The most exciting phrase to hear in science, the one that heralds new discoveries,

is not 'Eureka!' but 'That's funny…'

- Isaac Asimov

University of Alberta

MUC1 is a novel costimulatory and coinhibitory molecule of T cells

by

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Abbreviations

7-AAD	7-aminoactinomycin D
AP-1	activator protein 1
BTLA	B and T lymphocyte attenuator
CCR	chemokine receptor
CPM	counts per minute
CTLA-4	cytotoxic T lymphocyte associated antigen 4
CXCR	CXC chemokine receptor
DC	dendritic cell
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
Foxp3	forkhead box P3
GITR	glucocorticoid-induced TNFR family-related protein
HVEM	herpesvirus entry mediator
ICAM	intracellular adhesion molecule
ICOS	inducible T cell costimulator
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ITAM	immunotyrosine activation motif
ITIM	immunotyrosine inhibitory motif
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LAT	linker of activated T cells
LFA-1	lymphocyte function-associated antigen 1
MACS	magnetic affinity column separation
MAPK	mitogen-activated protein kinase
MHC	major histocompatability class
μg	microgram
μl	microliter
NAC	non-adherent cell
NF-AT	nuclear factor of activated T cells
NF-kB	nuclear factor κB
NK	natural killer
NKT	natural killer T
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD-1	programmed death 1
PD-L1	programmed death ligand 1
PHA	phytohemagglutinin
PLCγ1	phospholipase C gamma 1
PKC	protein kinase C

SDS	sodium dodecyl sulphate
SHP	src homology phosphatase
TGF	tumor growth factor
TNF	tumor necrosis factor
T_{reg}	regulator T cell
ZAP-70	ζ-associated protein 70

Chapter-1

General Introduction

1.1 THESIS OVERVIEW

T cell immunoregulation is a phenomenon highly controlled by the human immune system. A variety of protein-protein interactions are required to take place in order to safeguard the body from aberrant immune responses which could lead to autoimmunity. In the absence of any one of these highly regulated interactions, T cells will either become anergic or undergo apoptosis (1). These controls are necessary both during the processes of activation and deactivation in response to a pathogen.

In recent years, many of these protein regulators have been discovered and brought to light, though many of their functions still remain a mystery. Other proteins have been predicted to have similar effects, though for many reasons have never been thoroughly studied so as to bring them to the same echelons as known immune regulators such as CD28, CD25 and CTLA-4.

One such protein, Mucin-1 (MUC1), has primarily only been studied for its vaccine potential in treating epithelial-based carcinomas. Several papers, however, have shown its expression on T cells (2, 3) and its potential therein for immunoregulation (2, 4, 5). Not only has MUC1 ligation been shown previously to inhibit the proliferation caused by typical T cell stimulation (4, 5), but a variety of intracellular signaling molecules have also been found to bind to its cytoplasmic tail (6, 7, 8, 9). Despite this evidence, MUC1's immunoregulatory role on T cells has never been fully explored and a model has never been

proposed. This project originated by seeking to answer these outstanding questions regarding MUC1's role in T cell immunoregulation.

1.2 MUC1 – EXPRESSION AND FUNCTION

MUC1 is a large, >200kDa transmembrane glycoprotein (10). Its extracellular domain consists of a variable number of 20 amino acid tandem repeats (VNTRs). These VNTRs are heavily glycosylated with *o*-linked oligosaccharides, which make up the majority of MUC1's molecular weight (11). The intracellular portion of MUC1, the cytoplasmic tail, is non-covalently bound to the rest of the molecule (12). It contains numerous binding and signaling motifs (13) and, based on the cell type expressing MUC1, will bind to different cell signaling proteins and transcription factors (6, 7, 8, 9, 14, 15).

MUC1 is mainly expressed on the majority of epithelial cell types where, as a mucin protein, it acts to protect the cell surface from pathogens and irritants (10, 16, 17). However, MUC1 has also been found to be expressed on other cell types such as monocytes (18), dendritic cells (19) and T cells (2).

Of most interest to researchers studying MUC1 is the fact that epithelial-derived carcinomas express an aberrantly-glycosylated version of MUC1, either with different motifs glycosylated than non-tumor MUC1 or less glycosylation entirely (19, 20). Tumor cells also express differentially spliced isoforms of MUC1, including a secreted version of MUC1 (21, 22).

By studying MUC1 expression on tumor cells, many alternate functions ranging far beyond cellular protection have been found. The first and most important was the discovery of a ligand for MUC1 in ICAM-1 (23). Recently, it was found that MUC1 can bind to ICAM-1, with underglycosylated MUC1 binding to it with much greater efficiency than ICAM-1 (24). Tumor cells, expressing MUC1 with fewer *o*-linked oligosaccharides, utilizes MUC1 to undergo transendothelial migration, potentially acting as a mediator of metastasis (25).

Several other roles for MUC1 have also been shown, including the ability of its cytoplasmic tail to bind to the transcription factor β-catenin (14). Normally, β-catenin is bound and regulated by the transmembrane surface protein e-cadherin on epithelial cells (26). Dysregulation of this cycle, through mutation of e-cadherin, has been shown to cause tumorigenesis (27). When MUC1 on tumor cells is given an extracellular stimulus, the cytoplasmic tail has been shown to dissociate from the transmembrane domain and enter the nucleus, carrying β-catenin with it (12). Since β-catenin is an oncoprotein that results in cell cycle progression (28), MUC1 has been dubbed a proto-oncogene for its newly-established role in tumorigenicity (29). Also, secreted MUC1 from tumor cells has been shown to have immunoregulatory effects (21, 30), inhibiting the immune response against the tumor cells releasing this differentially-spliced version of MUC1. This was the first suggestion that MUC1 might be able to influence the immune system.

1.3 MUC1 AND T CELLS

The reasons for the expression of MUC1 on human T cells have long been overlooked, with very few authors providing data to elucidate its further function. The interaction with ICAM-1 remains a potential explanation, allowing for the movement of white blood cells to distal sites, but those studies have never been performed. One function of tumor MUC1 that cannot be mirrored by T cells is the regulation of β-catenin, as β-catenin is only expressed in T cells during their developmental stages as thymocytes and not in later cell life in the periphery (31, 32).

Agrawal et al. (4) first showed that when T cells were given a CD3-based antibody stimulus as well as a MUC1 stimulus with the addition of a crosslinking antibody, the proliferation that would normally be induced by the anti-CD3 antibody was partially inhibited. Treatment afterwards with IL-2 reduced the observed inhibition (30). This was the first evidence which led researchers to first believe that MUC1 could act as a negative regulator of T cells.

1.4 REGULATION OF T CELLS - COSTIMULATION

T cells are regulated by a variety of proteins. Some provide positive stimuli, leading to cellular activation and proliferation, while others provide negative stimuli, leading to the inhibition of proliferation and the onset of anergy.

Both are important in ensuring a proper immune response to pathogens as well as a proper downregulation of cellular functions afterwards.

T cell activation occurs when the T cell recognizes its antigen bound to the MHC molecule of a target cell, with $CD8^+$ T cells recognize antigen in the context of MHC class I while $CD4^+$ T cells recognize antigen in the context of MHC class II (33). The recognition of the appropriate peptide bound to MHC occurs through the T cell receptor complex (TCR), including CD3, with CD4 or CD8 specifying for the MHC class itself. Once recognized, a variety of intracellular events take place.

The first event is the phosphorylation of the $CD3_\zeta$ chain – the predominant signaling chain of the CD3 complex (34). This intracellular chain contains six signaling motifs called 'Immunotyrosine Activation Motifs' or 'ITAMs' (35). Upon receiving an extracellular stimulus, these ITAMs will become phosphorylated by the signaling proteins Lck and Fyn (36). Once phosphorylated, the ITAM domains recruit the signaling protein ZAP-70, beginning the intracellular signaling cascade (37).

ZAP-70, a kinase, is then activated and phosphorylates a number of other proteins. Most importantly, it activates the protein Linker of Activated T cells (LAT) (38). LAT then allows the binding and activation of numerous other signal cascade proteins and pathways, including the Ras pathway of T cell activation (39) and the NF-AT pathway (40). The Ras pathway leads to the activation of MAP kinases and, downstream, activation and nuclear migration of the Fos/Jun AP-1 transcription factor complex (41) while other signaling proteins activated by

LAT, such as PLCγ, lead to the activation of the NF-AT transcription factor and its nuclear migration (40). Both the NF-AT and AP-1 transcription factors are necessary for proper T cell activation and proliferation as they function in tandem to produce pro-inflammatory cytokines such as IL-2, IFN-γ and TNF-α (42).

Also important during CD3 signaling is the CD28 costimulatory protein. During recognition of the MHC by CD3, a second signal is also required. This comes from the T cell protein CD28 binding to CD80/CD86 (also named B7.1/B7.2, respectively) expressed on the antigen presenting cell (43). This signal activates the Jun amino-terminal kinase (JNK), a member of the MAPK superfamily, which phosphorylates the AP-1 complex, activating its transcriptional potential (44). In the absence of CD28, not enough AP-1 is able to activate the appropriate genes (45). NF-AT, however, is brought into the nucleus in sufficient quantities by CD3 stimulation alone and is able to activate its genes; combined with the absence of AP-1, this leads to the induction of anergy (46). In the absence of costimulation, the cell will not become activated or proliferate and will not be responsive to subsequent stimuli. The purpose of this is to prevent potential autoimmunity by permanently inactivating T cells which could become activated by cells not expressing CD80/86.

Several other molecules are expressed on T cells which provide costimulatory functions. These include CD27, 4-1BB, OX-40, ICOS, GITR and LFA-1, each with their own specific function in maintaining the T cell response to pathogens.

CD27, 4-1BB and OX-40, all members of the TNF family of receptors, are able to generate intracellular cascades that result in the phosphorylation of IKK which normally binds to and prevents the nuclear translocation of NF-kB, the transcription factor primarily involved in IL-2 production (24). They also inhibit pro-apoptotic molecules, ensuring that the T cell response is both enhanced and maintained (47). CD27 is naturally expressed on T cells in low amounts, with increased expression after CD3 stimulation, while its ligand CD70 is expressed on antigen presenting cells post-stimulation (48). Though less is known about CD27's function *in vivo, in vitro* costimulation of CD27 and CD3 result in enhanced proliferation and activation. Mice deficient in CD27 have difficulties in generating responses to influenza virus infection and have fewer memory T cells, suggesting an important role in T cell function (49). 4-1BB, upregulated after TCR stimulation, is thought to be a costimulatory protein of $CD8^+$ T cells, as 4-1BB stimulation in priming restores defective $CD8^+$ T cells in CD28 knockout mouse models (50). 4-1BB knockout mice also have poor CD8 memory and late-stage T cell responses and poor anti-tumor responses, making it a necessary costimulatory molecule for advanced $CD8^+$ T cell responses (51). OX-40, also upregulated after CD3 stimulation, has been found to be involved in maintaining the immune response, keeping effector cells alive and active so that they can eliminate the pathogenic insult (47). In fact, *in vivo* studies blocking OX-40 in murine autoimmunity models reduced disease symptoms and damage. Mouse knockout models for OX-40 have shown reduced antiviral effectiveness of T cells, along with fewer T cells being generated later in the immune response (52,

53). All three of these molecules appear to be necessary for a complete and proper T cell response, highlighting the importance of costimulation via this family of molecules.

Whereas CD28 is primarily responsible for the costimulation of naïve T cells, generating effector cells in order to eliminate pathogens, Inducible T Cell Costimulator (ICOS), a CD28 homologue, is mainly involved in the costimulation of memory T cells (54). However, rather than primarily result in IL-2 production, ICOS costimulation causes IFN-γ and IL-10 production (55). Blockade has prevented rejection in an allogeneic heart transplant model (56), while murine knockout models of ICOS show impaired production of Th2 lineage T cells while maintaining equivalent if not higher levels of IFN-γ production (57). ICOS provides a more specific role for costimulation in the reactivation of memory cells and the generation of Th2 cells.

The Glucocorticoid-induced TNFR Family-Related protein (GITR) is expressed at a low level in naïve T cells, increase in expression after CD3/CD28 stimulation (58). $GITR^{-/-}$ mice have normally-functioning T cells; however, *in vitro*, their T cells are hyperresponsive to CD3 stimulation (58), suggesting that it functions mainly to enhance a low level of CD3 stimulation (59) and, in $CD28^{-/-}$ studies, has actually been shown to act in a redundant manner to CD28, costimulating T cells in this scenario (60). This provides more roles that costimulation can fulfill – enhancing low-level stimulatory responses, generating an immune response when, generally, one would not occur, and acting

redundantly to CD28, ensuring that T cells can respond to pathogens when antigen presenting cells do not express the proper molecules, as is necessary.

LFA-1, though not commonly thought of as a costimulatory molecule, has several important functions which enhance CD3 stimulation of T cells. Normally identified as an adhesion molecule, LFA-1 binds to ICAM-1 and is responsible for transendothelial migration, where activated effector T cells move to distal sites in the body (61). LFA-1 – ICAM-1 interactions are also necessary for a proper immune response to occur. Through ligation of these adhesion molecules, the APC MHC and T cell TCR are brought within proximity to one another, generating the initial signal required for the immune response (62). In fact, both LFA-1 and ICAM-1 knockout mouse models show poor T cell priming and proliferation upon TCR stimulation (63). LFA-1 has also been found to activate the ERK pathway which is able to activate AP-1, an important transcription factor in the CD28 costimulation pathway (64). LFA-1 showcases a final ability for costimulatory function in addition to its ability to activate a specific intracellular pathway – to allow for prolonged and necessary contact of the immunological synapse in order to generate an immune response and reduce the antigen dose required.

1.5 REGULATION OF T CELLS - COINHIBITION

Coinhibition, the opposing process to costimulation, seeks to either directly or indirectly inhibit the costimulatory response. Many protein mediators

of coinhibition exist on T cells. Two such proteins, CTLA-4 and PD-1, both homologues of CD28 (65, 66), can produce these responses. Both are upregulated later in the life of an activated T cell with the purpose of deactivating the T cell and stopping the immune response, often due to the successful elimination of the pathogen of interest.

The primary function of CTLA-4 is in out-competing CD28 for CD80/CD86 ligation (65). CTLA-4 has a higher affinity for these proteins than CD28 does, preventing CD28 from receiving adequate stimulus to allow its downstream effector functions from taking place. CTLA-4 also contains immunotyrosine inhibitory motifs (ITIMs) (67) which function in opposition to the aforementioned ITAMs. Upon phosphorylation, these domains recruit phosphatases of the SHP family, either SHP-1 or SHP-2 (68). These proteins, after recruitment, proceed to dephosphorylate the signaling protein ZAP-70, preventing phosphorylation of LAT and other proteins, thereby preventing downstream activation (69) as well as dephosphorylate the $CD3_{\zeta}$ chain, preventing recruitment of other signaling molecules (70). In mice, CTLA-4 knockouts die soon after birth due to an uncontrolled lymphoproliferative disorder (71), strengthening the necessity of coinhibitory proteins in normal immunoregulation.

Similarly, PD-1 also contains an ITIM domain as well as an immunotyrosine switch domain (ITSM) for the recruitment of SHP phosphatases (66). However, rather than bind to CD80/CD86 on the antigen-presenting cell, PD-1 binds to homologues of CD80/CD86 named PD-L1 and PD-L2. This

binding can also have modulatory effects on anti-apoptotic factors, signaling for cellular death, though in T cells this effect has only been observed in maturing thymocytes (72). PD-1 knockout models, like CTLA-4 knockout models, show problems with T cells which culminate in large-scale autoimmunity (73).

BTLA, another coinhibitory protein of T cells, shows a similar function to these previous two proteins. BTLA on T cells, upon binding to its ligand HVEM on an APC, produces a SHP-based response, inhibiting the CD3-based signaling cascade (74). $BTLA^{-/-}$ mice reject allografts much more quickly (75), a similar phenotype to $PD\text{-}1^{-/-}$ mice receiving allografts .

Recently, an entire subset of T cells has been defined which allow for T cell regulation in the periphery. These cells, named 'T_{regs}', have been defined as cells with a phenotype $CD4^+/CD25^+/Foxp3^+$ (76). T_{regs} have often been shown to produce powerful inhibitory signals in both contact-dependent and contact-independent manners (77). The primary function of these cells is to prevent self-specific autoimmunity by suppressing cells which display these traits in the periphery (78), making them a powerful and important T cell subset.

Some molecules normally associated with costimulation also appear to have a dual role, also functioning as coinhibitory molecules in certain scenarios. ICOS, for example, causes production of IL-10, an inhibitory cytokine (79). Its expression on T_{regs} has been linked to an inhibitory role (79), as blocking it has been shown to reduce their regulatory abilities (80). 4-1BB, the costimulator of late-stage $CD8^+$ T cells, has been shown to have many additional coinhibitory effects *in vivo*, including deletion of autoreactive $CD4^+$ T cells and IDO

production leading to general immune suppression through tryptophan depletion (81). Likewise, CTLA-4 has also been shown to have costimulatory functions after a bivalent antibody was generated which causes CTLA-4-based enhancement of proliferation through TCR stimulation (82). It is unclear how important these secondary, anti-type functions are *in vivo* compared to the normal role of these molecules, but their existence suggests that the costimulatory/coinhibitory dynamic may be more complex than was originally thought.

1.6 PERIPHERAL TOLERANCE

After T cells leave the thymus, their TCR are able to bind to a specific antigen presented on a MHC molecule. They then enter the periphery, searching for this antigen so that they may generate an immune response and eliminate pathogen-infected or altered-self cells. Despite several mechanisms in place to eliminate autoreactive T cells in the thymus, some still persevere and will become active, damaging normal, healthy tissue. In most healthy individuals, several different methods exist to maintain peripheral tolerance and are successful at preventing autoimmunity. Some of the most basic methods used are antigen presenting cells which do not express costimulatory molecules, resulting in T cell anergy, inhibitory cytokines and induced apoptosis (83). However, often these are not enough by themselves, and the aforementioned coinhibitory molecules and regulatory T cells are used to further maintain peripheral tolerance.

CTLA-4 and PD-1, as previously mentioned, prevent the costimulatory response in T cells after antigen recognition by the CD3/TCR complex. They do this later in cell life, post-activation, in order to prevent the continued expansion and development of effector T cells. Both molecules have been linked to autoimmune disorders, as their knockout models both show severe autoimmunity (71, 73). Not only this, but they have both been shown to be necessary in the induction of peripheral tolerance, the initial onset of tolerance in T cells, as naïve autoreactive $CD8^+$ T cells in the periphery will become tolerized by resting dendritic cells, but not if these cells lack CTLA-4 or PD-1 (84). More specifically, PD-1/PD-L1 interactions have been shown to be necessary for the continued maintenance of tolerized T cells, with the T cells reactivating and becoming autoreactive after PD-1/PD-L1 or PD-L2 blockade (85, 86). CTLA-4 is necessary for tolerance induction, as transgenic murine T cells with CTLA-4 knocked out are unable to become tolerized (87). There is also evidence that CTLA-4, too, is involved in the maintenance of tolerance, as blockade in some autoimmune models can result in increased incidence and severity of that disorder (88, 89).

T_{reg} cells, through *in vitro* and *in vivo* studies, have been shown to have the ability to suppress cells in the periphery through both contact-dependent and contact-independent mechanisms (90). Contact-dependent mechanisms are still not fully understood; however, it is accepted that contact-dependent mechanisms are responsible for T_{reg} function *in vitro*, as cytokine knockouts have still shown the ability to suppress autoreactive T cells (91). Contact-independent

mechanisms, observed in *in vivo* models, utilize the T cell inhibitory cytokines IL-10 and TGF-β, the latter of which has been linked to increasing the number of T_{reg} cells (91). The importance of T_{regs}, like the importance of the coinhibitory molecules, can be observed in deficiency models for the T_{reg}-specific transcription factor, Foxp3. A lack of Foxp3 has been linked to the autoimmune disorder IPEX 'immune dysregulation, polyendocrinopathy, enteropathy, X-linked' (IPEX) syndrome, a disorder mirrored by the murine Foxp3 knockout model (92). IPEX leads to a massive insult of autoimmune disorders ranging from diabetes to thyroiditis and uncontrolled lymphoproliferation, showcasing the necessity of Foxp3 and T_{reg} cells in controlling autoreactive T cells (92).

