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A study of MUC1 mucin in tumor progression and immune modulation

by

Gabrielle Lee Zimmermann 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Immunology

Department of Medical Microbiology and Immunology

Edmonton, Alberta

Spring 2000

University of Alberta

Library Release Form

Name of Author: Gabrielle Lee Zimmermann

Title of Thesis: A study of MUC1 mucin in tumor progression and immune modulation.

Degree: Doctor of Philosophy

Year this Degree Granted: 2000

April 13th, 2000

Gabrielle Zimmermann

4423 49th Street, N.W.
Calgary, Alberta
T3A 0K3

Always Look On The Bright Side Of Life

As performed in Monty Python's Life of Brian

Always look on the bright side of life.
Always look on the light side of life.

If life seems jolly rotten, there's something you've forgotten,
And that's to laugh and dance and smile and sing.
When you're feeling in the dumps, don't be silly chumps.
Just purse your lips and whistle! That's the thing.

And always look on the bright side of life.
Always look on the right side of life.

For life is quite absurd and death's the final word.
You must always face the curtain with a bow.
Forget about your sin! Give the audience a grin.
Enjoy it! It's your last chance, anyhow.

So, always look on the bright side of death,
Just before you draw your terminal breath.

Life's a piece of sh*t, when you look at it.
Life's a laugh and death's a joke, it's true.
You'll see it's all a show. Keep 'em laughing as you go.
Just remember that the last laugh is on you!

And always look on the bright side of life.
Always look on the right side of life.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled A study of MUC1 mucin in tumor progression and immune modulation submitted by Gabrielle Lee Zimmermann in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Immunology.

B. Michael Longenecker, PhD

Kevin P. Kane, PhD

Timothy R. Mosmann, PhD

John Samuel, PhD

Katharine E. Magor, PhD

Michael A. Hollingsworth, PhD

Dedication

To My Family and Friends

Abstract

Introduction: Mucins are large molecular weight glycoproteins present on the apical surface of normal epithelial cells. Cancer-associated mucins, such as MUC1, are underglycosylated and are often present over the entire surface of the cell due to a loss of polarity. These cancer-associated mucins contain novel epitopes that can be potentially recognized as foreign by the immune system and they have been the focus of many forms of immunotherapy. There are many models in the literature to show that human MUC1 mucin is immunogenic in mice, but these relied on the rejection of MUC1 positive tumors and did not allow the investigation of the role of MUC1 in tumor progression.

Purpose: The purpose of this thesis research was to establish a relevant tumor model to study the mechanisms of tumor progression and immune modulation by MUC1.

Methods: A murine mammary adenocarcinoma cell line, 410.4, was transfected with a 42 tandem repeat of human MUC1 cDNA. The resultant transfectants were sorted by FACS into a high cell surface MUC1 expresser (GZHi) and a low cell surface MUC1 expresser (GZLo). The stability and efficacy of these cell lines as a tumor model was then tested by injecting them subcutaneously into different strains of mice and monitoring tumor progression, metastases, survival, cell surface MUC1 stability, serum MUC1 mucin levels and anti-MUC1 antibody levels.

The ability of either cell surface or soluble MUC1 mucin to interfere with murine immune responses was tested both *in vitro* and *in vivo* using standard CTL, NK, T-cell proliferation and IFN-γ production assays.

Results: GZHi tumors, while stable *in vitro*, showed a loss of MUC1 expression *in vivo*; the mice showed longer survival and had negligible serum MUC1 mucin levels. GZLo

challenged mice showed shorter survival and many showed rapidly increasing serum MUC1 mucin levels prior to death.

In vitro, it was shown that soluble MUC1, rather than cell surface MUC1, had an effect on inhibiting CTL killing of targets as well as decreasing IFN-γ production in T cell proliferation assays.

Conclusions: The GZLo cell line represents a relevant tumor cell to be used in animal models to study the immunomodulatory role of MUC1 mucin and to evaluate cancer vaccine candidates.

Acknowledgments

I would like to thank my supervisors Mike Longenecker and Kevin Kane. Mike for his undying enthusiasm for immunology and research, which brought me into the field in the first place and which always made me feel good about what I was doing; Kevin for keeping me in check and always providing me with a different perspective on things. I would like to thank my committee members, Tim Mosmann, for continually challenging me and John Samuel, for constant support and guidance. I would also like to thank Dr. Tony Hollingsworth and Dr. Kathy Magor for participating in my defense and making it interesting.

I would like to thank the original "Longenecker Lab" for teaching me the basics and for making the lab an enjoyable place to work. I would especially like to thank Jana for her valued friendship and for helping me through the rough days, the good days and the long days at the hood. The staff and scientists at Biomira have been an invaluable resource for me and I thank each and every one who has helped me learn and grow both scientifically and personally. I am thankful for the friends and contacts I have made there. I would also like to thank all my fellow grad students for their friendship and camaraderie over the years. I wish them all the best of luck. I would like to thank Dr. Judith Hugh and Jan for all their help and guidance with histology and my two favorite secretaries, Loreen Degenstein and Anne Hudson for making my life easier in countless ways.

Innumerable thanks go to my family and friends without whose support and encouragement I may not have made it through this trying adventure. Mom and Dad, who knew how important finishing was and who gave me constant support both emotionally and financially, I thank you. A huge thanks goes to my longtime friend and confidant, Sandra, who has always been there for all the ups and downs, the good and the bad, the long runs and margies! I also thank the rest of my good friends in the "running club" - Kelly, Stacey and Sarah – for their support, friendship and many enjoyable evenings filled with running, eating and talking! A great big thanks also goes to my dear friends Vanessa, Patti and Heather for always being there and for occasionally getting me away from it all by taking me to my favorite place, the mountains. I would also like to thank Tove for her continued friendship and support, Paula for a different view on life and Kent for being there and making me laugh.

Last but not least I thank my husband Brett for his friendship, love and patience, which have helped me grow over the years and for his continual support and gentle nudging to keep me on track to finish.

I thank you all for contributing to my success.

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^{3}H	Tritium
ABTS	2,2'-Azinobis (3-ethylbenzo-thiazoline-6-sulfonic acid), diammonium salt
ADCC	Antibody Dependent Cellular Cytotoxicity
APC	Antigen Presenting Cell
ASI	Active Specific Immunotherapy
BLT	Nα-benzyloxycarbonyl-L-lysine thiobenzyl ester
BSA	Bovine Serum Albumin
CIP	Complement Inhibitory Protein
Cr	Chromium
CTL	Cytotoxic T Lymphocyte
DAB	diaminobenzidine
DMEM	Dulbecco's Modified Eagle Medium
DNTB	5,5'-Dithio-bis(2-nitrobenzoic acid)
DTH	Delayed Type Hypersensitivity
E:T	Effector:Target
ECM	Extracellular Matrix
EDTA	Ethylene Diamine Tetraacetic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
EMA	Epithelial Membrane Antigen
ETA	Epithelial Tumor Antigen

FACS	Fluorescence Activated Cell Sorter
FasL	Fas Ligand
FBS	Fetal Bovine Serum
FITC	Fluorescein-isothiocyanate
HLA	Human Leukocyte Antigen
HSLAS	Health Sciences Lab Animal Services
i.p.	intraperitoneal
ICAM-1	Intercellular Adhesion Molecule 1
IFN-γ	Interferon - gamma
Ig	Immunoglobulin
IL	Interleukin
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif
K	Potassium
KIR	Killer Inhibitory Receptor
LAK	Lymphokine Activated Killer
LFA-1	Leukocyte Function Antigen
MAb	Monoclonal Antibody
MAC	Membrane Attack Complex
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MLR	Mixed Lymphocyte Reaction
MMP	Matrix Metalloproteinase
MUC1.Tg	MUC1 Transgenic

NK	Natural Killer
OD	Optical Density
OSM	Ovine Submaxillary Mucin
PBS	Phosphate Buffered Saline
PEM	Polymorphic Epithelial Mucin
PFA	Paraformaldehyde
Poly I:C	Polyinosinic:polycytidylic
RIA	Radioimmunoassay
SEM	Standard Error of the Mean
SER	Serine Esterase Release
STn	Sialyl Tn
T	Thomsen-Freidenreich
TAA	Tumor Associated Antigen
TGF-β	Tumor Growth Factor – beta
Th	T helper
Th1	T helper 1
TKPBS	Tween Potassium Phosphate Buffered Saline
TNF-α	Tumor Necrosis Factor - alpha
TNF-β	Tumor Necrosis Factor - beta
TPBS	Tween Phosphate Buffered Saline
TR	Tandem Repeat
VAF	Viral Antigen Free
VNTR	Variable Number Tandem Repeats

VV Vaccinia Virus

CHAPTER I

Introduction

A) Immune response to tumors

According to the immune surveillance theory, the immune system is our first line of defense against cancer. This theory was developed in the mid-1900s by Burnett and Thomas. Expanding on an idea put forth by Erlich in 1909, they proposed that the immune system, in particular cell-mediated immunity, continually surveyed the body for the presence of aberrant cells which, once recognized, were destroyed. However, the data to support this theory arose mainly from tumors induced by viruses. In this case the immune response may have been geared solely towards elimination of viral spread, rather than elimination of the tumor. This did not mean that the immune system was incapable of eliminating tumors, however. There is abundant evidence in the literature to show that the immune system is capable of destroying cancer cells. The problem in eliminating tumors arises in the immune system's lack of ability to recognize and respond to tumor cells and from the general lack of identifiable target antigens on tumor cells (apart from viral antigens on virally induced tumors).

i) Innate immunity to tumors

Innate or nonspecific immunity is considered the front line of defense against invading pathogens. The primary cells involved in innate immunity to tumor cells are natural killer (NK) cells, macrophages, neutrophils and eosinophils.

NK cells were found to recognize tumor cells in a non-major histocompatibility complex-restricted fashion which meant the absence of major histocompatibility complex (MHC) class I alleles or the presence of allogeneic MHC molecules on target cells were responsible for activating NK cell killing [Trinchieri, 1989]. NK cell "recognition" of a lack of MHC was referred to as the missing-self hypothesis [Ljunggren and Kärre, 1990]. This allows NK cells to kill tumor cells that may have down-regulated their MHC class I molecules. NK cells have several inhibitory receptors that recognize MHC class I molecules which prevent NK cells from killing normal cells [Moretta et al, 1996]. These are called killer-cell inhibitory receptors (KIRs) in humans [Long et al, 1996]. However, there are now also some triggering receptors that recognize MHC class I molecules such as, NKp44 and NKp46 in humans, which are able to activate the cytolytic machinery of NK cells [Vitale et al, 1998; Pessino et al, 1998].

In mice there is a family of receptors called Ly-49 that contain both inhibitory and activating receptors which bind to MHC class I molecules [Mason et al, 1996]. The inhibitory receptors contain sequences in their cytoplasmic portion called immunoreceptor tyrosine-based inhibitory motifs (ITIMs) which mediate their inhibitory effects, whereas activating receptors do not display these sequences [Biassoni et al, 1996; Vivier et al, 1997]. The FcγRIIIA receptors on NK cells are able to trigger NK cell

killing by binding to IgG antibody coated tumor targets, which is called antibody dependent cell-mediated cytotoxicity (ADCC) [Ravetch and Kinet, 1991]. There are other triggering receptors whose exact ligands are unknown, such as NKR-P1. It is postulated that these types of receptors can bind to carbohydrate ligands produced by tumor cells thereby activating NK cells [Yokoyama, 1995]. Several adhesion and co-stimulatory molecules are also involved in NK cell binding and killing of targets [Timonen and Helander, 1997; Martín-Fontecha et al, 1999]. It is likely that the triggering of NK cell lysis as well as inhibition of NK cell lysis involves multiple receptors [Timonen and Helander, 1997]. Upon activation, NK cells are able to lyse tumor cells through the release of granules containing perforin and granzymes as well as by secreting the potentially cytolytic cytokine, tumor necrosis factor-alpha (TNF-α). In addition, activated NK cells can mediate killing through Fas-Fas ligand (FasL) interactions [Oshimi et al, 1996].

Macrophages, neutrophils and eosinophils also express Fc receptors and can bind to antibody coated tumor cells initiating ADCC. Activation of macrophages and neutrophils by interferon-gamma (IFN-γ) leads to increased secretion of lytic enzymes that can destroy tumor cells. Neutrophils can be recruited to the site of a tumor by Interleukin-8 (IL-8) and CD95L (FasL), and the local inflammatory response generated contributes to eradication of tumor cells [Shimizu et al, 1999]. In addition, macrophages and neutrophils produce free radical species, like superoxide and nitric oxide, and secrete TNF-α which have potent antitumor activity. The production of peroxidase by eosinophils can synergize with macrophage reactive oxygen intermediates to kill tumor cells [Nathan et al, 1982]. It has recently been found that peroxidase can catalyze the

oxidation of nitrite to generate further cytotoxic radicals, providing additional evidence for the importance of eosinophils in tumor eradication [van der Vliet et al, 1997].

ii) Acquired immunity to tumors

Acquired or specific immunity arises from the specific recognition of tumor cells as foreign. There are two types of acquired immunity, humoral and cell-mediated.

Humoral immunity involves the production of antibody by B cells to specific tumor antigens. B cells are activated by cytokines produced by T helper (Th) cells in addition to recognition of tumor antigen by cell surface antibody on B cells. CD40-CD40L interactions between B cells and Th cells induce the expression of cytokine receptors on B cells aiding activation. Antibody activates the complement system by initiating the formation of the membrane attack complex (MAC or C5-9) on tumor cells leading to complement-mediated lysis. In addition, antibody facilitates ADCC by NK cells and macrophages producing an antigen specific response to an otherwise non-specific effector function.

Cell-mediated immunity against tumors involves the development of antigen-specific cytotoxic T lymphocytes (CTLs) through recognition of specific tumor peptide antigens presented by MHC class I on antigen presenting cells (APCs). Th, $CD4^+$ cells have an important role in priming CTLs against tumor cells. Th cells recognize tumor antigens presented by MHC class II on APCs and secrete cytokines, such as IL-2, which activate CTLs. In addition, CD40L, expressed on the surface of activated $CD4^+$ Th cells, may increase the antigen presenting and co-stimulatory capacity of APCs so that they

may prime CTLs in the absence of Th cells [Schoenberger et al, 1998]. Activated CTLs are then able to kill tumor targets through either a calcium-dependent mechanism involving release of cytotoxic granules, containing perforin and granzymes, or a calcium-insensitive mechanism involving receptor-mediated apoptosis. For example, the interaction between FasL on T cells with the Fas receptor on target cells can mediate apoptosis of the target cell.

Numerous studies in mouse models using adoptive transfer of purified T cell subsets or *in vivo* depletion have indicated the importance of $CD8^+$ CTLs in anti-tumor immunity [for review see Melief, 1992]. The role of $CD4^+$ T cells in the anti-tumor immune response has been recently expanded. In addition to activation of $CD8^+$ CTLs, $CD4^+$ T cells, specifically Th type 1 (Th1) cells, are able to activate eosinophils and macrophages, through the release of cytokines such as IFN-γ. Th1 and Th2 type responses are distinguished by their different cytokine production profiles [Mosmann et al, 1986]. In mice, Th1 cells produce IFN-γ, IL-2 and tumor necrosis factor-beta (TNF-β) resulting in cell-mediated and inflammatory responses whereas Th2 cells produce the cytokines IL-4, IL-5, IL-6, IL-10 and IL-13 and induce B cell class switching to IgE and IgG_1 which is associated with strong antibody and allergic responses [Mosmann et al, 1986; Mosmann and Sad, 1996]. Human Th cells produce similar patterns of cytokines [Mosmann and Sad, 1996].

These two responses are generally thought to be mutually exclusive since production of IL-10 by Th2 cells inhibits proliferation of Th1 cells and production of IFN-γ by Th1 cells inhibits proliferation of Th2 cells [Mosmann and Sad, 1996]. In addition, in many infections in humans and mice there is a strong bias towards either Th1

or Th2 responses [Mosmann and Sad, 1996]. It has been suggested that patients with advanced cancer may have impaired cell-mediated immunity caused by a switch from a Th1 response to a Th2 response. However, there is now evidence to show that it is not likely a switch from Th1 to Th2 that is responsible for tumor progression, but a malfunction in Th1 cells possibly caused by down-regulation of cytokines such as IL-12 and that both Th1 and Th2 responses are required for maximal systemic antitumor immunity [Elsasser-Beile et al, 1998; Handel-Fernandez et al, 1997; Hung et al, 1998]. In addition, IL-4 deficient mice have revealed an impaired cell mediated immunity to tumors indicating a need for both Th1- and Th2-type cells [Schuler et al, 1999]. There is also evidence that $CD4^+$ cells have cytolytic capabilities. Like $CD8^+$ cells, human $CD4^+$ CTLs are able to lyse target cells through a calcium-dependent mechanism as well as a calcium-insensitive mechanism [Susskind et al, 1996]. These data reveal the importance of $CD4^+$ cells in addition to $CD8^+$ cells in the elimination of tumor cells.

Cytokine secretion from various cell types also contributes to the elimination of tumor cells through antigen specific and non-specific means. IFN-γ produced by Th1 cells and NK cells enhances the activity of macrophages as well as increasing the expression of MHC class I and class II on many cell types. IL-12 produced by macrophages stimulates proliferation of NK and Th1 cells as well as inducing differentiation of CTLs. The production of IL-2 by Th1 cells induces proliferation of Th cells and CTLs as well as enhancing the activity of some NK cells and CTLs. Transduction of various cytokine genes into murine tumor cells not only led to rejection of these tumors but also elicited protective immunity in most cases [for review see Colombo and Forni, 1994].

While elimination of and protection against tumors is generally mediated by tumor-specific T cells, the local inflammatory response mediated by granulocytes, macrophages and/or NK cells is thought to promote presentation of tumor antigens to the immune system [Seino et al, 1997].

iii) Immune escape mechanisms

Despite the systems in place to eliminate cancer cells, tumors still arise. There are a number of different mechanisms by which tumor cells appear to evade the immune system. Many tumor cells can evade detection by CTLs through loss or down regulation of MHC class I molecules. In addition, loss or lack of adhesion molecules required for lymphocyte interaction, such as leukocyte function-associated antigen 1 (LFA-1) or intercellular adhesion molecule 1 (ICAM-1) may inhibit CTL activation. Lack of expression of costimulatory molecules by tumor cells can result in T cell anergy or tolerance despite recognition of MHC and tumor antigen by these T cells [Antonia et al, 1998]. Some tumor cells may acquire molecules that increase their metastatic capability, such as matrix metalloproteinases (MMPs), allowing them to move more easily through the extracellular matrix [for review see Westermarck and Kähäri, 1999]. Others may produce and/or secrete some type of immune inhibitory factor, such as transforming growth factor-beta (TGF-β), which may either down-regulate immune cells or prevent activation, in addition to promoting the process of tumorigenesis [Botti et al, 1998]. Both fresh and cultured human tumors have been shown to express FasL [Whiteside, 1998].

The interaction of FasL on tumor cells with Fas on T cells leads to signaling defects and apoptosis in the T cell.

Tumor cells are also able to take advantage of the mechanisms in place to prevent normal cells from being destroyed by the immune system. There are cell surface molecules present on normal cells called complement inhibitory proteins (CIP) which are able to inhibit complement-mediated lysis. Several studies have shown that not only are these molecules expressed on most tumor cells but they are up-regulated in several cases [see Yu et al, 1999].

In many cases the immune response generated against tumors is inappropriate or ineffective. Patients with advanced cancer have been found to produce higher levels of the Th2-type cytokines and/or lower levels of Th1-type cytokines [Lee et al, 1997; Sato et al, 1998]. Antibody that binds to a tumor cell can actually block recognition factors thereby inhibiting CTLs from recognizing and killing tumor cells [Baldwin and Robins, 1975]. Furthermore, the fact that tumor cells are self cells lends to the inability of the immune system to recognize and respond to them. However, in cases where tumors have tumor-associated antigens (TAAs), the immune response may be responsible for selection of antigen-negative tumor variants or tumor cells that have down-regulated MHC class I expression so as not to present these antigens [Naftzger and Houghton, 1991; Botti et al, 1998; Hersey, 1999].

iv) Immunotherapy of cancer

Until recently, it has been the absence of TAAs that has been the major block in developing specific immunotherapies for cancer. There are now several TAAs that have been identified and are being investigated for potential use in immunotherapy [Boon et al, 1994]. TAAs may come from normal nonmutated genes whose expression is limited to tumors and selected normal cells. These include melanocyte differentiation antigens such as MART-1/Melan A as well as antigens that are normally present only in immune priveleged sites such as the testis (MAGE-1) or only during development, such as carcinoembryonic antigen (CEA) and α-feto protein [Rosenberg, 1999]. In addition, TAAs may be expressed in higher quantities than normal antigens, such as HER2/neu, or the glycosylation patterns may be altered as in MUC1 [Boon et al, 1994; Finn, 1993].

While many TAAs have been shown to be antigenic, the trick lies in making them immunogenic. Many groups have developed genetically modified tumor vaccines using genes encoding MHC molecules, costimulatory molecules and cytokines, with varying degrees of success [for review see Greten and Jaffee, 1999]. Additionally, peptide or protein "vaccines" have been utilized with various adjuvants to help target them to APCs for processing and presentation to immune cells. Dendritic cells have been shown to be the most potent APCs and many groups are investigating several approaches using dendritic cells for active specific immunotherapy [for review see Greten and Jaffee, 1999].

Specific, passive immunotherapy involves the passive transfer of tumor-specific monoclonal antibodies (mAbs) to patients. These mAbs may be used either alone, or

coupled to drugs, pro-drugs, toxins, cytokines or isotopes [Naftzger and Houghton, 1991]. Limitations to antibody therapy include poor penetration into large tumor masses, binding to other cells and the fact that they themselves may be eliminated by the immune system. Non-specific, passive immunotherapy is achieved using lymphokine activated killer (LAK) cells or cytokines, however, these methods have resulted in significant toxicity, limiting their usefulness.

B) The MUC1 mucin in cancer

In an effort to find novel tumor-associated antigens, many laboratories developed antibodies by injecting tumor cells or tumor cell lysates into mice. In the early 1980s the development of monoclonal antibodies (mAbs) to epithelial cells and carcinomas indicated that mucin molecules could be immunogenic in mice. Many antibodies reactive to epithelial cells or carcinomas were found to react with a glycoprotein called polymorphic epithelial mucin (PEM, also know as Episialin, epithelial tumor antigen (ETA), epithelial membrane antigen (EMA), DF3 and now referred to as MUC1) [Taylor-Papidimitriou, 1993]. PEM is dramatically up-regulated in the mammary gland at lactation and in malignancy [Taylor-Papidimitriou, 1993]. In addition, it was found to be aberrantly glycosylated in breast and other carcinomas [Burchell et al, 1987]. PEM showed both qualitative and quantitative differences compared to normal mucin, unlike other tumor-associated antigens [Taylor-Papidimitriou, 1993]. In addition, certain mAbs, such as SM-3 (which recognizes the epitope PDTRP), were found to react specifically with carcinomas, but showed little or no reactivity on normal tissues [Burchell et al,

1987; Girling et al, 1989]. These results indicated that epitopes on the PEM molecule could potentially be recognized as foreign by the immune system and should therefore be investigated for development of novel immunotherapies for adenocarcinomas.

C) Mucins

i) Discovery, structure and expression

Mucins are high molecular weight glycoproteins expressed by a variety of normal and malignant epithelial cells. Mucins consist of 50-80% carbohydrate by weight that is O-linked through N-acetylgalactosamine to serine and/or threonine [Gendler et al, 1990]; however, cancer-associated mucins are underglycosylated revealing core carbohydrate structures such as Tn, STn and T (Thomsen-Friedenreich) antigen [Springer, 1984; Terada and Nakanuma, 1996]. The carbohydrate composition of MUC1 in different tissues, as well as different individuals and species, is highly variable due to differing amounts of glycosyl transferases [Patton et al, 1995].

MUC1 was the first human mucin to be cloned and sequenced [Gendler et al, 1990]. Although there are now at least 12 human mucin genes identified, only five have been found to be membrane-associated, including MUC1 [Gendler et al, 1990; Lagow et al, 1999; Moniaux et al, 1999; Williams et al, 1999a; Williams et al, 1999b]. Unlike MUC3, 4, 11 and 12 however, MUC1 does not contain epidermal growth factor-like domains [Gendler et al, 1990; Moniaux et al, 1999; Williams et al, 1999a; Williams et al, 1999b]. The polypeptide core of MUC1 mucin consists of a cytoplasmic domain, a

transmembrane domain and a large extracellular domain containing a variable number of tandem repeats (VNTR) of the 20 amino acid sequence (PDTRPAPGSTAPPAHGVTSA) [Gendler et al, 1990]. Polymorphism is generated through differences in the number of tandem repeats within two alleles on chromosome 1q21 [Gendler et al, 1990].

Normally mucins are expressed on the apical or lumenal surface of epithelial cells; however, in tumor cells mucins are expressed over the entire surface of the cell due to a loss of polarization [Hilkens et al, 1984]. Additionally, MUC1 mucin can be shed or secreted into the circulation. It has been reported that the MUC1 protein undergoes proteolytic cleavage soon after translation, generating two molecules, a large extracellular domain containing the tandem repeats and another smaller piece containing the cytoplasmic and transmembrane domains [Ligtenberg et al, 1992]. These two pieces are linked noncovalently forming a tight heterodimer complex. The extracellular domain is then postulated to be shed by an unknown mechanism [Boshell et al, 1992]. Another group identified an extracellular repeat domain devoid of a hydrophobic region and determined this to be a secreted form of MUC1 produced through alternative splicing [Wreschner et al, 1990]. Another differential splice form, MUC1/Y containing only the transmembrane and cytoplasmic portions has been reported to have the features of a receptor molecule and its ligand is thought to be the secreted form of MUC1 [Baruch et al, 1999].

