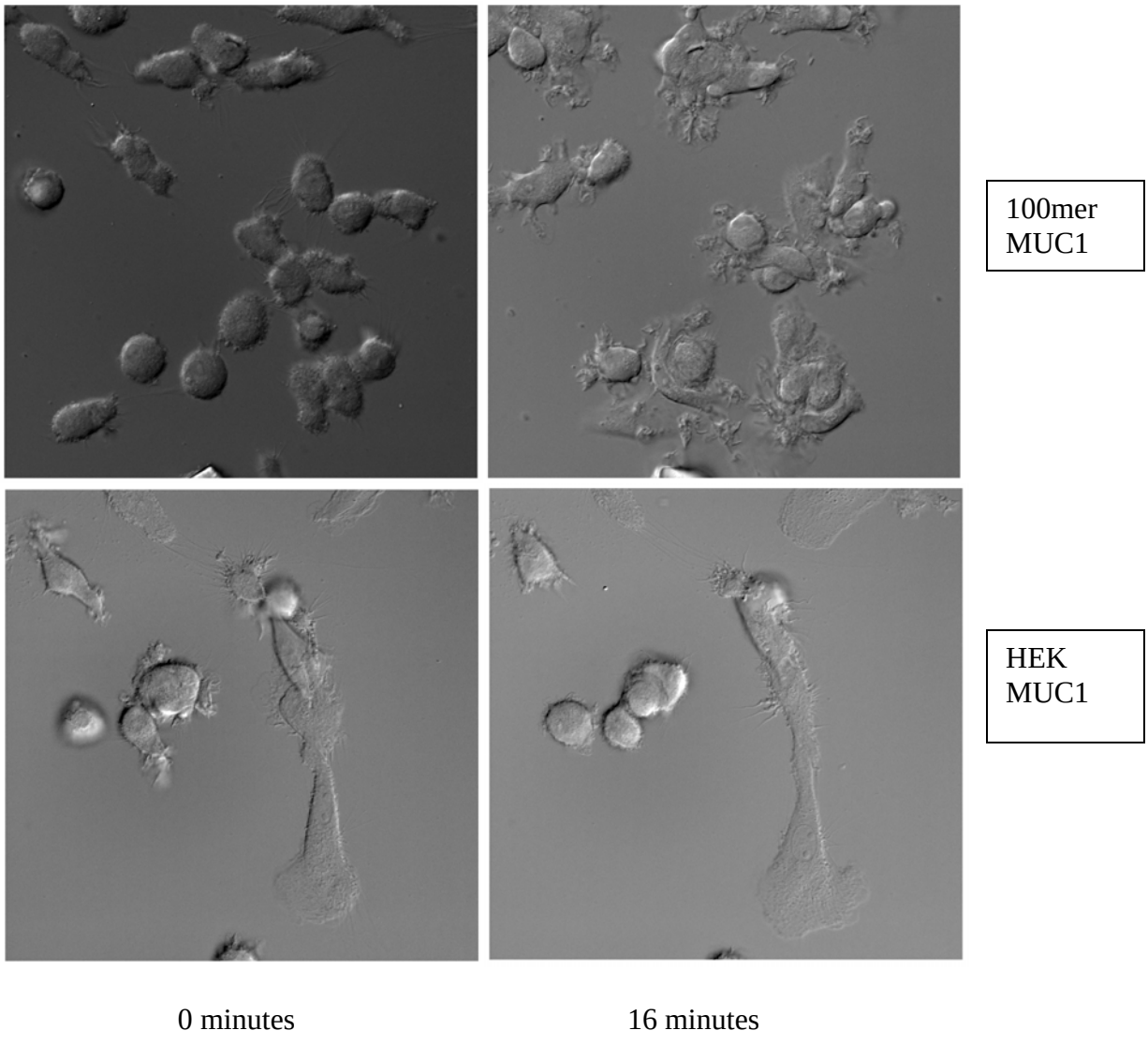

Figure 20: Pseudopods are induced by 100mer MUC1 but not HEK MUC1.

Closed chamber live microscopy was conducted as described in Materials and Methods. 100mer MUC1 or HEK MUC1 were injected at time zero and cells were observed for 30 minutes.

Within a gradient the dominant pseudopod, formed at the leading edge of the cell, inhibits the formation of pseudopods on other regions of the membrane. Although a true pseudopod and uropod, opposite the pseudopod, cannot be seen in these cells because of a lack of chemotactic gradient, there are clearly pseudopodial protrusions. It is likely that without a gradient, pseudopods are formed in all directions. There is also a possibility that the pseudopod-like structures seen in this experiment are a form of targeted antigen uptake. Proteins that bind to receptors on the DC surface and be recognized may also be able to up-regulate its own ingestion by the DC.