The maintenance of peripheral tolerance is of the utmost necessity, tolerizing autoreactive T cells which have escaped the central tolerance mechanisms of the thymus and preventing them from causing autoimmunity. Paramount in regulating peripheral tolerance are coinhibitory molecules and T_{regs}, both of which are necessary for the induction of peripheral tolerance and the ongoing maintenance. It is possible that other molecules exist, each just as necessary as those described, as peripheral tolerance is a broad area with numerous overlapping and, often, redundant mechanisms aimed at preventing the chance of self-reactive T cells becoming active.

1.7 MUC1 AND IMMUNOMODULATION

Though MUC1's true function on T cells remains a mystery, there have been several studies hinting at its potential. Not only have several signal cascade proteins been discovered to bind to its cytoplasmic domain (6, 7, 8, 9), but several putative motifs have also been found which could suggest an immunomodulatory function on T cells (13).

Through immunoprecipitation studies in T cells and the Jurkat T cell lymphoma cell line, the cytoplasmic domain of MUC1 has been shown to bind to Lck (6), ZAP-70 (6), Grb-2 (7) and ERK1/2 (9). As previously mentioned, Lck and ZAP-70 are both kinases activated immediately after TCR/MHC ligation, resulting in downstream activation of proliferation and activation-inducing transcription factors (35, 37). Grb-2 is an activator of the Ras pathway of activation (93) while ERK1/2 are members of the MAPK family and contribute to JNK activation (94).

All of these proteins contribute to the activation and proliferation of T cells. This is at odds, however, with other data which have shown that, when MUC1 and CD3 were costimulated, proliferation was inhibited (4, 5). However, upon analyzing the amino acid sequence of the cytoplasmic domain of MUC1, several hypotheses for these contrasting results come to light.

The cytoplasmic domain contains two sequence motifs very similar to the known ITAM and ITIM motifs (13). With dual ITAM and ITIM-like domains and data already showing that ITAM-related proteins bind to MUC1's cytoplasmic tail, an explanation for the apparently contrary costimulatory/coinhibitory abilities comes to light – MUC1 stimulation could,

theoretically, be able to produce both stimulatory and inhibitory signals in T cells. As previously mentioned, several signaling molecules appear to have a dual function in both positive and negative immunoregulation. Additionally, many more activation molecules are thought to have ITIM-like domains (95), suggesting that the dual function may be much more common that initially thought.

1.8 RATIONALE AND HYPOTHESES

This thesis is focused on understanding the potential immunomodulatory role of MUC1 on T cells with a goal of examining both costimulatory and coinhibitory functions. It builds on earlier work (4, 5) showing that MUC1 stimulation can inhibit the proliferation normally caused by CD3 stimulation on T cells, and expands it to encompass its costimulatory abilities predicted by studies showing it contains both an ITAM- and ITIM-like domain. The triggers which cause each will also be studied, as well as the downstream intracellular signals that occur. The hypotheses of this study are as follows:

i) MUC1 can function as a costimulatory molecule of T cells, enhancing the proliferation of T cells with specific phenotypes. The costimulation mediated by MUC1 utilizes a specific intracellular pathway which leads to an increased production of proliferation-inducing cytokines.

ii) MUC1 can function as a coinhibitory molecule of T cells in conditions distinct from its costimulatory capabilities. As a coinhibitory molecule, MUC1 is expressed more highly on the T-regulatory subset of T cells.

Each of these hypotheses will be tested in the following two chapters of my thesis.

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CHAPTER-2

MUC1 is a novel costimulatory molecule of human T cells and functions in an NF-AT-dependent manner

2.1 INTRODUCTION

Mucin-1 (MUC1) is a large, >200kDa, transmembrane glycoprotein expressed on the surface of most types of epithelial cells (1). Its extracellular domain consists of a variable number of 20 amino acid tandem repeats which are heavily glycosylated with *o*-linked oligosaccharides, making up the majority of MUC1's molecular weight. Its cytoplasmic domain contains many signaling motifs (2) and is non-covalently linked to the extracellular domain (3). Recent studies have suggested that it migrates to the nucleus upon extracellular ligation in epithelial cells, acting as a shuttle protein for transcription factors such as β-catenin (4).

MUC1 is expressed in an aberrantly-glycosylated form on the surface of epithelial-derived carcinomas (5, 6, 7). Its surface expression on these tumor cells has also been directly linked to prognostic outcome, with a high expression of MUC1 being a poor indicator of patient survival (8, 9). Many trials have been performed utilizing tumor-derived MUC1 as a cancer vaccination target in both mice (10, 11) and humans, with mixed results (12, 13, 14). MUC1 has also been associated with late-stage tumor cell metastasis through association with its currently known ligand, the adhesion molecule ICAM-1 (15).

However, MUC1 has been found to not only be expressed on epithelial cells and epithelial-derived carcinomas, but also on activated T cells (16, 17), dendritic cells (18) and monocytes (19). Previously, assays performed on purified T cells with soluble anti-CD3 and anti-MUC1 antibodies have shown that MUC1

may act as an inhibitory protein, as MUC1 crosslinking severely inhibited the proliferation of T cells normally caused by the anti-CD3 stimulus (16, 20).

Upon analysis of MUC1's cytoplasmic domain, two putative sequences of interest were identified – one very closely resembling an Immunotyrosine Inhibitory Motif (ITIM) and one resembling an Immunotyrosine Activation Motif (ITAM) (2). In T cells, ITAM sequences allow for the recruitment of signal cascade-inducing proteins such as Lck which cause phosphorylation events that lead to cellular activation and proliferation (21). ITIM sequences, however, allow for the recruitment of SHP-family phosphatases which dephosphorylate the $CD3_{\zeta}$ chain, preventing signal cascades from occurring and, thereby, cellular activation (22). Co-immunoprecipitations performed have shown that the majority of proteins that bind to the cytoplasmic tail of MUC1 in T cells are signal cascade proteins normally associated with the ITAM sequence, including Lck (23), Grb-2 (24) and ZAP-70 (23, 25). In lymphoma models, it has also been shown that, by transfecting Jurkat T cells with a chimeric protein consisting of a CD4 extracellular domain and MUC1's cytoplasmic tail, that ERK1 and ERK2 can also bind the cytoplasmic tail after providing an extracellular stimulus (26). Both of these kinases are members of the MAPK family and can lead to progression of the cell cycle through phosphorylation of transcription factors such as AP-1 (27).

In the original studies (16), it was shown that MUC1 was only expressed on a fraction of activated T cells at a given time. Therefore, we investigated the T cell subsets which preferentially express MUC1 upon activation. Interestingly,

we found that, upon mitogen activation, a higher percentage of $CD4^+$ T cells showed MUC1 expression whereas, in a non-activated state, $CD8^+$ T cells were expressing MUC1 to a significantly higher degree. MUC1 expression was only slightly increased on $CD8^+$ T cells upon mitogen stimulation. Due to the presence of the ITAM motif in the cytoplasmic tail of MUC1, we sought to investigate whether MUC1 can act as a costimulatory molecule on T cells in addition to acting as a coinhibitory molecule as reported previously (16, 20). CTLA-4, a well-studied coinhibitory molecule, has also been shown to have costimulatory functions when ligated with a manipulated antibody (28).

For the first time, we show that MUC1 is able to act as a costimulatory protein of T cells. Utilizing the NF-AT, calcium-dependent pathway of T cell activation, it is able to increase cytokine production and proliferation. These studies have significant implications in the immunotherapeutic intervention of both cancer and autoimmune diseases.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of non-adherent cells from human blood

Peripheral blood samples were obtained from individuals of both sexes 30-60 years of age with informed consent. Use of human blood samples was approved by institutional Health Research Ethics Board (HREB) at the University of Alberta, Canada. The blood was gently layered overtop of Lymphocyte Separation Medium (Cellgro, Herndon, VA) and was centrifuged at 1500-2000 g for 30 minutes at room temperature. The intermediate buffy layer containing the peripheral blood mononuclear cells (PBMCs) was removed and washed twice in PBS and resuspended in RPMI 1640 medium supplemented with 0.2% penicillin-streptomycin, 0.2% sodium pyruvate (Invitrogen, Carlsbad, CA) and 1% human AB serum (Sigma, St. Louis, MO). Cells were plated into 6-well plates at $3x10^7$cells/well and placed into an incubator for two hours at 37^{o}C and 5% CO_2 and 95% air (hereby just '37^{o}C'). Adherent monocytes and macrophages would adhere to the bottom of the plate while the non-adherent T-, B- and NK cells (consisting of approximately >60% T cells, based on Flow Cytometric analysis [see Figure 4-1]; hereby termed 'T cells') would not. The T cells were collected and resuspended in AIM-V medium (Invitrogen, Carlsbad, CA). T cells were then stimulated with 1μg/ml of PHA (Sigma, St. Louis, MO) and left at 37^{o}C for three days in order to induce optimal MUC1 expression.

2.2.2 Flow cytometry

T cells were separated into tubes at numbers between 800,000 and 1,000,000 per tube, resuspended into cold FACS buffer (PBS + 2% FBS) and kept at 4^{o}C for the remainder of the experiment. Cells were stained with fluorescent antibodies against CD4-QR, CD8-QR, MUC1 (anti-MUC1 antibody labeled with Alexa 647 via an Alexa Fluor 647 Protein Labeling Kit [Invitrogen, Carlsbad, CA]), CD27-PE, CXCR4-PE-Cy7, CCR5-PE-Cy7, CD45RA-FITC and CD45RO-FITC (all non-MUC1 antibodies were purchased from eBioscience, San Diego, CA) for thirty minutes before being centrifuged and washed twice in FACS buffer. Cells were fixed in FACS fixative solution (PBS + 2% paraformaldehyde) and resuspended in FACS buffer before analysis on FACSCanto (BDBiosciences, Franklin Lakes, NJ). Isotype control antibody was used for each fluorescent antibody and cells were gated to exclude 98% of the isotype control stained cells.

2.2.3 Proliferation assays

T cells from donors, either from frozen stocks or directly from blood, were incubated at 37^{o}C for three days with 1μg/ml PHA. They were washed twice in PBS and resuspended into AIM-V medium at a concentration of 4x10^{6}cells/ml. A 96-well plate was used for cell treatments, with 2x10^{5}cells/well, along with 10μg/ml B27.29 (anti-MUC1 antibody) (Biomira, Edmonton, AB) or 10 μg/ml mouse IgG_1 isotype, 1μg/ml goat anti-mouse crosslinking antibody (Sigma, St. Louis, MO), 20 μg/ml OKT3 (anti-CD3 antibody). Plates were incubated at 37 ^{o}C

for three days, with the addition of 0.5 μCi/well [^{3}H]thymidine (Amersham, Piscataway, NJ) at the end of the third day. The cells were harvested after 18 h and read on a Microbeta Liquid Scintillation Counter (PerkinElmer, Waltham, MA).

2.2.4 ELISA for cytokines

ELISA assays for IL-2, IL-10, TNF-α, IFN-γ (Biosource, Carlsbad, CA) were performed according to the manufacturer's protocol. In brief, 96-well plates were coated with anti-cytokine antibodies. Supernatants from proliferation assays were added at 1:20 and 1:50 dilutions in duplicate, along with standards, and incubated at room temperature before washing and the addition of a second anti-cytokine antibody linked with biotin. After another incubation and wash step, an enzyme-strepavidin conjugate was added along with the substrate. After development for 30 minutes, a stop solution was added and the plate was read. Plates were washed using the ELx405 ELISA plate washer (Bio Tek, Winooski, VT) and were read and analyzed on a Fluostar Optima Fluorimeter (BMG Labtech, Offenburg, Germany) within 30 minutes of development. Standard curves were run between 15-2000 pg/ml in each assay.

2.2.5 Microsphere preparation

Latex microspheres measuring 1μm (Polysciences Inc., Warrington, PA) were washed and resuspended in 0.1M borate buffer, pH 8.5 to a concentration of $1x10^9$ microspheres/ml and coupled with 150μg total of anti-MUC1, mouse IgG isotype and/or anti-CD3. The beads were left shaking overnight at room temperature and washed three times for 30 minutes each in 0.1M borate buffer, pH 8.5, with 10mg/ml bovine serum albumin in order to block any remaining unbound sites. The beads were stored at 4 ^oC in PBS + 10mg/ml bovine serum albumin + 0.1% sodium azide + 5% glycerol (storage buffer). Beads were washed three times with PBS before use in cell culture.

2.2.6 Microsphere-based proliferation assays

T cells from donors were kept at 37 ^oC for three days either without PHA or with 1μg/ml PHA to allow MUC1 expression on T cells (16). They were washed twice in PBS and resuspended into AIM-V medium at a concentration of $4x10^6$cells/ml. Microspheres were washed twice in PBS before being resuspended in AIM-V to required concentrations. A 96-well plate was used for cell culture, with cells added at $2x10^5$cells/well. Microspheres were then added to the cell suspensions at a ratio of 1000 microspheres to 1 cell; for separately-ligated beads, 500 microspheres to 1 cell were used in the experimental wells for each of the two bead types, giving 1000:1 total. Plates were incubated at 37 ^oC for three days, with the addition of 0.5 μCi/well [^{3}H]thymidine at the end of the third

day. The following day the cells were harvested and counted on a microbeta liquid scintillation counter.

2.2.7 Inhibition Assay

Cyclosporine A, Bisindolylmaleimide I and SB203580 (Invitrogen, Carlsbad, CA) were purchased in solid form and resuspended in DMSO. Cyclosporine A was diluted into PBS and then AIM-V medium to a final concentration in each treatment well of 42nM. Bisindolylmaleimide I was diluted into PBS then AIM-V medium to a final concentration of 30nM in each treatment well. SB203580 was diluted into PBS then AIM-V medium to a final concentration of 1μM in each treatment well. Proliferation assays were then performed as described.

2.2.8 Confocal microscopy

Glass slides were coated with poly-L-lysine (Sigma, St. Louis, MO) and washed with PBS and 90% ethanol three times each, then left to dry. T cells stimulated with PHA for three days were washed twice in PBS and added to the slides at 1-2x10^7 cells per slide. Cells were allowed to adhere for 30 minutes and then stimulated with either 20μg anti-CD3 or no antibody. The slides were then fixed in 4% paraformaldehyde + 120μM glucose for 30 minutes. The slides were washed with PBS before the addition of 0.1% Triton-X in PBS for 30 minutes.

Slides were washed twice with PBS before 1μg of CT2 (a gift from Dr. Sandra Gendler, Mayo Clinic, Scottsdale, AZ), an antibody against the cytoplasmic tail of MUC1, was added to the slides for 1 hour in 500μl of Dulbecco's Modified Eagle Medium. Slides were then washed twice with PBS and a goat anti-Armenian hamster IgG – Cy3 conjugate (Leinco Technologies, St. Louis, MO) was added at 1μg in 500μl of Dulbecco's Modified Eagle Medium to each slide for 1 hour. Slides were washed twice with PBS, then mounted with mounting solution containing a 60:40 ratio of glycerol: PBS, 2% of the antifadant 1,4-diazabicyclo(*2,2,2*)octane (Sigma, St. Louis, MO) and 1μl of DAPI dye (Invitrogen, Carlsbad, CA) per 500μl of solution. Coverslips were added on each slide and sealed with nail polish. Slides were analyzed via Confocal microscope (Zeiss LSM-510 Confocal Microscope, Zeiss, Toronto, Canada).

2.2.9 Lysates

T cells stimulated with PHA for three days were stimulated with antibody-bound plates (20μg/ml anti-CD3, 20μg/ml anti-CD3 and 10μg/ml anti-MUC1, or 20μg/ml anti-CD3 and 10μg/ml mouse IgG isotype) for 45 minutes at 37^{o}C in AIM V medium in a 6-well plate. Cells were then taken and lysed to obtain cytoplasmic and nuclear fractions as previously described (29). In brief, cells were first treated with Lysis Buffer A (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithithreitol, 0.2 mM PMSF) at 4^{o}C. After 10 minutes for swelling, the cells were vortexed and lysed, centrifuged and the

cytoplasmic lysate extract collected. Nuclei were treated with Lysis Buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithitheitol, 0.2 mM PMSF) at 4^{o}C for 20 minutes, then vortexed and centrifuged. The nuclei extracts were then removed. All lysates were stored at -80^{o}C until used. Lysate fractions were kept at -80 ^{o}C until required for use.

2.2.10 SDS-PAGE and Western blotting

Lysates were prepared for Western blotting with the reagents provided in the Protein G Immunoprecipitation Kit (Sigma, St. Louis, MO). After loading and running the samples on a 10% resolving gel, the gel was transferred to nitrocellulose overnight at 60V. The membrane was blocked for one hour with 5% skimmed milk, then incubated with anti-NFATc1, anti-c-Fos or anti-c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA) in Western wash buffer (0.1% Tween-20 in PBS) for 1 hour. After five 5 minute washes with Western wash, a secondary goat anti-mouse – HRP conjugated antibody (Novus Biologicals, Littleton, CO) was added in Western Wash for 2 hours. After five more 5 minute washes, an enhanced chemiluminescence (ECL) substrate (Fisher, Pittsburgh, PA) was added to the blot for 2 minutes. After removal of the substrate, the blot was imaged on x-ray film.

2.2.11 Statistical analyses

Statistics were performed using one-way analysis of variance (ANOVA) with Tukey's test for post-hoc analysis or independent sample T-test using SPSS 16.0 software (SPSS Inc., Chicago, IL). * represents a statistically-significant difference at the $p<0.05$ level to the closest appropriate control group. All error bars shown are indicative of standard error.

2.3 RESULTS

2.3.1 MUC1 expression increases on both $CD4^+$ and $CD8^+$ T cells after mitogen (PHA) stimulation

To investigate whether MUC1 is broadly expressed on all T cell subsets or expressed on a specific T cell subset, MUC1 expression was analyzed on naïve, memory, memory/effector and effector $CD4^+$ and $CD8^+$ T cells before and after mitogen (PHA) stimulation using flow cytometry. All cells were gated as shown in Figure 2-1A. $CD8^+$ T cells were gated for $CD45RA^+/CD27^+CCR5^-$ (naïve phenotype), $CD45RA^-/CD27^+/CCR5^+$ (memory phenotype), $CD45RA^-/CD27^{Low}/CCR5^-$ (memory/effector phenotype) and $CD45RA^+/CD27^-/CCR5^-$ (effector phenotype) while $CD4^+$ T cells were gated for $CD45RA^+/CD27^+/CCR7^+$ (naïve phenotype), $CD45RA^-/CD27^{+/-}/CCR7^+$ (memory phenotype), $CD45RA^-/CD27^-/CCR7^-$ (memory/effector phenotype) and $CD45RA^-/CD27^+/CCR7^-$ (effector phenotype) as previously described (54). It was found that a low percentage of $CD4^+$ T cells of all subsets expressed MUC1 (~5%) after isolation from fresh human blood before PHA stimulation (Fig. 2-1B). $CD8^+$ T cells isolated from fresh human blood, however, exhibited a higher percentage of $MUC1^+$ cells (15-40%), with naïve and memory subsets having the highest percentages (Fig. 2-1C). Three days after PHA stimulation, MUC1 expression in both the $CD4^+$ and $CD8^+$ T cells increased, with the largest increase occurring in

the $CD4^+$ T cell subsets (a 2-6 fold increase across all groups) and with the 'naïve' $CD4^+$ T cells having the largest increase, likely showing a progression of unstimulated cells into a more matured phenotype. Expression on $CD8^+$ T cells, however, did not increase very substantially (20-30% increase across all groups), though no $CD8^+$ effector cells were found to express MUC1 post-mitogenic stimulus.

2.3.2 CD3 and MUC1 coligation and crosslinking in T cell cultures causes enhanced cellular proliferation

In order to investigate whether or not MUC1 can act as a costimulatory molecule in addition to its purported coinhibitory properties, T cells were first stimulated with PHA for 72 hours to induce MUC1 expression. These T cells were then treated with antibodies against CD3, MUC1 or IgG isotype control and a crosslinking goat anti-mouse antibody. After a three day incubation, the cells in the MUC1-stimulated group proliferated in a greater manner than the anti-CD3-only group and the IgG isotype control group, with a statistical significance of $p < 0.01$ (Fig. 2-2*A*). This experiment provided the first evidence that crosslinking MUC1 is able to provide costimulation to enhance the proliferation normally generated by the anti-CD3 stimulus. Control cultures with anti-MUC1 and crosslinking goat anti mouse antibody only, in the absence of CD3 stimulation, did not show significant proliferation over background (Fig. 2-2*B*).