The murine homologue of MUC1 (designated Muc-1) was cloned in 1991 and unlike its human counterpart, was found to be nonpolymorphic [Spicer et al, 1991]. The extracellular domain contains 16 degenerate tandem repeats of 20-21 amino acids [Spicer et al, 1991]. The identity within the repeats between human and mouse is only 34%

[Spicer et al, 1991]. However, the transmembrane and cytoplasmic domains are highly conserved. There is evidence that the cytoplasmic tail contains tyrosine phosphorylation sites, however, the mechanism to induce phosphorylation and the subsequent result of intracellular signaling is unknown [Zrihan-Licht et al, 1994; Pandey et al, 1995].

ii) Normal function of mucins

Mucin glycoproteins are subdivided into secretory and membrane-associated forms. Secretory mucins remain at the apical surface and comprise the mucous layer covering lumenal surfaces of all epithelial organs [Gendler and Spicer, 1995]. This layer provides a protective barrier to the cell surface from microorganisms and parasites as well as protecting against dehydration and pH extremes [Ho and Kim, 1991]. Plasma membrane-associated mucin may be involved in cell-cell and cell-substratum interactions resulting in stabilization of the cytoskeleton through associations with actin [Ho and Kim, 1991; Parry et al, 1990]. Interactions with cytoplasmic molecules such as β- and γ-catenin may indicate a role in cytoskeletal remodeling [Yamamoto et al, 1997; Parry et al, 1990]. In addition, tyrosine-phosphorylation of the cytoplasmic tail and interactions with Grb2 and Sos/Ras may indicate a role for mucins in signal transduction and regulation of molecules involved in cell-cell, cell-substratum or membrane-cytoskeletal interactions [Parry et al, 1990; Zrihan-Licht et al, 1994; Pandey et al, 1995; Mockensturm-Gardner and Gendler, 1996; Yamamoto et al, 1997]. The MUC1 mucin is found in various normal tissues and organs and is closely correlated with epithelial differentiation [for

review see Patton et al, 1995]. It also appears to be hormonally regulated [for review see Patton et al, 1995].

Mucin-like glycoproteins which are found in nonepithelial tissues serve as ligands for selectins and are involved in trafficking of lymphocytes [Shimizu and Shaw, 1993]. Due to the dense array of O-linked glycosylation, mucins have a rigid extended structure. In addition, due to extensive sialylation mucins have a high negative charge [Hilkens and Buijs, 1988]. These two characteristics allow mucins to act as a repulsive barrier around the cell. However, with the proper ligand, mucins may act as receptors enabling adhesion to other cells and molecules [Shimizu and Shaw, 1993].

D) Role of mucins in tumor progression

Mucins extend 200-500 nm above the plasma membrane whereas other cell surface molecules, such as adhesion molecules, do not extend much more than 30 nm above the surface [Bramwell et al, 1986; Wesseling et al, 1995]. By virtue of its long extended rod-like structure, MUC1 mucin can mask other cell surface molecules. In addition, due to a loss of polarity in many adenocarcinomas, MUC1 mucin is found over the entire surface of the cell [Hilkens et al, 1984]. In some carcinomas, there is more than a tenfold increase in the expression of MUC1 mucin [Zaretsky et al, 1990]. This can lead to inhibition of cell-cell adhesion which may allow for increased metastatic potential of $MUC1^+$ tumor cells [Ligtenberg et al, 1992]. Cell surface MUC1 may also inhibit lymphocyte-target cell interactions preventing recognition of tumor cells by immune cells [van de Wiel-van Kemenade et al, 1993]. It has also been shown to cause inhibition of

MHC-restricted CTL-target cell interactions [van de Wiel-van Kemenade et al, 1993] as well as NK cell cytotoxicity [Ogata et al, 1992]. In Muc-1 null mice, tumor growth rate and metastasis is significantly decreased [Spicer et al, 1995]. Cell surface MUC1 therefore appears to aid in the evasion of immune responses against adenocarcinomas.

In cancer patients, there is a correlation between high serum MUC1 levels and poor prognosis [MacLean et al, 1997; Reddish et al, 1996]. Furthermore, STn which is a major component of the carbohydrate on MUC1 has been associated with poor prognosis in a number of different adenocarcinoma patients [Itzkowitz et al, 1990; Kobayashi et al, 1992; Werther et al, 1996]. Earlier work showed that injection of epiglycanin, a murine breast cancer associated mucin, leads to immunosuppression and shorter survival times in mice challenged with tumors [Fung and Longenecker, 1991]. In addition, it has been shown that circulating MUC1 could interfere with ADCC activity of eosinophils [Hayes et al, 1990]. MUC1 mucin secreted by carcinoma cells may also promote metastasis by inhibiting lysis of target cells by NK cells [Zhang et al, 1997a]. Recently it has been shown that soluble MUC1 mucin can inhibit human T-cell proliferation *in vitro* [Agrawal et al, 1998c; Chan et al, 1999]. These data together suggest an immunosuppressive role for soluble cancer-associated mucins.

MUC1 mucin has been shown to be a ligand for ICAM-1 [Regimbald et al, 1996] and E-selectin [Sawada et al, 1994] on endothelial cells. This association may facilitate intravasation and extravasation during metastasis of $MUC1^+$ tumor cells. Soluble MUC1 mucin may also inhibit leukocyte adhesion to E-selectin-expressing cells which could prevent lymphocytes from extravasating to the tumor [Zhang et al, 1997b].

The cytoplasmic domain of MUC1 is highly conserved across species and has been shown to be associated with a number of different intracellular molecules as well as having potential signalling capability [Parry et al, 1990; Spicer et al, 1991; Zrihan-Licht et al, 1994; Mockensturm-Gardner and Gendler, 1996; Yamamoto et al, 1997]. These interactions may be responsible for or may contribute to the tumor potentiating effects of MUC1. For example, MUC1 may compete with E-cadherin for binding of β-catenin resulting in loss of adherens junctions formation and thereby cell-cell adhesion [Yamamoto et al, 1997]. In addition, binding of MUC1 to the actin cytoskeleton may allow for cytoskeletal remodelling resulting in increased motility [Parry et al, 1990]. Furthermore, the fact that the MUC1 cytoplasmic tail can be tyrosine phosphorylated and can interact with Grb2 and the Sos/Ras exchange protein may indicate that signalling through MUC1 may play a role in tumor progression [Zrihan-Licht et al, 1994; Pandey et al, 1995].

E) Mucins in immunotherapy

Cancer-associated mucins are underglycosylated revealing normally cryptic epitopes that can be recognized by the immune system [Longenecker and MacLean, 1993; Taylor-Papadimitriou and Gendler, 1988]. In addition, due to a loss of polarity in adenocarcinomas, MUC1 mucin is expressed over the entire surface of the cell resulting in increased antigen expression [Hilkens et al, 1984]. This also contributes to potential recognition by the immune system, especially considering the large number of tandem repeats on each mucin molecule. Barnd et al [1989] have shown that human CTLs can

recognize tumor associated mucins on target cells in an MHC-unrestricted fashion, however, this is only seen following long term multiple antigenic stimulations in cultures. Reddish et al [1998] have found MHC class I-restricted CTLs in breast cancer patients immunized with the MUC1 peptide. MUC1 mucin antigens are presented by certain human lymphocyte antigen (HLA) alleles to CTL resulting in MHC class I-restricted lysis of the tumor target [Domènech et al, 1995; Apostolopoulos et al, 1997b; Hiltbold et al, 1999]. Mice also respond with class I-restricted CTLs to MUC1 immunizations [Apostolopoulos et al, 1995; Gong et al, 1997]. These results indicate that the MUC1 mucin is able to induce a cellular immune response.

Normally mucins are expressed on the apical surface of cells and are secreted into lumena or ducts where they are not expected to encounter the immune system. However, Agrawal et al [1995] have shown that pregnancy can induce T cells responsive to MUC1. It was proposed that altered glycoforms of MUC1 produced during pregnancy could be processed and presented by APCs to T cells which could then lead to protection against breast cancer. MUC1-transgenic (MUC1.Tg) mice, which express the human MUC1 gene product in a tissue-specific manner, were found to be tolerant to MUC1 [Peat et al, 1992; Rowse et al, 1998]. While similar numbers of MUC1-specific CTLs could be detected in wild-type and MUC1.Tg mice challenged with B16.MUC1 tumor cells, only wild-type mice were able to reject their tumors [Tempero et al, 1998]. MUC1-specific antibodies were not able to protect against MUC1 expressing tumors in MUC1.Tg mice [Tempero et al, 1999]. However, tolerance could be overcome in these mice by either adoptive transfer of MUC1-specific leukocytes or immunization with fusions of dendritic cells and carcinoma cells [Tempero et al, 1998; Gong et al, 1998]. There are similar

recognition specificities for both human and murine CTL found on the MUC1 VNTR [Apostolopoulos et al, 1997; Jerome et al, 1991]. B cell epitopes are also encoded within the VNTR, specifically APDTR [Xing et al, 1989].

A number of antibodies against MUC1 mucin have stronger reactions with tumors than with normal tissues. These antibodies have been used for both diagnostic and therapeutic purposes. The TRUQUANT BR®™ radioimmunoassay, which uses the monoclonal antibody B27.29 to quantitate MUC1 mucin, is used for predicting recurrent breast cancer as well as predicting the prognosis of patients with metastatic adenocarcinomas after active specific immunotherapy (ASI) [Muss et al, 1996; MacLean et al, 1997]. The CA 15-3 assay is used to detect circulating mucin in breast and ovarian cancer patients [Snijdewint et al, 1999]. Bispecific antibodies consisting of an anti-MUC1 antibody (MUSE 11) and either anti-CD3 or anti-CD28 have been created to enhance lymphocyte activated killer activity against a bile duct carcinoma [Katayose et al, 1996].

While antibody may be effective as an anti-tumor agent [MacLean et al, 1996], a cellular immune response is thought to be more effective at eliminating tumors. It has been shown that there are numerous CTL precursors against MUC1 in the human T cell repertoire [Agrawal et al, 1996], as well as in MUC1.Tg mice [Tempero et al, 1998]. Indeed, class I-restricted anti-MUC1 CTLs could be found in metastatic breast cancer patients who had been immunized with a synthetic MUC1 peptide [Reddish et al, 1998]. Likewise, MUC1-specific CTLs could be found in MUC1.Tg mice immunized with fusions of dendritic cells and MC-38/MUC1 cells [Gong et al, 1998]. However, it has also been shown that MUC1 mucin can inhibit human T cell proliferation *in vitro*

[Agrawal et al, 1998c; Chan et al, 1999]. Nonetheless, this inhibition could be overcome with IL-2 indicating a possible need for the incorporation of IL-2 when designing immunotherapies for $MUC1^+$ cancers [Agrawal et al, 1998c].

F) Animal models

Many mouse models have been developed in an attempt to study the efficacy of MUC1 based immunotherapeutic cancer vaccines. The murine adenocarcinoma cell line, 410.4, was transfected with a genomic fragment of the human MUC1 gene and tested in Balb/c mice for immunogenicity [Lalani et al, 1991]. MUC1 transfection resulted in reduced incidence of tumor development suggesting that cell surface MUC1 stimulated an anti-tumor immune response in mice. In addition, mice which rejected a low number of transfected cells did not develop tumors after a subsequent inoculation of a large number of transfected cells [Lalani et al, 1991]. These transfectants were also used in CAF1 mice to test the immunogenicity and anti-tumor activity of synthetic MUC1 peptide vaccines [Ding et al, 1993]. Immunization resulted in either complete rejection of the tumor or delayed tumor development in addition to the generation of specific delayed type hypersensitivity (DTH) reactions against specific MUC1 sequences and MUC1-transfected cells [Ding et al, 1993].

Balb/c 3T3 fibroblast cells transfected with MUC1 were also used to test the immunogenicity of various MUC1 antigens [Apostolopoulos et al, 1994]. This system was the first to describe murine anti-MUC1 CTL. The presence of a $CD8^+$ CTL response or a Th1-type response, as opposed to a Th2-type response appeared to be more effective

in tumor rejection [Ding et al, 1993; Apostolopoulos et al, 1994]. It is interesting to note that in the two preceding cases, tumor cells isolated from non-rejected tumors were mostly negative for MUC1 expression.

Other cell lines have been transfected with human MUC1 and shown to be stable for up to 2 weeks *in vivo* [Acres et al, 1993]. These were used to examine the immune response of vaccinia virus (VV)-MUC1 immunized mice [Acres et al, 1993]. VV-MUC1 immunization did not produce MUC1-specific CTL and only managed to delay tumor growth somewhat. There was only 30% tumor rejection in H-2^d mice and the authors suggested that H-2^b mice might be better responders. Apostolopoulos et al [1995] could find no MUC1 non-responder mouse strains and could produce MHC-restricted CTLs to MUC1 peptide sequences in all strains tested. In fact, nine different MUC1 peptide epitopes are recognized in the context of different H-2 molecules [Apostolopoulos et al, 1997]. In yet another model, anti-Id antibodies were used as immunogens against a murine tumor cell line transfected with MUC1 [Smorodinsky et al, 1994]. Growth of these tumors in C3Heb mice was not affected by transfection with MUC1.

Mice transgenic for human MUC1 have been developed and have been shown to express MUC1 in a tissue specific manner [Peat et al, 1992; Rowse et al, 1998]. The uninhibited growth of $MUC1^+$ tumors and the lack of antibody following immunization with MUC1 peptides revealed these mice to be tolerant to MUC1 [Rowse et al, 1998]. However, this tolerance could be overcome by immunization with fusions of dendritic and $MUC1^+$ carcinoma cells resulting in cellular and humoral immunity against MUC1 [Gong et al, 1998]. Importantly, generation of an anti-MUC1 immune response in these mice has, as yet, not induced autoimmunity [Tempero et al, 1998; Smith et al, 1999].

G) Hypothesis/objective

The focus of this thesis is to establish a relevant tumor model with which to study the mechanisms of immune modulation by MUC1 mucin. A relevant model should show stable expression of cell surface MUC1 mucin, secretion of MUC1 mucin *in vivo*, which is associated with a poor prognosis [Agrawal et al, 1998; Maclean et al, 1997; Reddish et al, 1996], and metastatic tumor growth. Expression of MUC1 mucin by tumor cells is postulated to confer an advantage over control tumor cells by enhancing tumor progression and decreasing host survival. The role of both cell-surface and soluble MUC1 in tumor progression and host survival is hypothesized to be an increase in metastatic potential of the tumor cells and inhibition of cell-mediated immune responses against the tumor cells.

In the first phase, I developed stable MUC1-transfected cell lines that did not lose MUC1 expression *in vivo* under immune pressure. Two transfected cell lines have been developed, a high cell surface MUC1 expresser and a low cell surface MUC1 expresser. These two cell lines allowed the investigation of whether higher levels of MUC1 on a tumor cell would contribute to greater lethality or increased immunogenicity in mice than lower levels of MUC1.

Phase II involved testing tumor progression, metastasis, host survival, cell-surface MUC1 stability and serum MUC1 mucin levels in normal mice challenged with the MUC1-high and -low transfected tumor cell lines. SCID-BEIGE mice, which lack T cell, B cell and NK function and SCID mice, which lack T cell and B cell function, were used in addition to normal mice to evaluate the role of the immune response in the growth of

the MUC1-transfected tumors. The ability of soluble MUC1 mucin to inhibit the induction of an immune response *in vivo* was tested by immunizing mice that had high levels of circulating MUC1 mucin. T cell proliferation and IFN-γ production from antigen specific T cell responses as well as allo-mixed lymphocyte reactions (MLR) were tested *in vitro* following *in vivo* exposure to MUC1.

As a result of the *in vivo* studies, Phase III employed *in vitro* assay systems to further delineate possible mechanisms of action for cell-surface and soluble MUC1 in modulating the immune response to tumors. These assays included CTL, NK, T cell proliferation and IFN-γ production assays.

A good tumor model and a better understanding of the immune modulating properties of cancer-associated MUC1 mucin will facilitate the development of novel immunotherapies for adenocarcinomas.

CHAPTER II

General experimental methods

A) Materials

410.4 cells were a gift from Dr. Joyce Taylor-Papadimitriou (London, UK). CTL clones, EL4, Yac-1 and A20 cells were provided by Dr. Kevin Kane (Edmonton, AB). Plasmid DNA (MUC1-pHβAPr-1-neo) was a gift from Dr. Michael Hollingsworth (Omaha, Nebraska) [Batra et al, 1991]. RPMI-1640, Dulbecco's Modified Eagle's Medium (DMEM), 0.25% Trypsin - Ethylene Diamine Tetraacetic Acid (EDTA), Fetal Bovine Serum (FBS), L-glutamine (100x), Gentamicin, Geneticin (G-418), LIPOFECTAMINE™ Reagent, Hepes buffer, Sodium Pyruvate, Non-essential amino acids, Penicillin/Streptomycin and 2-Mercaptoethanol were all obtained from GIBCO BRL Life Technologies, Inc. (Grand Island, NY). All tissue culture plates, dishes and tubes were obtained from either Canadian Life Technologies (Burlington, ON), Fisher (Nepean, ON) or VWR Canlab (Edmonton, AB). C57Bl/6, BALB/c, CB6F1, CAF1, C3H-H2, B10.LG, CBA, A and DBA/2 mice were all obtained from Jackson Laboratories (Bar Harbour, ME). C.B-17, C.B-17 SCID and C.B-17 SCID-BEIGE mice were all obtained from Taconic Farms, Inc. (Germantown, NY). The SCID-BEIGE mice used in **CHAPTER IV**, Section D, were obtained from the University of Alberta Health Sciences Lab Animal Services (HSLAS) (Edmonton, AB). ^{3}H-Thymidine and ^{51}Chromium (^{51}Cr) were obtained from Amersham Canada (Mississauga, ON). Microtainer brand serum separator tubes were obtained from Becton Dickinson Canada (Mississauga, ON).

Polyinosinic:polycytidylic acid (Poly I:C), Nα-benzyloxycarbonyl-L-lysine thiobenzyl ester (BLT), 5,5'-Dithio-bis(2-nitrobenzoic acid) (DNTB) and diaminobenzidine (DAB) were all obtained from Sigma Chemical Company (St. Louis, Missouri). 2,2'-Azinobis (3-ethylbenzo-thiazoline-6-sulfonic acid), diammonium salt (ABTS) was obtained from Aldrich Chemical Co. (Milwaukee, WI). Flu peptide ($NP_{365-380}$) was provided by Dr. Kevin Kane (Edmonton, AB); all other peptides and affinity purified MUC1 mucin [see Agrawal et al, 1998c for purification details], as well as liposomes were provided by Biomira Inc. (Edmonton, AB). Ovine submaxillary mucin (OSM) was obtained from Cedarlane Labs (Hornby, ON). Peroxidase conjugated goat anti-mouse immunoglobin (Ig, IgG_1, IgG_{2a}) antiserum were obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD). Peroxidase conjugated streptavidin was obtained from Jackson ImmunoResearch Laboratories Inc. (BIO/CAN, Mississauga, ON). XMG1.2 was a gift from Dr. Tim Mosmann (Rochester, NY) and Y3 (anti-H-$2K^b$) antibody was provided by Dr. Kevin Kane (Edmonton, AB); all other antibodies were provided by Biomira Inc. (Edmonton, AB). The IFN-γ standard was obtained from PharMingen Canada (Mississauga, ON) and the positive control IFN-γ was obtained from Genzyme (Cambridge, MA). All other reagent grade chemicals were obtained from Fisher (Nepean, ON).

B) Immunoperoxidase staining of adherent cells

Cells (410.4 and transfectants) were seeded in NUNC 8 well chamberslides to obtain 80-90% confluence by the following day. Confluent cells were washed with warm

phosphate buffered saline (PBS) then fixed for 10 minutes in cold methanol/acetone (1:1, v/v) mixture and air dried. Antibody (anti-MUC1 mAbs BCP8 or B27.29 and negative control mAb BB7.2) was added onto the dried cell layers at a concentration of 10 μg/ml in PBS and incubated for 1 hour at 37°C in a humidified incubator. (For staining of live cells, the fixation step was omitted before staining and cultures were fixed with methanol/acetone (1:1) after staining). The slides were washed for 5 minutes in PBS, followed by a 1 hour incubation at 37°C with peroxidase-conjugated goat anti-mouse Ig antiserum diluted 1:200 in PBS with 15% FBS and 3% human serum. These slides were then washed 3-4 times in PBS for 30 seconds with intermittent agitation. Substrate solution (0.03% H_2O_2 in PBS and 1mg/ml diaminobenzidine (DAB)) was then added and the reaction allowed to continue for 5-8 minutes. Slides were washed with PBS and counterstained with hematoxylin (with acetic acid) for 30 seconds, followed by 2 or 3 changes of dH_2O (until water was clear). Slides were then immersed in saturated lithium carbonate in dH_2O for 90 seconds, followed by washing in two changes of dH_2O. The slides were dehydrated by successive immersion for 30 seconds each into graded alcohol (70% ethanol in ddH2O, 90 % ethanol in ddH2O and absolute ethanol) and mounted with permount.

C) Fluorescence Activated Cell Sorter (FACS) Analysis

Confluent cultures of the transfectants were harvested using trypsin-EDTA and stained for FACS analysis as previously described [Lalani et al, 1991] using the anti-MUC1 antibody B27.29. Briefly, after trypsinization cells were resuspended in 2%

FBS/PBS at a concentration of 5 x 10^5 cells / tube in a 100 µl volume and centrifuged at 350 x g for 3 minutes. The supernatant was removed and cells were resuspended with 0.5 µg antibody (anti-MUC1 mAbs B27.29 or BCP8 and negative control mAb B70.3) diluted in 100 µl cold 2% FBS/PBS and incubated on ice for 30 minutes. Following centrifugation at 350 x g for 3 minutes, cells were washed 3 times with cold 2% FBS/PBS. Fluorescein-isothiocyanate (FITC)-labelled goat anti-mouse Ig was diluted 1:100 in 2% FBS/PBS and 100 µl added to each tube. The tubes were incubated for 30 minutes on ice and then washed as above. Cells were pelleted (350 x g, 3 minutes) and resuspended in 100 µl of 2% paraformaldehyde (PFA)/PBS and stored at 4°C until analyzed by FACS. (For sorting, 1 x 10^6 cells were stained with double the amount of reagents used and they were resuspended in 1 ml of DMEM FACS media.)

D) Animal models

All animal use, housing, experimental manipulations and euthanization had been approved by the Health Sciences Animal Welfare Committee at the University of Alberta, and conform to the standards set by the Canadian Council of Animal Care.

C.B-17, C.B-17 SCID and C.B-17 SCID-BEIGE mice were housed in the VAF (Viral Antigen Free) facility at the University of Alberta by HSLAS and were given Novotrimel (2%) in their water weekly to prevent infection. All other mice were housed conventionally.

i) Subcutaneous tumor model

Groups of ten mice (except a preliminary experiment, which used groups of five,) were injected subcutaneously in the left inguinal area with a volume of 100 µl containing different doses of cells (3×10^4, 1×10^5, 3×10^5, 1×10^6, 3×10^6). Tumor size was measured weekly and recorded using Vernier calipers as a length by width measurement (mm^2) to assess tumor growth. Tail bleed samples (described below) were taken from half of each group each week, so that each individual mouse was only bled every two weeks. Ten random mice had tail bleed samples taken the day before challenge to be used as control. Tail veins were nicked with a razor blade and anywhere from 50-500 µl blood was collected and centrifuged in Microtainer brand serum separator tubes. The serum was then transferred to a 0.5 ml microfuge tube, labelled and frozen for testing at a later date. As the mice became sick, they were monitored daily and were euthanized when they became moribund (ie. fur became scruffy/absence of grooming, lack of movement/response and/or difficulty breathing), using CO_2 asphyxiation or an overdose of Halothane anaesthetic. At this time a final serum sample was taken via a cardiac puncture. A detailed autopsy was performed and recorded to document locations of metastases. Samples of various organs and metastases were fixed in 10% formalin, embedded in parafin by a tissue processer (Tissue-Tek VIP, Miles, Scientific) after which 0.5 µ slices were made and transferred to poly-L-Lysine coated slides for staining. In addition, samples of primary tumor, spleen and metastases were gently ground between the ends of two sterile, frosted microscope slides to disperse the cells, then washed (with DMEM plus 5% FBS) and cultured in DMEM supplemented with 10% FBS and 2 mM

L-glutamine. When the number of cells reached 3 - 4 x 10^6, (after an average of one week in culture) they were stained as in Section C above and analyzed by FACS for cell surface expression of MUC1.

ii) MUC1 suppression model

Groups of mice were injected intraperitoneally (i.p.) with either affinity purified MUC1 mucin [see Agrawal et al, 1998c] or OSM (20 μg in a total volume of 100 μl PBS) or an equal volume of PBS every day for three, six or ten days. In the experiments where the mice were also immunized they were injected with vaccine subcutaneously on the second day of i.p. injections 2-4 hours after the i.p. injection. Vaccines were either BLP24 (MUC1 24mer in liposomes) or BP1-109 (HIV-V3 34mer) as a liposomal formulation. The liposomal delivery vehicle consisted of multilamellar large vesicles (MLV) composed of dipalmitoyl phosphatidyl choline (DPPC), dimyristoyl phosphatidyl glycerol (DMPG) and cholesterol at a molar ratio of 44.1:5.9:50 and a concentration of 20 mg/mL. These MLV also contained synthetic lipopeptide and Lipid A as active ingredients, typically at concentrations of 400 mg/mL and 200 mg/mL, respectively. The day after the last i.p. injection the mice were euthanized, a serum sample was taken and their spleens and inguinal lymph nodes removed for proliferation assays.