4.3.4. 100mer MUC1 induces DC Ca^{2+} flux

Most chemokine receptors respond to ligand binding through a variety of signaling pathways. One of the most common pathways results in a flux in the concentration of intracellular calcium ions. Because this is a common pathway, we sought to determine whether the MUC1 chemotactic receptor induced Ca^{2+} flux similar to other chemokine receptors. Day 6 monocyte-derived DC were labeled with fura-2AM dye prior to live cell microscopy. Live cell microscopy was conducted as described in section 4.3.3. Fura-2AM is an ultraviolet (UV) excitable fluorescent probe for Ca^{2+}. As shown in Figure 21 and in the movie accompanying this dissertation (Calcium flux.avi), 100mer MUC1 induced Ca^{2+} flux.

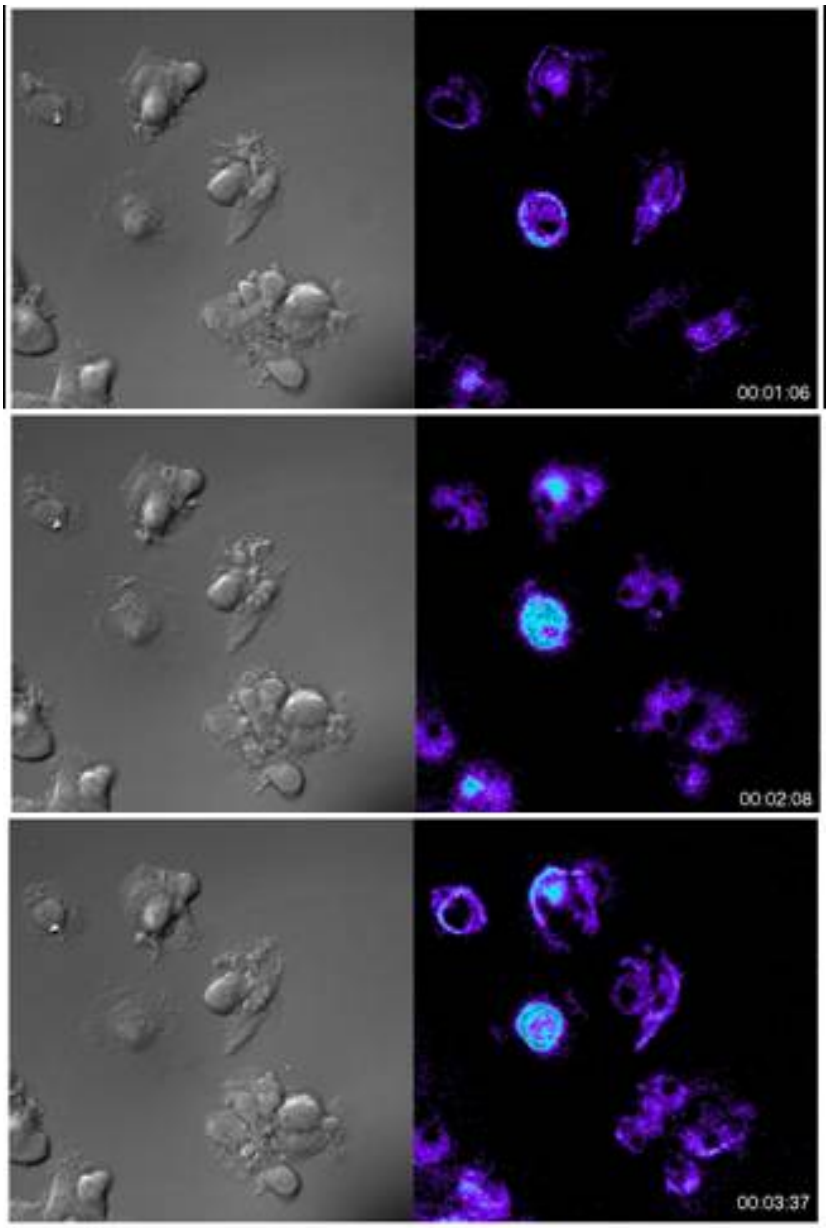

Figure 21: 100mer MUC1 induced Ca^{2+} flux in human DC.