2.3.3 CD3 and MUC1 costimulation leads to an increase in $CD4^+$ memory, $CD8^+$ memory and memory/effector cells

The apparent costimulatory effects of MUC1 stimulation on T cells in the presence of CD3 stimulation encouraged us to determine what T cell subsets, within either $CD4^+$ or $CD8^+$ cells, were increased after anti-CD3 and anti-MUC1 costimulation. The T cells costimulated with anti-CD3 and anti-MUC1 antibody were stained for various markers of memory, memory/effector and effector T cells in both $CD4^+$ and $CD8^+$ T cell subsets. The flow cytometry analysis demonstrated that there was a statistically significant increase in $CD4^+$ memory cells (~70%), $CD8^+$ memory (~35%) and memory/effector (~60%) cells after MUC1 costimulation as compared to isotype costimulation (Fig. 2-3). All other subsets were not significantly different from 0% and, thus, did not change significantly after MUC1 costimulation compared to the isotype group.

2.3.4 MUC1-mediated costimulation requires CD3 and MUC1 coligation

Most of the costimulatory molecules of T cells often require CD3 within close proximity due to the sharing of intracellular kinases, phosphatases and other

proteins (30, 31). Therefore, we hypothesized that MUC1 may function in a similar manner. Using 1μm latex microspheres coligated with anti-CD3 and either anti-MUC1 or isotype or, instead, beads ligated separately with anti-CD3 and anti-MUC1 or anti-CD3 and isotype, it was found that T cells showed enhanced proliferation with the anti-CD3 and anti-MUC1 coligated group as compared to the other groups ($p < 0.05$) (Fig. 2-4). There was no statistically significant enhancement of proliferation when cells were treated with the separately-ligated beads rather than the coligated beads ($p > 0.05$), compared to the isotype control.

2.3.5 MUC1-based costimulation increases the expression and release of TNF-α, IFN-γ and IL-2, but not the inhibitory cytokine IL-10

Activation of each of the calcineurin, PKC_θ and p38 MAPK pathways has been shown to induce a unique cytokine production profile: IL-2, IFN-γ, TNF-α for the calcineurin-dependent NF-AT pathway (32); IL-2 for the PKC_θ-dependent NF-kB pathway (33); and IL-10 for the p38 MAPK pathway (34). Therefore, in order to further delineate the intracellular pathway involved with MUC1 costimulation, supernatants were collected from representative MUC1 costimulation cultures (Fig. 2-2*A* and similar experiments) and analyzed via sandwich ELISA. The anti-CD3 and anti-MUC1-treated group produced more

TNF-α, IFN-γ and IL-2 into the supernatant at a statistically significant level compared to the control groups with anti-CD3 alone or anti-CD3 with isotype control and crosslinking antibody ($p < 0.05$) (Fig. 2-5). For IL-10, however, there was no statistically significant difference between the anti-CD3 and anti-MUC1-treated group and the isotype control. These experiments further suggested that MUC1 costimulation functions independent of the p38 MAPK pathway but involves the NF-AT pathway.

2.3.6 MUC1 costimulation is unaffected by a specific inhibitor of the NF-kB pathway, but not the NF-AT or p38 MAPK pathways

To determine the pathway utilized by MUC1-mediated costimulation resulting in enhanced proliferation, intracellular inhibitors of different signaling pathways were added to MUC1-costimulated cultures. These inhibitors included Cyclosporine A (an inhibitor of calcineurin, a necessary molecule in the NF-AT pathway), Bisindolylmaleimide I (an inhibitor of PKC_{θ}, a necessary molecule in the NF-kB pathway) and SB203580 (an inhibitor of p38 MAPK). Optimal concentrations of inhibitors were first determined, with final values of 42nM Cyclosporine A, 30nM Bisinolylmaleimide I and 1μM SB203580. The T cells were treated with the optimal concentrations of inhibitors for 10 minutes and then further treated with antibodies against CD3 alone, CD3 and MUC1 or CD3 and isotype along with the crosslinking goat anti-mouse antibody as per prior

experiments. After three days, there was no statistically significant difference between the anti-CD3 and anti-MUC1-treated group and either of the control groups in the presence of Cyclosporine A or SB203580 (Fig. 2-6). However, there was a statistically significant difference between the anti-CD3 and anti-MUC1-treated group compared to the controls in the Bisindolylmaleimide I-treated group ($p<0.01$), showing a partial to complete reversal of the inhibition and/or no effect of Bisindolylmaleimide I on MUC1 costimulation. These results demonstrate that MUC1 mediated costimulation functions independent of the PKC_{θ} pathway, but likely involves calcineurin and/or p38 MAPK pathways.

2.3.7 The cytoplasmic tail of MUC1 migrates into the nucleus upon CD3 stimulation in T cells

In tumor cells, the cytoplasmic tail of MUC1 has been shown to migrate into the nucleus along with transcription factors. Therefore, we hypothesized that MUC1 on T cells may be functioning in a similar manner. T cells were placed on slides, stimulated and stained for the MUC1-cytoplasmic tail and the nucleus. Slides were then analyzed by confocal microscopy, with approximately 300 cells being analyzed per experimental group and representative pictures taken. Without anti-CD3 stimulation, the cytoplasmic tail of MUC1 remained at the cell membrane and appeared to be clustered in what closely resembled the staining profile of lipid rafts (35, 36) (Fig. 2-7). However, with anti-CD3 stimulation

regardless of MUC1 costimulation, cytoplasmic tail could be clearly seen in the nuclei of the cells.

2.3.8 The cytoplasmic tail of MUC1 binds to the transcription factors c-Jun and c-Fos

The cytoplasmic tail of MUC1 migrated to the nucleus after CD3 or CD3/MUC1 costimulation; therefore, we hypothesized that the cytoplasmic tail of MUC1 would bring transcription factors into the nucleus in T cells. With the earlier results supporting the calcineurin-dependent NF-AT pathway as an intracellular pathway used in MUC1 costimulation, we blotted the transcription factors NF-ATc1, c-Jun and c-Fos after immunoprecipitation of the cytoplasmic tail of MUC1 from T cell lysates. For NF-ATc1, only the positive control (T cell whole cell nuclear lysate) showed a band at the appropriate molecular weight; there were no bands present in any of the experimental groups (see Figure 4-3). For c-Jun, bands of the appropriate molecular weight of 35-39kDa were found in both the control group (T cell whole cell nuclear lysate) and in the anti-CD3 and anti-MUC1 pre-treated cytoplasmic and nuclear fractions. No other pre-treatment had a band appear indicative of c-Jun co-immunoprecipitation with the cytoplasmic tail of MUC1 (Fig. 2-8*A*). For c-Fos, a band between 60-70kDa representative of the 62kDa weight of c-Fos was found in the positive control group and all the anti-CD3 stimulated cytoplasmic and nuclear fractions, as well

as small amounts in the unstimulated groups (Fig. 2-8*B*). However, there was a clearly visible increase in the c-Fos band in the nuclear fraction of the MUC1 costimulated group.

2.4 DISCUSSION

MUC1 has been identified and researched as a tumor-associated antigen in most of the studies to date. Tumor cells express aberrantly-glycosylated versions of MUC1 (5, 6, 7), dysregulating its cytoplasmic tail (3) and, thereby, β-catenin regulation (4). This then allows it to interact with other nuclear proteins, such as p53 (37), further enhancing tumor growth and success. Tumor-associated soluble MUC1 has also been shown to have immunomodulatory activity (38). The role of MUC1 in T cell activation, regulation and homeostasis as an activation-induced transmembrane glycoprotein in T lymphocytes is being recognized in recent studies (16, 17, 20, 23). Until recently, crosslinking MUC1 has been found to inhibit the proliferation of T cells when given a CD3-based stimulation (16, 20). However, the presence of putative ITAM and ITIM domains, as well as evidence of signaling proteins binding to its cytoplasmic tail, suggest that MUC1 may have a dual costimulatory as well as coinhibitory function in T cells.

In our studies, we found that MUC1 expression increases significantly on $CD4^+$ T cells after mitogen stimulation, with 'naïve' phenotype T cells having the largest increase. This supports the hypothesis that MUC1 plays a role in T cell immunoregulation, as naïve T cells begin to express maturation markers after mitogen stimulation (39), preparing them to become activated upon recognition of their antigen. Indeed, treating T cells with antibodies against CD3 and MUC1, under crosslinking conditions, led to enhanced proliferation of the T cells. This is the first evidence obtained in characterizing MUC1 as a costimulatory protein of

T cells, as previous evidence (16, 20) has only suggested a coinhibitory role for MUC1 in T cell immunoregulation. Earlier studies utilized a partially purified T cell population (>80% $CD3^+$ T cells, see Figure 4-2) and demonstrated coinhibition mediated by MUC1 (16, 20). However, in the current study, a non-adherent T cell population (consisting of >60% $CD3^+$ T cells, see Figure 4-1) was used to demonstrate a costimulatory effect of MUC1. These inverse results suggest a role of accessory cells in MUC1-mediated T cell costimulation and/or coinhibition. In fact, in our experiments, when we used highly pure $CD3^+$ T cells (~99% $CD3^+$ T cells) and stimulated them with anti-CD3 with or without anti-MUC1, no proliferation of T cells was observed unlike when anti-CD28 costimulation was included. However, in partially purified T cell (~90-94% $CD3^+$ T cells) cultures, addition of irradiated autologous $CD3^-$ accessory cells resulted in a costimulatory effect proportional to the amount of accessory cells added (data shown in Chapter 3). These experiments suggest that in order for the MUC1 to function as a costimulatory molecule, another signal/interaction is required.

After determining the conditions that allow MUC1 ligation to result in a costimulatory response, we examined its effects on different $CD4^+$ and $CD8^+$ T cell subsets. With $CD4^+$ T cells, the percentage of memory cells increased greatly while, with $CD8^+$ T cells, naïve, memory and memory/effector cells increased after CD3 and MUC1 costimulation compared to CD3 and isotype control. These results are interesting, since we observed that mitogenic stimulation results in higher MUC1 expression on naïve $CD4^+$ T cells, whereas MUC1 costimulation allows antigen-experienced memory $CD4^+$ T cells to expand vigorously compared

to other $CD4^+$ T cell populations. In contrast, MUC1 costimulation allows the expansion of memory, memory/effector and effector $CD8^+$ T cells. These results suggest that MUC1 costimulation does not just enhance the proliferation of T cells in a generic manner, but explicitly causes a specific subset of cells to divide or increase, allowing a fine-tuned regulation of T cell responses. Not only this, but like other costimulatory proteins, we discovered that MUC1 is required to be in close proximity to the $CD3_\zeta$ chain, a result that is likely due to shared kinases/phosphatases (30, 31), further allowing antigen-specific TCR-mediated stimulation as a requirement to provide its immunoregulatory role.

Three of the major intracellular signaling pathways used by T cells are the NF-AT, NF-kB and p38 MAPK pathways. The calcium-dependent NF-AT pathway is activated by CD3 stimulation and results in an increase in pro-inflammatory cytokine production – namely IFN-γ, TNF-α and IL-2 (32). The NF-kB pathway requires both CD3 and CD28 costimulation and results primarily in the production of the proliferation-inducing cytokine IL-2 (33). Finally, the p38 MAPK pathway results in, after T cell activation, production of Th_2 cytokines such as IL-4 and IL-13 as well as the proliferation-inhibiting cytokine IL-10 (34). In our results, proliferation did not differ with MUC1-mediated costimulation in comparison to the controls in the p38 MAPK and NF-AT- inhibited groups, suggesting either pathway could be used by MUC1 costimulation. MUC1-mediated costimulation however, was either not affected by or was able to completely reverse the inhibition caused by an inhibitor of PKC_θ, suggesting that MUC1 either uses a separate pathway from NF-kB to enhance T cell proliferation

or is somehow able to reverse or abrogate the inhibition caused by the inhibitor. By examining the cytokine profile produced by T cells stimulated by anti-CD3 and anti-MUC1, it was determined that MUC1 costimulation functions through the calcium-dependent NF-AT pathway, as IFN-γ, TNF-α and IL-2 are the main cytokines produced. This data is also supported by our observation that CD3/MUC1 costimulation causes an increase in the number of memory $CD4^+$ T cells which are producers of IFN-γ and both memory and memory/effector $CD8^+$ T cells which are producers of IFN-γ and TNF-α (40).

The most likely proteins to enhance proliferation at the nuclear level are transcription factors, several of which MUC1 has already been shown to bind to in tumor cells (3, 4, 37). The most significant of these is β-catenin. Unlike tumor cells, however, T cells do not typically produce β-catenin (41); only during development as thymocytes do they transcribe it (42). Thus, the most likely transcription factors, based on our data supporting the NF-AT pathway as the one MUC1 utilizes in T cells, were NF-AT family members such as NF-ATc1, c-Fos and c-Jun. Indeed, we found that c-Jun and c-Fos both bind to the cytoplasmic tail of MUC1 and enter the nucleus with them while NF-ATc1 does not. Both c-Fos and c-Jun are imperative in the NF-AT pathway, dimerizing together after phosphorylative activation to produce the transcription factor AP-1 (43). However, our data showed that c-Fos constitutively bound to the cytoplasmic tail of MUC1, with more binding after CD3 stimulation and slightly more after MUC1 costimulation, while c-Jun only bound to the cytoplasmic tail after CD3/MUC1 costimulation. Since we found that CD3 stimulation alone is

sufficient to cause migration of the cytoplasmic tail of MUC1 into the nucleus, this provides a role for MUC1 stimulation: phosphorylation of c-Fos and/or c-Jun to allow for c-Jun binding so that it may form the AP-1 dimer and be brought into the nucleus. This theory is supported by previous observations by Gendler et al. which showed that transfection of a tumor cell line with a MUC1 analogue resulted in an intracellular increase in AP-1 (44), as well as previous research showing ERK1/2 binding to MUC1's cytoplasmic domain (26). Since ERK1/2 phosphorylates c-Fos, leading to its further stability and enhancing its activity (27), it is possible that this leads to c-Fos/c-Jun dimerization either directly or indirectly.

AP-1 is vital in the early immune response, binding to specific genes alongside the NF-AT family of transcription factors. This results in cytokine production, cellular activation and proliferation (45). Normally c-Jun is transcribed after the initial CD3 response, with c-Jun dimers then leading to c-Fos production after CD28 costimulation (46). c-Jun then forms dimers with c-Fos or other members of the c-Fos family through its leucine zipper, all of which are known as AP-1 transcription factors (47). These AP-1 dimers then migrate into the nucleus where they bind to several promoter regions, generally for the production of pro-inflammatory and proliferation-inducing cytokines (48). In the absence of AP-1, anergy-inducing genes are transcribed, resulting in a T cell non-response to stimuli (49). This brings up a question as to why we were unable to observe NF-ATc1 binding despite the binding of the AP-1 transcription factor. It is possible that the cytoplasmic tail of MUC1 dissociates from AP-1 before it

binds to DNA, possibly due to size restriction or the binding of the cytoplasmic tail to the DNA-binding sites of AP-1, though future studies will have to be performed to determine which answer is correct.

In T cells, the nuclear entry of c-Fos and c-Jun is regulated by their respective nuclear localization signals which allow them to bind to nuclear transport proteins (50, 51). However, it has been shown that c-Fos has a weaker nuclear localization sequence than c-Jun; small quantities of c-Fos are able to migrate into the nucleus in the absence of c-Jun, meaning it is dependent on dimerization with c-Jun to enter the nucleus in a significant fashion (52). By binding to the cytoplasmic tail of MUC1, c-Fos is able to circumvent this regulation as it is provided with an alternate pathway of nuclear translocation. One obstacle, however, lies in the fact that PKC_{θ} is required to begin the phosphorylation cascade which leads JNK to phosphorylate c-Jun and c-Fos, allowing for their dimerization (48). Since, in our results, we observed that MUC1 costimulation was largely unaffected by PKC_{θ} inhibition, this suggests that the cytoplasmic tail of MUC1 either has phosphorylative abilities or is able to recruit other proteins which are able to phosphorylate c-Jun/c-Fos in a PKC_{θ}-independent manner.

These results provide evidence that MUC1 is a novel costimulatory protein on T cells. With both CD3 and MUC1 costimulation, MUC1's cytoplasmic tail is modified, resulting in the binding of c-Jun and c-Fos followed by subsequent nuclear translocation. By enhancing the amount of c-Jun and c-Fos

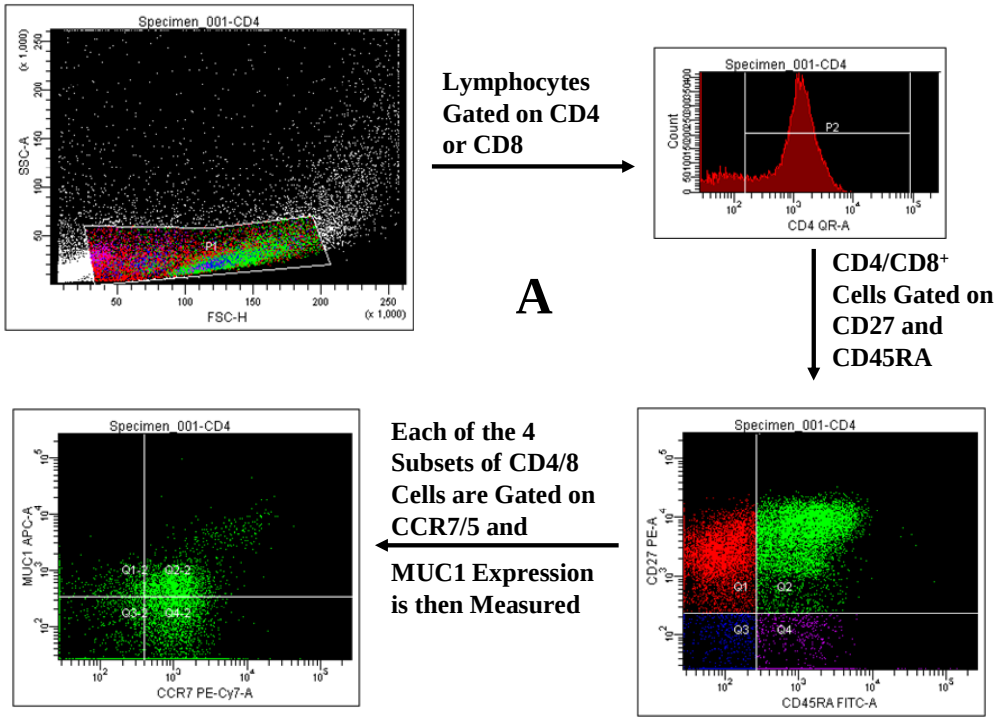
dimers entering the nucleus in a PKC_{θ}-independent manner, MUC1 costimulation is able to further activate genes which cause the production of pro-inflammatory and proliferation-inducing cytokines, resulting in an overall enhanced proliferative response. MUC1 could exist on T cells in the same manner as a protein such as OX40 which maintains the immune response after a primary activation, allowing them to become active again when presented with their antigen (53). MUC1 could also serve to enhance lower levels of CD3 and/or costimulatory molecule stimulation, increasing the amount of nuclear AP-1 when, normally, the amount would be too low. These are all possibilities which must be tested in future research.