E) Immunoassays for detection of soluble MUC1 mucin

MUC1 mucin in the serum of the mice used in **CHAPTER IV** Section A) was determined using a sandwich radioimmunoassay (RIA). Serum samples were diluted 1:100 and were set up in duplicate in mAb B27.29 coated tubes in a 200 µl volume of BR standard: Na Acetate (1:1). Tubes were shaken for three hours on an orbital shaker. Supernatants were aspirated and tubes washed 2 X with H_2O. Radioactive tracer (B27.29-^{125}I) was added to each tube in a 200 µl volume and the tubes were shaken for another three hours on an orbital shaker. Samples were washed twice with H_2O and counted on a gamma counter. Results were expressed in cpm. For all other samples, MUC1 in serum and cell culture supernatants was determined with a sandwich enzyme immunoassay (EIA) employing mAb B27.29, as previously described [Agrawal et al, 1998]. Serum samples were assayed at a dilution of 1:100 in protein buffer and cell culture supernatants were assayed undiluted. Results were calibrated to Units/ml (one Unit/ml is equal to 50 ng/ml polypeptide.)

F) ELISA for detection of antibody and cytokines

i) Anti-MUC1 antibody titres

Ninety-six well ELISA plates (Nunc Maxisorb) were coated overnight with 5 µg/ml BP1-7-HSA or BP1-65-HSA (Biomira Inc.), or 10 Units/ml MUC1 mucin in PBS. The peptide BP1-7 comprises the sequence GVTSAPDTRPAPGSTA of the MUC1

tandem repeat and BP1-65 is TAPPAHGVTSAPDTRPAPGSTAPP. After overnight coating, plates were washed with PBS and blocked for one hour at room temperature with 0.8% (w/v) gelatin solution using TweenPBS (TPBS) as diluent. Serial doubling dilutions of sera were made (starting at 1/80) and added to the plates in duplicate after washing four times with TPBS. After a one-hour incubation at room temperature, plates were washed again with TPBS. Peroxidase labelled goat anti-mouse IgG_1 or IgG_{2a} were diluted 1:1000 in 0.2% gelatin (w/v) in TPBS, added and incubated for one hour at room temperature. Plates were then washed as above and enzymatic activity measured by adding ABTS substrate and taking a kinetic reading of optical density at 405 nm on an ELISA microplate reader. Antibody titres were determined by taking the highest dilution giving a kinetic rate value of at least 2x the kinetic rate value of the pre-bleed serum and > 2 mOD (optical density)/min. Antibody titres were reported as $\log_2$ titre. Sera from mice injected with 410.4 cells served as background.

For the determination of anti-MUC1 antibody that binds to MUC1 expressed by the transfectants, 410.4 and GZHi (MUC1-transfectant) cells were harvested by trypsinization, washed with RPMI-1640 and plated in 96 well v-bottomed plates at a concentration of 5×10^5 per well. Mouse sera was diluted 1:25 in RPMI-1640 and added to each cell line in the wells along with appropriate controls. After a 25-minute incubation on ice the cells were washed with RPMI-1640 and incubated with goat-anti-mouse IgG-FITC for another 25 minutes on ice. The cells were washed again and resuspended in 300 µl PBS for analysis by FACS.

ii) Interferon-gamma

The inner 60 wells of a 96 well plate were coated with 1.5 μg/ml rat anti-mouse IFN-γ monoclonal antibody, R46A2, (diluted in PBS containing potassium (K)) at 50 μl/well and 50 μl/well of KPBS was added to the outer wells (for each step). The plates were sealed with an adhesive cover and incubated at 37°C for 35 ± 5 minutes (plates were gently shaken half way through each incubation) after which time they were washed once with 0.05% Tween-KPBS (TKPBS). (Each time following washing, the plates were flicked into a sink and then tapped on a paper towel to remove residual washing liquid.) The IFN-γ standard was diluted serially from 10 ng/ml to 312.5 pg/ml in RPMI-1640 plus 5% FBS and the positive control IFN-γ was diluted to 5000 pg/ml in RPMI-1640 plus 5% FBS. Each was added at 50 μl/well. Samples were added 50 μl/well undiluted (unless they were previously out of range in which case they were diluted 1:5). The plates were incubated at 37°C for 40 ± 5 minutes after which time they were washed 2 times with TKPBS. The detecting antibody, biotinylated rat anti-mouse IFN-γ monoclonal antibody XMG1.2 was diluted to 0.045 μg/ml in 1% bovine serum albumin (BSA)/TKPBS (50 μl/well) and incubated at 37°C for 35 ± 5 minutes after which time the plates were washed 5 times with TKPBS. Peroxidase conjugated streptavidin was diluted in 1% BSA/TKPBS to an optimal working concentration that was determined in a previous titration and added at 50 μl/well. The plates were incubated at 37°C for 30-35 minutes, but not longer than 40 minutes after which time they were washed 6 times with TKPBS. Substrate was prepared fresh each time by weighing out 50 mg of ABTS and adding 11 ml Na_2HPO_4 [0.2 M], 14 ml citrate [0.1 M], 25 ml water and 5 μl 30% H_2O_2.

Substrate was added at 50 µl/well and the plates read immediately on an ELISA microplate reader in kinetic mode at an OD of 405 nm for 10 minutes. Values for all samples were read off the standard curve in ng/ml.

G) In vitro killer cell recognition assays

i) Cytotoxic T-Lymphocyte (CTL) assay

Target cells (5×10^5) were labelled with 50 µCi (50 µl) of Chromium-51 (^{51}Cr) + 10 µl FBS in 15 ml conical tubes for 1 hour at 37°C, with periodic shaking. ^{51}Cr-labelled target cells were then washed with 12 ml of RPMI-1640 plus 10% FBS 3 times, counted, resuspended at 1×10^5/ml and added to 96 well V-bottom plates at 100 µl/well. Cytotoxic T cells were harvested, counted, resuspended to a concentration depending on the final effector to target ratio, and added to the targets in the 96 well V-bottom plates at 100 µl/well. 2% Trition X-100 was added to target cells alone to measure maximum release of ^{51}Cr and 100 µl of media was added to the target cells to measure spontaneous release. Plates were centrifuged for 3 minutes at 30 x g and incubated at 37°C for 4 hours (unless a previous experiment dictated a longer time) after which time 100 µl of the supernatant was collected, placed into small tubes and counted on a gamma counter. Values were expressed as Percent (%) Specific Lysis = 100 x [(experimental release - spontaneous release) / (maximum release - spontaneous release)].

ii) Natural Killer cell assay

Mice were injected with polyI:C 200 µl, 40 µg i.p. 24 hours before the experiments. On the day of the experiment spleens were removed and the cells isolated by gently grinding between the ends of two sterile microscope slides. Cells were washed, counted, resuspended at 5 x 10^6 cells/ml in RPMI-1640, plated in tissue culture dishes at 7ml/plate and incubated at 37°C for 1 hour. The non-adherent cells were removed, washed, counted and resuspended at concentrations to reflect final effector to target ratios (100:1, 50:1, 25:1 and 12.5:1). NK cells were added at 100 µl/well in 96 well V-bottom plates. Target cells were harvested, counted and labelled as above for the CTL assay. They were washed, resuspended at 10^5 cells/ml (RPMI-1640 with 10% FBS and 2mM L-glutamine) and plated at 100 µl/well. Plates were centrifuged for 3 minutes at 30 x g and incubated for 4 hours at 37°C after which time supernatants were harvested and counted as above for the CTL assay. Values for percent specific lysis were calculated as above.

iii) Serine Esterase Release assay

Target cells and CTLs were harvested, counted, washed 3 times (wash and assay medium was RPMI-1640 with 2% FBS and 2mM L-glutamine), resuspended at 4 x 10^6 cells/ml (a separate tube of CTLs were resuspended at 2 x 10^6 cells/ml) and added to 96 well flat-bottom floppy plates (Falcon) at 50 µl/well each. The plates were parafilmed, centrifuged at 50 x g for 3 minutes and incubated at 37°C for 2.5 hours. Immediately before harvesting the plates, fresh BLT-DTNB was made up by weighing out 4.5 mg of

BLT and 4.5 mg of DTNB, dissolving in a small amount of PBS and adding this up to a final volume of 50 ml PBS. 20 µl of supernatant was removed from each well of the original plate and placed into the corresponding well of a new plate and 150 µl of the BLT-DNTB reaction buffer was added. The reaction was allowed to proceed for about 20 minutes (judged by color development) and read on an ELISA microplate reader at an OD of 405 nm.

H) Binding assay

96 well flat-bottom plates were coated overnight at 4°C with 100 µl/well of antigen (Y3, MUC1, OSM, BSA – each at 0.25, 0.5, 1 and 2 µg/ml in triplicate). The next day, supernatants were removed from the plates and 100 µl of 3% FBS/PBS was added to each well for 30 minutes at room temperature. Plates were washed twice with 3% FBS/PBS before the addition of cells. CTLs were harvested, counted and 3 x 10^6 cells were resuspended in 100 µl media in 15 ml conical tubes. 100 µCi of ^{51}Cr (100 µl) and 10 µl FBS was added and the tubes were incubated for 1 hour in a 37°C water bath. Cells were washed 3 times, resuspended at 5 x 10^5 cells/ml in RPMI-1640 plus 2% FBS and were added at 100 µl/well to the above coated plate. Plates were centrifuged for 4 minutes at 40 x g and incubated in a 37°C incubator for 1 hour. After incubating, the plates were put in an ice bath and another 100 µl/well of cold RPMI-1640 plus 3% FBS was added and mixed gently 6 times. The full 200 µl of supernatant was removed and the binding of cells to antigen was visually inspected under the microscope before

counting the wells with a gamma counter. Values were expressed as % Specific Binding = [(well count – background) / maximum cell per well count] x 100.

I) Proliferation assay

Spleens and inguinal lymph nodes were removed from each mouse at the time of sacrifice and placed in cold RPMI-1640 supplemented with 5% FBS. Cells were teased out by gently grinding between the frosted ends of sterile microscope slides. The suspension was pipetted into a 50 ml tube and washed with fresh RPMI-1640 plus 5% FBS. The cells were counted and resuspended in RPMI-1640 plus 10% FBS to be added to a nylon wool column. Nylon wool columns were prepared ahead of time by weighing out either 0.5 g (for 1 x 10^8 cells) or 1 g (for 1-2 x 10^8 cells) of nylon wool (Robbins Scientific), teasing it apart into long thin pieces and placing into the barrel of either a 5 cc syringe (0.5 g) or a 10 cc syringe (1 g). The columns were covered with aluminum foil, placed in autoclave bags and autoclaved. Prior to use, the columns were removed, a sterile 20 G-1" needle attached and placed over a 50 ml tube. The columns were prewashed with 20 ml of warm RPMI-1640 plus 5% FBS and all air bubbles were removed. Ten ml of warm RPMI-1640 plus 10% FBS was added and once the columns stopped dripping, they were covered with foil and allowed to equilibrate at 37°C for a minimum of 30 minutes. The cells were added to the columns in either 1.5 ml media (for 5 cc column) or 2.5 ml media (for 10 cc column) and overlayed with 1 ml of FBS. Columns were covered again and placed in a 37°C incubator for 45 minutes, after which time the T cells were washed off using 10-15 ml of warm RPMI-1640 plus 10% FBS.

The T cells were counted and resuspended in complete media (RPMI-1640 with 5% FBS, 2 mM L-glutamine and 55 μM 2-Mercaptoethanol) at a concentration of 5 x 10^6 cells/ml. APCs were harvested the same way from spleens, without separation on columns, and were either irradiated at 2500 Rads or Mitomycin C treated at a concentration of 60 μg/ml (with the cells at a concentration of 2 x 10^7 /ml) for 120 minutes at 37°C followed by several washes. APCs were resuspended in complete media at a concentration of either 5 x 10^6 cells/ml or 1 x 10^7 cells/ml.

T cells and APCs were set up in 96 well plates each at 100 μl/well in replicates of 3-6 wells. Antigens, when used, were added at a concentration of 20 μg/ml in 100 μl of complete media to each well. All wells were equated to a total volume of 300 μl with complete media. Plates were incubated for 5-7 days at 37°C in a 5-7% CO_2 incubator. On the second to last day, 200 μl of supernatant from each well was collected and frozen for cytokine testing and 1 μCi of ^{3}H-Thymidine (in 50 μl complete media) was added to each well. On the last day, 18-24 hours after addition of ^{3}H-Thymidine, the plates were harvested on Skatron filter paper using a Skatron cell harvester and each disc was placed into a labelled scintillation vial containing 3 ml of Cytoscint scintillation fluid. The vials were counted on a Beckman Scintillation counter.

J) Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM); n = number of observations. Comparison between groups was performed using the unpaired Students 't' test. Multiple comparisons were made by ordinary ANOVA and correlation determined

by simple regression analysis. Survival curves were determined using Kaplan-Meier analysis and the Mantel-Cox Logrank Test was used to determine the significant p-values for survival.

CHAPTER III

Development of human MUC1-expressing murine cell lines

A) Assessing potency of Geneticin on cell lines to be transfected

Before transfection, the lethal dose of Geneticin (G418 - an aminoglycoside related to neomycin) was determined for each cell line used. The plasmid used for transfection, pHβAPr-1-neo, is driven by the β-actin promoter for constitutive expression and contains the neomycin resistance gene, which allows the cells that were successfully transfected to grow in the presence of the drug [Batra et al, 1991]. The lethal dose was defined as the lowest dose required to kill all cells within 8 days, as the first effects of the drug are visible only after several days of exposure. The active concentration of Geneticin was calculated as follows: 661 μg/mg x 100 mg/ml = 66100 μg/ml. A stock solution of Geneticin was made up by adding one vial (5 g) of Geneticin (lot # 77N2730) to 40 ml DMEM supplemented with 10 ml FBS and 0.5 ml L-glutamine. A 12 well plate was labelled with concentrations of 0-1000 μg/ml in 100 μg/ml increments to which the appropriate amount of Geneticin stock was added. The cell line used (for **CHAPTERS III** and **IV**) was an adherent murine mammary adenocarcinoma cell line, 410.4, which originated from a Balb/cfC3H mouse [Fulton and Heppner, 1985]. The cells were trypsinized with Trypsin-EDTA, counted and added to the 12 well plate in 2 ml/well of DMEM media with 10% FBS and 2mM L-glutamine (complete media) at a concentration of 1 x 10^4 cells/ml. Plates were kept at 37°C in a 7% CO_2 incubator. On day 4 through day 8, the cells were observed with an inverted microscope and the percent confluence

was recorded for each well. The values representing percent confluence of cells, in each concentration of Geneticin, on day 8 was graphed as percentage of control growth versus concentration of active Geneticin. The value of 600 μg/ml was deduced as the lethal dose of Geneticin (for this lot) to be used in all selective media for 410.4 transfectants.

B) Transfection by lipofection of 410.4 cells with human MUC1 cDNA

Since the cell line 410.4 was adherent, the process of transfection by lipofection was chosen over electroporation. The protocol provided with the LIPOFECTAMINE™ reagent was used as a guideline, but the conditions for this cell line needed to be optimized. In order to obtain 50-80% confluence for transfection, 410.4 cells were seeded in 6 well culture plates at concentrations of 5 x 10^4/well for one plate and 1 x 10^5/well for a second plate. After 3 days, the plate seeded at 5 x 10^4/well was at 55-60% confluence and the plate seeded at 1 x 10^5/well was at 70-75% confluence. Both plates of cells were transfected with the same conditions so that one plate was a duplicate of the other (except for starting cell concentration). Solution A was prepared by diluting either 2 or 4 μg of EcoR1-linearized plasmid (pHβApr-1-neo containing full length human MUC1 cDNA with 42 tandem repeats (TR)) into 200 μl of serum free DMEM media. Solution B was made by diluting 4, 8 or 16 μl of LIPOFECTAMINE™ reagent (lot # DHD103) into 200 μl of serum free DMEM media. Solutions A and B were then mixed together in the six possible combinations of DNA and LIPOFECTAMINE™ reagent and incubated for 45 minutes at room temperature to allow DNA-liposome complexes to form. The volume was brought up to 2 ml/tube with serum free media and the tubes were

mixed gently. One ml of diluted complexes was overlayed onto cells that were previously rinsed with 2 ml of serum free media. (The second ml was added to the second plate, in the corresponding well). Cells were incubated with DNA-liposome complexes for 5 hours at 37°C in a 7% CO_2 incubator. Following incubation, 1 ml of growth medium containing twice the normal concentration of serum was added without removing the transfection mixture. This medium was then replaced with fresh complete medium (DMEM, 10% FBS and 2mM L-glutamine) 21 hours after transfection. 72 hours after transfection, the cells were counted for viability using Trypan blue. The plate seeded at 1 x 10^5/well was overgrown and therefore not used. The plate seeded at 5 x 10^4/ well had healthy cells and were passaged into selective media (complete media containing 600 μg/ml Geneticin). Media was replaced as necessary. As clumps or colonies appeared, they were removed using a 1-ml pipette by gentle scraping and each clump was placed in a new well of a 6 well plate. The colonies that were removed from each well were grown up as separate lines. Although there is no assurance that the colonies arose from a single cell, they were likely only a few cells at most. The colony from the first well of the original 6 well plate was renamed A1.1, the colony from the second well of the original 6 well plate was renamed A2.1, etc. After 2 days, the transfectant A5.1 was outgrowing the well and was transferred to a 100-cm tissue culture dish. The original well represented by A5.1 used 2 μg of DNA and 8 μl of LIPOFECTAMINE™ reagent. Once there was a sufficient number of cells (about 1 x 10^6 cells), A5.1 was investigated for MUC1 expression by immunoperoxidase staining as described in **CHAPTER II** and is shown in Figure 3-1. (Other colonies were analyzed however, A5.1 was the first because it grew the fastest).

C) Selection of transfectants for use in model

The transfectant A5.1 was stained and analyzed by FACS as described in **CHAPTER II.** There was a significant proportion of MUC1-negative cells as shown in Figure 3-2 and therefore the MUC1-positive cells needed to be sorted out. The cells were sorted according to their surface expression of MUC1. A gate on the top 10% of the positive population were sorted out as high MUC1 expressers and a gate on the lower 10% of the positive population were sorted out as low MUC1 expressers (Figure 3-2). The high and low MUC1 expressing cells were each collected in 6-50 ml tubes (previously blocked with a 5% BSA solution) containing 0.5 ml FBS. After centrifugation, the cells were combined (low and high separately) in 2 ml of selective media and plated in one well of a 6 well plate. 15,600 cells were collected for low and 22,000 cells were collected for high out of 300,000 cells sorted each time. The high expressers, A5.2H, were grown for 2 weeks and the cells were again stained and sorted using a gate on the top 5%. The newly sorted cells, A5.3H, were plated at a concentration of 0.3 cells per well in a 96 well plate to develop single cell clones. The low expressers, A5.2L, were sorted twice more (A5.3L, A5.4L), using a gate on the lowest 5% of the positive population each time and A5.4L was cloned out as for the high expressers. Several clones appeared for each of the high and low MUC1 transfectants and these were tested for stability of cell surface MUC1 expression, along with the original lines from each FACSort step, by growing in side by side cultures in selective and non-selective media for two months. Clone 4 of the high MUC1 expressers (A5.3H.4) remained stable for the two month period and maintained a high mean

fluorescence intensity. This clone was chosen for all further studies and is herein referred to as GZHi (Figure 3-3). A5.4L of the low expressers remained stable for the two month period and was more stable with respect to mean fluorescence intensity than any of the clones. This line was chosen for all further studies and is herein referred to as GZLo (Figure 3-3). On average, there is a 1.7 log difference in mean fluorescence intensity between GZHi and GZLo.

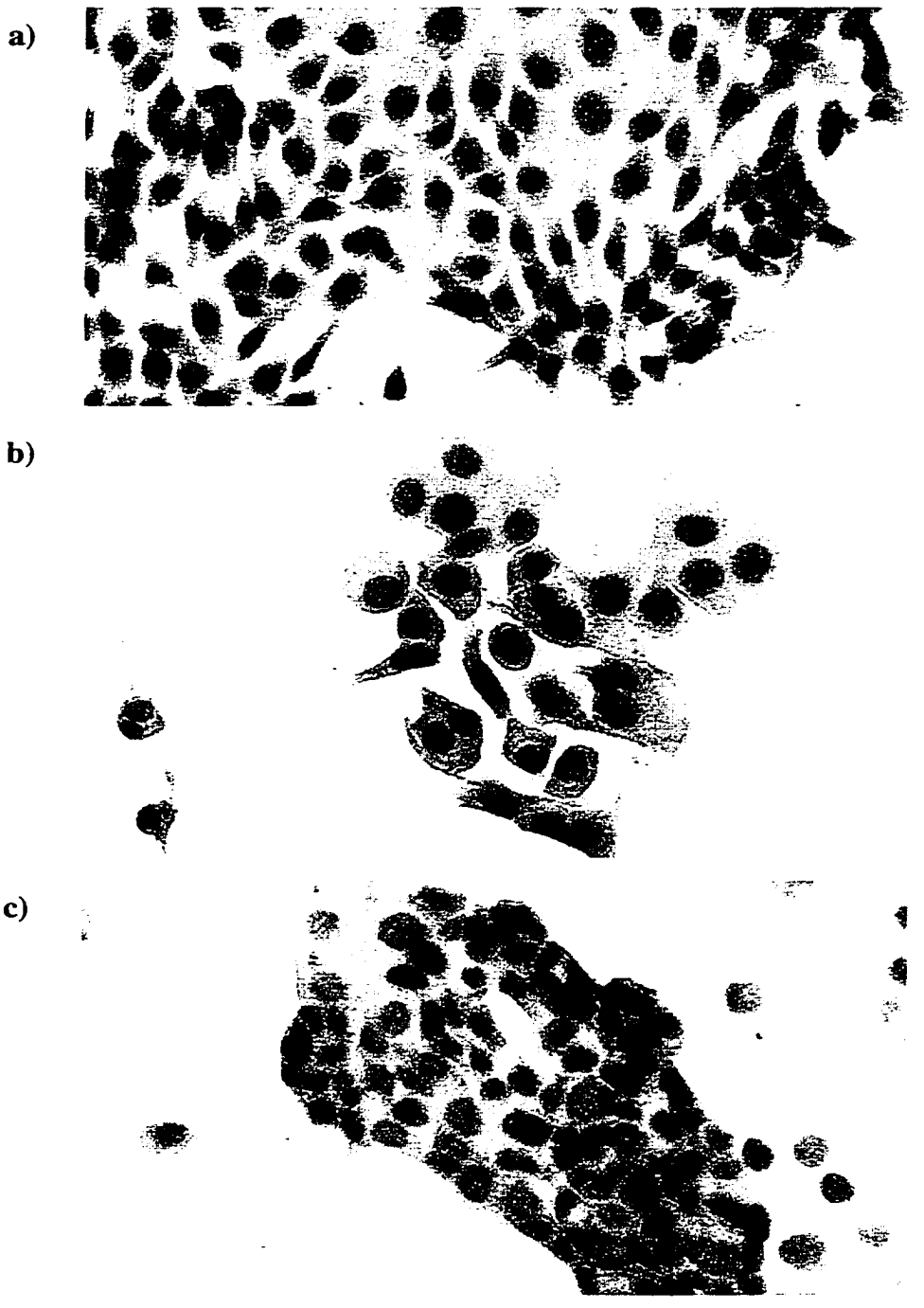


Figure 3-1: Immunoperoxidase staining of the MUC1 transfectant A5.1.

Transfectants were immunohistochemically stained with the anti-MUC1 mAb BCP8 as described in **CHAPTER II**. Briefly, cells were incubated with 100 µl antibody (10 µg/ml) for 1 hour followed by peroxidase-conjugated goat anti-mouse Ig antiserum (1:200) for 1 hour. Substrate (1 mg/ml DAB with 0.03% H_2O_2) was added for 5-8 minutes. Slides were washed and counterstained with hematoxylin and mounted with permount. a) 410.4 cells, b) A5.1 cells, and c) Positive control (CAPAN-1) cells.

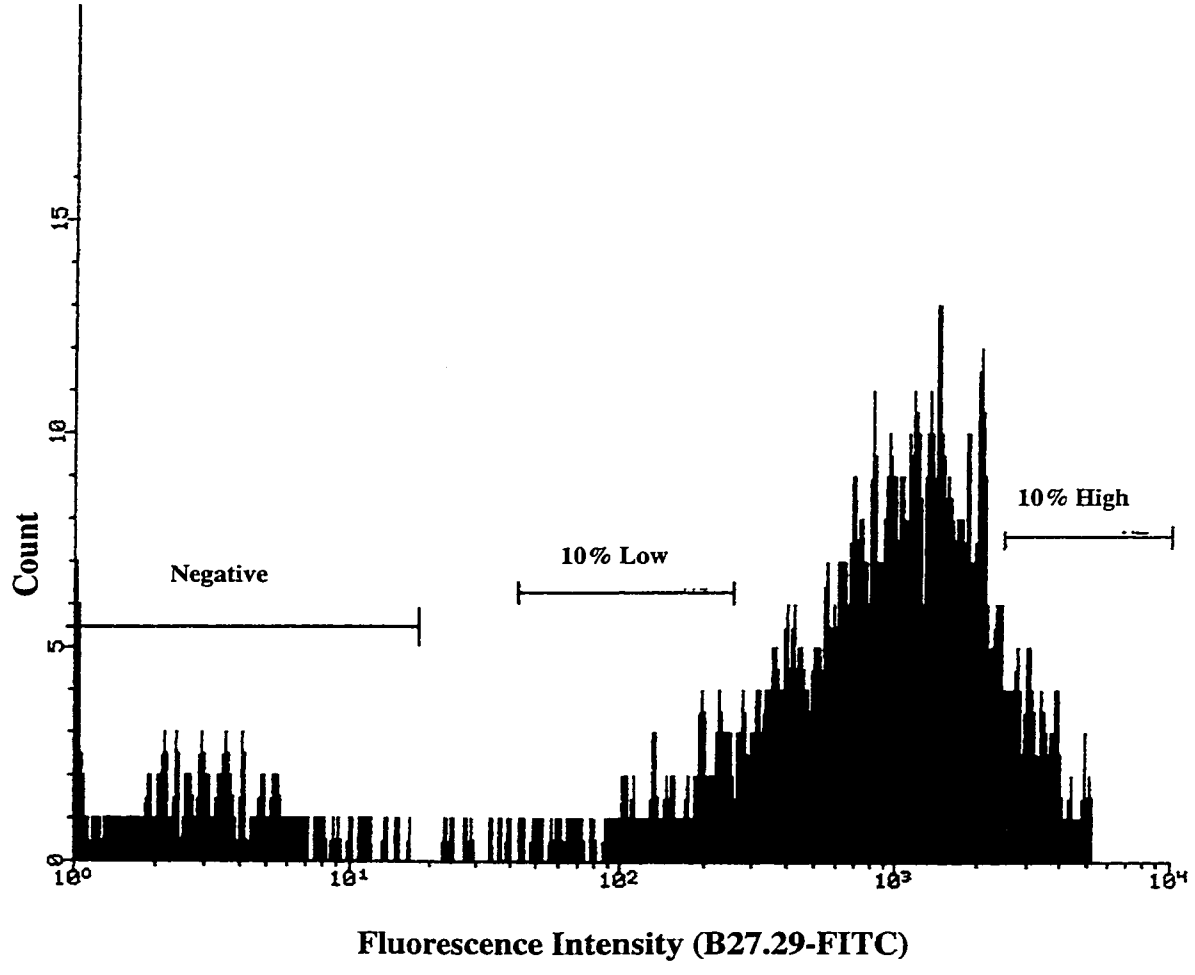


Figure 3-2: Cell surface MUC1 expression on the transfectant A5.1.