Day 6 monocyte-derived DC were plated on coverslips and loaded with fura-2AM prior to assembly of the closed chamber microscopy apparatus. 100mer MUC1 was injected and UV light was used to excite the fura-2AM. The time (hours:minutes:seconds) after injection of 100mer MUC1 is shown in the lower right hand corner. On the left is the differential interference contrast (DIC) image and on the left is the image in fluorescence.

The Ca^{2+} flux can be seen as an increase in the blue intensity in the cells. In the movie of these cells, the level of blue emission changes with time. The flux is seen as a switch in the cell color from purple to blue and back again. With a strong signal, all the cells can be synchronized in their Ca^{2+} flux. This was not the case for 100mer MUC1 treated DC. Again, as seen best in the movie, each individual cell fluxed independent of the next cell. The lack of synchronization indicates that either the response to MUC1 is weaker than for other stimuli, such as LPS, or that it may take more time to generate the signal necessary for Ca^{2+} flux from MUC1 binding.

4.4. Discussion

This study has shown that the MUC1 chemotactic receptor can preliminarily be classified as part of the greater chemokine family of G protein-coupled receptors. Its function is inhibitable by pertussis toxin and its signaling results in the formation of pseudopods and Ca^{2+} flux. This classification is important because it greatly restricts the group of receptors that are candidates for the MUC1 chemotactic receptor. The characteristics stated above and its expression on immature DC but not mature DC, lymphocytes, NK cells, or monocytes, fit closely the characteristics of CCR6 receptor. This possibility has already been tested and CCR6 is not the MUC1 chemotactic receptor. Therefore, it is most likely an orphan receptor expressed on the DC, which still needs to be defined.

100mer MUC1 can bind to the surface of the DC. However, the binding was not inhibitable by the addition of unlabeled 100mer MUC1. This result is very unusual and we have considered two possibilities. The less interesting one is that there was unbound fluorochrome in the purified fluorochrome-conjugated 100mer MUC1 that was able to bind to the unlabeled 100mer MUC, thus increasing the overall fluorescence of the cell. We ruled that out because any unconjugated fluorochrome should bind to cellular proteins with equal affinity as to the

unlabeled protein. Since the unconjugated fluorochrome is added at the same concentration to both groups of cells, there should be no increase in fluorescence in the presence of unlabeled 100mer peptide. The second scenario is that 100mer MUC1 is capable of a homotypic interaction. In this case, unlabeled MUC1 could bind both to the receptor and to the labeled MUC1 and hence increase the overall binding of labeled MUC1 to the cell surface. This hypothesis is supported by other work done in our lab showing that the MUC1 dependent binding of tumor cells to collagen was also increased in the presence of soluble 100mer MUC1 (P. Ciborowski, personal communication).

The ability of pertussis toxin to block the DC chemotaxis shows that the MUC1 chemotactic receptor is a G protein-linked receptor. G protein-linked receptors have been extensively studied and, as mentioned above, most chemokine receptors belong to this family. Based on this, MUC1 chemotaxis receptor would be expected to similarly use other signaling pathways common to chemokine receptors. It will be interesting and relevant to analyze tyrosine phosphorylation especially of members of the MAP kinase and STAT pathways to see if they are modified by MUC1 binding.

The formation of a pseudopod is a complicated process. It contains a large concentration of actin filaments and usually the uropod, at the opposite end, contains a large number of myosin filaments. The live microscopy experiments show pseudopod formation in response to 100mer MUC1 but, because of the inability to set up a true concentration gradient, the pseudopods were formed in all directions. For this same reason, a clearly defined uropod could not be seen. It may also be possible that these membrane arms are extending in a form of targeted antigen uptake. In either case, there were distinct differences between the chemotactic forms of MUC1 and the non-chemotactic forms. Hence, it will be important to study the cell morphology in a

concentration gradient and also to determine if the morphological changes seen in DC are similar to the change seen in fibroblasts.

The identification of the MUC1 chemotactic receptor is important for cancer immunotherapy. A tumor specific chemokine has a potential to be exploited and blocked to prevent DC dysfunction. This preventative measure would help maintain a healthy pool of circulating DC that are not drawn into the suppressive environment of the tumor. Conversely, a chemokine receptor that draws cells specifically to the tumor site could be used to target cytolytic cells or transfected and activated cells to the tumor site. Finally, the injection of chemotactic forms of MUC1 with an adjuvant may be a more effective MUC1 vaccine than forms that are not chemotactic. All of these issues must be considered in the formulation of MUC1 targeted cancer vaccines.