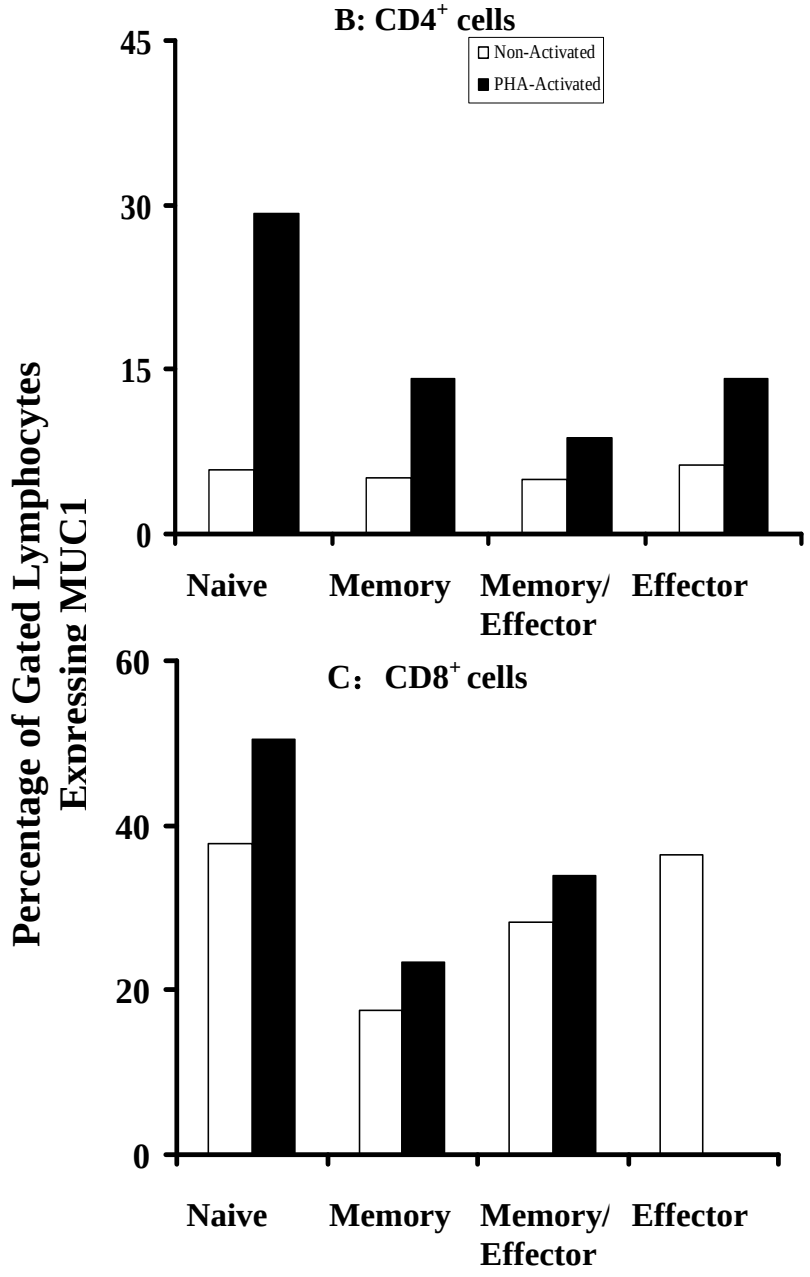
In conclusion, our study establishes MUC1 mucin as a novel T cell activation molecule with significant role as a costimulatory molecule. Our results also point towards a novel paradigm by which MUC1 adopts a costimulatory or coinhibitory function on T cells. Identifying the ligand for MUC1 expressed on activated T cells is imperative to explain the dual role played by MUC1. Further characterization of the costimulatory abilities of MUC1 may prove useful in the treatment of certain diseases where the immune system is inhibited, such as in many tumor microenvironments, or in diseases where the immune system is hyperactive, such as with many autoimmune disorders. For the former, through modulation of the MUC1 costimulatory pathway, it may be possible to partially reverse this inhibition. For the latter, blocking MUC1 with either an antibody or a specifically-designed inhibitor may be able to reduce the damage caused by autoimmune T cells. However, further studies on the expression and function of

MUC1 are needed before novel treatments and therapies can be created based on MUC1 and T cells.

2.5 FIGURES

Figure 2-1: MUC1 expression increases on both $CD4^+$ and $CD8^+$ T cells after mitogen (PHA) stimulation

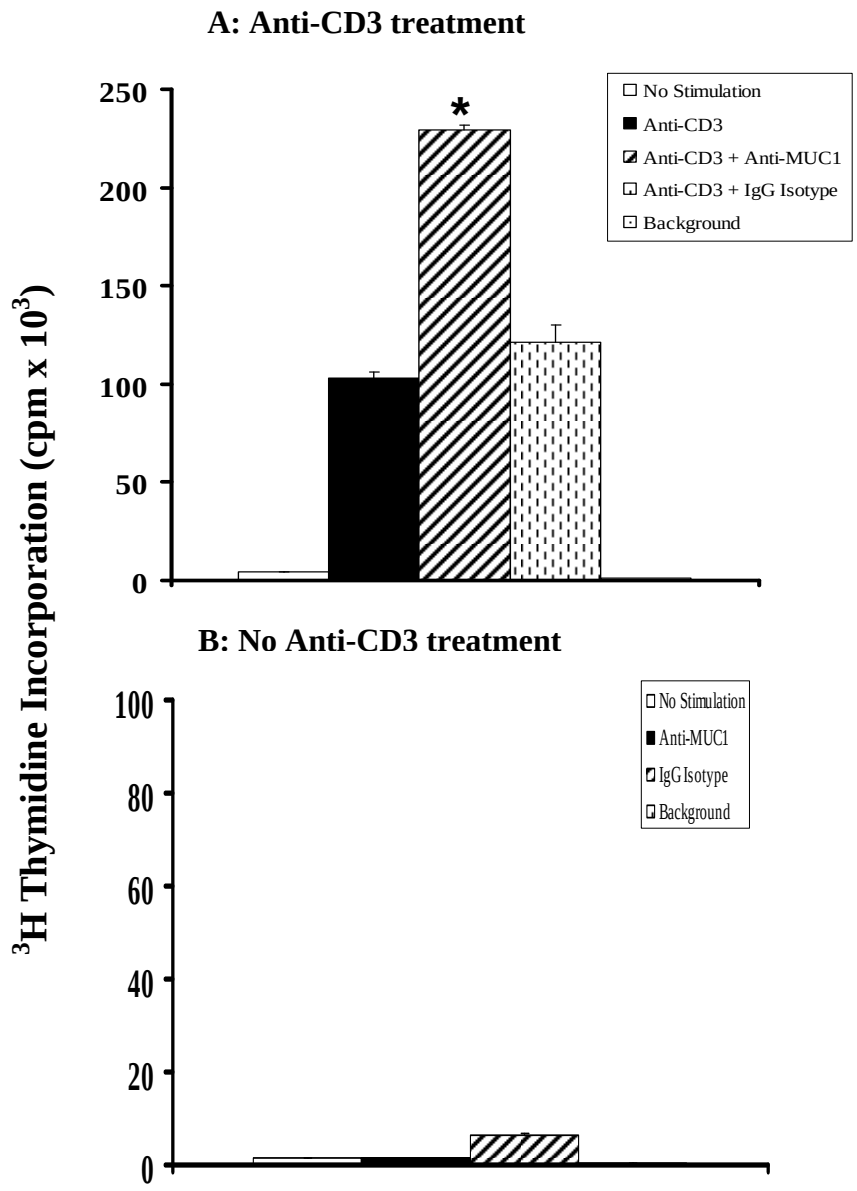




T cells were isolated from fresh human blood and either stained immediately or treated with 1μg/ml of PHA for three days then stained afterward. A gate was set on the lymphocyte population for analysis. $CD4^+$ T cells were analyzed for $CD45RA^+/CD27^{+/}CCR5^-$ (naïve phenotype), $CD45RA^-/CD27^+/CCR5^+$ (memory phenotype), $CD45RA^-/CD27^{Low}/CCR5^-$ (memory/effector phenotype) and $CD45RA^+/CD27^-/CCR5^-$ (effector phenotype) while $CD8^+$ T

cells were analyzed for $CD45RA^{+}/CD27^{+}/CCR7^{+}$ (naïve phenotype), $CD45RA^{-}/CD27^{+/-}/CCR7^{+}$ (memory phenotype), $CD45RA^{-}/CD27^{-}/CCR7^{-}$ (memory/effector phenotype) and $CD45RA^{-}/CD27^{+}/CCR7^{-}$ (effector phenotype) as previously described (54) and shown in *A*. The percentages of the gated T cell phenotypes were then compared from pre-stimulated (white) and three day PHA-stimulated (black) for $CD4^{+}$ T cells (*B*) and $CD8^{+}$ T cells (*C*). Results are representative of three separate experiments on three different donors. ND stands for 'not detectable'.

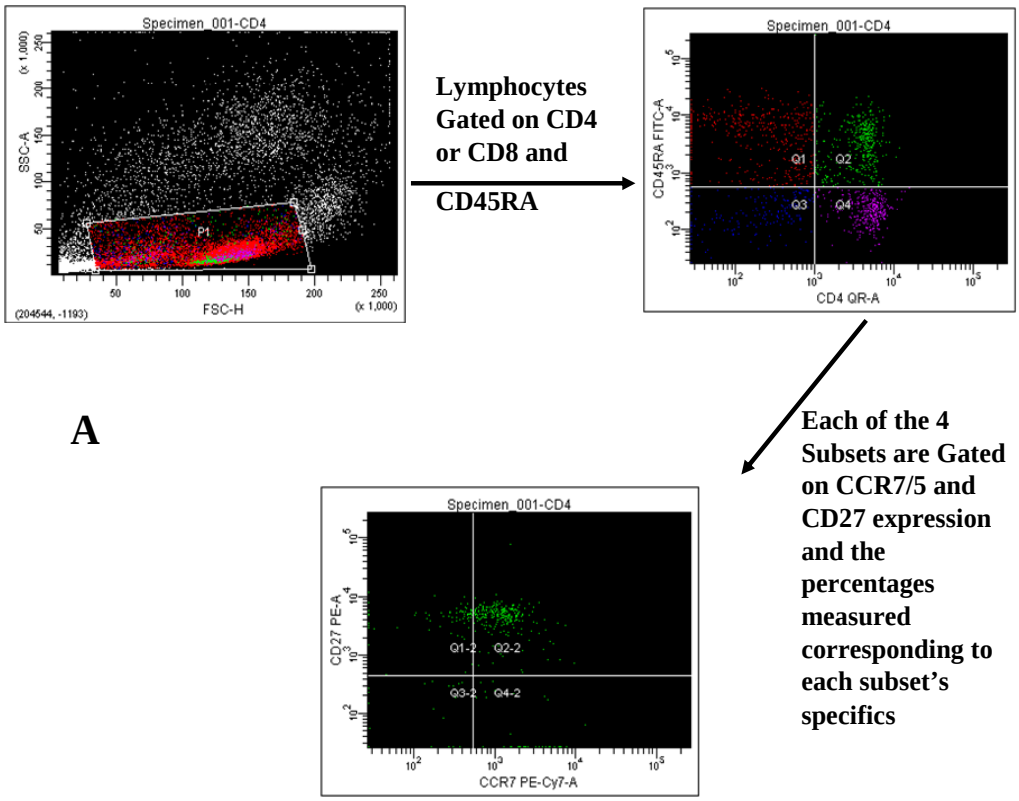
Figure 2-2: CD3 and MUC1 coligation and crosslinking causes enhanced cellular proliferation



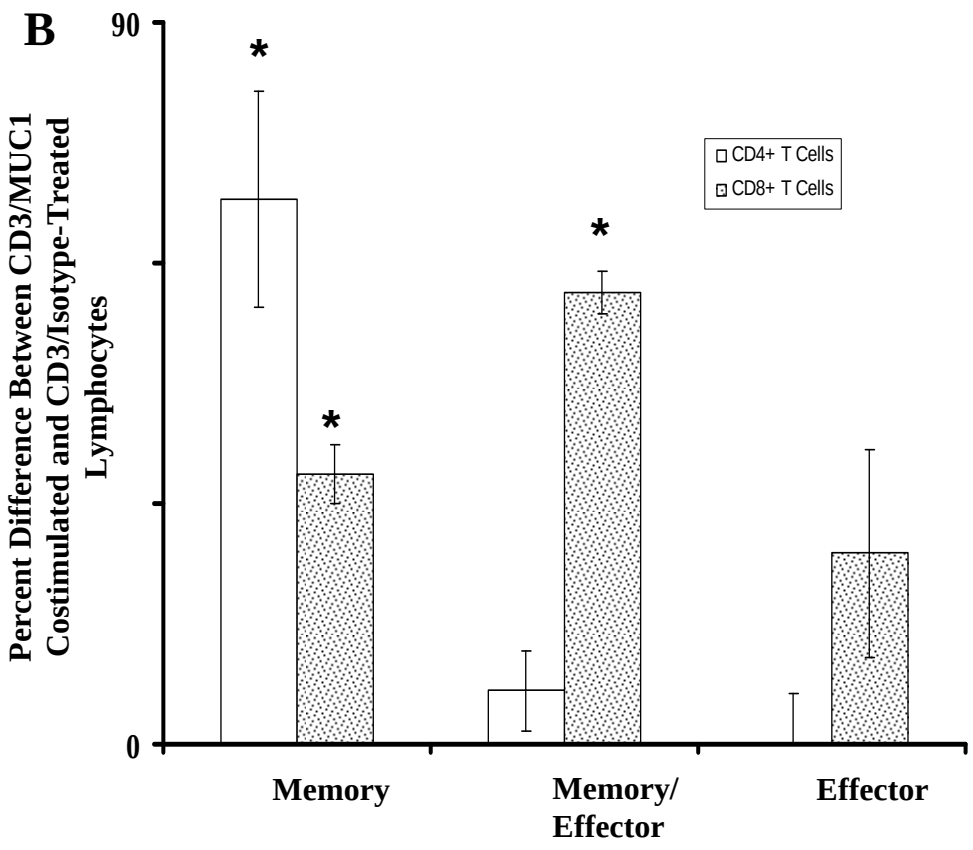
Proliferation was measured in 3-day PHA-stimulated T cells treated with: *A*, no antibodies (white bar), 20μg/ml of soluble anti-CD3 antibody (black bar), 20μg/ml anti-CD3 and 20μg/ml of the anti-MUC1 (hatched bar) or 20μg/ml anti-CD3 and 20μg/ml of mouse IgG isotype control (dashed

bar) or *B*, no antibodies (white bar), 20μg/ml of the anti-MUC1 (black bar) or 20μg/ml of the mouse IgG isotype control (hatched bar). Each antibody-treated group was also treated with 1μg/ml of goat anti-mouse antibody to cross-link by binding to both antibodies. There was a statistically significant difference between the anti-CD3 plus anti-MUC1-treated group compared to the other treatment groups, with $p<0.05$. There was no significant difference between the anti-MUC1-only treated group and the controls groups. Data is representative of >10 experiments on >10 different donors.

Figure 2-3: CD3 and MUC1 costimulation leads to an increase in $CD4^+$ memory, $CD8^+$ memory and memory/effector cells



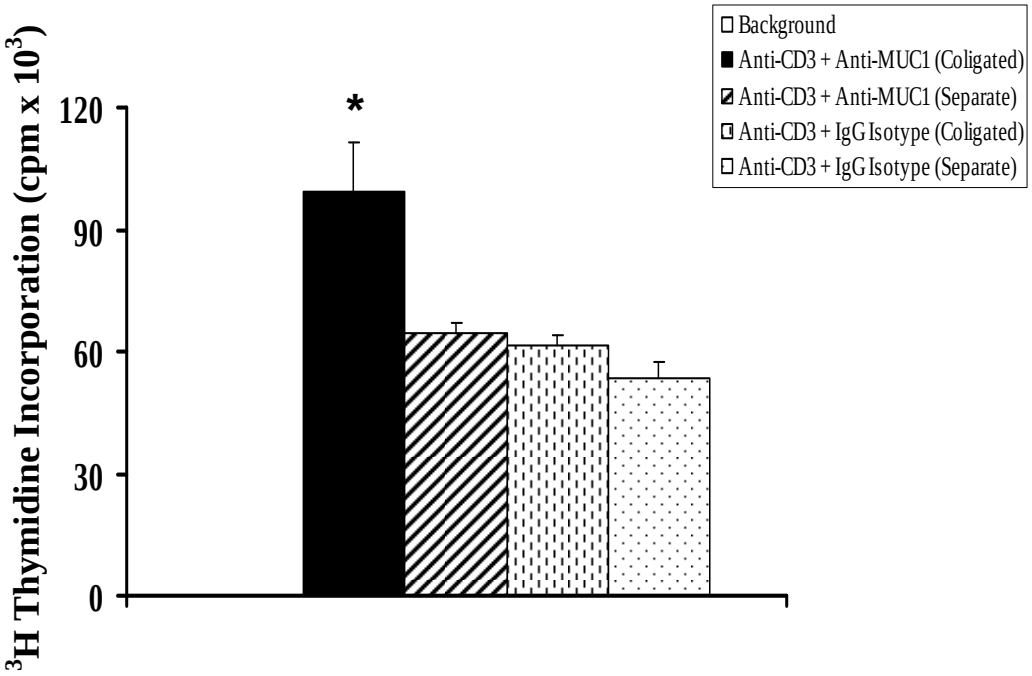
A



T cells were stimulated with 1μg/ml of PHA for three days to induce MUC1 expression before being stimulated for three more days with 20μg/ml anti-CD3, 10μg/ml anti-MUC1 and 1μg/ml goat anti-mouse IgG or 20μg/ml anti-CD3, 10μg/ml IgG isotype control and 1μg/ml goat anti-mouse IgG. A gate was set on the lymphocyte population for analysis. $CD8^+$ T cells were analyzed for $CD45RA^-/CD27^+/CCR5^+$ (memory phenotype), $CD45RA^-/CD27^{Low}/CCR5^-$ (memory/effector phenotype) and $CD45RA^+/CD27^-/CCR5^-$ (effector phenotype) while $CD4^+$ T cells were analyzed for $CD45RA^-/CD27^{+/-}/CCR7^+$ (memory phenotype), $CD45RA^-/CD27^-/CCR7^-$ (memory/effector phenotype) and $CD45RA^-/CD27^+/CCR7^-$ (effector phenotype) as shown in *A*. The percentages of cell phenotypes in the anti-MUC1-stimulated cells were then subtracted by the IgG isotype-stimulated results, divided by the IgG isotype-stimulated results ([observed-expected]/expected) and graphed, showing the percentage difference between anti-MUC1 and isotype stimulation in $CD4^+$ and $CD8^+$ T cell subsets *B*. Statistics were performed by comparing

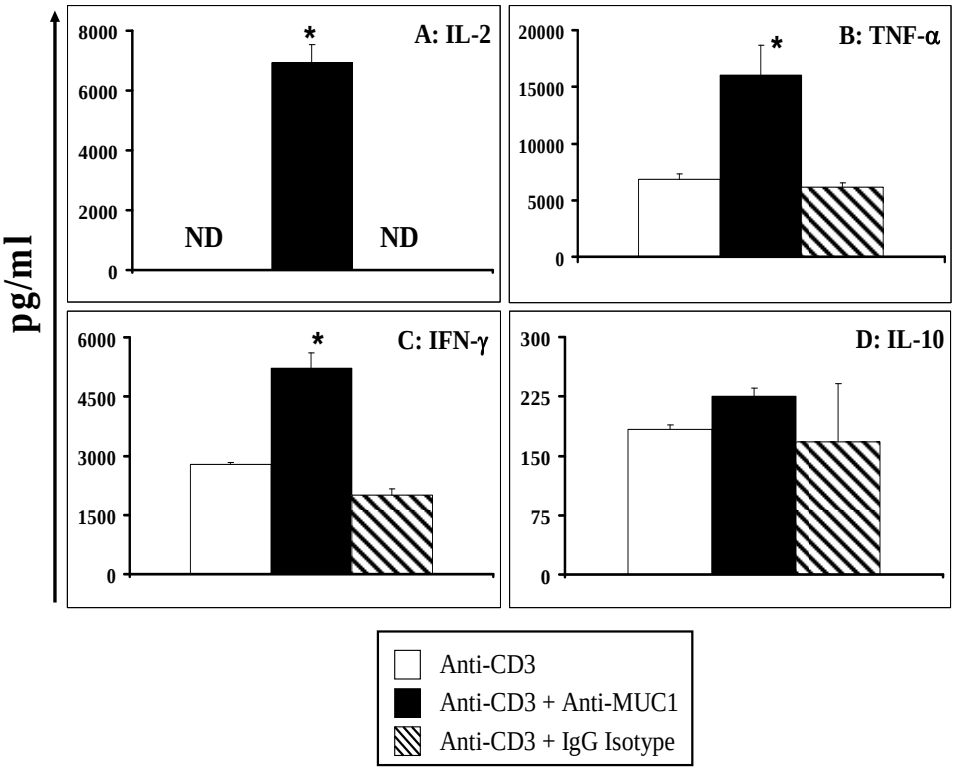
the values to a 0% change between the compared groups. Results are the average between three individual experiments on three different donors.