Cells were stained with the anti-MUC1 mAb B27.29 and analyzed by FACS as described in **CHAPTER II**. The MUC1-positive cells were sorted out by FACSort using an initial 10% gate on the high and 10% gate on the low ends (represented by the markers shown) resulting in two separate populations of cells, high MUC1 expressers and low MUC1 expressers. (Further sorts employed tighter gates of 5% of either high or low.)

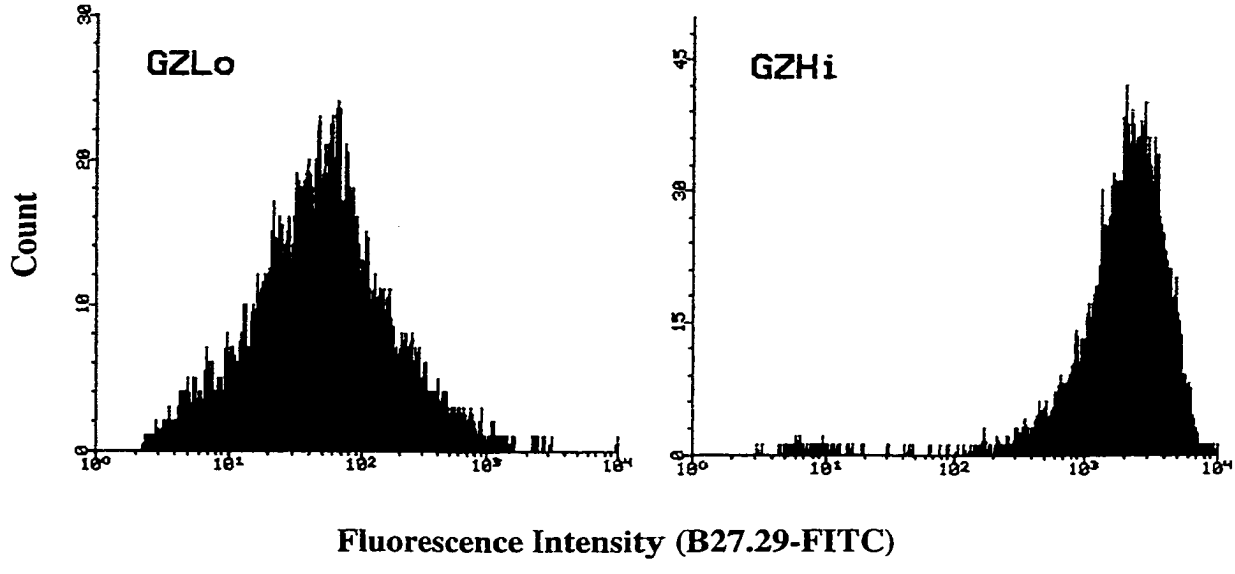


Figure 3-3: Cell surface expression of MUC1 on GZHi and GZLo after 2 months *in vitro*.

Transfectants were sorted for high and low expression of surface MUC1 and tested for stability *in vitro* in non-selective media for two months as outlined in **CHAPTER II**. The resultant stable high MUC1 expresser was named GZHi and the resultant stable low MUC1 expresser was named GZLo.

CHAPTER IV

Subcutaneous tumor models

A) Stability and lethality of transfected cell lines in vivo – Pilot study[1]

i) Survival and tumor growth

In order to test the stability and lethality of GZHi and GZLo *in vivo*, a pilot study was performed whereby a subcutaneous tumor model in CB6F1 mice (BALB/c x C57BL/6) was evaluated. Groups of five mice were injected subcutaneously in the inguinal area with 100 µl of either GZHi, GZLo or 410.4 in tumor cell doses of 1×10^5, 3×10^5, 1×10^6 and 3×10^6. Survival and tumor growth were monitored for two months (until day 63) at which time all remaining mice were terminated and examined by autopsy. Spleens and lymph nodes were removed and T cells isolated to use in T cell proliferation and CTL assays. However, there was high background and no MUC1 specific responses could be detected in either type of assay (data not shown), possibly due to the timing of the assays. There were no significant differences in the number of mice with tumors or tumor growth among the various doses during this 9-week time frame therefore all four doses were grouped together (for each cell line) for analysis. These preliminary results showed that surprisingly, GZLo injected mice started dying earlier than mice injected with either GZHi or the parental 410.4 tumor cells (Figure 4-1).

[1] These results were presented in an abstract at the 9th International Congress of Immunology in San Francisco, July, 1995

However, there was no significant difference in tumor area between GZHi and GZLo injected mice (Figure 4-2). GZLo injected mice had significantly smaller tumor areas than 410.4 injected mice ($p < 0.05$) from days 14-35&56 despite having shorter survival. GZHi groups had significantly smaller tumor area than 410.4 groups ($p < 0.01$) from days 21-56 although survival was similar (Figure 4-2).

Lower tumor area for the transfected cell lines was not unexpected since they carry a foreign protein and this type of rejection has been reported [Lalani E-N, et al., 1991]. However, despite the presence of a foreign tumor antigen, GZLo challenged mice had a shorter survival time than parental 410.4 challenged mice while GZHi challenged mice, also with lower tumor area than parental cells, did not differ in survival from 410.4 challenged mice.

ii) MUC1 expression by transfectants in vivo

A possible reason for the difference in survival between GZHi and GZLo groups became apparent when examining the cell surface expression of MUC1 on tumors removed from either GZHi or GZLo injected mice. Tumors or metastases removed from mice injected with GZHi cells were found to have a high percentage of MUC1 negative cells compared to those found in mice injected with GZLo cells (Figure 4-3). This result is compatible with the hypothesis that GZHi cells induced an anti-MUC1 immune response leading to elimination of $MUC1^+$ cells and the outgrowth of $MUC1^-$ cells. Higher levels of MUC1 mucin could be detected in the serum of the mice that died before day 63 (8 GZLo challenged mice and 1 GZHi challenged mouse) compared to those that

survived to day 63 (752 ± 94 cpm versus 581 ± 19 cpm, respectively; unpaired T-test, p = 0.0073). Of the nine mice that died before day 63, the 4 mice with the highest serum MUC1 mucin levels had shorter survival than the 5 mice with lower serum MUC1 levels (Figure 4-4; Logrank, p = 0.0116).

The results from this preliminary study indicated that both the presence of cell surface MUC1 on established tumors plus the presence of soluble MUC1 mucin in the serum correlated with decreased survival.

B) Elaboration of tumor model in CB6F1 mice

Based on the data from the pilot study, a long term repeat study was performed using larger experimental groups to expand on and verify the differences between GZHi and GZLo with respect to MUC1 expression *in vivo*, in addition to determining the suitability of GZLo as a tumor model.

i) Survival and tumor growth

Groups of ten CB6F1 mice were injected subcutaneously in the inguinal area with 100 µl of either GZHi or GZLo tumor cells at doses of 3 x 10^4, 1 x 10^5, 3 x 10^5 and 1 x 10^6. The cell surface expression levels of MUC1 on GZHi and GZLo at the time of injection are shown in Figure 4-5. The parental cell line, 410.4, was injected at a dose of 1 x 10^6 as a control. In agreement with the results of the pilot study, mice challenged with GZLo cells had significantly decreased survival compared to mice challenged with

GZHi cells at the three highest doses (Figure 4-6; Logrank, $1x10^5$, $p = 0.0312$; $3x10^5$, $p < 0.0001$; $1x10^6$, $p = 0.0006$). Survival of mice challenged with 410.4 did not differ significantly from survival of mice challenged with GZLo at a dose of 1×10^6 in this study. In the pilot study at a dose of 1×10^6, 3 out of 5 mice injected with GZLo cells survived to day 63, whereas 4 out of 5 mice injected with 410.4 cells survived to day 63.

Tumor growth was monitored weekly and reported as a bidimensional measure. Figure 4-7 shows the tumor growth for each of the cell lines at all four doses in CB6F1 mice. There were no statistically significant differences in tumor area between GZHi and GZLo in CB6F1 mice except at day 70 with the dose of 3×10^5 and day 77 with the dose of 3×10^4 ($p = 0.0402$ for both). In addition, at a dose of 1×10^6, there was no significant difference in tumor area between the transfectants and the parental cell line.

ii) Tumor cell metastasis

Complete autopsies were performed at the time of death to attempt to evaluate the extent of metastasis of the tumor cells. It was very difficult to quantify metastases, however, there were no obvious differences in the location or extent of grossly detectable metastases among the groups at the time of death. All tumors, regardless of dose or cell line, metastasized extensively to several organs including but not limited to spleen, lymph nodes (most notably inguinal, axillary and mesenteric), liver, lungs, kidneys, adrenal glands, ovaries, pancreas and thymus. Not all organs were involved in all mice and each mouse had varying levels of involvement of each organ. Histological examination of samples verified the presence of tumor cells. There was no correlation between the

number of grossly evaluable metastases and tumor area at death or number of metastases and survival.

iii) Cell surface expression of MUC1 on established tumors and metastases

Samples of the primary tumor, spleen and one of the largest metastases were taken at the time of death from each mouse and the tumor cells isolated in culture to be examined for cell surface expression of MUC1. The cells were immunohistochemically stained with anti-MUC1 mAb B27.29 and examined by FACS as described in **CHAPTER II**. Of the 14 mice examined from the groups challenged with GZHi tumor cells, 11 mice developed tumors which no longer expressed detectable cell surface MUC1 in either primary tumor or metastases or both (mouse #1, 4, 5, 6, 7, 8, 10, 11, 12, 13 and 14; Figure 4-8). Approximately 84% of primary tumor cells remained positive for cell surface MUC1 in two mice (mouse #2 and 9), but with less than half the mean fluorescence intensity (MFI) of GZHi cells grown *in vitro* (337 and 352, respectively, compared to 717). One mouse (#3) had only 32% MUC1 positive cells with a MFI of 1001. Metastases from the 3 mice with $MUC1^+$ primary tumors also showed low expression of MUC1 (mouse #2, 25% positive at MFI of 185; #3, 61% positive at MFI of 479; #9, 72% positive at MFI of 463). Of the 19 mice examined from the groups injected with GZLo cells, 16 mice retained cell surface MUC1 expression on more than 73% (average 92%) of the tumor cells from either the primary tumor or metastases or both, with MFI's ranging from 132-738 (GZLo grown *in vitro* compared at 154). Two mice (mouse #18 and 27) from the GZLo groups lost expression of MUC1 on the tumor cells,

and the remaining mouse (#32) expressed very low MUC1 levels on approximately 25% of the tumor cells.

Samples of primary tumor were also fixed in 10% formalin at the time of autopsy and processed as described in **CHAPTER II.** Tumor samples were then immunohistochemically stained with the anti-MUC1 mAb, B27.29, to verify expression of MUC1 on the tumors *in situ.* As shown in Figure 4-9, GZHi tumor cells were negative for cell surface MUC1 expression at the time of autopsy, while GZLo tumor cells were clearly positive.

iv) Serum MUC1 mucin levels

The serum from the final bleed of each mouse at autopsy was tested for MUC1 mucin levels as described in **CHAPTER II.** For comparison, cell culture supernatants from the cell lines grown *in vitro* were also tested for soluble MUC1 mucin. GZHi cultures secreted approximately 10-fold more soluble MUC1 mucin than GZLo cultures (Table 4-1). Levels of serum MUC1 mucin at the time of death were significantly higher in GZLo challenged CB6F1 mice compared to GZHi challenged mice ($p < 0.0001$) as seen in Figure 4-10. The serum MUC1 mucin levels started to rise anywhere from 1 to 2 weeks prior to death in most of the GZLo challenged mice, but only one of the GZHi challenged mice had a rising serum MUC1 level before death (Figure 4-11). There was no correlation between serum MUC1 mucin levels and subcutaneous tumor area ($p = 0.91$).

As seen in Figure 4-11, the vast majority of the mice challenged with 10^6 or $3x10^5$ GZLo tumor cells had rising serum MUC1 levels. In mice challenged with $3x10^4$ or 10^5 GZLo tumor cells (low dose groups) the 7 (out of 20 mice) which had rising serum MUC1 levels had shorter survivals compared to the 13 mice which had no detectable serum MUC1 levels (Figure 4-12, p = 0.0031, Logrank test).

v) Anti-MUC1 antibody titres

Anti-MUC1 antibody titres were measured to see if antibody concentration correlated with tumor progression or survival and if a certain isotype would prevail. Serum from the final bleed of each mouse at autopsy was tested for anti-MUC1 antibody titres of both the IgG_1 and IgG_{2a} isotypes using a standard ELISA method as described in **CHAPTER II**. Anti-MUC1 IgG_1 levels were higher than IgG_{2a}, in all groups, although the two were directly correlated to each other (p = 0.0002; Table 4-2). Generally anti-MUC1 antibody levels were directly proportional to the dose of tumor cells inoculated (IgG_1: GZHi p = 0.0468, GZLo p = 0.0328; IgG_{2a}: GZHi p = 0.6389, GZLo p = 0.0034; Table 4-2). Overall there were equivalent titres of anti-MUC1 antibodies in GZHi and GZLo injected mice (unpaired t-test: IgG_1, p = 0.7974 and IgG_{2a}, p = 0.4532).

In GZHi challenged mice there was no correlation between anti-MUC1 antibodies and survival (IgG_1, p = 0.1113 and IgG_{2a}, p = 0.1079). However, in GZLo challenged mice there was a significant correlation between high levels of anti-MUC1 antibodies and decreased survival (p < 0.0001 for both isotypes) in those mice with low serum MUC1 levels, while GZLo challenged mice with rising serum MUC1 levels had no correlation

between anti-MUC1 antibody titres and survival (Figure 4-13). At the time of death there was a significant positive correlation between tumor area and level of IgG_1 anti-MUC1 antibody in both GZHi and GZLo challenged mice (GZHi, $p = 0.0031$ and GZLo, $p < 0.0001$). Higher levels of IgG_{2a} anti-MUC1 antibody correlated with larger tumor area at death in GZLo challenged mice only ($p < 0.0001$, GZHi, $p = 0.1318$).

Serum antibodies were also tested for capacity to bind to MUC1 mucin on transfected cells. Anti-MUC1 antibody (reactive to MUC1 on GZHi cells) in sera of mice was detected as early as 2 weeks post tumor challenge at a 1/25 dilution (Figure 4-14). There was no significant difference in antibody binding to MUC1 on GZHi cells of mouse sera from GZHi versus GZLo tumor challenged groups.

The results from this extended study verified results of the pilot in that, a) mice challenged with GZLo cells had shorter survival than GZHi challenged mice, b) GZLo cells maintained their level of cell surface MUC1 expression *in vivo* while GZHi cells did not, and c) GZLo challenged mice had high levels of serum MUC1 which correlated with decreased survival while GZHi challenged mice did not have detectable levels of serum MUC1. In addition, there was no correlation between tumor area or metastases and survival.

C) SCID-BEIGE mouse model to investigate differences between GZHi and GZLo

i) Survival and tumor growth

To evaluate the role of the immune response in the growth of GZHi and GZLo tumors, survival and tumor growth were examined in SCID-BEIGE mice. Groups of 10 SCID-BEIGE mice were injected subcutaneously in the inguinal area with 10^5 GZHi, GZLo or 410.4 cells. Figure 4-15 shows that GZLo injected SCID-BEIGE mice had the shortest survival compared to GZHi and 410.4 groups ($p < 0.0001$, Logrank test). GZHi injected mice had the longest survival, although not significantly longer than 410.4 challenged mice, and the smallest tumor area as seen in Figure 4-16.

ii) MUC1 expression by tumor cells in SCID-BEIGE mice

Samples of the primary tumor and one of the largest metastases were taken at the time of death from each mouse and the tumor cells were isolated in culture to be examined for cell surface expression of MUC1 as outlined in Section B)iii). In SCID-BEIGE mice both GZHi and GZLo cell lines retained cell surface expression of MUC1 on all tumor cells which was comparable to their respective *in vitro* expression levels (Figure 4-17). The serum from the final bleed of each mouse at autopsy was also tested for MUC1 mucin levels as described in **CHAPTER II.** SCID-BEIGE mice injected with GZHi cells had high levels of MUC1 mucin in their serum as shown in Table 4-3. These

levels were found to be 10-fold higher than those detected in the serum of SCID-BEIGE mice injected with GZLo cells, which is comparable to the difference seen *in vitro* (Table 4-1).

These results indicated that GZHi cells, in the absence of an immune response, could retain cell surface MUC1 expression and could produce high levels of serum MUC1 *in vivo*. However, GZLo cells with a lower level of both cell surface and serum MUC1 remain more lethal than GZHi under the same conditions indicating that MUC1 mucin may not be the only contributor to decreased survival in mice challenged with GZLo cells.

D) Investigation of immune modulation by MUC1 mucin in vivo

In the pilot study, GZLo injected CB6F1 mice, challenged with a range of tumor cell doses, died earlier than parental 410.4 injected mice. At the tumor cell dose of 1 x 10^6 in the pilot study, 4 out of 5 410.4 challenged CB6F1 mice survived to day 63, while 3 out of 5 GZLo challenged mice survived to day 63. In the repeat study at a tumor cell dose of 1 x 10^6, both GZLo and 410.4 injected mice appeared to die at the same rate in semisyngeneic, CB6F1 mice. In order to further investigate the differences between $MUC1^+$ and $MUC1^-$ tumor cell lines in tumor progression and host survival, syngeneic, C.B-17 mice were used in comparison to C.B–17 SCID-BEIGE and C.B-17 SCID mice. This might allow investigation of possible immune modulation by MUC1 mucin produced on or secreted by GZLo cells.

i) Survival and tumor growth

Groups of ten C.B-17, SCID or SCID-BEIGE mice were injected subcutaneously in the inguinal area with 10^5 GZLo or 410.4 cells. In all three strains of mice, those that were challenged with GZLo cells had a shorter survival than those challenged with 410.4 cells (Figure 4-18). In addition, as expected SCID and SCID-BEIGE mice challenged with either GZLo or 410.4 had significantly shorter survival than C.B-17 challenged mice (Figure 4-18). Table 4-4 outlines the differences in median survival among these strains. Most notably there was a slight but statistically significant difference in median survival between SCID and SCID-BEIGE mice challenged with GZLo ($p = 0.0421$), but not 410.4 cells ($p = 0.2429$).

Tumor growth was monitored as outlined above in Section B) i). Figure 4-19 shows the tumor growth for both GZLo and 410.4 in all three strains of mice. There were no significant differences in tumor growth between GZLo and 410.4 in normal, C.B-17 mice. In SCID mice there was a statistically significant difference in tumor area between GZLo and 410.4 only on days 14 ($p = 0.0142$) and 35 ($p = 0.0373$) and in SCID-BEIGE mice on day 21 ($p = 0.0099$).

Differences in tumor area between SCID and SCID-BEIGE mice challenged with GZLo cells only occurred on days 14 ($p = 0.0142$) and 28 ($p = 0.0039$). There were no significant differences in tumor growth between SCID and SCID-BEIGE mice challenged with 410.4 cells.

ii) MUC1 expression in vivo

Samples of the primary tumor and one of the largest metastases were taken at the time of death from each mouse and the tumor cells isolated in culture to be examined for cell surface expression of MUC1 as outlined in Section B)iii). All tumor cells taken from any of the three strains challenged with GZLo cells expressed cell surface MUC1 comparable to *in vitro* expression levels (Figure 4-20). There were no significant differences in cell surface MUC1 expression on GZLo cells between the strains. Similar levels of serum MUC1 mucin were found in all three strains of mice challenged with GZLo cells (Table 4-5). In addition, rising levels of serum MUC1 approximately 1 to 2 weeks prior to death could be found in all mice challenged with GZLo cells (Figure 4-21).

GZLo challenged C.B-17 mice have significantly shorter survival than 410.4 challenged C.B-17 mice supporting the hypothesis that MUC1 contributes to decreased survival. Additionally, in the absence of an immune response in SCID-BEIGE and SCID mice, GZLo challenged mice have shorter survival than 410.4 challenged mice indicating a role for MUC1 in tumor progression that is non-immune related. The fact that SCID mice challenged with GZLo cells, but not 410.4 cells, had significantly longer median survival than similarly challenged SCID-BEIGE mice suggests that MUC1 expressed by GZLo cells may interact with NK cells resulting in the inhibition of growth of $MUC1^+$ tumors.

Table 4-1: Cell culture supernatant soluble MUC1 mucin levels

	Tumor Cells in Culture		
	GZHi	**GZLo**	**410.4**
Day 1	0.040 ± 0.001	0.009 ± 0.001	NT
Day 2	0.102 ± 0.002	0.016 ± 0.002	NT
Day 3	0.419 ± 0.003	0.045 ± 0.001	0.010 ± 0.001
Day 4	0.822 ± 0.010	0.097 ± 0.006	0.009 ± 0.001

Values are mean Units/ml of three separate cultures ± SEM. All cultures were started at $5x10^4$ cells/ml on day 0. Supernatant samples (500 µl) were taken each day from separate cultures; NT = not tested.

Table 4-2: Anti-MUC1 antibody titres in CB6F1 mice

Tumor Cell Line	Tumor Cell Dose	IgG_1 (Log_2 titre)		IgG_{2a} (Log_2 titre)		n
		Mean	SEM	Mean	SEM	
GZHi	1×10^6	14.7	1.51	8.1	1.47	7
	3×10^5	9.9	2.00	6.0	1.81	10
	1×10^5	10.8	1.45	5.4	1.77	8
	3×10^4	10.4	1.60	6.8	1.49	10
GZLo	1×10^6	15.7	0.48	11.7	0.72	7
	3×10^5	15.1	0.53	11.7	0.76	10
	1×10^5	10.4	2.94	7.2	1.64	5
	3×10^4	7.5	1.86	4.8	1.60	9
410.4	1×10^6	6.8	0.94	Not detectable		8

Serum samples were obtained at the time of death and frozen until tested. Sera were serially diluted and antibody was detected using standard ELISA as described in **CHAPTER II**.

Table 4-3: Serum MUC1 mucin levels in SCID-BEIGE mice

Group	Units/ml	# mice
GZHi	50.26 ± 8.98	20
GZLo	5.06 ± 1.17	15
410.4	0.59 ± 0.07	17

Values are mean Units/ml ± SEM for each group. Serum samples were obtained at the time of death and frozen until tested.

Table 4-4: Summary table of median survival (in days)

Cell line	Strain of mice		
	C.B-17	**SCID-BEIGE**	**SCID**
410.4	$68^{a,b}$	$37^{a,c}$	$40^{b,c}$
GZLo	$41^{d,e}$	$28^{d,f}$	$32^{e,f}$

^{a,b,d,e} $p < 0.0001$; ^{c} $p = 0.2429$; ^{f} $p = 0.0421$, $n = 10$

Table 4-5: Serum MUC1 mucin levels in C.B-17, SCID and SCID-BEIGE mice

	GZLo groups	
Strain	**Units/ml**	**# mice**
SCID	4.795 ± 1.242	10
SCID-BEIGE	4.292 ± 1.235	10
C.B-17	7.021 ± 1.500	10

Values are mean Units/ml ± SEM. Serum samples were obtained at the time of death and frozen until tested. Unpaired T-tests: SCID vs C.B-17, $p = 0.265$; SCID-BEIGE vs C.B-17, $p = 0.177$, SCID vs SCID-BEIGE, $p = 0.776$

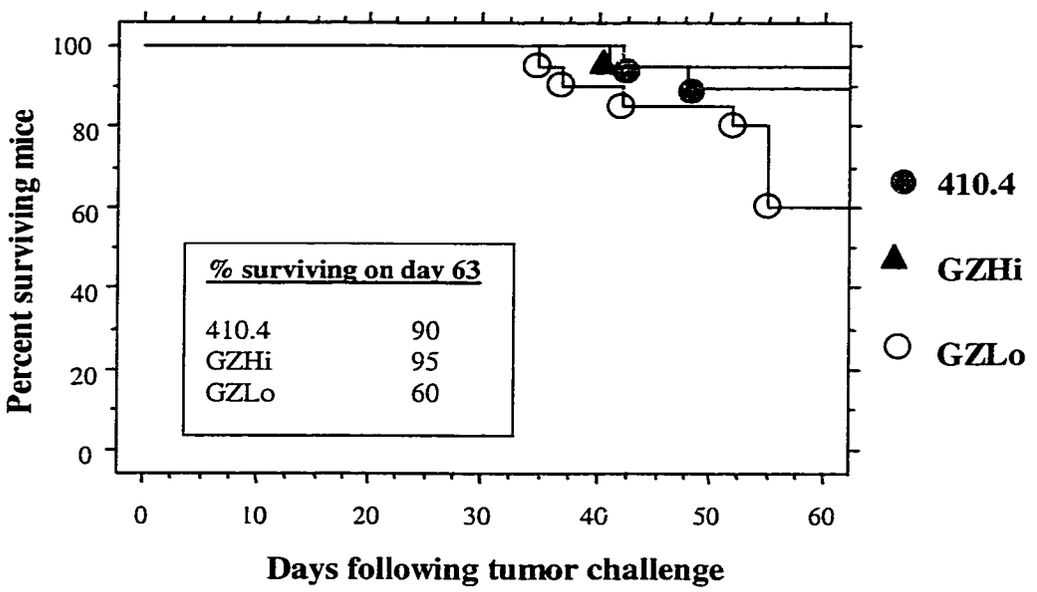


Figure 4-1: Survival of mice challenged with GZHi and GZLo transfectants versus parental cell line.