5. Summary

The work described in this dissertation has elucidated the effects of the tumor antigen MUC1 on human DC. Previous work has shown that glycosylated MUC1 can bind to the mannose receptor and enter early endocytic vesicles. These vesicles fail to fuse with late endosomes and hence tumor MUC1 fails to be processed and presented on MHC Class II molecules although it does get presented on a small number of MHC Class I molecules. Here we broaden the role of MUC1 in altering the immune response to MUC1 and tumors in general. We show that the unglycosylated VNTR region of MUC1 is chemotactic to immature DC but not to other cells of the immune system. Through the expression and shedding of MUC1, tumors can induce the immature DC, which should be the start of the anti-tumor response, into the immunosuppressive environment of the tumor. After the DC is at the tumor site, the sialylated epitopes on MUC1 induce DC maturation. However, this maturation does not induce a strong production of IL-12. IL-12 is a critical cytokine for the support of Th1 polarization and

accordingly the MUC1 treated DC fail to activate Th1 cells. Instead they induce naïve T cells to produce IL-13 and IL-5. Both of these cytokines have been shown to promote the growth of tumors. Finally, the chemotactic receptor for MUC1 has been classified as a typical chemokine receptor. The receptor has the mechanisms, such as pertussis toxin sensitivity, the formation of pseudopods, and Ca^{2+} flux, of other chemokine receptors but, the signaling on only immature DC of all the cell types tested, does not fit with any of the known chemokine receptors. The identity of this receptor and its mechanisms could be used to modulate the chemotactic effects of $MUC1^{+}$ tumors. These conclusions are illustrated in Figure 22.

It is interesting to note that the induction of an immune response with semi-mature DC in mice corresponded very well to the human cancer immune response. A semi-mature DC population was attained by treating immature DC with TNF-α and were indistinguishable from LPS treated DC in terms of cell surface markers but differed in their cytokine production. These DC, when loaded with peptide and injected into mice induced $CD4^{+}$ T cell tolerance and a partially activated $CD8^{+}$ T cells with some cytolytic function. Even when the $CD4^{+}$ T cells are stimulated by mature but not truly activated DC, they fail to induce Th1 polarization (455). MUC1 induces TNF-α production by DC, a phenotype similar to this semi-mature DC, and in patients with $MUC1^{+}$ tumors, this is the type of immune response found (456).

Since MUC1 is shed from the tumor cell surface, it could also affect the general immune status of cancer patients. Proteins that enter the afferent lymphatics can reach the lymph node and be taken up by resident dendritic cells for presentation (457). In fact, MUC1 has been shown to bind to sialoadhesin, which is highly expressed on macrophages in the lymph node. The lymph node could, through this mechanism, become a depot for MUC1 and its immunosuppressive effects. Efficient and strong T helper cell responses are an absolute

requirement for the establishment of a strong and lasting anti-tumor response. However, as we have shown here, in the presence of MUC1, these responses will be difficult to generate.