Figure 2-4: MUC1-mediated costimulation requires CD3 and MUC1 coligation



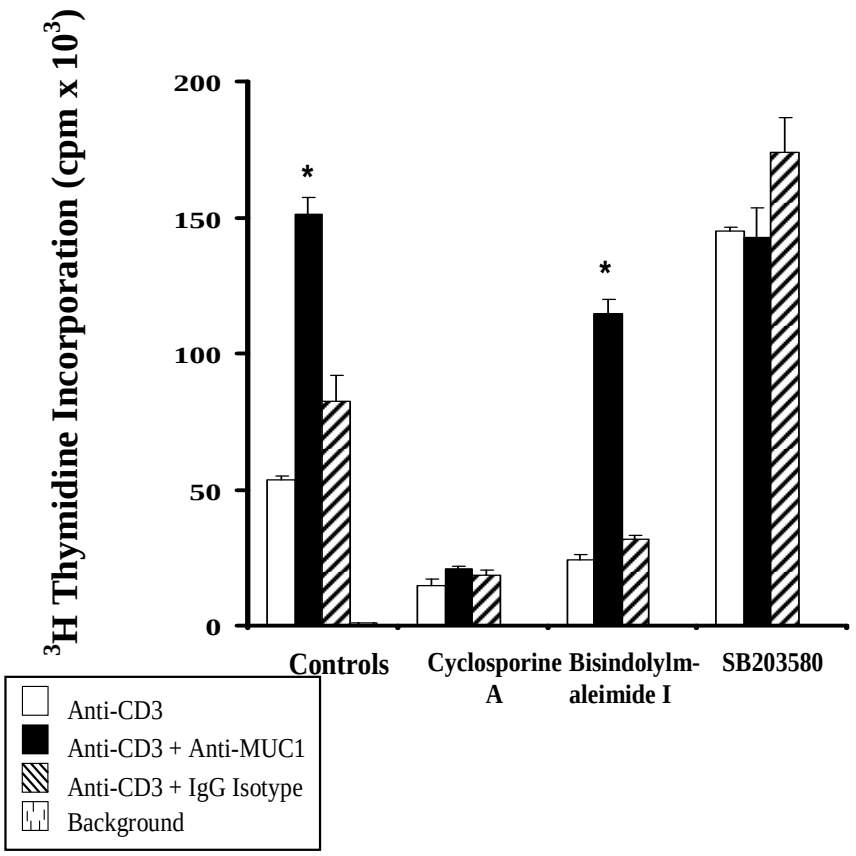
3-day PHA-stimulated T cells were treated with microspheres ligated separately with anti-CD3 and either anti-MUC1 or isotype or, instead, coligated with anti-CD3 and either anti-MUC1 or isotype. Microspheres were added to a final amount of $2x10^8$ per $2x10^5$ cells; for beads with separate antibodies bound, $1x10^8$ beads of both types were added to culture to achieve the final amount of $2x10^8$. The coligated anti-CD3 plus anti-MUC1-treated group had a statistically significant increase in proliferation over the other groups ($p<0.05$). Data is one experiment representative of two separate experiments on two different donors.

Figure 2-5: MUC1-based costimulation increases the release of TNF-α, IFN-γ and IL-2, but not the inhibitory cytokine IL-10



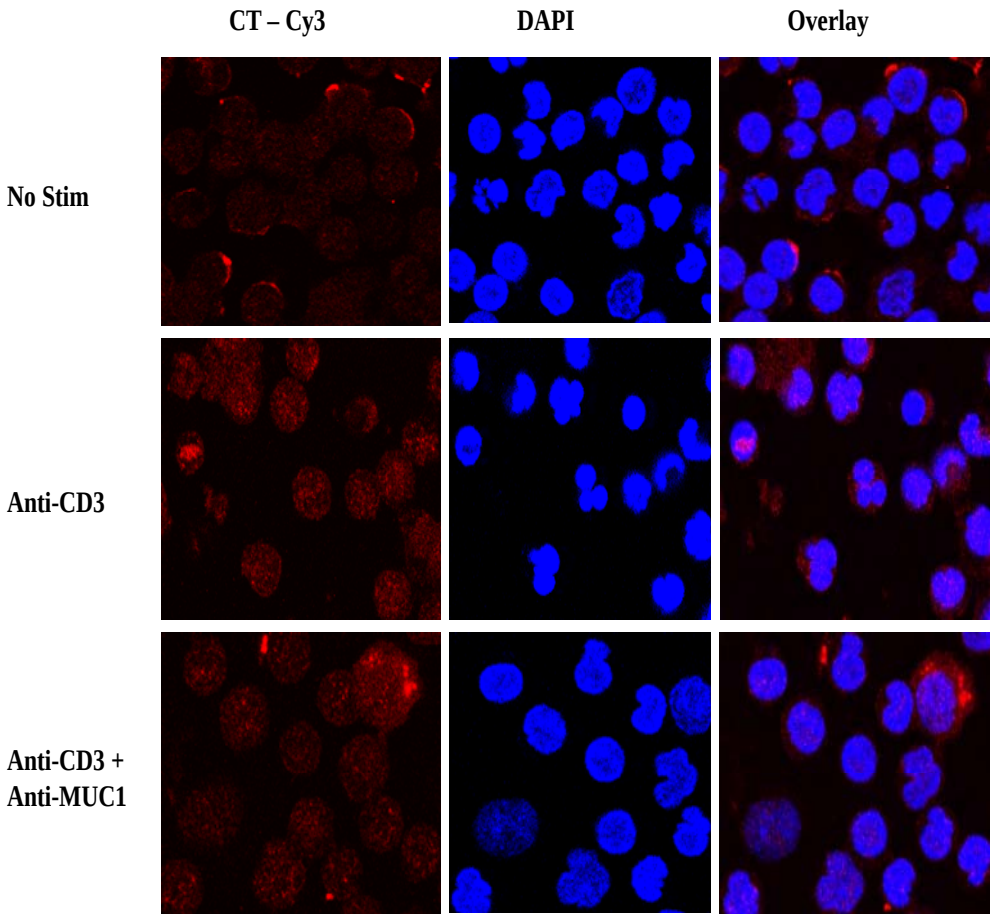
Supernatants taken at three days from Fig. 2-2*A* and similar experiments were analyzed via ELISA for the cytokines: *A*, IL-2, *B*, TNF-α, *C*, IFN-γ and *D*, IL-10. The anti-CD3 plus anti-MUC1 antibody treatment produced statistically higher amounts of the proliferation-inducing cytokine IL-2 as well as the pro-inflammatory cytokines TNF-α and IFN-γ ($p<0.05$). Amounts of the inhibitory cytokine IL-10, however, were not statistically significant amongst the groups ($p>0.05$). All cytokine amounts are in pg/ml. IL-2 data is representative of two experiments performed on two different donors while the other cytokines are representative of three experiments performed on three different donors. ND stands for 'not detectable'.

Figure 2-6: MUC1 costimulation is unaffected by a specific inhibitor of the NF-kB pathway, but not the NF-AT or p38 MAPK pathways



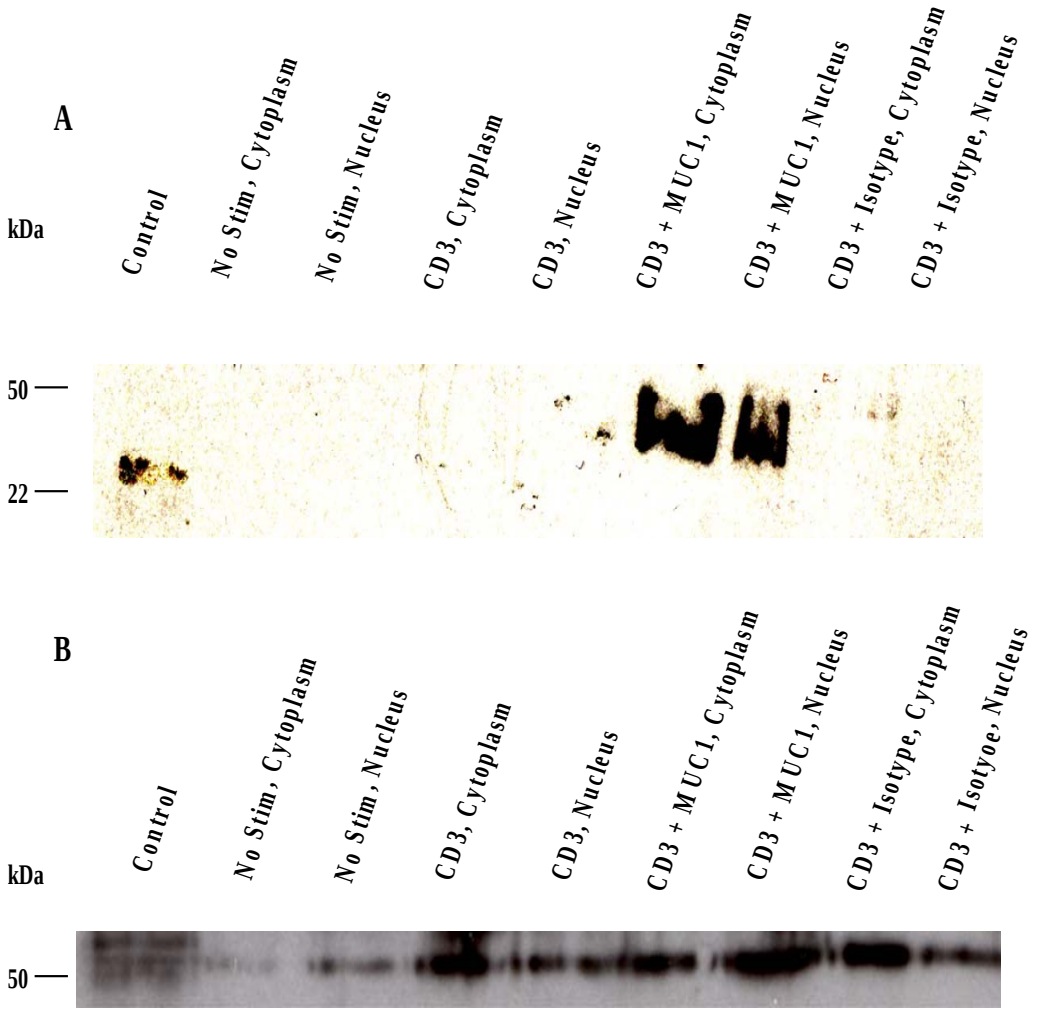
T cells were stimulated with PHA for 3 days before being treated with intracellular inhibitors along with 20μg/ml anti-CD3 (white bars), 20μg/ml anti-CD3 and 20μg/ml anti-MUC1 (black bars), 20μg/ml anti-CD3 and 20μg/ml isotype (hatched bars), and untreated (dashed bar). Each group was also given 1μg/ml of goat anti-mouse antibody to cross-link. There was no statistically significant difference between groups in the data for the intracellular inhibitors Cyclosporine A or SB203580. However, there was a statistically significant increase in proliferation in the anti-CD3 plus anti-MUC1-treated group given the PKC_θ inhibitor Bisindolylmaleimide I ($p<0.05$), compared to the inhibitor-treated control groups. Data is representative of four separate experiments on four different donors.

Figure 2-7: The cytoplasmic tail of MUC1 migrates into the nucleus upon CD3 stimulation in T cells



T cells stimulated with PHA for 3 days were given either no treatment, 20μg/ml anti-CD3, or 20μg/ml anti-CD3, 10μg/ml anti-MUC1 and 1μg/ml goat anti-mouse IgG antibody for 30 minutes before being stained against the cytoplasmic tail of MUC1 (Cy3, in red) and the nucleus (DAPI, in blue). Images are shown in single colors and overlaid, either as cells with no stimulation, with only anti-CD3 stimulation or with anti-CD3 and anti-MUC1 stimulation. Pictures are representative of three separate experiments on three individual donors. Approximately 300 cells were analyzed in each group and pictures are representative of those observations.

Figure 2-8: The cytoplasmic tail of MUC1 binds to the transcription factors c-Jun and c-Fos



Western blots using anti-MUC1 cytoplasmic tail (CT2) to precipitate and *A*, anti-c-Jun or *B*, anti-c-Fos to blot. For c-Jun, bands of the appropriate molecular weight (~39 kDa) appeared for the positive control (pure cellular lysate run without a precipitating antibody) and both of the anti-CD3 plus anti-MUC1 treatment's cytoplasmic and nuclear fractions. For c-Fos, bands of the appropriate molecular weight (~62 kDa) appeared for the positive control (pure cellular lysate run without a precipitating antibody) and all treatment groups in both the cytoplasmic extracts and the

nuclear extracts. In the untreated (non-stimulated) group, however, only a small amount of c-Fos was detected bound to MUC1 compared to the other groups.

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CHAPTER-3

MUC1 can act as a coinhibitory molecule of T cells in the absence of accessory cells

3.1 INTRODUCTION

Regulation of the immune response by the inhibition of T cells is an important and necessary facet of the immune system. Without inhibitory molecules, T cell responses to their antigens become uncontrolled, resulting in constitutive proliferation and the death of the organism (1). The ability to inhibit the immune response, therefore, is paramount in every individual and is achieved through multiple methods.

One of the most commonly studied mediators of T cell inhibition is CTLA-4. CTLA-4 is expressed on activated T cells in order to deactivate them after the pathogen of interest has been cleared. By outcompeting the coexpressed costimulatory molecules, CTLA-4 prevents the costimulatory response, leading to T cell anergy (2). Several other molecules perform similar functions, all with the same result.

Recently, a cell type has been defined and studied which is responsible for the crux of T cell regulation in the periphery. These regulatory T cells (T_{regs}) express regulatory proteins and secrete T cell-inhibiting cytokines such as IL-10 and TGF-β (3). These cells have been implicated in a variety of necessary roles, as their absence has been noted in disorders such as multiple sclerosis (4) and type 1 autoimmune diabetes (5), amongst many others, showing the necessity of T_{regs} in human T cell immunoregulation.

Mucin-1 (MUC1) is a member of the mucin family and is generally identified as both a protective surface protein of epithelial cells and an

oncoprotein in epithelial-derived carcinomas (6). In these carcinoma cells, several tumorigenic signaling proteins have recently been found to bind to its cytoplasmic domain (7) as well as its extracellular domain showing a potential role in tumor cell metastasis (8). A MUC1 isoform has also been found to cause T cell inhibition when secreted, suggesting an immunoregulatory role for MUC1 (9).

MUC1 has recently been found to not only be expressed on T cells, but also to play an inhibitory role by downregulating the proliferative response when crosslinked with CD3 (10). More recently, MUC1 has been discovered to have costimulatory capabilities on T cells, enhancing the proliferative response of T cells to CD3 stimulation in a NF-AT-dependent manner (Chapter 2). These contradictory observations on the role of MUC1 as a costimulatory and a coinhibitory molecule are supported by the observation that both a putative immunotyrosine activation motif (ITAM) and immunotyrosine inhibitory motif (ITIM) are present on MUC1's cytoplasmic tail (11). However, the conditions or the environment that determine which response occurs have yet to be elucidated. In addition, the role MUC1 plays as a whole in regulating T cells needs to be investigated.

First, we investigated the conditions under which MUC1 can act as a costimulatory or coinhibitory molecule of T cells. From there, we determined whether MUC1-induced T cell inhibition results in apoptosis or whether the observed 'inhibition' is the result of an entirely different mechanism. Next, we investigated MUC1's expression and influence on the T_{reg} subset of T cells (12),

in order to determine if MUC1 could be playing a role in their function as a novel immunoregulatory protein of T cells.

3.2 MATERIALS AND METHODS

3.2.1 Isolation of Non-Adherent Cells (NACs) from human blood

Peripheral blood samples were obtained from individuals of both sexes 30-60 years of age with informed consent. Use of human blood samples was approved by the institutional Health Research Ethics Board (HREB) at the University of Alberta, Canada. The blood was gently layered overtop of Lymphocyte Separation Medium (Cellgro, Herndon, VA) and was centrifuged at 1500-2000 g for 30 minutes. The intermediate buffy layer containing the PBMCs was removed and washed twice in PBS and resuspended in RPMI 1640 medium supplemented with 0.2% penicillin-streptomycin, 0.2% sodium pyruvate (Invitrogen, Carlsbad, CA) and 1% human AB serum (Sigma, St. Louis, MO). Cells were plated into 6-well plates at $3x10^7$cells/well and placed in an incubator for two hours at 37^{o}C with 5% CO_2 and 95% air (hereby just '37 ^{o}C'). Adherent monocytes and macrophages would adhere to the bottom of the plate while the non-adherent T-, B- and NK cells (termed NACs or unpurified T cells) would not. The unpurified T cells were collected and resuspended in AIM-V medium (Invitrogen, Carlsbad, CA). Unpurified T cells requiring activation were stimulated with 1μg/ml of PHA (Sigma, St. Louis, MO).

3.2.2 Nylon wool enrichment of T cells

Ten millilitre syringes were filled with 0.8-1g of nylon wool (Polysciences Inc., Warrington, PA) and autoclaved for sterility. The columns were then washed with 10ml of supplemented RPMI 1640 medium (Hyclone, Logan, UT) with 2% fetal bovine serum, 1% sodium pyruvate and 1% Penicillin/Streptomycin (Sigma, St. Louis, MO), removing any trapped air. The columns were equilibrated at 37 ^{o}C for 45 minutes before removal. Unpurified T cells were washed twice with warm PBS and resuspended into supplemented RPMI medium at 1x10^{8} cells/ml. A maximum of 2x10^{8} cells were added per syringe and returned to the incubator at 37 ^{o}C for 45 minutes. After, supplemented RPMI medium was added to each syringe until 15ml of cell suspension had eluted from each. Cells were then counted and resuspended in AIM-V medium. These nylon wool-purified T cells are termed 'enriched T cells'. Enriched T cells, after analysis via FACS for CD3, consisted of >80% CD3^{+} cells (see Figure 4-2).

3.2.3 Proliferation assays

Nylon wool-purified T cells were washed twice in PBS and resuspended into AIM-V medium at a concentration of 4x10^{6}cells/ml. A 96-well plate was used for cell treatments, with 2x10^{5}cells/well, along with 3.25μg/ml of B27.29, an anti-MUC1 antibody (Biomira, Edmonton, AB) or 3.25μg/ml mouse IgG$_1$ isotype, 1μg/ml goat anti-mouse (Sigma, St. Louis, MO), 20μg/ml OKT3 (anti-CD3 antibody). Plates were incubated at 37 ^{o}C for three days, with the addition of 0.5 μCi/well [^{3}H]thymidine (Amersham, Piscataway, NJ) at the end of the third day.

The cells were harvested after 18 hours and read on a Microbeta Liquid Scintillation Counter (PerkinElmer, Waltham, MA).

3.2.4 MACS purification

T cells were purified according to the protocol provided using CD3 MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). After FACS analysis, these cells (henceforth known as 'purified T cells') consisted of >99% $CD3^+$ cells.

3.2.5 Reconstitution Assay

T cells were purified using CD3 MACS beads. The eluted $CD3^-$ accessory cells were resuspended into AIM-V medium and irradiated with 24 Greys (courtesy of the Cross Cancer Institute of Alberta, Alberta, Canada). Purified T cells were added at $2x10^5$ cells/well in triplicate with $4x10^5$, $2x10^5$, $4x10^4$ or $2x10^4$ irradiated accessory cells. The different purified T cell : irradiated accessory cell ratios were treated with 20μg/ml anti-CD3, 20μg/ml anti-CD3 and 1μg/ml goat anti-mouse IgG, 20μg/ml anti-CD3 and 20μg/ml anti-MUC1, 20μg/ml anti-CD3 and 20μg/ml anti-MUC1 and 1μg/ml goat anti-mouse IgG, 20μg/ml anti-CD3 and 20μg/ml mouse IgG isotype, or 20μg/ml anti-CD3 and 20μg/ml mouse IgG isotype and 1μg/ml goat anti-mouse. Plates were kept at 37 ^oC for three days, with the addition of 0.5 μCi/ml [^3H]thymidine. After 18 hours, the cells were harvested and read on a microbeta liquid scintillation counter.