Groups of 5, 8-week-old, female, CB6F1 mice were challenged subcutaneously with GZHi, GZLo or 410.4 cells (all doses combined, n=20). All remaining mice were terminated on day 63. (Logrank, p = 0.0116).

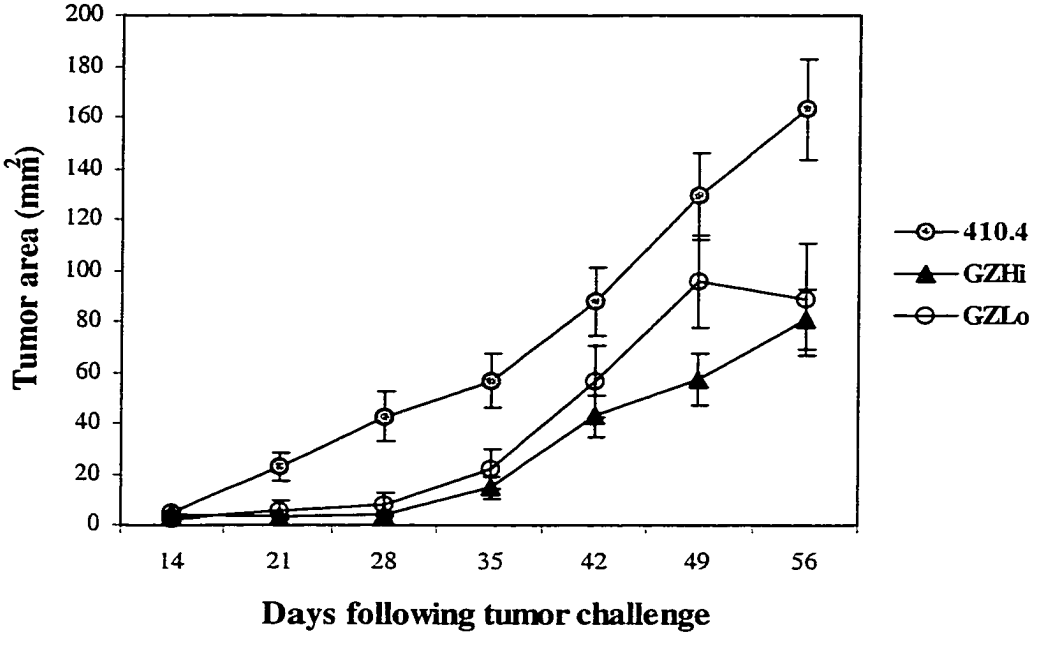


Figure 4-2: Tumor growth of transfectants versus parental cell line in CB6F1 mice.

Weekly bidimensional tumor measurements were taken for each mouse. Values are mean tumor area ± SEM for each cell line (all doses combined for each cell line, n=20 until day 35, when GZLo started dying). GZHi and GZLo groups are not significantly different for any day; GZLo and 410.4 groups are significantly different ($p < 0.05$) from days 14-35&56; GZHi and 410.4 groups are significantly different ($p < 0.01$) from days 21-56.

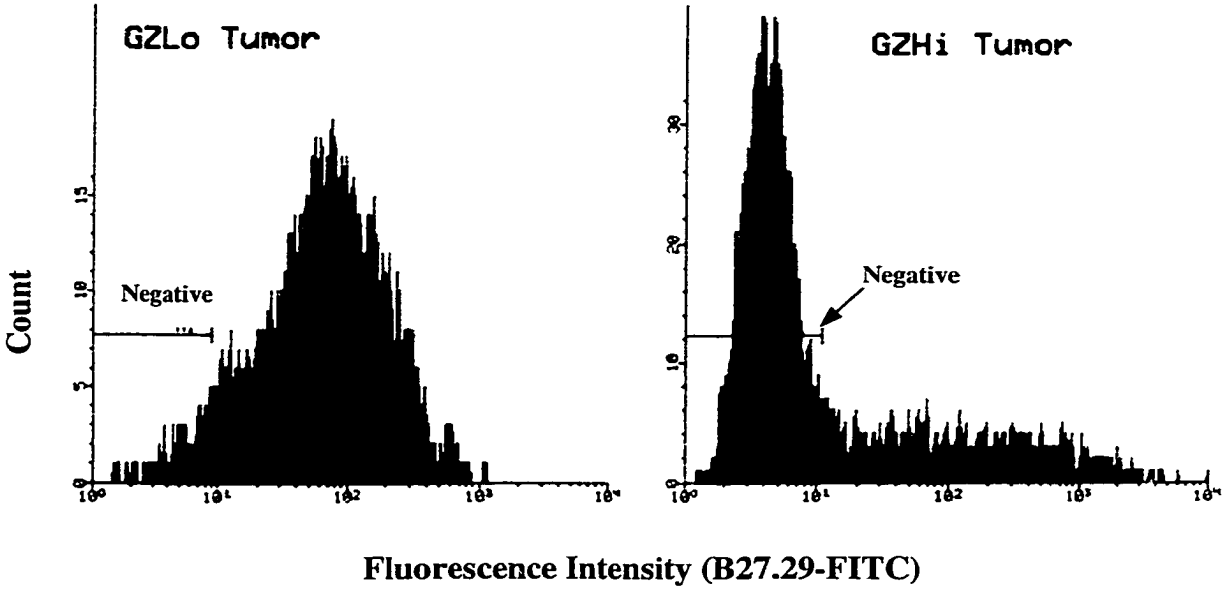


Figure 4-3: Cell surface MUC1 expression on transfectants after 2 months *in vivo.*

Samples of primary tumor and metastases were taken and stained with the anti-MUC1 mAb B27.29 as described in **CHAPTER II.** A representative example of a primary tumor from each group is shown. The marker (Negative) represents the background.

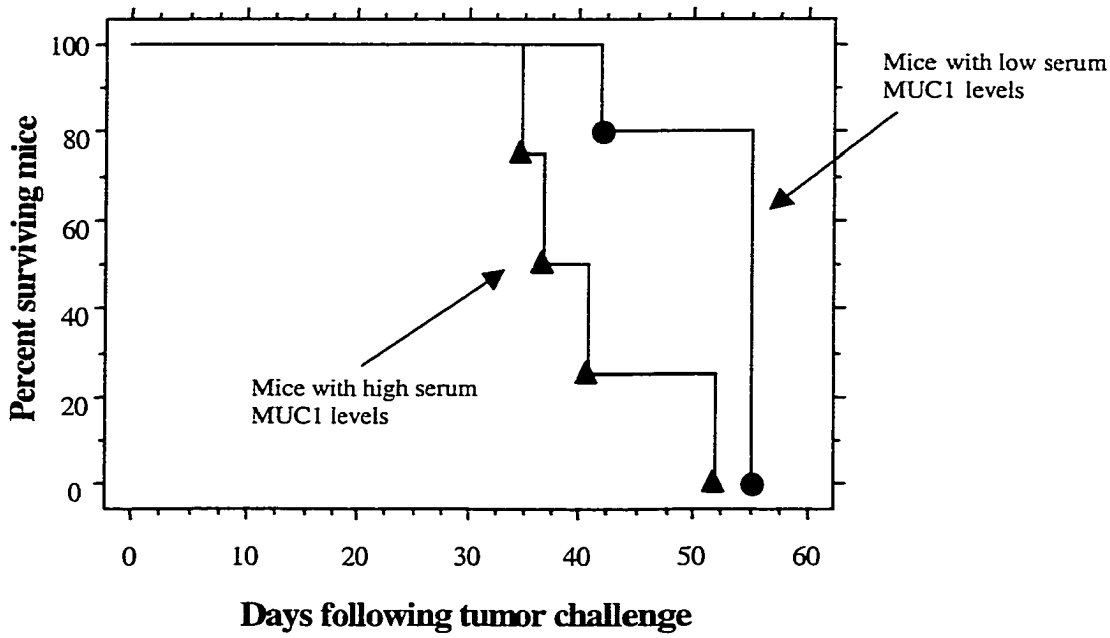


Figure 4-4: Presence of MUC1 mucin in the serum correlates with decreased survival.

Of the nine mice (injected with MUC1 transfected cells, 8 GZLo and 1 GZHi) that died before day 63, the day of sacrifice, the 4 mice with the highest levels of serum MUC1 died earlier than the 5 mice with lower serum MUC1 levels (Logrank, $p = 0.0108$).

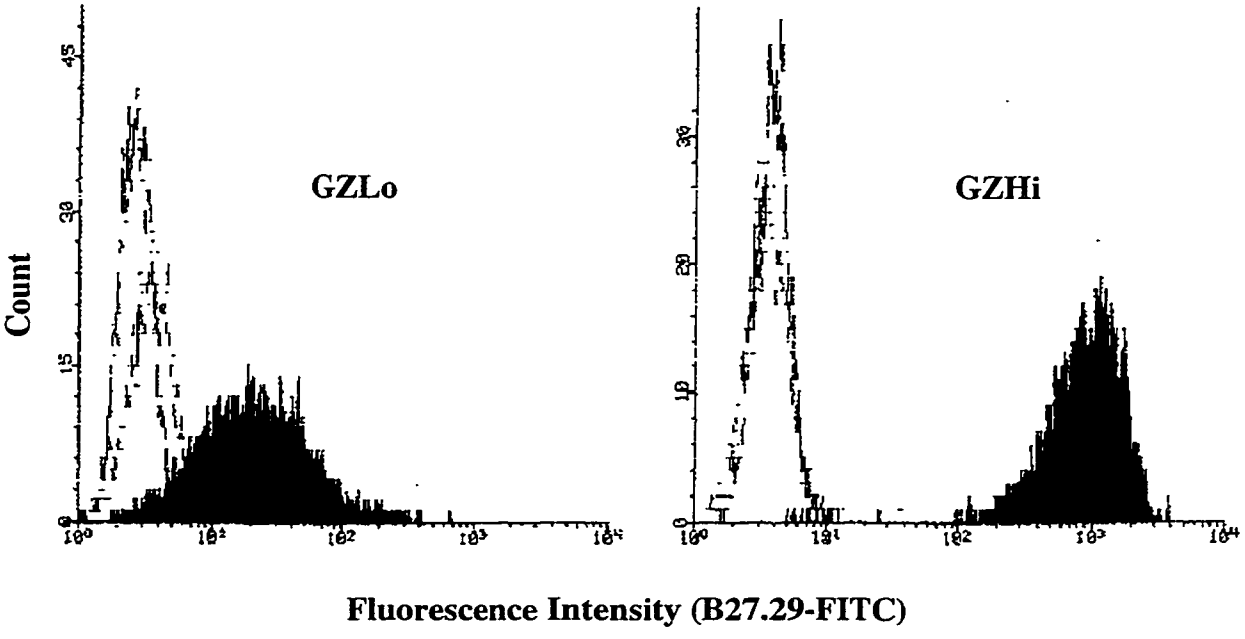


Figure 4-5: MUC1 expression on GZHi and GZLo at time of injection.

Cells were stained with the MUC1 specific antibody, B27.29 (shaded) and an isotype control (outline). Parental 410.4 shown stained with B27.29, as control (dotted outline).

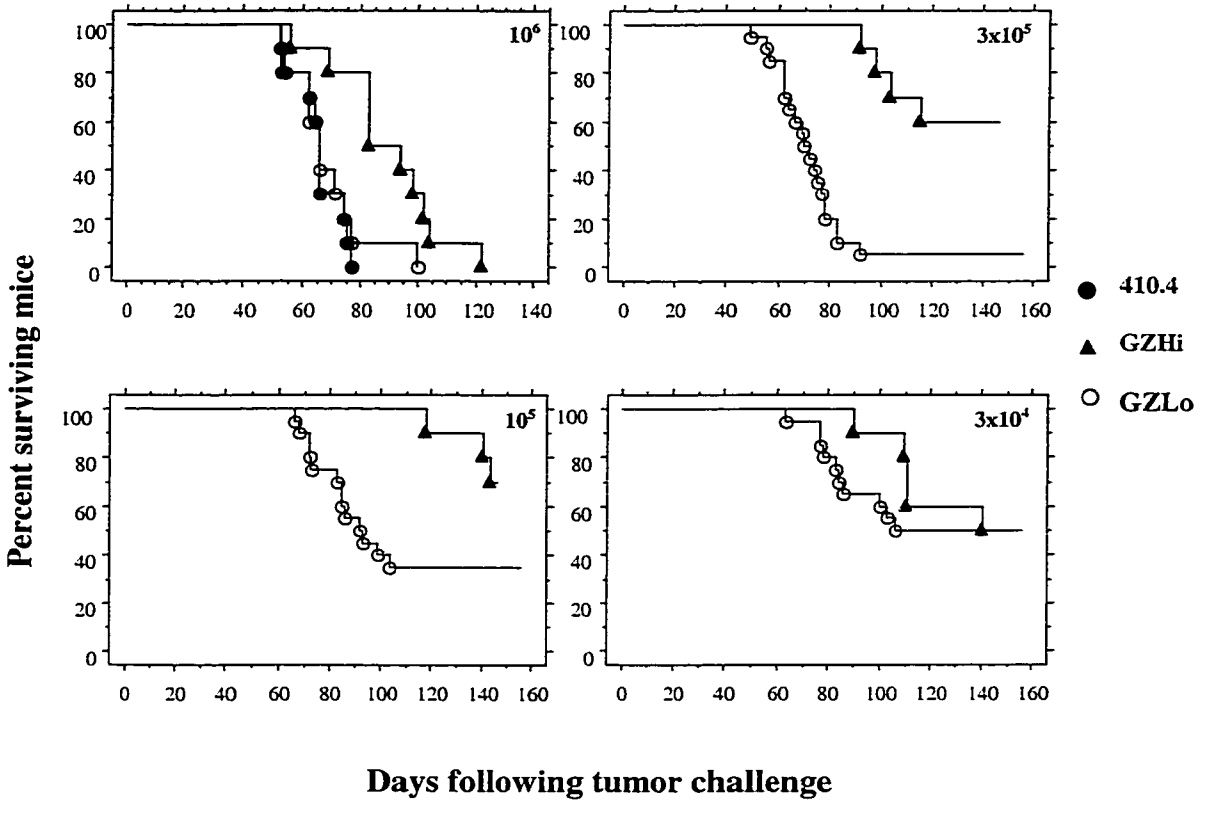


Figure 4-6: Survival of mice challenged with either GZHi or GZLo in CB6F1 mice.

Both GZHi and GZLo were tested in groups of ten, 8-week-old, female, CB6F1 mice over a range of four subcutaneous cell doses, $3x10^4$, $1x10^5$, $3x10^5$, $1x10^6$. 410.4 was used as a control at a dose of $1x10^6$. (Logrank, $3x10^4$, $p = 0.5751$; $1x10^5$, $p = 0.0312$; $3x10^5$, $p < 0.0001$; $1x10^6$, $p = 0.0006$.)

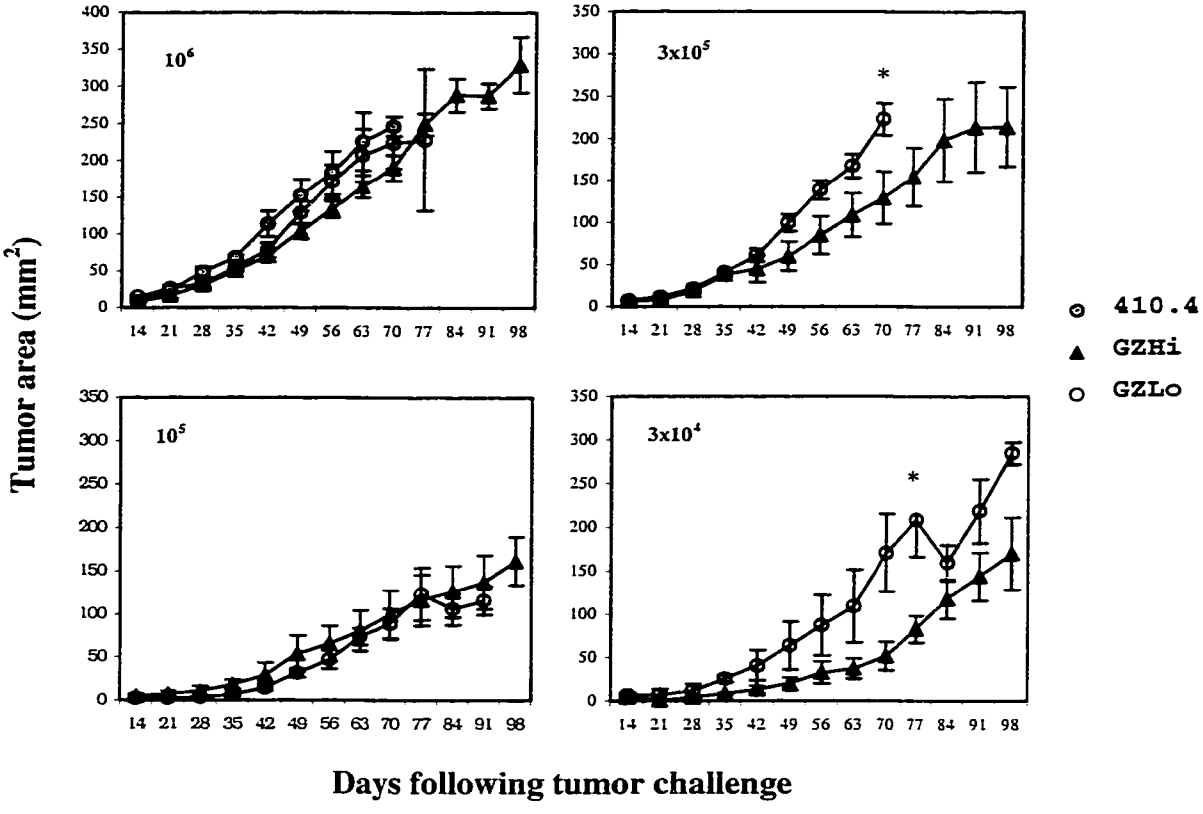
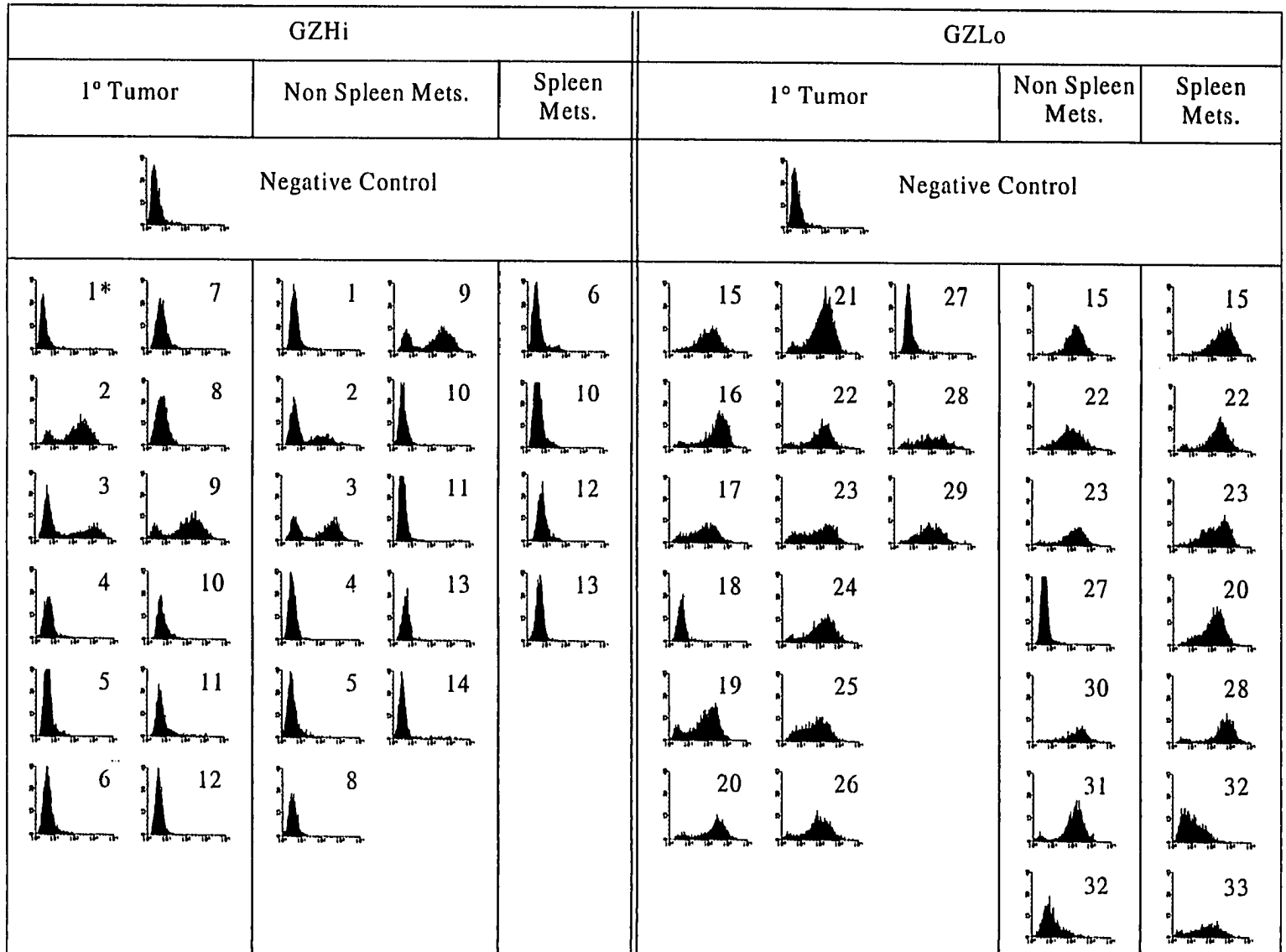


Figure 4-7: Tumor growth in CB6F1 mice.

Tumor area was measured weekly as a bidimensional measure in mm^2. Results are shown as mean ± SEM for each group (n = 10, until mice started dying). * p = 0.0402, GZHi versus GZLo.

Figure 4-8: Cell surface MUC1 expression on established tumors and metastases in CB6F1 mice.

Samples of primary tumor and metastases were cultured and indirectly stained with anti-MUC1 MAb B27.29 followed by FITC-labelled goat anti-mouse Ig as described in **CHAPTER II.** Samples are grouped in columns as 1° tumor, non-spleen metastases and spleen metastases, as well as an isotype control (first histogram). Each histogram is numbered according to the mouse from which it came and can be compared across columns.



* Mouse number.

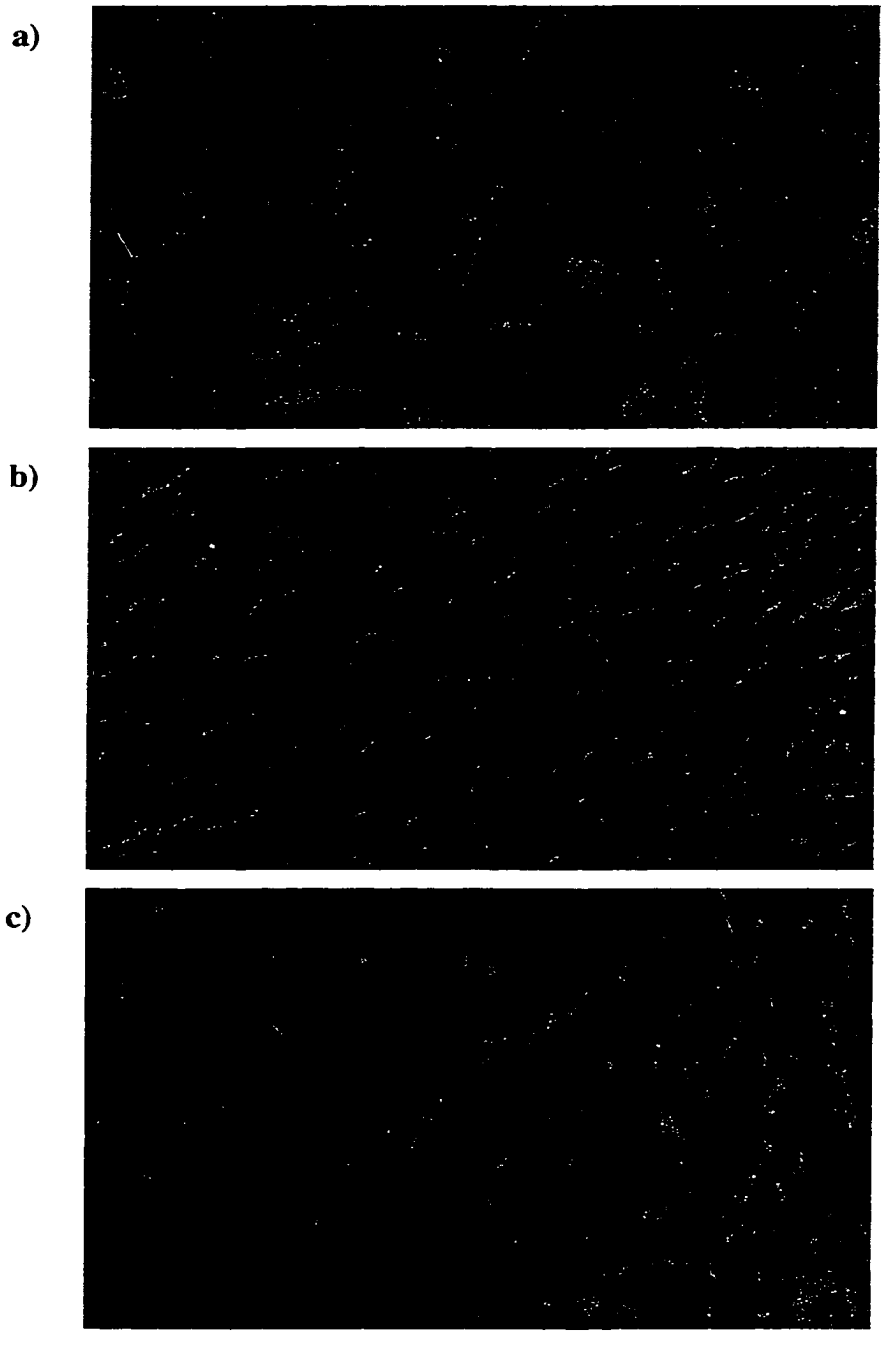


Figure 4-9: *In situ* expression of MUC1 on tumor cells.