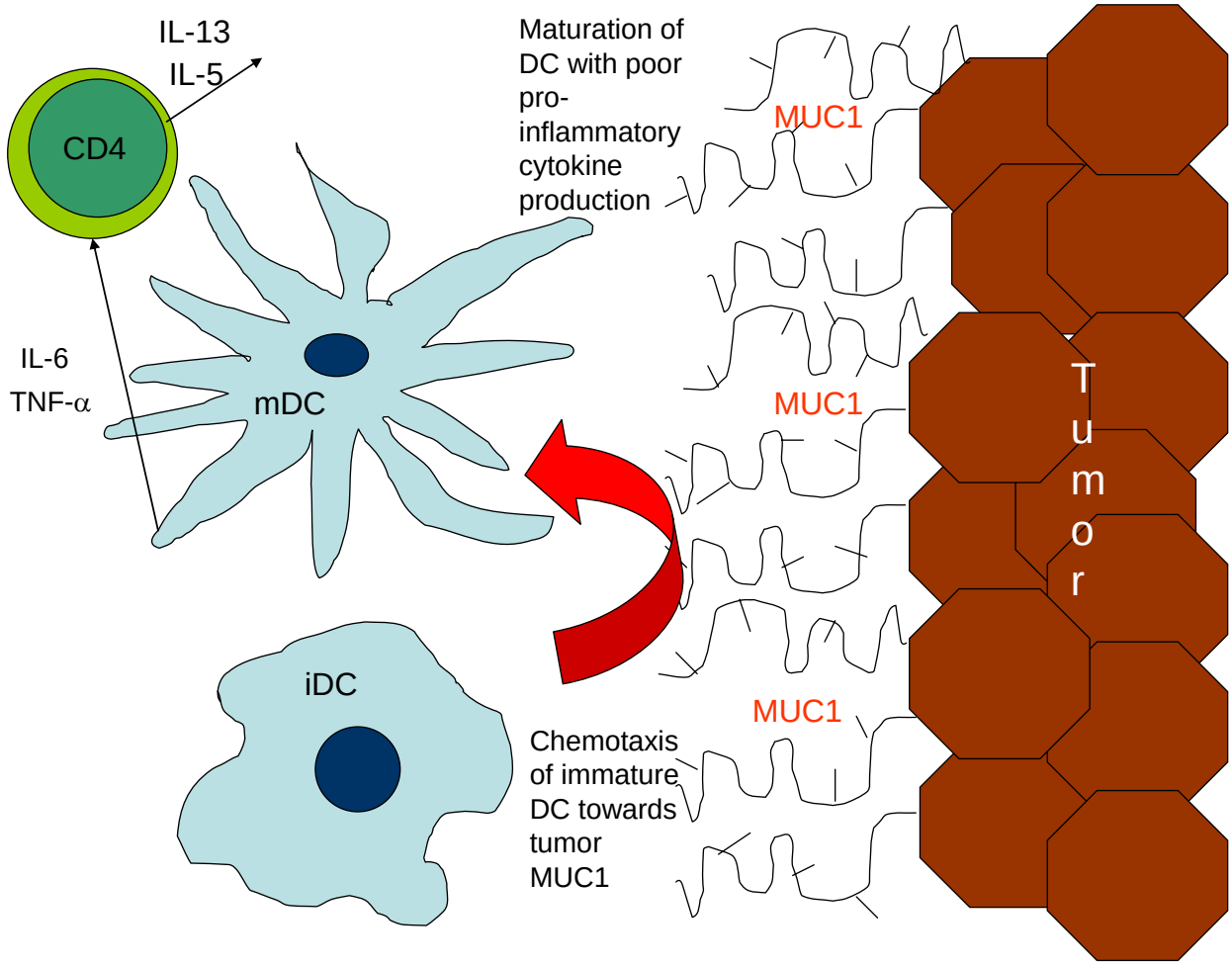

Figure 22: Summary of effects of MUC1 on human DC.
iDC=immature DC and mDC=mature DC.

This work has also shown the importance of glycosylation, in general, and sialic acid, more specifically, in signaling the immune system. Groups that have studied free oligosaccharides from human milk have shown that the acidic fractions *in vitro* increase the number of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells that produce IFN-γ and also the number of $CD3^+CD8^+$ T cells that produce IL-13 (458). In addition, the acidic fraction increased the number of $CD3^+CD4^+CD25^+$ T cells. We have shown here that MUC1 isolated form human

milk fat is capable of inducing DC semi-maturation. Perhaps the addition of sialic acid to proteins is a broad mechanism for inducing IL-13 production and suppressing a strong Th1 immune response.

In conclusion, although the targeting of MUC1 for immunotherapy is still a promising new therapy for cancer, the work shown here suggests that the forms of MUC1 and the timing of MUC1 vaccines must be considered very carefully. Pre-malignant lesions express MUC1 meaning that at very early points in the growth of tumors, MUC1 is already influencing the immune response to MUC1 and tumors as a whole. Therefore, a prophylactic cancer vaccine will be the most effective approach for anti-MUC1 cancer vaccines. It would have a chance to elicit a strong Th1 and CTL response prior to their exposure to MUC1 in the tumor cell environment. The chemotactic effects of MUC1 should be of benefit to a cancer vaccine. The use of 100mer MUC1 to draw into the vaccination site immature DC from the circulation, in combination with a strong adjuvant, would be predicted to be a strong vaccine. Even better would be the combination of the chemotactic 100mer MUC1 with the Tn-100mer MUC1 with an adjuvant, as this vaccine would target both peptide and glycopeptide specific T cells. However, even though there is a large concentration of sialylated epitopes on tumor MUC1, the sialylated forms of MUC1 should not be used in vaccines because of their immunomodulatory effects on DC.

APPENDIX. PUBLICATIONS

Vlad, A.M., J.C. Kettel, N.A. Alajez, **C.A. Carlos** and O.J. Finn. 2004. MUC1 immunobiology: from discovery to clinical applications. *Adv Immunol 82:249.*

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