3.2.6 Apoptosis assays

Three day PHA-stimulated, nylon wool-purified T cells were plated in 96 well plates at $100x10^3$, $50x10^3$, $25x10^3$, $12.5x10^3$, $6.25x10^3$, $3.13x10^3$ and $1.61x10^3$ cells per well before treatment with antibodies against CD3 (20μg/ml) alone, with anti-MUC1 (10μg/ml) or with IgG isotype (10μg/ml). A crosslinking goat anti-mouse IgG antibody was also added to the antibody-treated cultures (1μg/ml). Cells treated with Kanamycin (1μM) were used as positive control for the apoptosis assay. After 2 hours of treatment, the cells were removed and stained for Annexin V and 7-AAD according to the protocol provided with the Annexin V: PE Apoptosis Detection Kit I (BD Pharmingen, Mississauga, ON) and read immediately on FACSCanto (BD, Franklin Lakes, NJ**)** . From parallel plates, the cells were treated in the well according to the protocol provided with the CytotoxFluor Kit (Promega, Madison, WI) and read immediately on the Fluostar Optima Fluorimeter (BMG Labtech, Offenburg, Germany).

3.2.7 Flow cytometry

Unpurified T cells were stimulated with PHA for three days before being prepared for staining. Cells were separated into tubes at numbers between 800,000 and 1,000,000 per tube, resuspended into cold FACS buffer (PBS + 2% FBS) and kept at 4^oC for the remainder of the experiment. Cells were stained with fluorescent antibodies against CD4-QR, MUC1 indirectly labeled with AlexaFluor 647 (attached via AlexaFluor 647 Antibody Labeling Kit, Invitrogen,

Carlsbad, CA) or goat anti-mouse-FITC, and CD25-PE (all non-MUC1 antibodies from eBioscience, San Diego, CA) for thirty minutes before being spun down and washed twice in FACS buffer. Cells were then permeabilized using 0.1% saponin (Sigma, St. Louis, MO) in FACS buffer before being stained intracellularly using a Foxp3-APC antibody (eBioscience, San Diego, CA). Cells were next fixed in FACS fixative solution (PBS + 2% paraformaldehyde) and resuspended in FACS buffer before analysis on FACSCanto. Isotype control antibody was used for each fluorescent antibody and cells were gated to exclude 98% of the isotype control stained cells.

3.2.8 Statistical analyses

Statistics were performed using One-way ANOVA with Tukey's test for post-hoc analysis or independent sample T-test using SPSS 16.0 software (SPSS Inc., Chicago, IL). * represents a statistically-significant difference at the $p<0.05$ level to the closest appropriate control group. All error bars shown are indicative of standard error.

3.3 RESULTS

3.3.1 Nylon wool-purified T cells show an inhibition of proliferation when CD3 and MUC1 are crosslinked

The main difference between experiments performed previously (9, 10) and our own data was the use of T cells enriched through nylon wool columns rather than unpurified, non-adherent cells. Therefore, to resolve the issue of coinhibition vs. costimulation mediated by MUC1, unpurified T cells were isolated from fresh human blood and stimulated with the mitogen PHA for three days to induce MUC1 expression. The cells were then enriched via the nylon wool column as previously described (9) in order to remove the majority of monocytes, macrophages and B cells, leaving the T-, NK and NKT cells. FACS analysis of the cell populations for CD3 expression showed >60% $CD3^+$ cells in the unpurified culture (see Figure 4-1) and >80% $CD3^+$ cells after nylon wool enrichment (see Figure 4-2). After treatment with antibodies as described, it was found that the proliferation normally caused by CD3 stimulation was greatly inhibited at a statistically significant level ($p<0.05$) when anti- MUC1 antibody was added and only when the two antibodies were crosslinked (Fig. 3-1). Without crosslinking, proliferation was comparatively normal to the controls in the enriched T cell cultures. These results suggest that both CD3 and MUC1 must be stimulated in tandem and crosslinked for inhibition to occur and that the switch

between a stimulatory and inhibitory response may be determined by the number of antigen presenting cells (non-CD3 cells, hereby just 'APCs') present with the enriched T cells.

3.3.2 Addition of irradiated APCs leads to restoration of costimulation via MUC1 in purified T cells

In order to test out the effect of APCs on MUC1-mediated immunoregulation, we attempted to obtain T cells at a high (>99%) purity through MACS separation. The eluted $CD3^-$ cells (APCs) were irradiated so that the purified cultures could be reconstituted with a defined proportion of APCs. It was determined that, at a lower proportion of APCs (10 T cells : 1 APC and 5 T cells : 1 APC), there was no statistically significant difference between the experimental groups (Fig. 3-2). However, at higher ratios (1:1 and 1:2), the enhanced proliferative response normally observed in unpurified T cell cultures occurred at a statistically significant level ($p<0.05$ for each group). This supports the earlier hypothesis that CD3 and MUC1-based coinhibition or costimulation on T cells is dependent on the number of APCs present, as the addition of APCs increased the proliferation in the MUC1 costimulated group in a proportional manner.

3.3.3 Coinhibition of T cells by MUC1 crosslinking is not due to apoptosis

To determine whether the apparent 'inhibition' of T cell proliferation after MUC1/CD3 coligation in purified T cell culture was due to apoptotic death of the target cells, we stimulated the purified T cells with anti-CD3 and anti-MUC1, as well as the appropriate controls, for several hours before performing three different assays to determine apoptosis – Annexin V staining (for early cell death) (Fig. 3-3*A*), 7-AAD staining (for late cell death) (Fig. 3-3*B*) and a fluorimetric assay for general apoptosis (Fig. 3-3*C*). None of the three assays showed a significant difference in apoptosis with the MUC1/CD3 coligated group compared to the control isotype/CD3 group, while the positive control (Kanamycin-treated purified T cells) showed significant apoptosis in all studies performed. This suggests that MUC1/CD3 coligation does not result in apoptotic death and that the mechanism of inhibition is non-apoptotic, perhaps by regulating cytokine production and/or proliferation. This inference is supported by previous observation that exogenous IL-2 can reverse the coinhibition mediated by MUC1 (10).

3.3.4 MUC1 is expressed on the majority of T_{regs} ($CD4^+CD25^+Foxp3^+$) after CD3-based stimulation

Since earlier studies demonstrated that CD3 and MUC1 crosslinking can result in both an enhancement and inhibition of proliferation, it was speculated that MUC1 may play some role on T_{reg} cells, the primary peripheral regulatory class of cells. After PHA stimulation for three days to allow the T cells to express

an optimal amount of MUC1, the cells were then treated with an anti-CD3 antibody in order to mimic antigen dependent stimulation. We observed that without CD3 stimulation, up to 60% of the T_{reg} population (whose phenotype is considered as being $CD4^+CD25^+Foxp3^+$) express MUC1, depending on the donor. After CD3 stimulation, two out of the three experimental groups had 100% of the T_{reg} population positive for MUC1 expression, with the donor who had less still having a 60-fold increase in T_{reg} MUC1 expression (Fig. 3-4), with all three $CD4^+CD25^+Foxp3^+$ groups expressing MUC1 post-stimulation corresponding to between 200-400 cells out of a gated total of 5000-8000. Since, in a normal population of activated T cells, MUC1 expression is typically between 30-75% post-CD3 stimulus (our own observations), the majority of the T_{reg} population expressing MUC1 is unexpected. Like other immunoregulatory molecules such as CTLA-4, this suggests that MUC1 may have an immunoregulatory role on T_{regs}.

3.3.5 The percentage of T_{regs} ($CD4^+CD25^+Foxp3^+$) increases after CD3 and MUC1 costimulation

Since MUC1 has immunoregulatory functions and is expressed on almost all T_{reg} ($CD4^+CD25^+Foxp3^+$) cells after CD3 stimulation, we sought to determine whether CD3 and MUC1 costimulation would increase or decrease the number of T_{reg} cells present in culture. As before, cells were matured for three days with PHA to induce MUC1 expression and then treated with either no

antibody, anti-CD3, anti-CD3 and anti-MUC1 or anti-CD3 and isotype control antibodies. A crosslinking goat anti-mouse antibody was also added to these cultures. The results demonstrated that CD3 and MUC1 costimulation generates a larger number of T_{regs} over the control groups (two to four fold increase, depending on the donor) (Fig. 3-5). This suggests that, in an unpurified population of T cells, CD3 and MUC1 ligation leads to an enhancement of proliferation with an end result of a larger population of T_{regs}. One explanation for this observation is that the MUC1-mediated enhancement of proliferation leads to a higher production of T_{reg} cells in order to downregulate the immune response after the potential pathogen of interest has been dealt with, similar to how CTLA-4 becomes upregulated late in T cell life in order to prevent a constant state of immune activation (13).

3.4 DISCUSSION

MUC1 has been shown previously to have both positive and negative immunoregulatory effects on T cells, but the conditions that determine which result occurs have not been discerned. This study was designed to investigate what these conditions are, as well as whether MUC1 plays any role with the T_{reg} subset, the primary regulatory immune cell type in humans. By using nylon wool columns to partially deplete the T cell population of accessory cells, it was determined that CD3/MUC1 crosslinking inhibits the proliferation seen in the control groups, compared to an enhancement of proliferation in the same conditions using unpurified T cells (Chapter 2). By reconstituting MACS-purified T cells with irradiated accessory cells, we showed that the enhancement of proliferation increases as the number of accessory cells re-added to the culture increases. It was also discovered that, after CD3 stimulation, the vast majority of T_{regs} ($CD4^+CD25^+Foxp3^+$) express MUC1 and, if T cells receive both a CD3 and MUC1 stimulus, an increase in the percentage of T_{regs} results over the controls.

The fact that MUC1's costimulatory/coinhibitory capabilities appear to be dependent on the presence of accessory cells suggests that more molecules than just CD3/MUC1 are required for MUC1-mediated costimulation. When T cells are stimulated with an anti-CD3 antibody in the total absence of accessory cells, T cell anergy and/or apoptosis will occur, as a secondary costimulation between T cells and accessory cells, the interaction of CD28 and CD80/86, is required (14). With even a small number of accessory cells, such as after nylon wool enrichment

of T cell culture, an anti-CD3 antibody addition will generally have sufficient additional costimulation with it to result in a proliferative response from the T cells. However, when MUC1 is cross-linked along with CD3 stimulation, this proliferation is significantly reduced. When a larger number of accessory cells are reconstituted in a more stringently purified culture of T cells, enhancement of proliferation occurs instead. This suggests that MUC1, in the absence of a large amount of costimulation from accessory cells, will downregulate the proliferative response. The reason for this could be that crosslinking MUC1 on T cells results in inhibiting T cell proliferation when the cell both recognizes its antigen expressed on the appropriate MHC class and receives a small but sufficient amount of costimulation. Further studies will have to be performed on the inhibited T cells to determine if they are truly anergic or just inhibited with the capacity to still respond to stimulation. The fact that MUC1 crosslinking results in costimulation when a sufficient number of accessory cells are present suggests that there is a coordinated interaction with the signals produced by CD3/accessory cells and MUC1, some of which have been elucidated previously (Chapter 2). The specific interactions leading to coordinated costimulation should be investigated in future studies.

In humans, the most common set of markers used to define the T regulatory subset of T cells is $CD4^+/CD25^+/Foxp3^+$, based on previous studies in mice (12). However, there remains much controversy as to whether or not these are bona-fide markers in humans, with many studies showing conflicting results regarding the regulatory aspects of these cells (15, 16). Regardless, these three

markers remain the most widely-used T_{reg} markers for humans. MUC1, as we have shown, is expressed on almost all T_{reg} cells given a CD3-based stimulus, supporting a potential role for MUC1- mediated immunoregulation. However, when stimulated along with CD3 in unpurified T cell cultures, an enhancement in proliferation results (Chapter 2) and an increase in the T_{reg} population occurs with it. It is possible that MUC1 acts to enhance the proliferation of T_{regs} so that they may downregulate the immune response post-infection. However, another explanation lies in the fact that MUC1, CD25 and Foxp3 all become upregulated after either mitogen or CD3 stimulation (17, 18, 19). It may not be that the $CD4^+/CD25^+/Foxp3^+$ cells MUC1 is expressed on are T_{regs} but, instead, are mature T cells that are expressing these molecules as activation markers. Further studies will have to be performed on the regulatory function of these MUC1-expressing 'T_{regs}' to determine which of these explanations is correct.

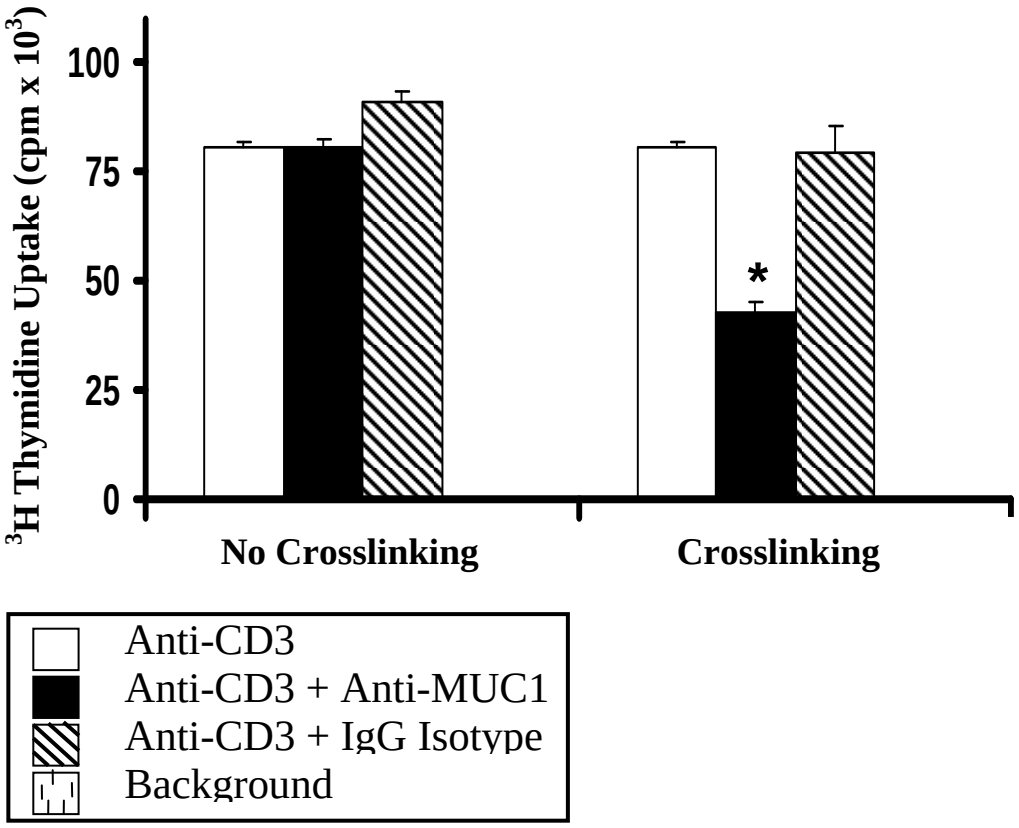
The identity of the pathway and factors that contribute to MUC1's coinhibitory capabilities are still unknown. When tested for the induction of apoptosis, MUC1 coligation with CD3 was found to not increase apoptosis in the inhibited cell population. With previous research showing that MUC1 costimulation functions in an NF-AT dependent manner (Chapter 2), it is possible instead that a lack of accessory cells prevents the activation of this pathway, potentially through preventing the binding of c-Jun to the cytoplasmic tail of MUC1. It is also possible that a lack of accessory cells prevents nuclear migration of the cytoplasmic tail, sequestering the AP-1 complex at the

membrane, though it is unclear whether this would be sufficient enough to induce the inhibition seen.

In conclusion, MUC1 is a novel immunoregulatory protein of T cells and is able to provide a costimulatory or coinhibitory signal based on the T cell to accessory cell ratio present in the cellular milieu. MUC1 costimulation also produces a larger number of putative T_{reg} cells ($CD4^+/CD25^+/ Foxp3^+$), with the majority of T_{regs} being $MUC1^+$ post-stimulus, supporting its role as a regulator of T cells. By knowing how MUC1 functions in T cell immunoregulation, we can potentially develop treatments against both diseases of an over-stimulated immune system (such as in autoimmune disorders) and diseases of immune inhibition (such as in many tumor microenvironments). The ability to manipulate MUC1 costimulation/coinhibition of T cells could be an invaluable tool in counter-acting these disorders.

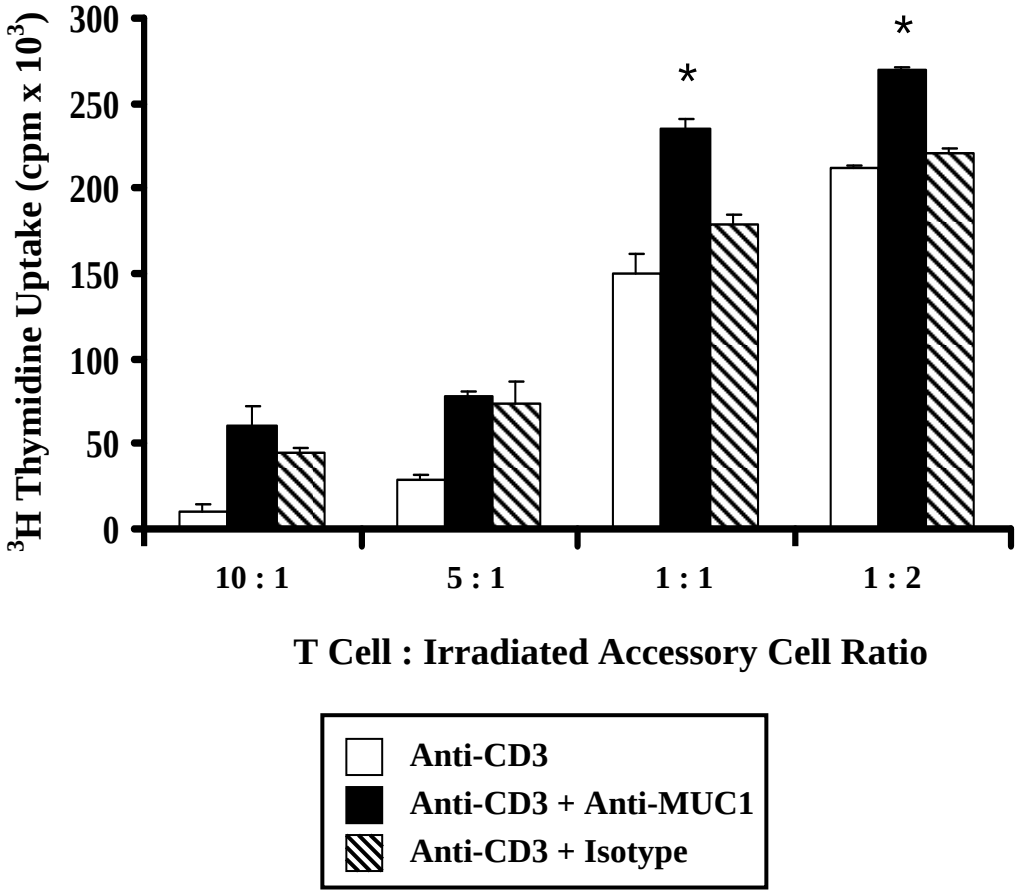
3.5: FIGURES

Figure 3-1: Nylon wool-purified T cells show an inhibition of proliferation when CD3 and MUC1 are crosslinked