Samples of primary tumor were fixed in 10% formalin at the time of death and were processed as described in **CHAPTER II.** Tumor samples were then immunohistochemically stained with the anti-MUC1 mAb, B27.29 (represented by dark pink) as described in Figure 3-1. A representative example is shown for each group: a) 410.4 challenged mouse, b) GZHi challenged mouse, and c) GZLo challenged mouse.

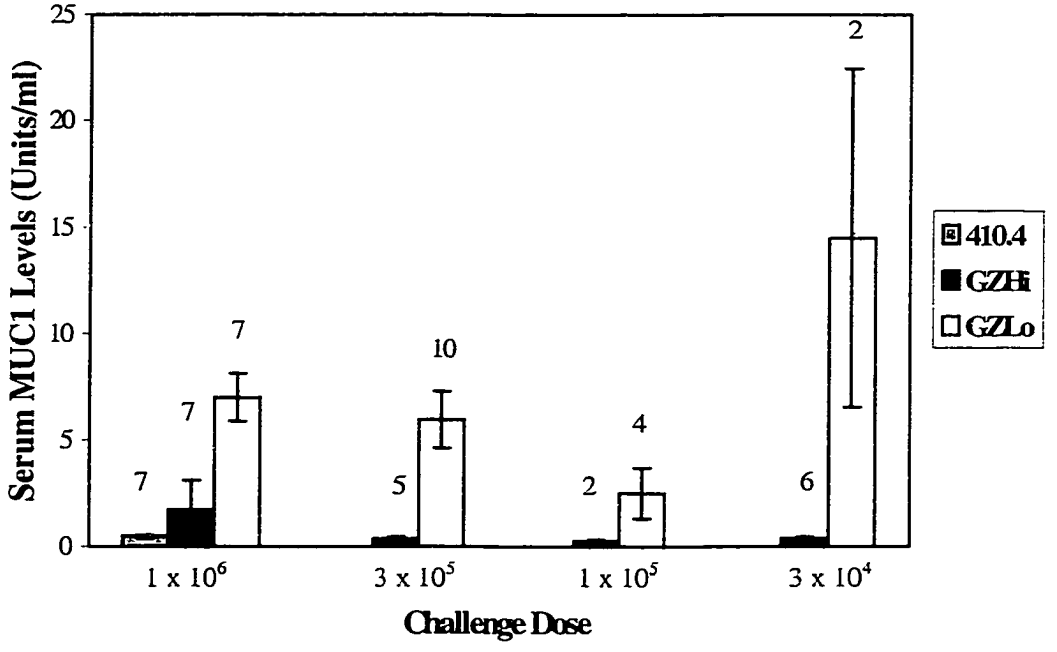


Figure 4-10: Serum MUC1 mucin antigen levels are higher in GZLo challenged CB6F1 mice.

Sera from the final bleed of each mouse at the time of death were analyzed by EIA. The number of mice tested in each group is shown above each bar. Values are mean Units/ml of MUC1 ± SEM for each group.

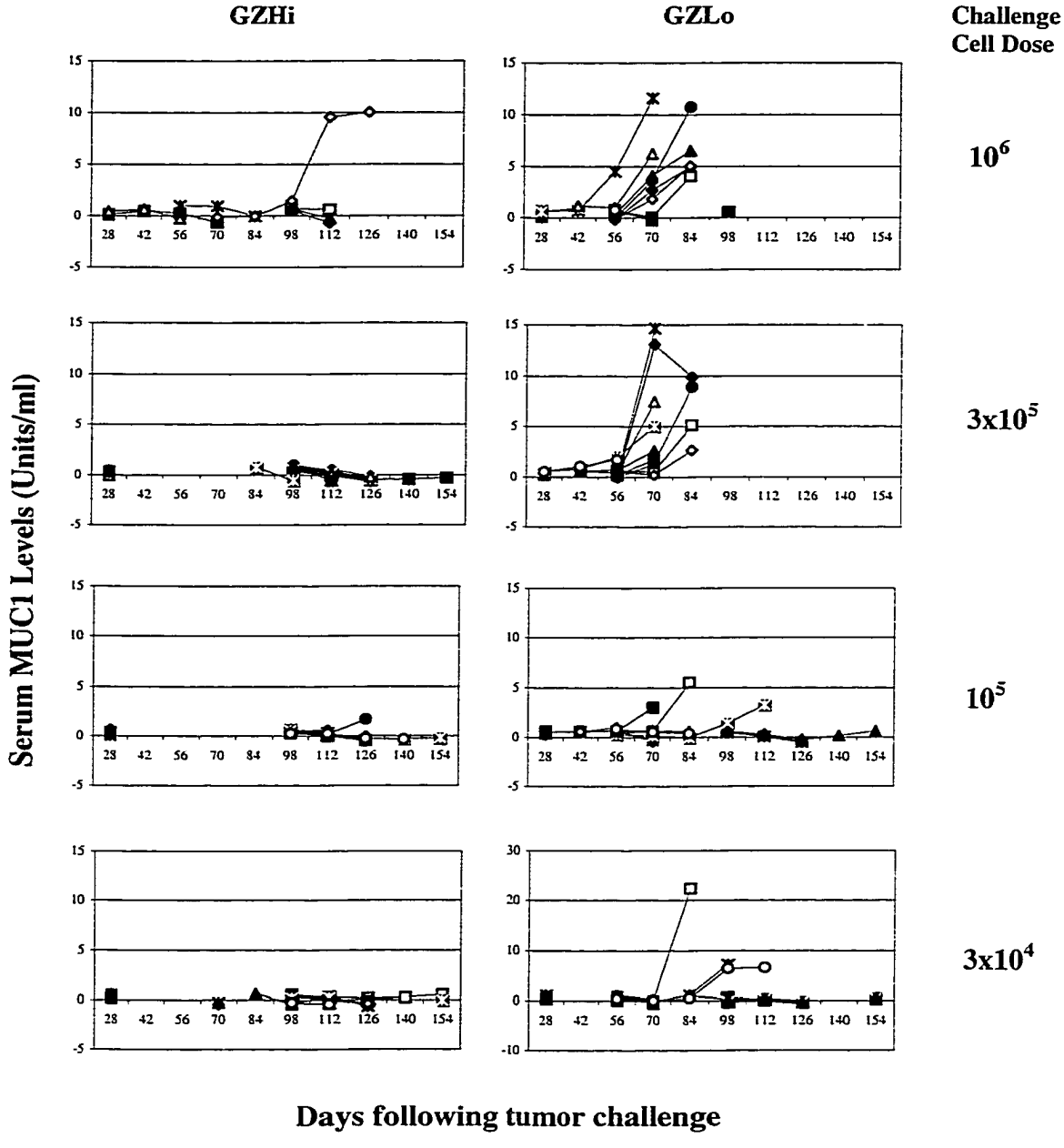


Figure 4-11: Serum MUC1 mucin levels start rising anywhere from 1 - 2 weeks prior to death in GZLo challenged CB6F1 mice.

Sera were collected every two weeks from each mouse and tested for MUC1 mucin by EIA. Results are represented in Units/ml and are shown as individual lines for each mouse.

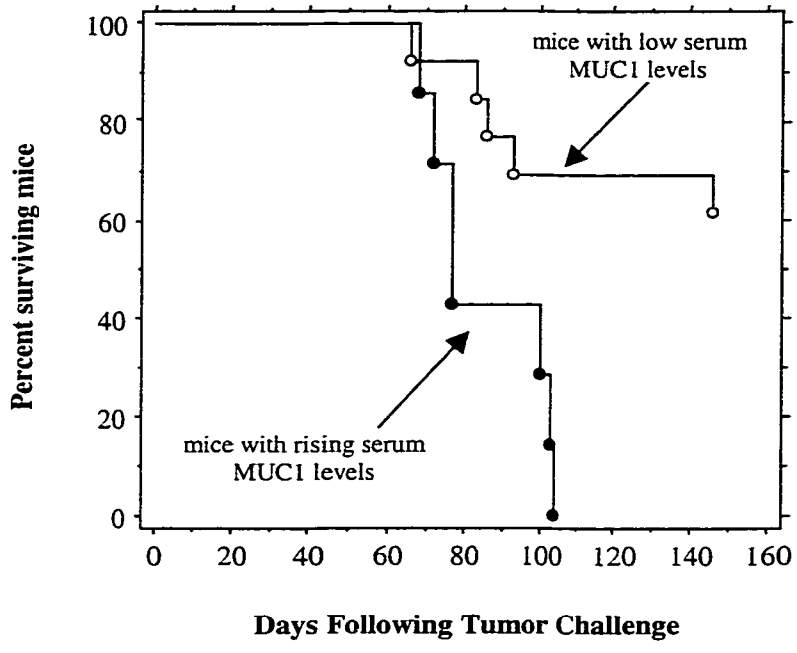


Figure 4-12: Survival of GZLo challenged CB6F1 mice at low tumor cell doses (3×10^4 and 1×10^5).

Mice with rising serum MUC1 levels had significantly shorter survival times than mice with non-detectable serum MUC1 levels (Logrank, $p = 0.0031$).

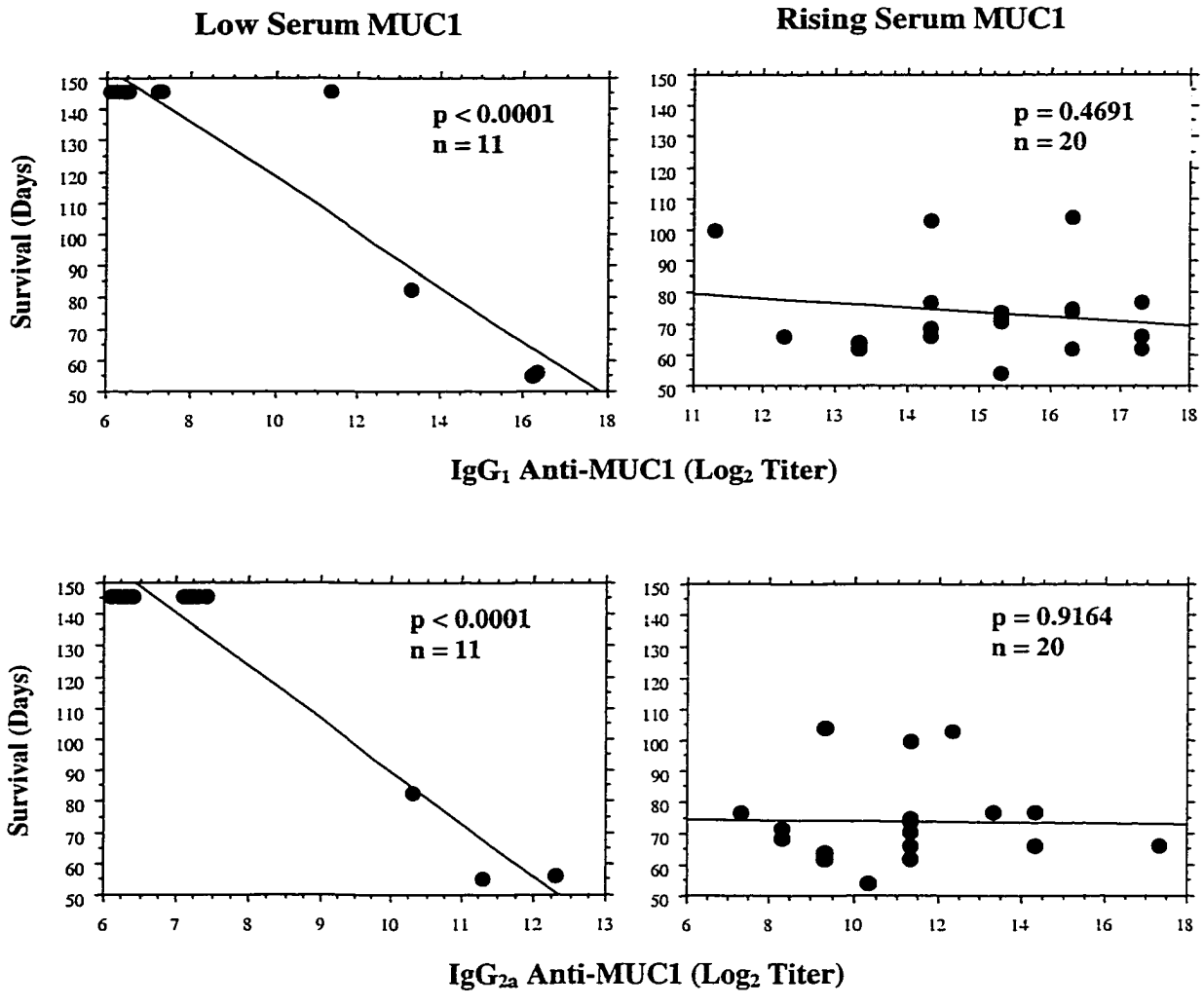


Figure 4-13: High anti-MUC1 antibody titres correlate to decreased survival in GZLo-challenged mice with low levels of serum MUC1.

Data were compared using simple Regression analysis.

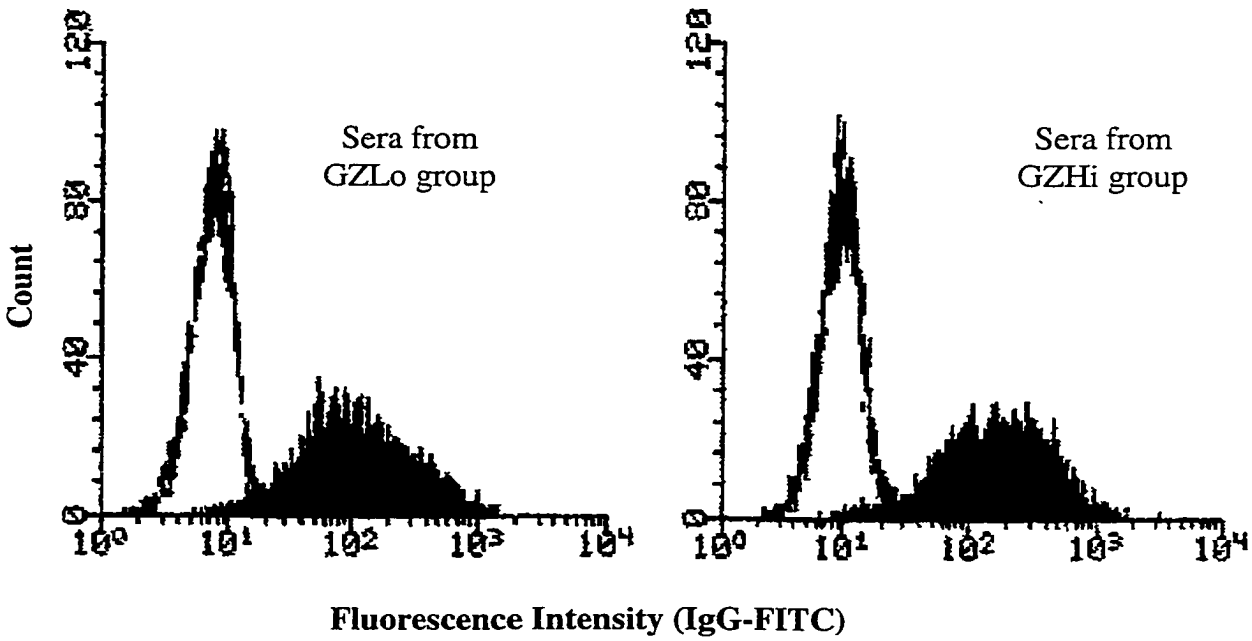


Figure 4-14: Antibodies in serum bind to GZHi cells.

Serum from the 2nd bleed of a representative mouse from both the GZHi and GZLo groups (of the 1 x 10^6 dose) was diluted 1:25 and added to either 410.4 cells (outline) or GZHi cells (shaded), detected by a goat-anti-mouse-IgG-FITC antibody and analyzed by FACS.

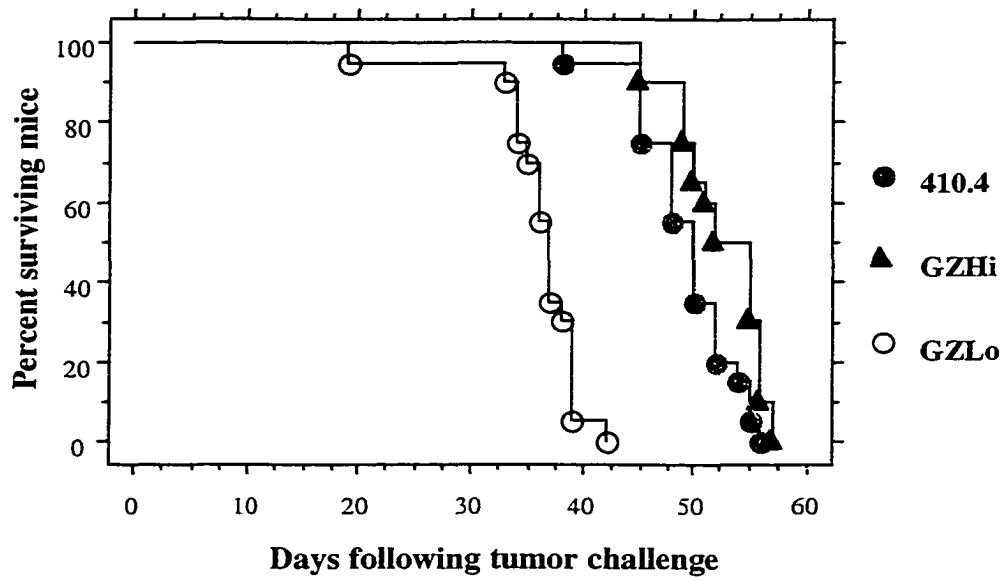


Figure 4-15: Survival of SCID-BEIGE mice challenged with GZHi, GZLo or 410.4. Mice were injected with a dose of 1 x 10^5 tumor cells subcutaneously (n = 20). (Logrank, $p < 0.0001$)

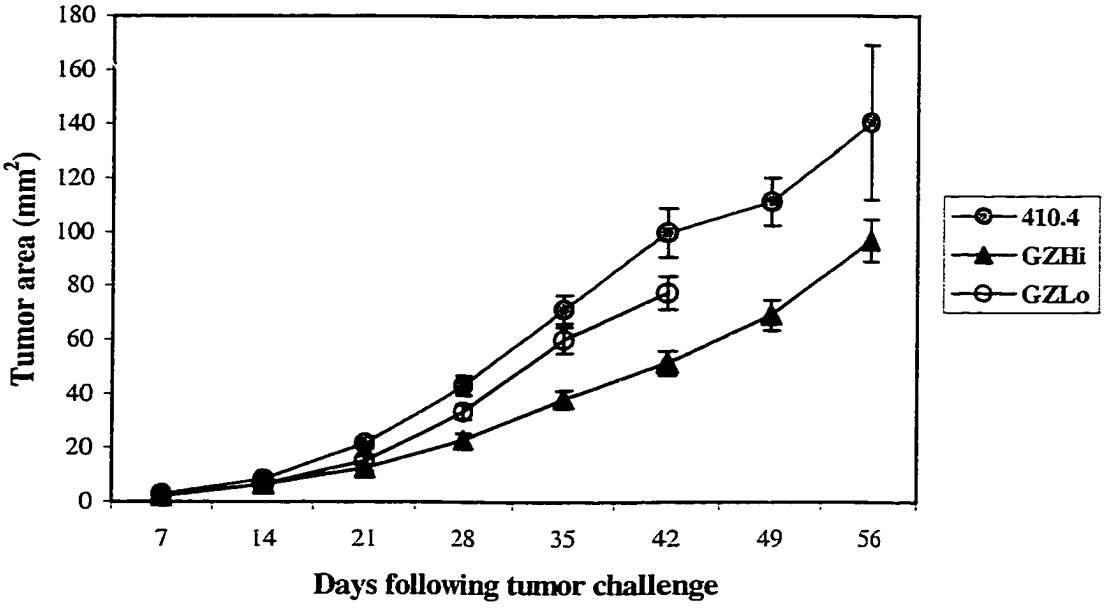


Figure 4-16: Tumor growth in SCID-BEIGE mice.

Weekly bidimensional tumor measurements were taken for each mouse. Values are mean tumor area ± SEM for each group (n = 20, until mice started dying). (unpaired t-tests: GZHi versus GZLo: 28 days, $p = 0.0066$, 35 days, $p = 0.0008$, 42 days, $p = 0.0037$; GZHi versus 410.4: 28, 35, 42 days, $p < 0.0001$, 49 days, $p = 0.0002$, 56 days, $p = 0.075$; GZLo versus 410.4: no significant differences.) This experiment was repeated with the same number of mice and the results were superimposable, therefore results of the two experiments were combined, n = 20 in each group of mice.

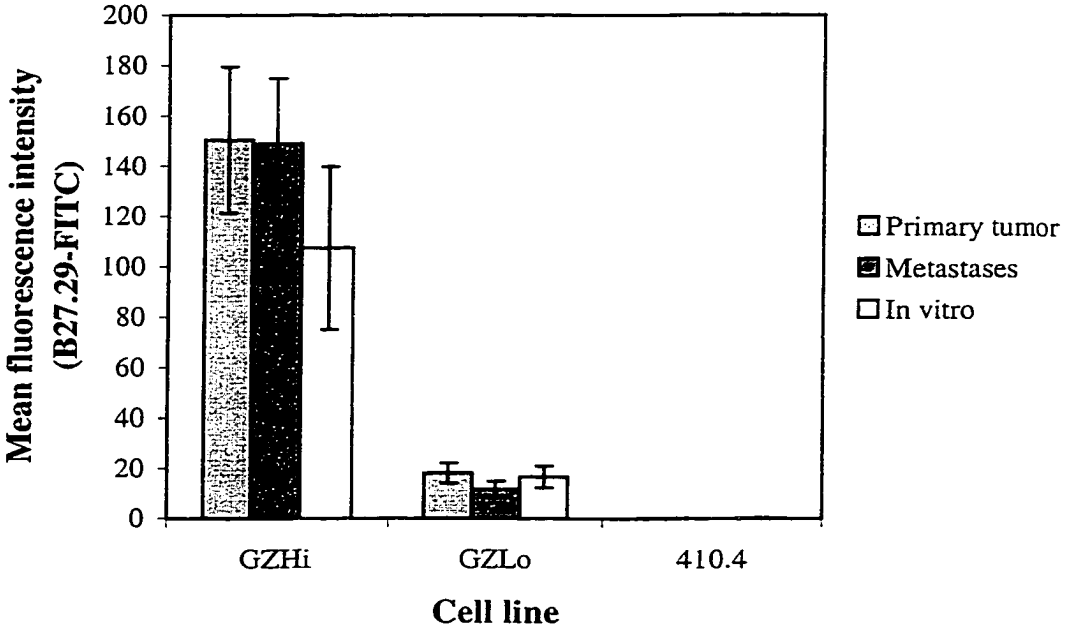


Figure 4-17: GZHi and GZLo tumor cells retain cell surface MUC1 expression in SCID-BEIGE mice comparable to their respective *in vitro* levels.

Tumor cells were harvested as in Fig. 4-8, but were stained directly with FITC-B27.29, resulting in lower mean fluorescence intensity (MFI). Values are MFI ± SEM (n = 20).