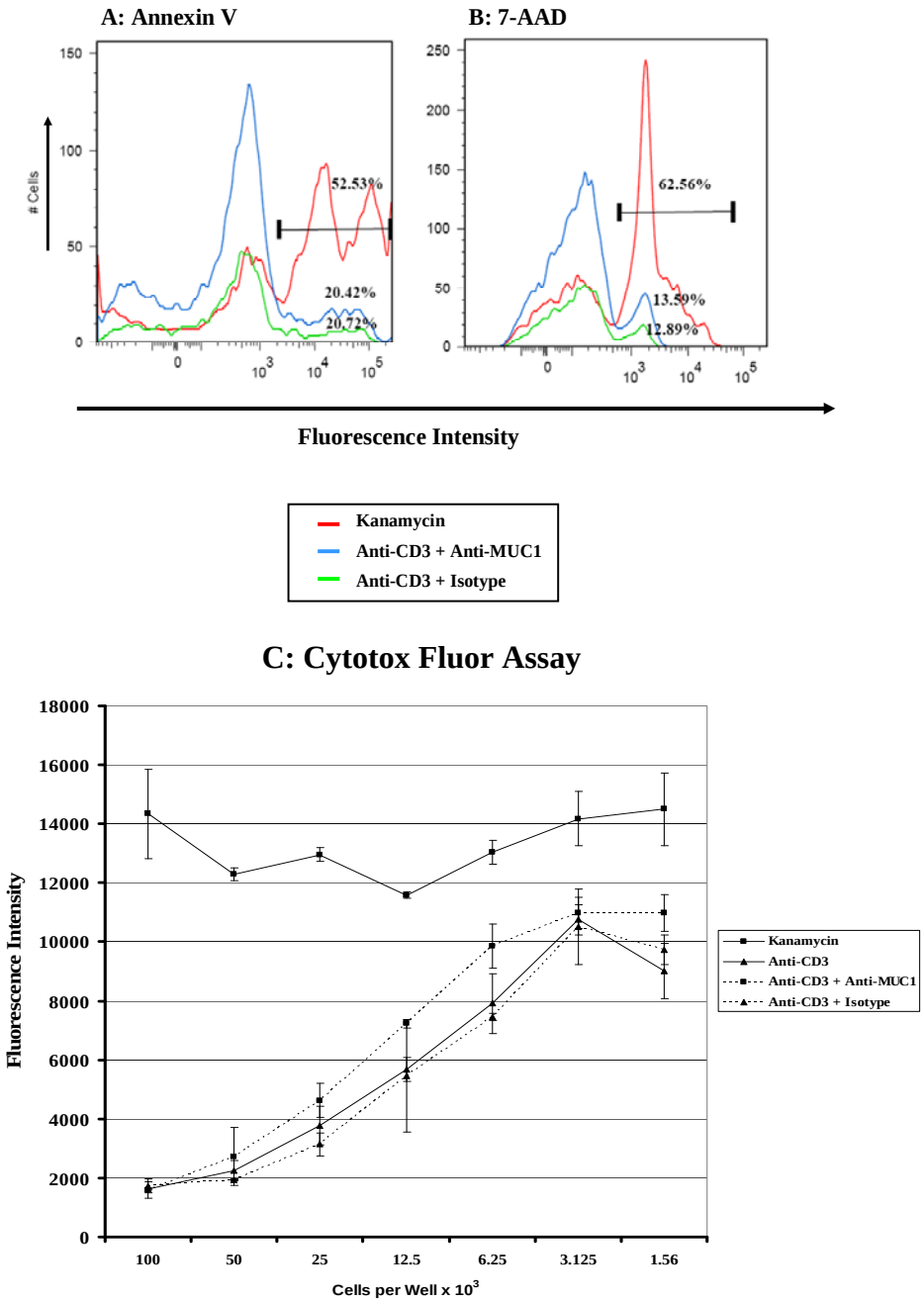
Non-adherent cells from whole human blood were stimulated with PHA for three days before nylon wool purification. Cells were then treated with anti-CD3 (white bars), anti-CD3 and anti-MUC1 (black bars) or anti-CD3 and isotype (hatched bars). The purified cells were also treated with or without a crosslinking antibody. Data is representative of two separate experiments on two different donors.

Figure 3-2: Addition of irradiated APCs leads to restoration of costimulation via MUC1 in purified T cells



Non-adherent cells from whole human blood were stimulated with PHA for three days before MACS separation by CD3-bound magnetic beads to obtain a population of $CD3^+$ T cells and $CD3^-$ accessory cells. The accessory cells were irradiated and reconstituted into culture with the $CD3^+$ T cells at ratios of 10:1, 5:1, 1:1 and 1:2 $CD3^+$ T cells to CD3-irradiated accessory cells. Cells were then treated with anti-CD3 (white bars), anti-CD3 and anti-MUC1 (black bars) or anti-CD3 and isotype (hatched bars). All groups were also treated with a crosslinking antibody. Data is representative of four different experiments on two different donors.

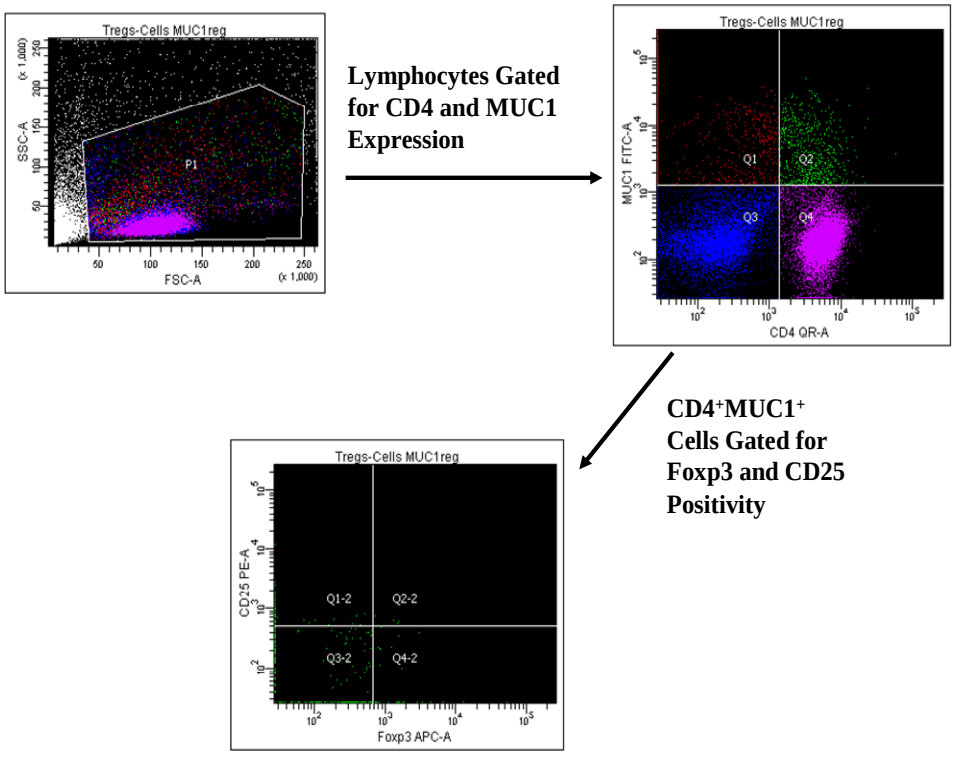
Figure 3-3: Coinhibition of T cells by MUC1 crosslinking is not due to apoptosis

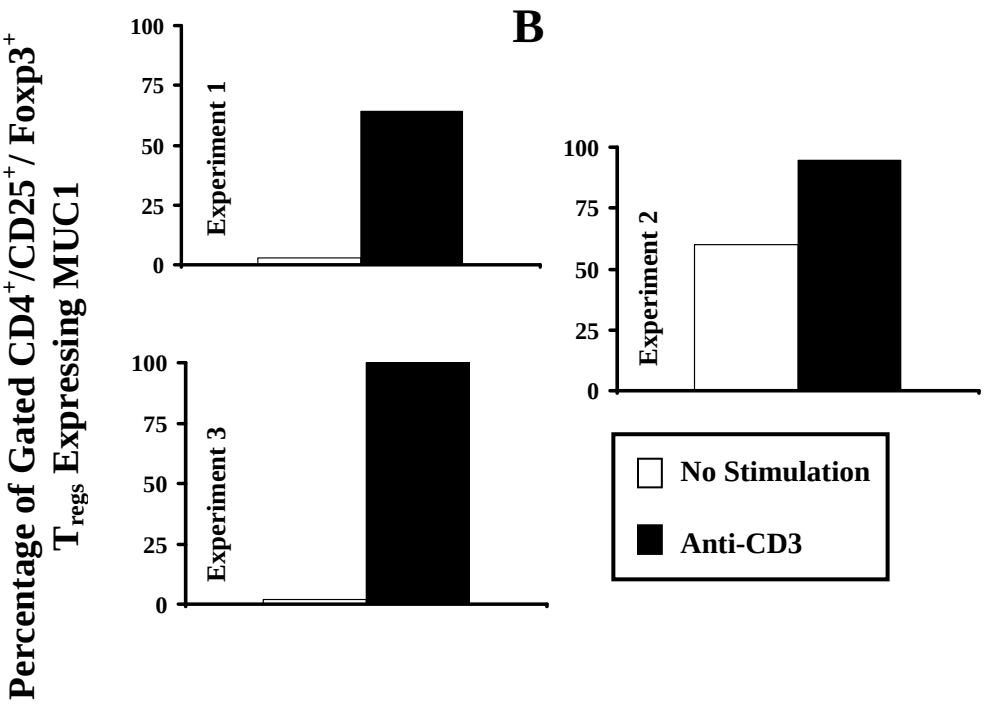


Three day PHA-stimulated nylon wool-purified T cells were treated with Kanamycin, anti-CD3, anti-CD3 and anti-MUC1 or anti-CD3 and isotype control along with a crosslinking antibody for

four hours before either: *A.* Staining for Annexin-V *B.* Staining for 7-AAD *C.* Treatment with the Cytotox Fluor detection kit for apoptosis. In *A* and *B*, the gated population is the total lymphocyte population. Data for *A* and *B* are representative of three separate experiments on three different donors. Data for *C* is representative of a single experiment on a single donor.

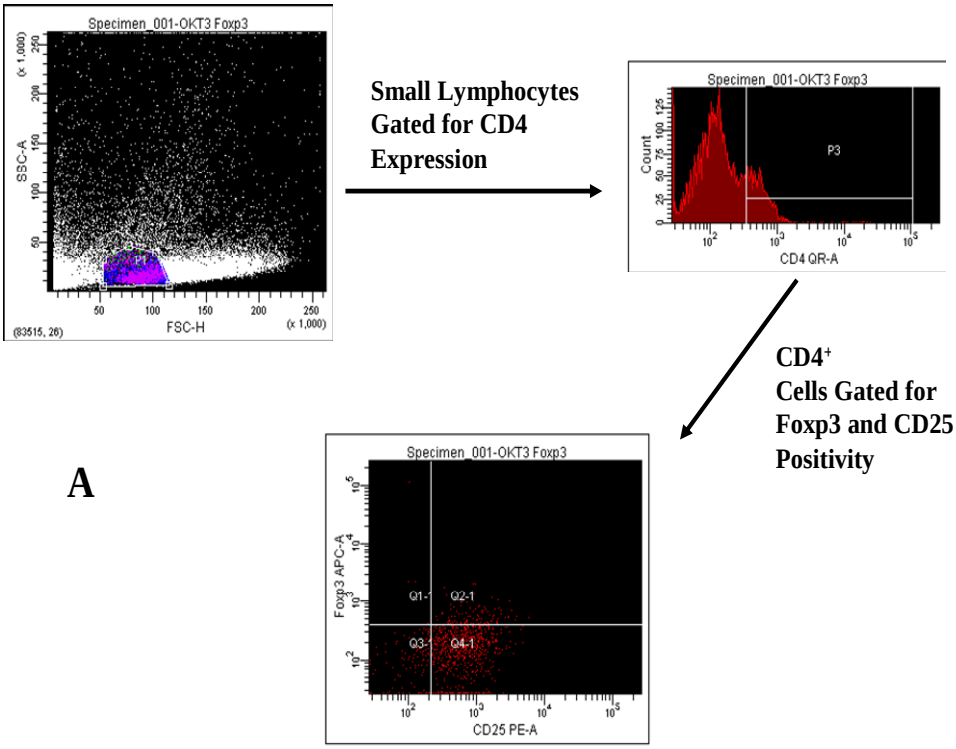
Figure 3-4: MUC1 is expressed on the majority of T_{regs} ($CD4^+CD25^+Foxp3^+$) CD3-based stimulation



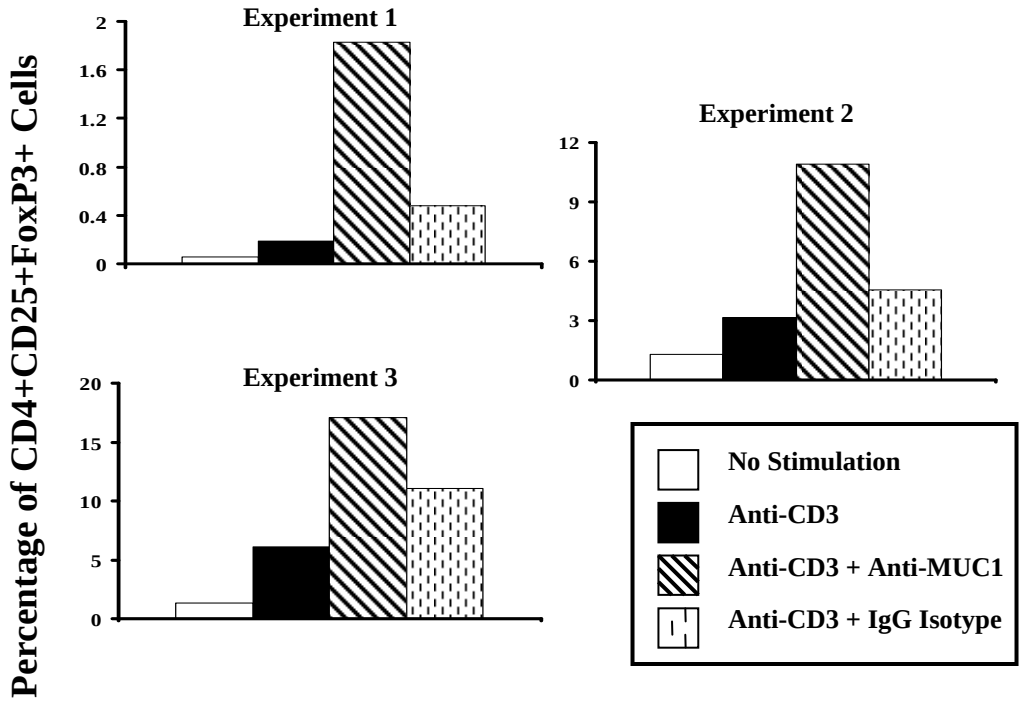


Non-adherent cells from whole human blood were stimulated with PHA for three days. Cells were then either treated with no antibody or anti-CD3. After three days, the cells were stained with antibodies against CD4, CD25, Foxp3 and MUC1 and gated as shown in *A*. *B* Data is given as a percentage of the gated small lymphocytes expressing CD4, CD25, Foxp3 and MUC1, comparing non-stimulated T cells (white) and CD3-stimulated T cells (black). Specifically, Experiment 1 corresponds to 0.10% and 0.24% of the gated lymphocyte population consisting of T_{regs} for the unstimulated and CD3-stimulated groups, respectively; 0.08% and 0.03% in Experiment 2; 0.10% and 2.62% in Experiment 3. Data consists of three separate experiments on three different donors.

Figure 3-5: The percentage of T_{regs} ($CD4^+CD25^+Foxp3^+$) increases after CD3 and MUC1 costimulation



A



Non-adherent cells from whole human blood were stimulated with PHA for three days. After three days of antibody treatment, the cells were stained with antibodies against CD4, CD25 and Foxp3 and analyzed via FACS analysis as shown in *A*. *B* the percentages of T_{regs} ($CD4^+CD25^+Foxp3^+$ cells) in the gated small lymphocyte population were compared based on their treatment with: no antibody (white), with anti-CD3 (black), with anti-CD3 and anti-MUC1 (hatched) or anti-CD3 and isotype (dashed). All antibody-treated groups were also given a crosslinking antibody. Data consists of three separate experiments on three different donors.

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CHAPTER-4

Discussion

In my studies, I have focused on T cell immunoregulation by the mucin protein, MUC1. Earlier studies have shown MUC1 to be involved in T cell inhibition; I have demonstrated an alternate, opposing function, its pathway of action and the conditions that determine which function occurs. These observations provide evidence towards MUC1 being a novel immune regulator of T cells capable of delivering both costimulatory and coinhibitory signals, and divulge the ways that T cell responses are controlled. In the following sections, I have provided an overall discussion of my results and how they affect our understanding of T cell immune regulation.

4.1 MUC1 CAN FUNCTION AS A COSTIMULATORY PROTEIN OF T CELLS

MUC1, a protective protein of epithelial cells (1, 2), is expressed on activated T cells (3). Previous studies provided a role for MUC1 as a potential coinhibitory protein of T cells, showing that MUC1/CD3 coligation with antibodies and cross-linking partially inhibited the proliferation normally observed with CD3 ligation alone (4, 5). However, upon performing our own studies, we instead observed an enhancement of proliferation rather than the previously observed inhibition. Given this new result, we investigated MUC1 in the context of a costimulatory protein on T cells. In this manner, we discovered that MUC1 is required to be within close proximity to CD3 in order for the costimulatory enhancement of proliferation to take place and that MUC1/CD3

costimulation causes the production of large, significant amounts of IFN-γ, TNF-α and IL-2, all pro-inflammatory and proliferation-inducing cytokines. This data showed that MUC1 can act on T cells in a costimulatory manner, enhancing proliferation through an increased production of cytokines and requiring close proximity to a CD3 stimulus, in a manner similar to other costimulatory molecules.

4.2 MUC1 COSTIMULATION UTILIZES THE NF-AT PATHWAY

T cells utilize a variety of overlapping intracellular signaling pathways in order to become activated and proliferative upon antigen recognition. The most well-known, well-studied and important pathways in T cells are the NF-kB and NF-AT pathways. NF-AT, the first pathway activated after antigen recognition, is primarily responsible for the production of pro-inflammatory cytokines (6). NF-kB, activated after the NF-AT pathway, is responsible for the production of the proliferation-inducing cytokine IL-2 (7). Other important pathways also exist, including several members of the broad MAPK category of cell signaling pathways. Several of these pathways are responsible for the induction of the NF-AT and NF-kB pathways (8), while some, such as the p38 MAPK pathway, have more independent functions. The p38 MAPK pathway is primarily responsible for the production of the proliferation-inhibiting cytokine IL-10 as a method of downregulating the T cell response after the elimination of the pathogen insult (9). By using inhibitors towards these primary pathways, we discovered that

CD3/MUC1 costimulation is able to reverse the inhibition of the NF-kB pathway while having no effect on the NF-AT or p38 MAPK pathways. With this evidence, combined with the cytokine data, we determined that the NF-AT pathway was largely responsible. By Western blotting for several transcription factors involved in NF-AT signaling, we discovered that c-Fos constitutively bound to MUC1's cytoplasmic tail while c-Jun bound only after CD3/MUC1 costimulation. By confocal microscopy, we also found that the cytoplasmic tail of MUC1 migrates into the nucleus of the T cell after CD3 stimulation, regardless of whether MUC1 is stimulated in conjunction. This all suggests a novel pathway of T cell costimulation by MUC1 where MUC1 is able to bind to both of the AP-1 transcription factors, c-Fos and c-Jun, after MUC1 stimulation and migrate into the nucleus with them after CD3 stimulation, presumably increasing their intranuclear concentration to a level higher than just CD3 stimulation alone, leading to the increase in proliferation and pro-inflammatory cytokine production.

4.3 MUC1 IMMUNOREGULATION OF T CELLS IS DEPENDENT ON THE NUMBER OF ACCESSORY CELLS PRESENT

With MUC1 defined as a costimulatory protein of T cells, we investigated the difference between our data which showed costimulation and the previous data by Agrawal et al. (4) which showed apparent coinhibition. The main difference discovered was the purity of T cells – we used unpurified, non-adherent cells consisting of ~60% $CD3^+$ cells (see Figure 4-1) while previous studies (4, 5)

used nylon wool-purified peripheral blood leukocytes consisting of >80% $CD3^+$ cells (see Figure 4-2). Thus, by repeating the proliferation assay with nylon wool-purified T cells instead of unpurified non-adherent cells, the results were similar to those observed previously (4) – T cell proliferation was partially inhibited by MUC1 coligation. We then isolated $CD3^+$ T cells and reconstituted them with irradiated $CD3^-$ cells, observing that MUC1 costimulation reappeared after reconstitution with higher numbers of $CD3^-$ accessory cells when compared with both control groups. This data shows that MUC1 costimulation of T cells is dependent on the number of $CD3^-$ accessory cells present, as a higher number of accessory cells leads to an enhancement of proliferation. The possible accessory cells include monocytes, macrophages, B cells, NK cells and NKT cells, as all granulocytes are eliminated in the cell separation process (10).

4.4 MUC1 COSTIMULATION ON T CELLS LEADS TO AN INCREASE IN SPECIFIC T CELL SUBSETS

T cell subsets fall into four main categories: effector cells, activated cells which carry out their effector functions (stimulating cells or killing target cells); memory cells which remain long after the initial infectious insult in order to maintain an immunologic memory; memory/effector cells which are memory cells capable of quickly becoming effectors; naïve cells, which are cells that have yet to be exposed to their antigen and are non-activated and non-proliferative (11, 12). Both 'helper' $CD4^+$ T cells and 'cytotoxic' $CD8^+$ T cells can be identified by

these subsets, with specific markers for the correct identification of each type. A 'regulatory' subset of cells also exists, which is commonly considered to be a lineage of T cells that are $CD4^+/CD25^+/Foxp3^+$ (13), termed 'T_{regs}'. Since MUC1 appears to be a novel immunoregulatory protein on T cells, it was speculated that T_{reg} cell type may express MUC1 more preferentially. Thus, through the use of flow cytometry, we sought to determine which subsets, if any, MUC1 was preferentially expressed on and/or was able to stimulate more than other types. After stimulation with the mitogen PHA, we discovered that MUC1 expression increases on all cell types, with $CD4^+$ T cells of all subsets having the largest upregulation of expression. With CD3/MUC1 costimulation, $CD4^+$ memory and $CD8^+$ memory and memory/effector cells all increasing in percentages over their control counterparts, it suggested that MUC1 may be more involved in the activation/maturation process which develops T cells into the matured memory subset. Finally, MUC1 was found to be expressed on the majority (>95%) of T_{reg} cells after CD3 stimulation, further suggesting a regulatory role. However, after CD3/MUC1 costimulation, the percentage of T_{reg} cells increased substantially over the controls. It is possible that, since CD25 and Foxp3 are activation markers of T cells and MUC1 is expressed after activation as well (14, 15), that these are activated T cells rather than true T_{regs}. However, it is also possible that MUC1 costimulation is able to expand the T_{reg} subset, allowing for more significant regulation of T cell activation and proliferation later on in the cellular response. This data clearly shows that MUC1 is expressed on a variety of T cell

subsets, with a preference for CD4 subsets, and is able to expand primarily cells of the memory phenotype as well as putative T_{reg} cells.

4.5 MUC1 COINHIBITION IS NOT A RESULT OF T CELL APOPTOSIS

After observing coinhibition by MUC1 in purified T cell populations, we attempted to relate our costimulatory results to our coinhibitory results. Knowing that c-Jun/c-Fos AP-1 dimers bind to the cytoplasmic tail of MUC1 for costimulation, we theorized that AP-1 might also be involved in the coinhibitory response. With previous literature having shown c-Jun being involved in FasL upregulation and activation-induced cell death (AICD) of T cells (10), we sought to determine whether this inhibition was the result of T cell apoptosis or whether MUC1 inhibits T cells in a different manner. Through both FACS analysis of Annexin V and 7-AAD staining for both early (16) and late stage apoptosis (17), respectively, as well as a fluorescence-based general apoptosis assay, it was determined that, in purified T cell populations, neither showed statistically significant apoptosis in the CD3/MUC1 coligated groups. This suggests an alternate mechanism for MUC1-based coinhibition, potentially by modifying its effects on the NF-AT pathway observed in MUC1-based costimulation. This result is further supported by earlier observations that IL-2 is able to reverse the MUC1 mediated coinhibition of T cells.

4.6 FUTURE STUDIES

My studies have elucidated a number of key regulatory functions for MUC1. Several questions remain regarding MUC1's function both as a costimulatory and a coinhibitory protein of T cells. In both cases, questions arise regarding the role that accessory cells, namely monocytes and macrophages, play in the MUC1-based responses. Monocytes and macrophages, as previously discussed, express MUC1 regardless of activation status (18). Though our evidence clearly shows that MUC1 manipulates the NF-AT pathway during costimulation, it is possible that monocyte and macrophage stimulation via MUC1 also plays some sort of role, potentially regarding cytokine production. It is unclear whether the enhancement of AP-1 translocation in MUC1 costimulation would be sufficient to produce both the increase in cytokines and the enhancement of proliferation seen in our assays. Monocytes and macrophages, as either the primary source or as a secondary source, could be producing cytokines in sufficient quantities to enhance this response, as they have been known to produce both IFN-γ and TNF-α (19), potentially increasing the activation of the surrounding T cells (20). This would also provide an alternate explanation as to why accessory cell reconstitution generates an enhanced proliferative response in MUC1-stimulated groups. Though no response has ever been observed when MUC1 is stimulated on APCs (21), cytokine levels in the surrounding media will have to be measured in order to either confirm or rule out this possibility.

Though MUC1 costimulation was found to increase certain subsets of CD4 and CD8 cells, it is unclear why those cells increase as well as what their

function is after MUC1 costimulation. Functional studies will have to be performed, both in mixed lymphocyte culture and killing culture in order to determine if there is any difference in function. Likewise, MUC1-expressing T cells, as well as MUC1 non-expressing T cells, will have to be purified and tested in culture to see how each responds to MUC1/CD3 costimulation, though previous attempts through MACS-based assays have failed (our own observations). FACS-based cell sorting will be attempted next, gating on $MUC1^+$ cells of proper size, so that both $MUC1^+$ and $MUC1^-$ cultures can be obtained and experimented on. Also, we will have to test the functional capabilities of MUC1-expressing 'T_{reg}' cells to determine whether they are bona-fide regulatory T cells or whether they are activated cells expressing late-stage activation markers. Finally, a microarray or proteomic experiments could be performed comparing the $MUC1^+$ and $MUC1^-$ cells. All of these experiments will determine the degree of influence which MUC1 has on the functionality of T cells, including their activation, anergic induction, protein expression (costimulatory molecules, coinhibitory molecules, cytokine/chemokine receptors, Fas/FasL) as well as the cytokine secretion and proliferation observed.

Despite having discovered that MUC1 costimulation functions through the NF-AT pathway, we are still unsure as to the pathway that MUC1 coinhibition utilizes. Since eliminating the apoptosis pathway, despite the relation of AICD to c-Jun upregulation (10), an explanation that lies either within the ITIM domains or an alternate portion of the NF-AT pathway seems the most likely. However, in our own unpublished observations, we failed to find SHP-2 bound to the

cytoplasmic tail of MUC1 both in unpurified and nylon wool-purified T cell cultures (see Figure 4-4), suggesting that the NF-AT-based explanation is likely to be correct. Since CD3 stimulation, with a lack of proper costimulation, generally results in T cell anergy (22), it is here where we will look next. MUC1-expressing T cells will have to be purified and collected and then determined whether they can be reactivated with a proper CD3/CD28 stimulus as similar experiments with T_{reg}-inhibited T cells have shown (23). Further on, Western blots will have to be performed on the coinhibited, nylon wool-purified T cells in a similar manner to those of costimulated, unpurified T cells in order to determine if there is any change in c-Fos and/or c-Jun binding. It is possible that, during coinhibition, an event (dephosphorylation, conformational change) occurs which prevents either or both of c-Fos and c-Jun from binding. With a lack of AP-1 dimer entering the nucleus, the threshold for activation and IL-2 production may not be reached, leading to inhibition and, possibly, anergy. Confocal studies can also be performed to determine if there are any changes in localization of the cytoplasmic tail of MUC1 or either of the AP-1 transcription factors. Should MUC1 stay at the membrane, it is possible that MUC1 coinhibition could be functioning by sequestering AP-1 dimers, preventing their nuclear migration and reducing the activation potential of the T cells. Failing these, a different aspect of the NF-AT pathway may be involved. This includes calcium release, as calcium is required for the activation of calcineurin and, thereby, NF-AT (24). A multitude of other possibilities exist, given the broadness of the NF-AT pathway

and its overlap with others, though these explanations seem the most likely to occur in MUC1-mediated coinhibition.

Several other possible interpretations of our results exist which must be either accounted for taken care of experimentally. Perhaps the most important one is the role of antigen presenting cells and accessory (non-$CD3^+$) cells in the MUC1 costimulatory response. As previously mentioned, monocytes and dendritic cells express MUC1 (21, 25). Since these cell types would have been present in our numerous non-purified T cell assays, it is possible that MUC1 stimulation and crosslinking is affecting them in some unknown way, perhaps through cytokine production or upregulation/downregulation of costimulatory/coinhibitory molecules on their surface. The fact that removal of the accessory cells results in inhibition only strengthens this possibility.

Despite our discovery that c-Fos and c-Jun, the proteins that make up the AP-1 transcription factor dimer, bind to the cytoplasmic tail of MUC1, we were unable to find NF-ATc1 binding to the cytoplasmic tail as well (see Figure 4-3). Since we observed c-Fos and c-Jun bound to MUC1's cytoplasmic tail in the nucleus, AP-1 should have dimerized with a NF-AT transcription factor, as AP-1 readily binds to undimerized NF-AT (26). It is possible that the cytoplasmic tail of MUC1 may dissociate from c-Fos and c-Jun either before or during NF-AT binding, explaining why we were unable to observe NF-ATc1 in our Western blots. Another explanation is that NF-ATc1 is the incorrect NF-AT family member to test; NF-ATc2, along with NF-ATc1, is the predominant NF-AT transcription factor expressed in T cells (26). It is possible that, due to some

interaction with the cytoplasmic tail of MUC1, NF-ATc2 preferentially binds to the c-Fos and c-Jun that it carries. In the future, both of these possibilities will have to be tested.

Though the majority of T cells express MUC1 after mitogen stimulation (3), a small population does not. We had sought to determine the difference between these populations and whether the enhancement of proliferation is primarily in $MUC1^+$ T cells by isolating $MUC1^+$ T cells versus $CD3^+$ T cells and performing concurrent reconstitution assays. However, through use of the MACS bead system for isolation of $MUC1^+$ cells (using B27.29, the anti-MUC1 antibody, and goat anti-mouse IgG MACS beads post-nylon wool purification), we found that we would consistently obtain <5% viable $MUC1^+$ T cells. This led us to investigate whether MUC1 ligation and crosslinking is able to trigger apoptosis and/or necrosis in purified T cell populations, with little success (see Figure 4-5). Due to successful purification of $CD3^+$ T cells using the goat anti-mouse MACS beads and OKT3, the anti-CD3 antibody, we were unable to prove why $MUC1^+$ T cells showed such low viability. Future studies are planned using flow cytometry to sort out $MUC1^+$ T cells instead.

Unfortunately, in Figure 3-2, we were unable to show inhibition of T cell proliferation in the presence of a reduced number of irradiated accessory cells, definitively showing a gradient of T cell costimulation/coinhibition in the face of MUC1/CD3 coligation. One possibility explaining this is that the MACS bead system used to isolate the T cells, a CD3 positive selection kit, uses magnetic beads with the stimulatory antibody CD3. After isolation of these cells, the

magnetic beads remain. Since these beads bind and stimulate CD3, the isolated T cells are receiving large amounts of constitutive CD3 stimulation through crosslinked antibodies. Not only this, but these beads likely prevent our own OKT3 antibodies from binding to the cells as many if not all of the epitopes are already bound. This would also prevent our goat anti-mouse antibody from crosslinking the anti-CD3 and anti-MUC1 antibodies. Finally, due to simple size issues, the bound beads may have prevented T cell interactions with accessory cells, resulting in either reduced proliferation (if the accessory cells have molecules to costimulate) or enhanced proliferation (if the accessory cells have molecules to coinhibit). All of these scenarios are unaccounted for variables that may have affected the proliferation of T cells in the presence of irradiated accessory cells of different ratios in a very different manner than predicted. In future experiments, an untouched $CD3^+$ T cell isolation kit (negative selection by using magnetic beads against all non-CD3-bearing cells) will be used to eliminate these variables.

4.7 THE MUC1 MODEL OF T CELL REGULATION

The sum of this work suggests that MUC1 can act as both a positive and a negative regulator of T cells, with specific conditions to trigger either function and specific cell types which are affected by its ligation. In the case of positive regulation, MUC1 modifies the calcium-dependent NF-AT signaling pathway by binding to the AP-1 transcription factors, with CD3 stimulation facilitating its

nuclear migration, thereby increasing the amount of intracellular AP-1 and increasing the potential for cellular activation and proliferation. In the case of negative regulation, in the absence of a sufficient number of $CD3^-$ accessory cells, MUC1 partially inhibits the CD3-mediated proliferative response. This inhibitory role would prevent autoimmune T cells from being activated in the absence of appropriate signals from professional APCs, similar to how a lack of APC costimulation leads to T cell anergy in the periphery (27).

These findings bring to light an entirely novel role for MUC1, with a new mechanism and model for its function on T cells. No longer just important in cancer research, MUC1 may provide new therapeutic targets for the treatment of autoimmune disorders, providing an inhibitory signal if given the proper stimulus, or in the treatment of diseases of immune inhibition, such as in tumor microenvironments, providing a stimulatory signal instead. As well, our data has provided much in the way of further understanding the complexities of T cell immunoregulation by providing a new ligand which functions in a way that no other T cell signaling molecule, to date, has been observed to.

With further research into MUC1's immunoregulatory abilities on T cells, it is hopeful that new methods to treating disease will emerge. Much work remains to be done before this can be accomplished, but the promise that our *in vitro* data shows for the MUC1 model of T cell regulation is strong.

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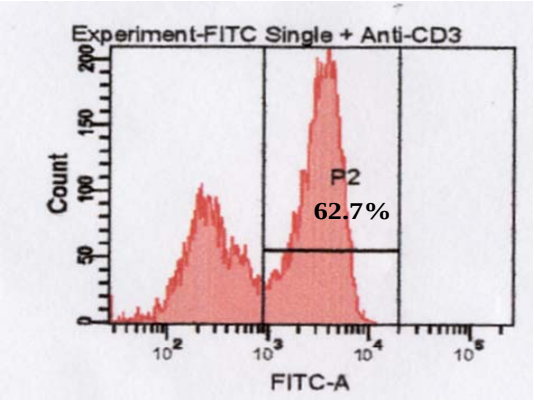
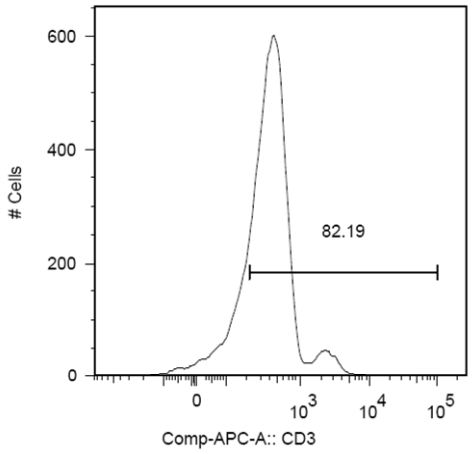
4.9 APPENDIX

Figure 4-1: The percentage of $CD3^+$ T cells in an unpurified, non-adherent cell population



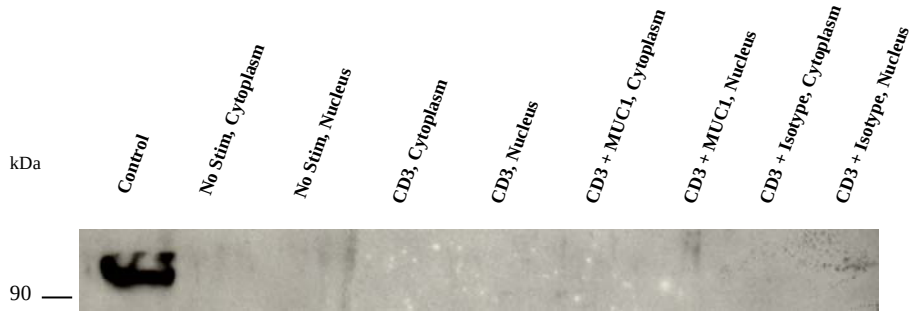
Non-adherent, unpurified T cells were stained with an anti-CD3 APC antibody (eBioscience, San Diego, CA) as described in section 2.2.2 before being read on a flow cytometer. Greater than 60% of the gated lymphocyte population stained positive for CD3.

Figure 4-2: The percentage of $CD3^+$ T cells in a PBMC population purified with nylon wool



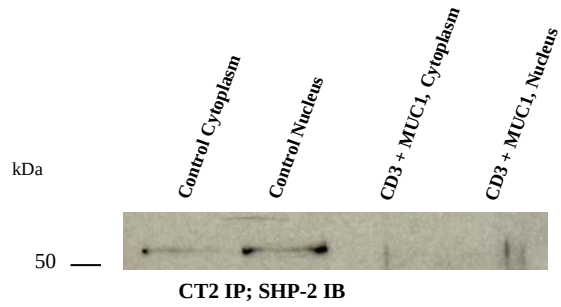
Nylon wool-purified T cells were stained with an anti-CD3 APC antibody (eBioscience, San Diego, CA) as described in section 2.2.2 before being read on a flow cytometer. Greater than 80% of the gated lymphocyte population stained positive for CD3.

Figure 4-3: NF-ATc1 does not bind to the cytoplasmic tail of MUC1



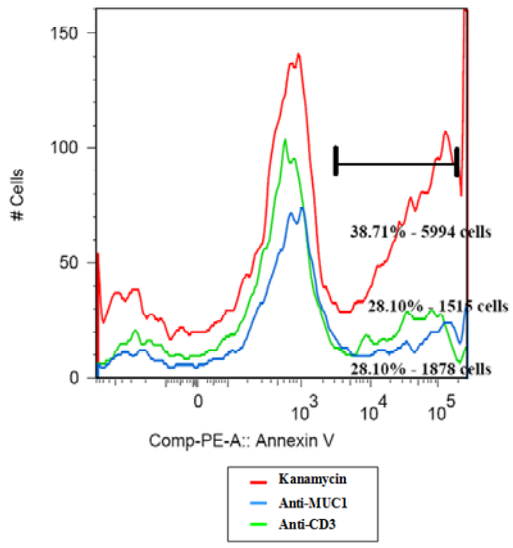
Cell lysates were generated and immunoprecipitations were performed as described in sections 2.2.9 and 2.2.10, precipitating with an antibody generated against MUC1's cytoplasmic tail (CT2) and with Western blotting performed using a monoclonal antibody generated against NF-ATc1 (Santa Cruz Biotechnology, Santa Cruz, CA). A band of the appropriate molecular weight (90-120 kDa) was found in the pure lysate control group while no bands were found in the immunoprecipitated experimental groups.

Figure 4-4: SHP-2 does not bind to the cytoplasmic tail of MUC1



Cell lysates were generated and immunoprecipitations were performed as described in sections 2.2.9 and 2.2.10, precipitating with an antibody generated against MUC1's cytoplasmic tail (CT2) and with Western blotting performed using a monoclonal antibody generated against SHP-2 (Santa Cruz Biotechnology, Santa Cruz, CA). Bands of the appropriate molecular weight (68 kDa) were found in the pure lysate control groups while no bands were found in the immunoprecipitated experimental groups.

Figure 4-5: Anti-MUC1-bound beads do not cause an increase in apoptosis in a nylon wool-purified T cell population



Three day PHA-stimulated, nylon wool-purified cells were treated for three hours with Kanamycin or beads bound with either anti-CD3 or anti-MUC1. Beads were generated as described in section 2.2.5 and cells were treated as described in 2.2.6. Cells were then stained for Annexin V as described in section 3.2.6 and analyzed via flow cytometry. The Kanamycin-treated positive control group showed a high level of apoptosis, with 38.71% of the gated population (5994 cells) testing positive for Annexin V. Both the anti-CD3-treated negative control group and the anti-MUC1-treated experimental group showed an equivalent percentage of the gated population staining positive for Annexin V (28.10%), with similar numbers of cells also testing positive (1515 in the anti-CD3 group versus 1878 in the anti-MUC1 group). This difference was not enough to account for the previously-observed apoptosis/necrotic recovery percentage of >95% from MACS-purification of $MUC1^+$ T cells.