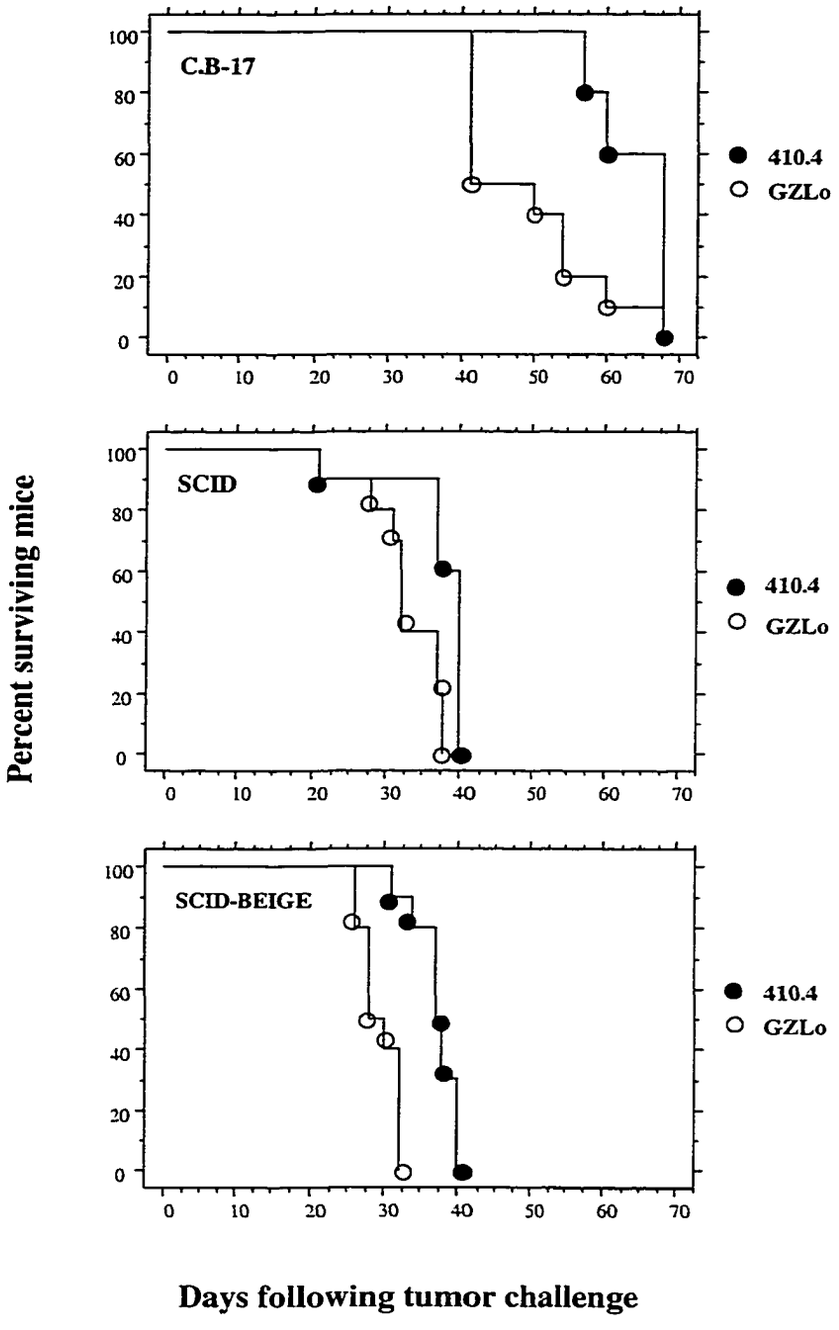


Figure 4-18: Survival of C.B-17, SCID and SCID-BEIGE mice challenged with GZLo cells is shorter than when challenged with 410.4 cells.

Mice were injected with a dose of 10^5 GZLo or 410.4 tumor cells subcutaneously (n = 10). (Logrank, C.B-17 $p = 0.0017$; SCID-BEIGE $p < 0.0001$; SCID $p = 0.0040$.)

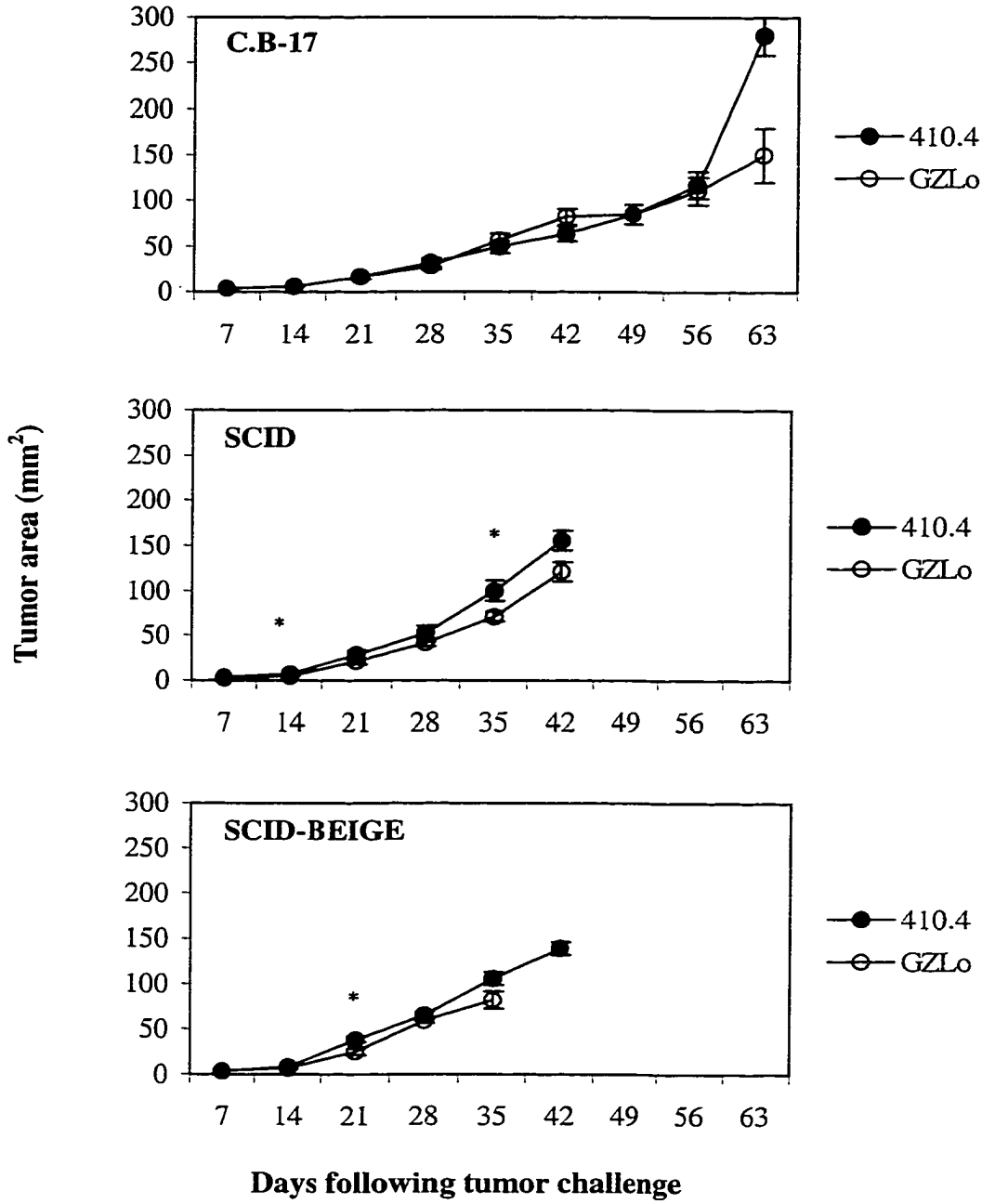


Figure 4-19: Tumor growth of 410.4 and GZLo in C.B-17, SCID and SCID-BEIGE mice.

Weekly bidimensional tumor measurements were taken for each of the mice. Values are mean tumor area ± SEM for each group (n = 10, until mice started dying). Tumor area was not significantly different between GZLo and 410.4 in C.B-17 mice. In SCID mice there was a significant difference in tumor area between GZLo and 410.4 only on days 14 and 35 and in SCID-BEIGE mice on day 21; * $p < 0.05$.

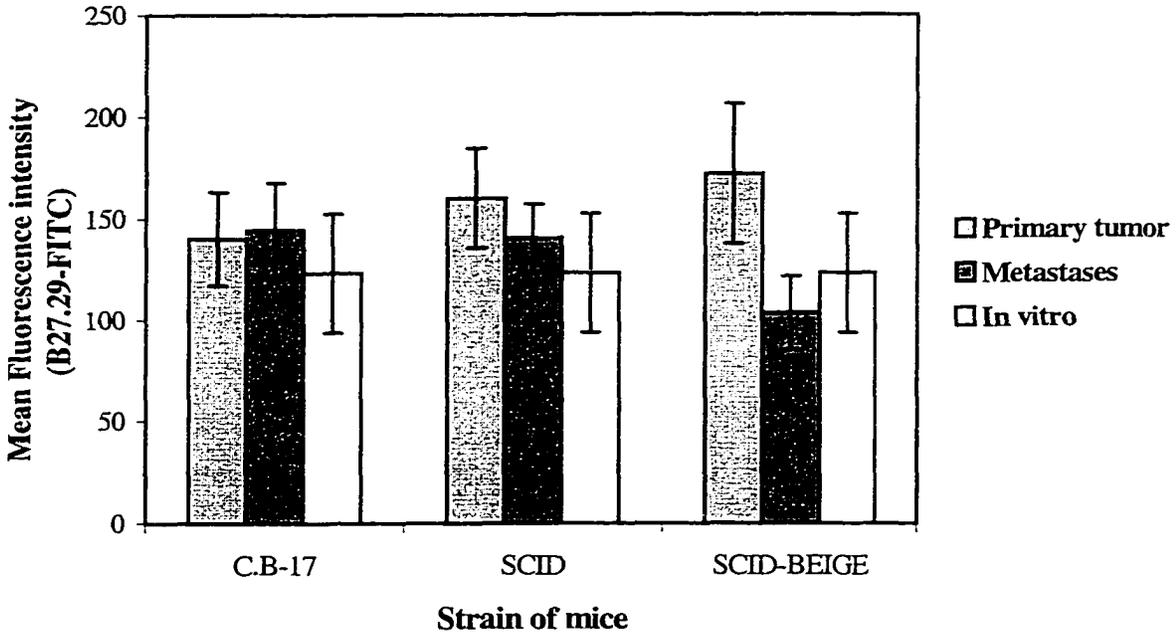


Figure 4-20: Cell surface MUC1 expression on GZLo tumors was equivalent among mouse strains.

Tumor cells were harvested as in Fig. 4-8, but were stained directly with FITC-B27.29. Values are mean fluorescence intensity ± SEM (n=10).

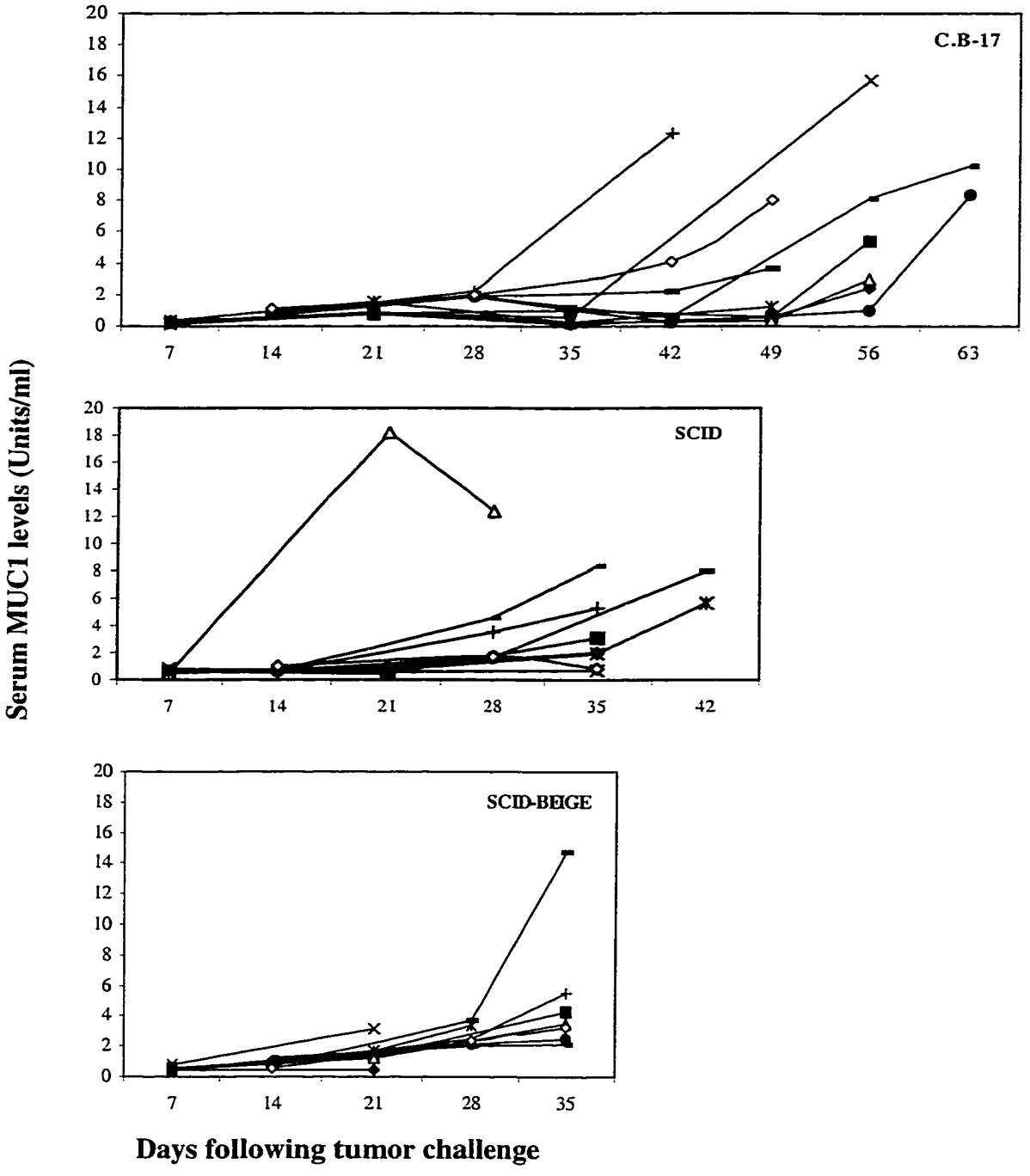


Figure 4-21: Rising serum MUC1 levels prior to death can be seen in all mouse strains challenged with GZLo cells.

Sera was collected every two weeks from each mouse and tested for MUC1 mucin by EIA. Results are represented in Units/ml and are shown as individual lines for each mouse.

CHAPTER V

Effects of soluble MUC1 mucin on generation of an immune response *in vivo*

To investigate if human MUC1 mucin might be immunosuppressive in mice as it may be in humans [Agrawal et al, 1998c], MUC1 mucin was injected intraperitoneally into mice for either 3 or 10 days after which time spleens and lymph nodes were removed to test T cells for specific responses (T cell proliferation and IFN-γ production) to either peptide immunizations or an allogeneic mixed lymphocyte reaction (allo-MLR). Figure 5-1 demonstrates that following i.p. injection of 400 units of MUC1 mucin there is a serum peak of MUC1 mucin 1 hour later and a rapid decline to background levels by 48 hours.

A) MUC1 specific recall response

Groups of five CB6F1 mice were injected i.p. with 400 units per day of human affinity purified MUC1 mucin or saline for 10 consecutive days. These mice were additionally immunized with liposomal vaccine containing human MUC1 peptide, one day after mucin injection was started in order to evaluate antigen-specific T-cell proliferation and IFN-γ levels in these mice. In 3 experiments, MUC1 mucin injected mice were compared to control mice for their immune response to a MUC1 peptide vaccine as measured in a 5-day T cell proliferation assay by ^{3}H-Thymidine uptake and by IFN-γ production (**CHAPTER II**). Table 5-1 demonstrates that MUC1 mucin injected mice showed significantly lower antigen specific IFN-γ production in T-cell proliferation

assays compared to control mice. Only one experiment (#3) showed a decrease in T cell proliferation when exposed to MUC1 *in vivo* compared to control mice.

B) HIV-V3 specific recall response

Groups of three C57Bl/6 mice were injected i.p. with 400 units per day of human affinity purified MUC1 mucin, OSM mucin, or saline for 10 consecutive days. These mice were additionally immunized with an HIV-V3 loop peptide (BP1-109) formulated with liposomes, one day after mucin injection was started in order to evaluate antigen-specific T-cell proliferation and IFN-γ levels in these mice. Nylon wool purified T cells were mixed with syngeneic APCs plus 20 μg/ml BP1-109 peptide and cultured for 5 days. Immune response was measured using T cell proliferation, as measured by ^{3}H-Thymidine uptake, and IFN-γ production as described in **CHAPTER II**. There was no significant inhibition of T cell proliferation following exposure to MUC1 or OSM *in vivo* compared to saline (Figure 5-2). However, *in vivo* exposure to MUC1 resulted in significantly decreased T cell proliferation compared to *in vivo* exposure to OSM (Figure 5-2, $p = 0.0015$). There was no significant production of IFN-γ by these mice in two different experiments (< 1 ng/ml or not detectable in MUC1 and Saline groups; 1.23 ± 0.2183 ng/ml in the OSM group); therefore it was difficult to observe any difference between the groups in IFN-γ production.

C) Allo-mixed lymphocyte reaction (MLR)

Groups of three C57Bl/6 and Balb/c mice were injected i.p. with 400 units per day of human affinity purified mucin, OSM mucin, or saline for 3 consecutive days. Nylon wool purified T cells from the C57Bl/6 mice were mixed with Mitomycin C-treated Balb/c spleen cells, and cultured for 6 days. T cell proliferation (as measured by ^{3}H-Thymidine uptake) and IFN-γ (from T cell proliferation supernatants) were tested as described in **CHAPTER II.**

Exposure of C57Bl/6 T cells to MUC1, OSM or saline *in vivo* resulted in no significant difference in T cell proliferation or IFN-γ production when mixed with normal (saline exposed) Balb/c APCs in two different experiments (Figure 5-3a). However, exposure of Balb/c APCs to MUC1 *in vivo* (compared to saline-exposed APCs), while not inhibiting proliferation of C57Bl/6 T cells, was able to significantly inhibit IFN-γ production from these T cells ($p = 0.004$, Figure 5-3b). Even more striking was the decrease in IFN-γ production from these same T cells when mixed with Balb/c APCs which were exposed to OSM *in vivo* ($p < 0.0001$, Figure 5-3b).

In these experiments, T cell function *in vitro*, as measured by proliferation and IFN-γ production was not affected by previous exposure of the responding T cells to MUC1 mucin *in vivo*. On the other hand, APCs previously exposed to MUC1 mucin *in vivo* significantly decreased the production of IFN-γ from normal T cells while not affecting their proliferation. Interestingly, APCs exposed to OSM *in vivo* were able to decrease IFN-γ production by normal T cells to a greater extent than MUC1 exposed APCs. These results suggest that not only MUC1 mucin, but also a control mucin, OSM,

may have an effect on APCs *in vivo* resulting in decreased production of IFN-γ from T cells.

Table 5-1: Effect of i.p. injection of human MUC1 mucin on the murine immune response[1]

		Antigen specific	
Experiment	**Mouse Treatment**	**T cell proliferation (cpm)**	**IFN-γ (ng/ml)**
1	PBS	53637 ± 3472	15.06 ± 0.04[b]
	MUC1 mucin	48512 ± 4160	2.70 ± 0.10[b]
2	PBS	19291 ± 2635	0.75 ± 0.17[c]
	MUC1 mucin	12757 ± 3098	0.00 ± 0.00[c]
3	PBS	52917 ± 1771[a]	12.68 ± 2.97
	MUC1 mucin	17127 ± 1626[a]	6.70 ± 0.44

[1]Produced in collaboration with Vladyslaw Budzynski, Biomira, Inc.
Values are mean ± SEM (n = 5); [a,b] $p < 0.0001$; [c] $p = 0.0107$; (The remaining MUC1/PBS pairs are not significantly different.)

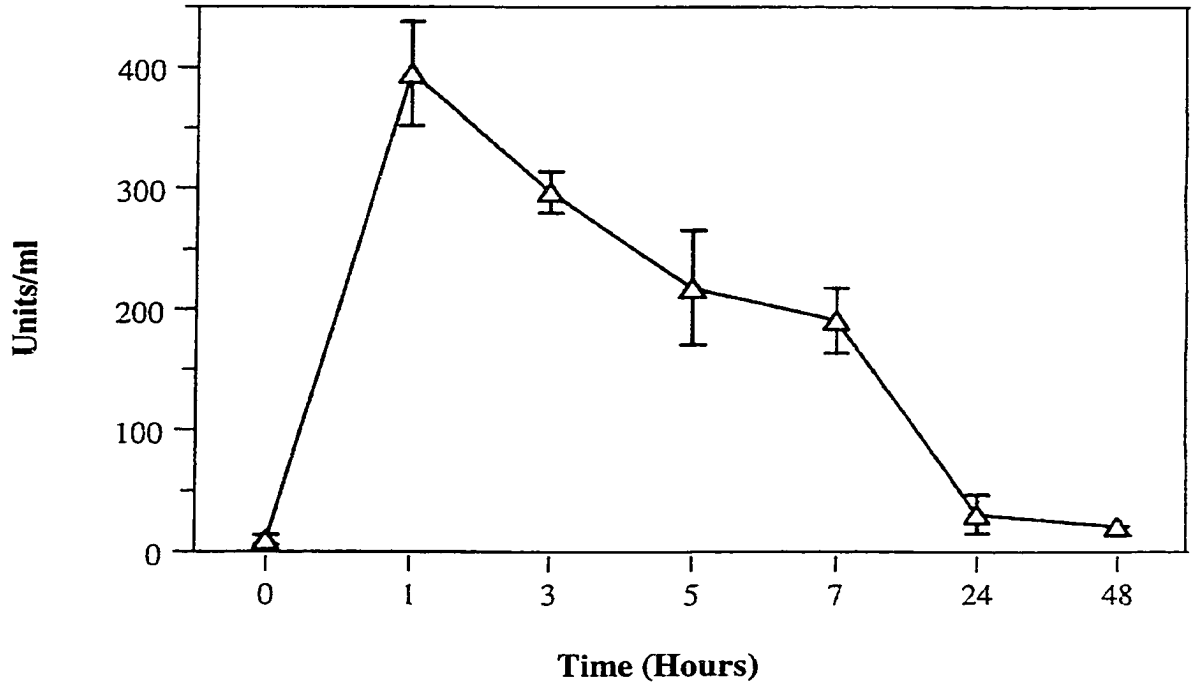


Figure 5-1: Serum levels of MUC1 mucin in mice over 48 hours.

18 mice were injected intraperitoneally with 400 units of MUC1 mucin at time 0. Serum samples were taken from three different mice at the times shown and tested for MUC1 mucin by EIA as described in **CHAPTER II.** Three mice injected with saline served as a background control. (Produced in collaboration with Vladyslaw Budzynski, Biomira, Inc.)

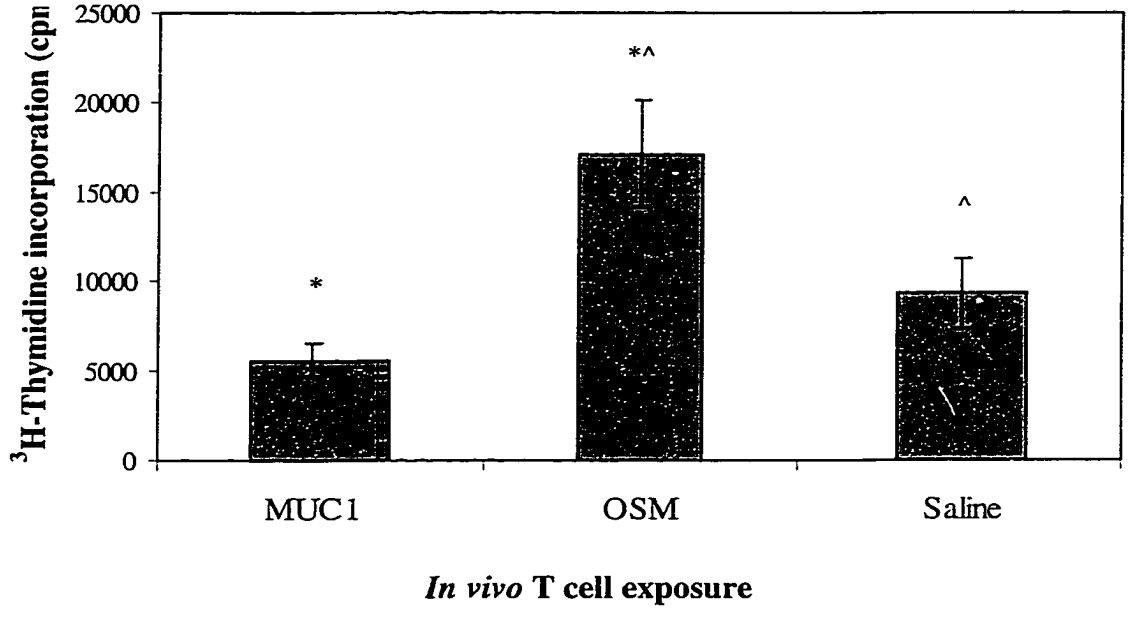
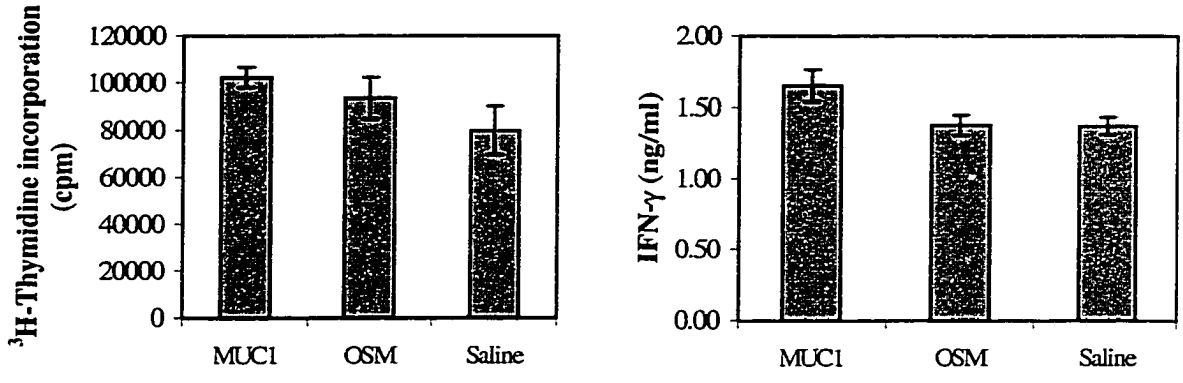


Figure 5-2: HIV-V3 specific recall response following *in vivo* exposure to mucin.

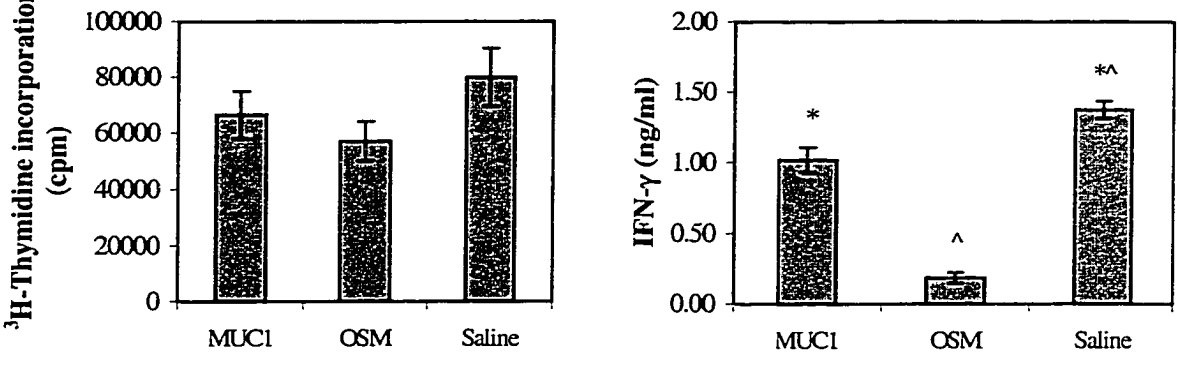
Groups of three C57Bl/6 mice were injected i.p. with 400 units per day of human affinity purified mucin, OSM mucin., or saline for 10 consecutive days. These mice were additionally immunized with an HIV-V3 loop peptide, BP1-109, mixed with liposomes, one day following the first mucin injection. Nylon wool purified T cells were added to syngeneic APCs plus 20 μg/ml BP1-109 and cultured for 5 days. Antigen-specific T-cell proliferation measured by ^{3}H-Thymidine incorporation in cpms. Values are mean ± SEM (n = 20); *p = 0.0015; ^p = 0.0369.

a)



***In vivo* T cell exposure**

b)



***In vivo* APC exposure**

Figure 5-3: Allo-MLR response following *in vivo* exposure to mucin.

C57Bl/6 and Balb/c mice were injected intraperitoneally with 400 units per day of MUC1 mucin for 3 consecutive days. Nylon wool purified T cells from the C57Bl/6 mice were mixed with Mitomycin C-treated Balb/c spleen cells, and cultured for 5 days. a) Exposure of T cells to MUC1, OSM or Saline *in vivo*. b) APCs exposed to either MUC1 OSM or Saline *in vivo*. T cell proliferation (^{3}H-Thymidine uptake in cpms) and IFN-γ production (ng/ml). Values are mean ± SEM (n = 12); * $p = 0.0040$, ^ $p < 0.0001$ (MUC1 vs OSM, $p < 0.0001$).

CHAPTER VI

Effects of cell-surface and soluble MUC1 mucin on murine *in vitro* immune response

To investigate possible mechanisms of MUC1 induced immunosuppression, several types of *in vitro* assays were performed using either target cells transfected with the MUC1 mucin or target cells/effector cells mixed with soluble MUC1 mucin.

A) Cytotoxic T-Lymphocyte killing of MUC1$^+$ target cells (as measured in a 4 hour ^{51}Cr-release assay)

To determine whether the presence of cell surface MUC1 on target cells could interfere with cytotoxic T-lymphocyte (CTL) killing of these targets, 4 hour ^{51}Cr-release assays were set up using CTL clones as well as using allo-CTL cultures. 410.4, GZHi and GZLo cells were used as targets for the H-2^d (class I major histocompatibility complex (MHC)) specific CTL Clone 10/1 compared to the positive control target cell line, A20 and the negative control cell line, EL4. There was no indication that cell-surface MUC1 on GZHi or GZLo target cells inhibited CTL killing compared to 410.4 (Figure 6-1). However, the percent specific lysis of these target cells was relatively low possibly due to a low level of MHC class I expression (Figure 6-2). Therefore other target cell lines with higher expression levels of MHC class I were transfected with the human MUC1 cDNA using the same procedure as outlined for 410.4 cells in **CHAPTER III**. The target cell line, EL4, was transfected with MUC1 cDNA and sorted for high (E-

M-Hi) levels of surface MUC1 by FACSort (Figure 6-3). These targets were then tested with the H-2^b specific CTL Clone 11, which was raised against EL4. In this case there was equivalent percent specific lysis of EL4 and E-M-Hi by CTL clone 11 (Figure 6-4).

EL4 is a strong target for CTL clone 11 and addition of cell surface MUC1 to EL4 may not produce significant inhibition in this case. Therefore, less aggressive allo-CTL lines were created and tested on the same targets. The allo-CTL line, CBAαB6, was created by mixing CBA spleen cells with irradiated C57Bl/6 spleen cells for 7 days. This line was maintained by weekly stimulation with irradiated C57Bl/6 spleen cells. These CBAαB6 allo-CTLs were then tested on EL4, E-M-Hi and A20 with much the same results as seen with CTL clone 11 (Figure 6-5). There was no difference in percent specific lysis between $MUC1^+$ and $MUC1^-$ cell lines. Another allo-CTL line, B6αBalb/c, was created against the H-2^d background (as described above, using C57Bl/6 spleen cells mixed with irradiated Balb/c spleen cells) to test the original cell lines, GZHi and 410.4. There was no specific lysis of any of these targets above background by B6αBalb/c (Figure 6-6). Attempts were made to raise an allo-CTL line against the parental 410.4 cell line, in order to test the original transfectants, but they were unsuccessful.

These experiments demonstrate that cell surface MUC1 is not able to inhibit lysis of targets by CTLs *in vitro* using murine EL4 cells. It also demonstrates that the 410.4 cell line and its MUC1-transfectants are not easily killed by CTLs.

B) CTL killing of MUC1$^+$ target cells (as measured in a serine esterase release assay)

To more fully investigate the role of cell surface MUC1, its ability to inhibit lysis of targets by CTLs was tested using the more sensitive serine esterase release assay. This assay measures the activation of CTLs and is able to identify earlier events, such as degranulation before actual lysis of targets. There was no difference in CTL activation by MUC1$^+$ or MUC1$^-$ targets (Figure 6-7). These results together with the ^{51}Cr-release assays suggest that cell surface MUC1 on murine target cells does not inhibit activation or killing by CTLs.

C) CTL killing in the presence of soluble MUC1 mucin in a 4-hour ^{51}Cr-release assay.

While cell surface MUC1 was not able to inhibit CTL killing of targets, it was possible that soluble MUC1 could. Therefore, ^{51}Cr-release assays were set up using different targets in the presence or absence of soluble MUC1 mucin. EL4 and A20 target cells were labelled with ^{51}Cr and mixed with either Clone 11 CTLs or B6αBalb/c allo-CTLs at an effector to target ratio of 4:1 (Clone 11) or 20:1 (B6αBalb/c). MUC1 mucin was added to each set of effector-targets at concentrations of 1, 5, 10 or 20 μg/ml. Assays were run for 4 hours. Clone 10/1 CTLs were used with the ^{51}Cr-labelled targets A20, EL4, Yac-1 and Y-MUC1 (Yac-1 was transfected with human MUC1 cDNA in the same method as for 410.4 in **CHAPTER III**; Figure 6-3) in the presence or absence of

20 μg/ml MUC1 at an effector to target ratio of 25:1 for 4 hours. Regardless of the target or effector cell used, there was a small but consistent, dose dependent inhibition of percent specific lysis in the presence of soluble MUC1 in each case as shown in Figure 6-8.

To ensure that the effect seen was MUC1 specific and not a function of the amount of protein present, a control mucin, OSM, as well as BSA and PBS were each independently added to the Clone 11 – EL4 assay. As shown in Figure 6-9, only MUC1 was able to produce an inhibitory effect on lysis of target cells, again in a dose dependent fashion. In fact the other reagents appeared to increase the percent specific lysis of targets somewhat.

In order to determine if this effect could be seen in a more physiological relevant system a syngeneic assay was employed. The flu-specific Clone 3/4 CTL was used to kill syngeneic, EL4 targets pulsed with the flu peptide ($NP_{365-380}$) in the presence or absence of MUC1 mucin. As seen with the allo-CTL assays, there was a consistent, dose dependent inhibition of lysis of targets in the presence of MUC1 mucin (Figure 6-10).

A regression analysis was performed on the dose range of MUC1 used in each CTL assay to show that the dose dependent inhibition was significant (Table 6-1). These results indicate a possible role of soluble MUC1 mucin in inhibiting the lysis of targets by CTLs.

D) Adhesion of CTLs to immobilized MUC1 mucin.

To determine if soluble MUC1 mucin could be binding to the CTLs thereby inhibiting CTL function, MUC1 mucin was immobilized on plastic 96 well plates as outlined in **CHAPTER II,** as well as a control mucin (OSM), a control protein (BSA) and the positive control, anti-class I MHC ($H\text{-}2K^b$) antibody, Y3. Clone 3/4 cells were harvested, labelled with ^{51}Cr and added to the immobilized antigens for one hour. Percent specific binding was calculated and as shown in Figure 6-11, the binding of CTL Clone 3/4 cells to MUC1 mucin in this assay format was negligible.

E) Killing of targets by NK cells in the presence of either cell surface or soluble MUC1 mucin.

To determine if the presence of MUC1 mucin, either cell-surface or soluble, could affect killing of targets by natural killer cells, NK assays were employed. Yac-1 target cells were transfected with human MUC1 cDNA (Y-MUC1) in the same manner as for 410.4 cells (see **CHAPTER III**). NK cells were prepared as described in **CHAPTER II** and added to ^{51}Cr-labelled targets in the presence or absence of soluble MUC1 mucin in the same type of assay as described for the CTLs above. Half of the NK cells were preincubated with 10 μg/ml MUC1 mucin for 30 minutes prior to addition to target cells.

Regardless of whether the MUC1 was cell surface bound or soluble there was no difference in percent specific lysis by NK cells, although preincubation of NK cells with 10 μg/ml MUC1 appeared to increase percent specific lysis (Figure 6-12). This indicates

that there is no significant inhibitory effect of MUC1 mucin on murine NK cell killing *in vitro* at the concentrations of MUC1 used.

F) T cell proliferation and IFN-γ production in the presence of soluble MUC1 mucin.

It has been shown that soluble MUC1 mucin can inhibit human T cell proliferation *in vitro* [Agrawal, et al., 1998]. To determine if MUC1 was performing a similar role in the murine system, T cell proliferation assays were used. Primary allo-MLRs were employed using nylon wool purified C57Bl/6 T cells mixed with irradiated (or Mitomycin C treated) Balb/c spleen cells in the presence or absence of MUC1 or OSM mucin. In 4 different experiments allo-MLR cultures were set up in 10-mL round-bottom tubes and the cells were counted after 7 days in culture (Table 6-2). Cell counts varied from experiment to experiment. In the first experiment proliferation was not affected by addition of MUC1. In the second experiment with either 5 or 10 μg/ml MUC1 the cell counts increased over background, while at 20 μg/ml the counts were decreased compared to 5 or 10 μg/ml of MUC1, but not below background. The third experiment showed reduced cell counts in the presence of 20 μg/ml MUC1 only, while the fourth experiment showed reduced cell counts at all concentrations of MUC1 used. Supernatant samples were removed from each culture on day 6 and tested for IFN-γ. In the first two experiments (Expt #1&2), the background levels of IFN-γ were low and the presence of 5 or 10 μg/ml of MUC1 actually increased IFN-γ production. However, addition of 20 μg/ml MUC1 resulted in decreased levels of IFN-γ. The third experiment

showed lower levels of IFN-γ in the presence of MUC1 compared to control, while the fourth experiment produced little or no IFN-γ in the presence of MUC1.

In allo-MLR cultures in 96 well plates, there was no significant inhibition of T cell proliferation seen over a number of different experiments (Figure 6-13a). However, when testing the supernatants of these T cell proliferations for presence of IFN-γ, significant inhibition of IFN-γ production was seen in the presence of 15, 20 and 50 μg/ml MUC1, as well as with 20 μg/ml OSM (Figure 6-13b).

To determine if soluble MUC1 mucin could have an effect on an antigen-specific T cell proliferation, mice were immunized with the 25mer MUC1 peptide, BP1-148, in a liposomal formulation (Biomira, Inc.). Nylon wool purified T cells from the spleens of these mice were added to cultures containing APCs plus 20 μg/ml BP1-148 peptide in the presence or absence of MUC1 or OSM mucin for 5 days. There was neither inhibition of T cell proliferation nor inhibition of IFN-γ production in these antigen-specific responses (Figure 6-14).

These results indicated that, unlike human T cell proliferation, soluble MUC1 mucin was not having an effect on murine T cell proliferation but rather it was able to inhibit IFN-γ production in some primary T cell proliferations.

Table 6-1: Regression summary of MUC1 dose dependent inhibition in CTL assays.

Experiment	r^2	p-value	count
Clone 11 (Figure 6-8a)	0.277	0.0082	24
B6αBalb/c (Figure 6-8b)	0.705	< 0.0001	15
Clone 11 (Figure 6-9)	0.604	0.0029	12
Clone 3/4 (Figure 6-10)	0.304	0.0005	36

Table 6-2: Allo-MLR T cell counts and IFN-γ levels in the presence or absence of mucin.

	Cell Counts from 7-Day Allo-MLR Proliferation (x 10^5)				ng/ml of IFN-γ			
mucin \ Expt #	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
None	7.20	3.40	13.40	8.40	0.561	0.480	2.192	2.468
5ug/ml OSM			16.20	7.80			1.818	2.717
10ug/ml OSM	7.28	9.40	11.60	9.20	12.469	5.120	2.106	3.870
15ug/ml OSM			14.40	10.60			1.549	2.885
20ug/ml OSM			16.80	4.80			1.896	0.936
5ug/ml MUC1	5.46	9.00	12.00	3.20	10.201	7.692	1.714	0.000
10ug/ml MUC1	5.98	8.60	12.20	2.80	8.364	4.209	1.858	0.000
15ug/ml MUC1			13.00	2.80			1.515	0.120
20ug/ml MUC1	6.24	3.40	8.00	3.00	0.329	0.136	1.417	0.000

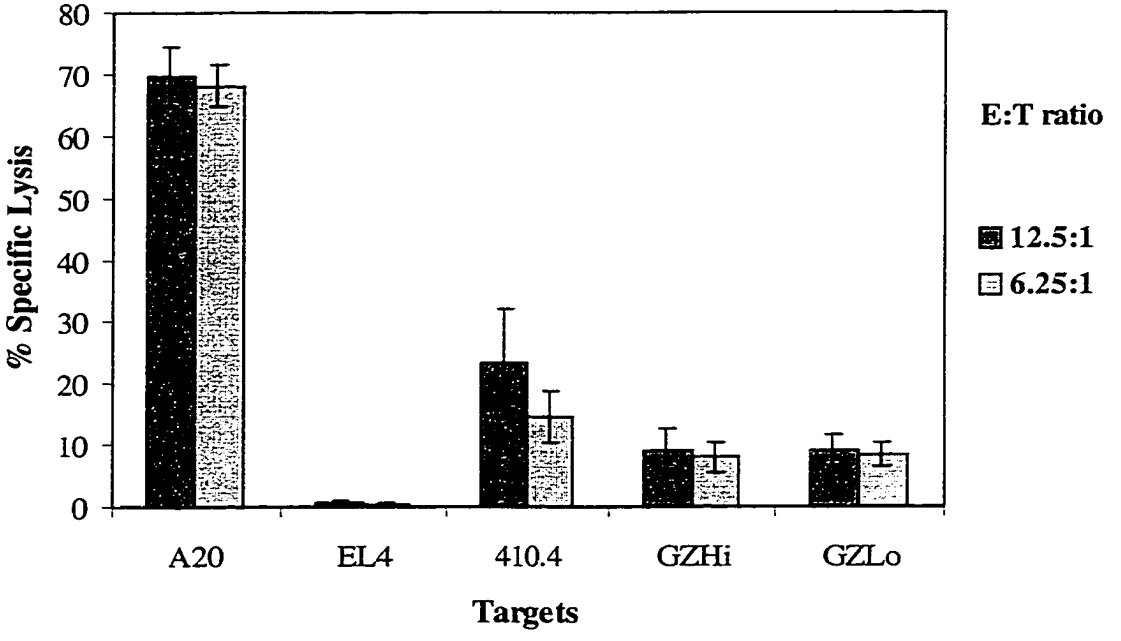


Figure 6-1: Specific lysis of the parental cell line 410.4 and its MUC1 transfectants by CTL clone 10/1 is low.

The CTL clone 10/1 was added to ^{51}Cr- labelled A20 (positive control), EL4 (negative control), GZHi, GZLo and 410.4 target cells for 4 hours at effector:target (E:T) ratios of 12.5:1 and 6.25:1 after which time supernatants were collected and measured for amount of ^{51}Cr released. Results are shown as mean percent specific lysis ± SEM (n = 6).

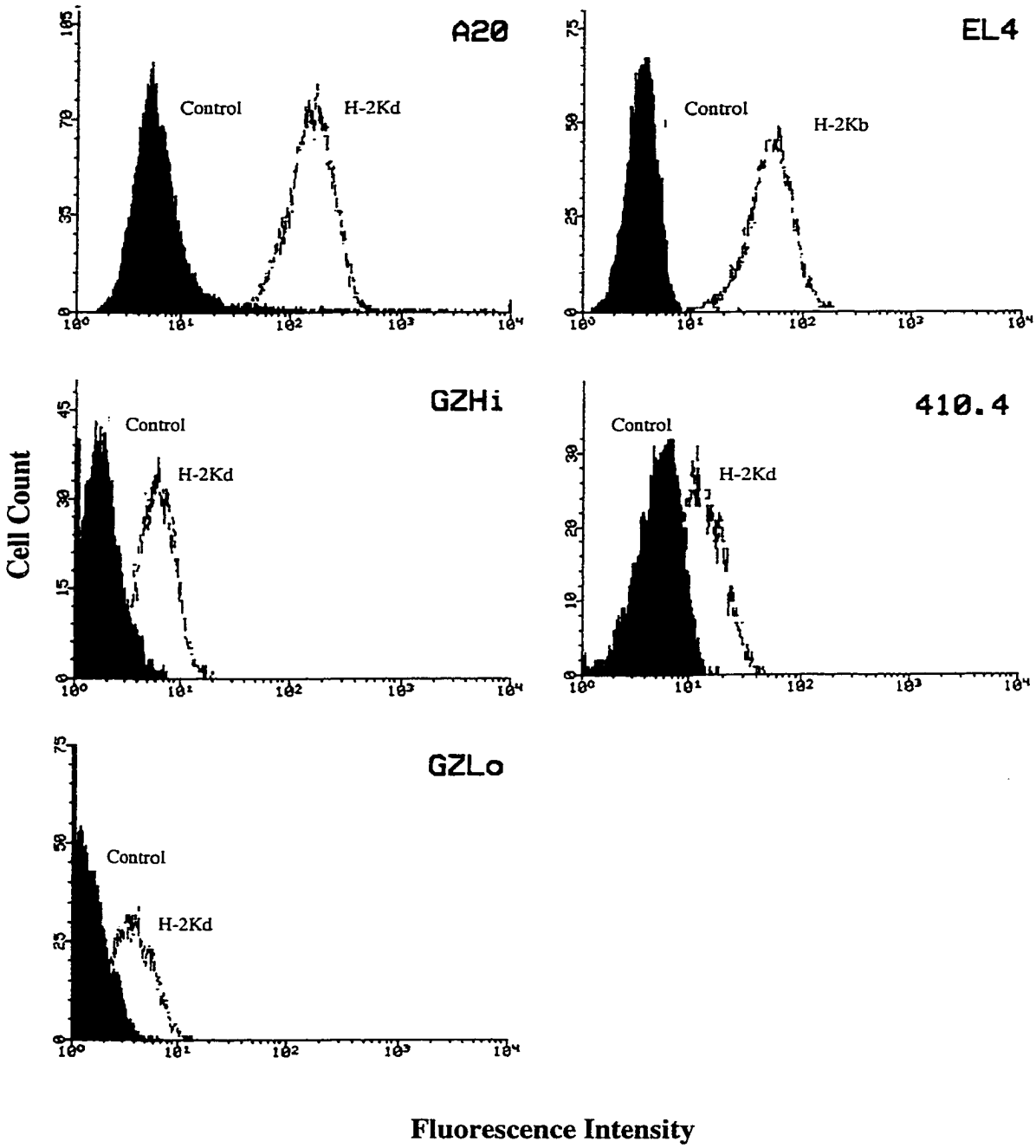


Figure 6-2: MHC class I expression on target cells.

Cells were stained with either anti-H-2K^d (A20, GZHi, GZLo and 410.4) or anti-H-2K^b (EL4) antibody and analyzed by FACS as described in **CHAPTER II**. Mean fluorescence intensity: A20 = 159.4, EL4 = 50.9, GZHi = 4.8, GZLo = 2.7, 410.4 = 7.9.

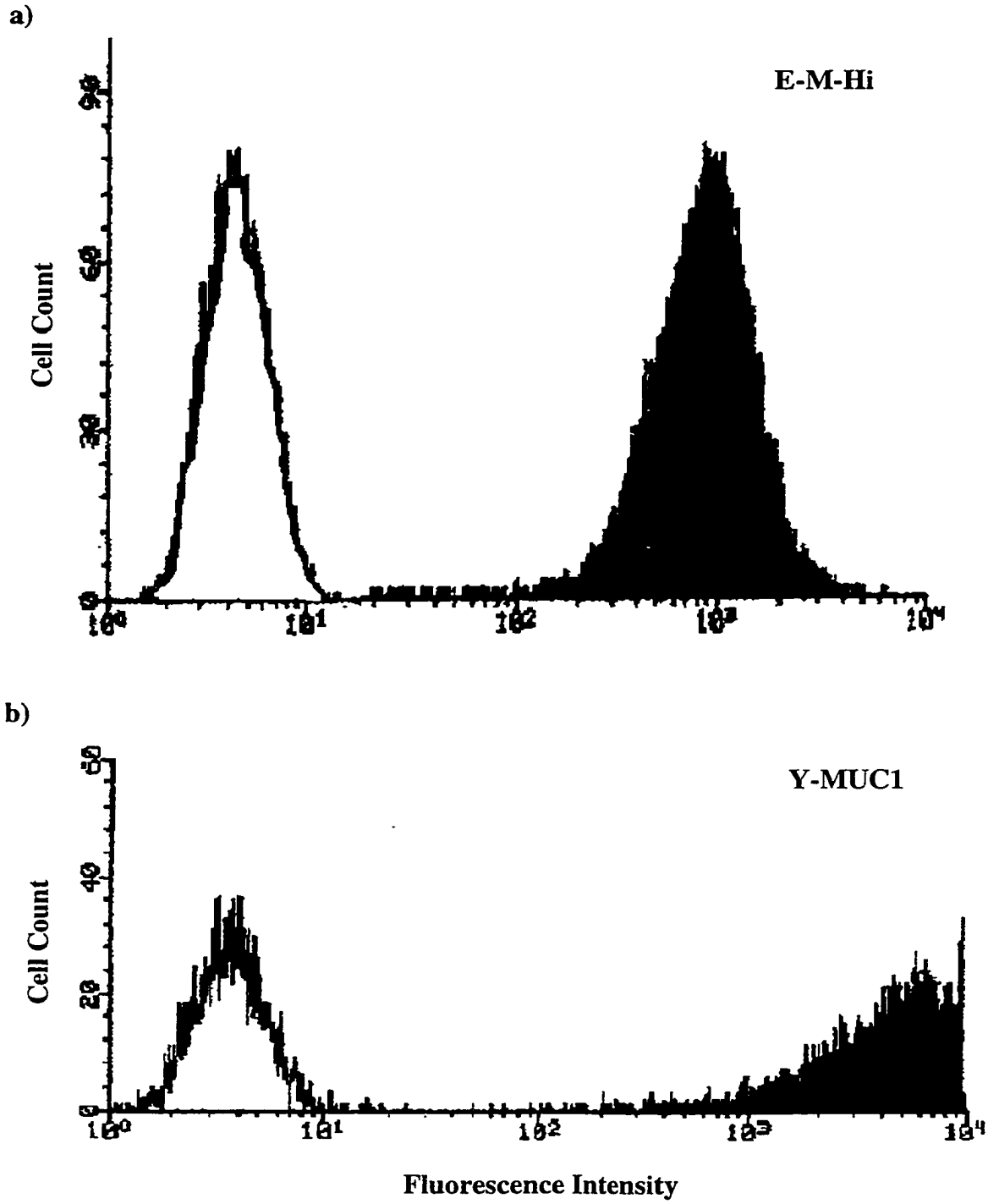


Figure 6-3: MUC1 transfectants, E-M-Hi and Y-MUC1.

EL4 (a) and Yac-1 (b) cells were transfected in the same manner as were 410.4 cells in **CHAPTER III.** These cells were then sorted by FACSort for high expression of cell surface MUC1. Outline is control antibody (B80.3) and shaded is anti-MUC1 antibody (B27.29).

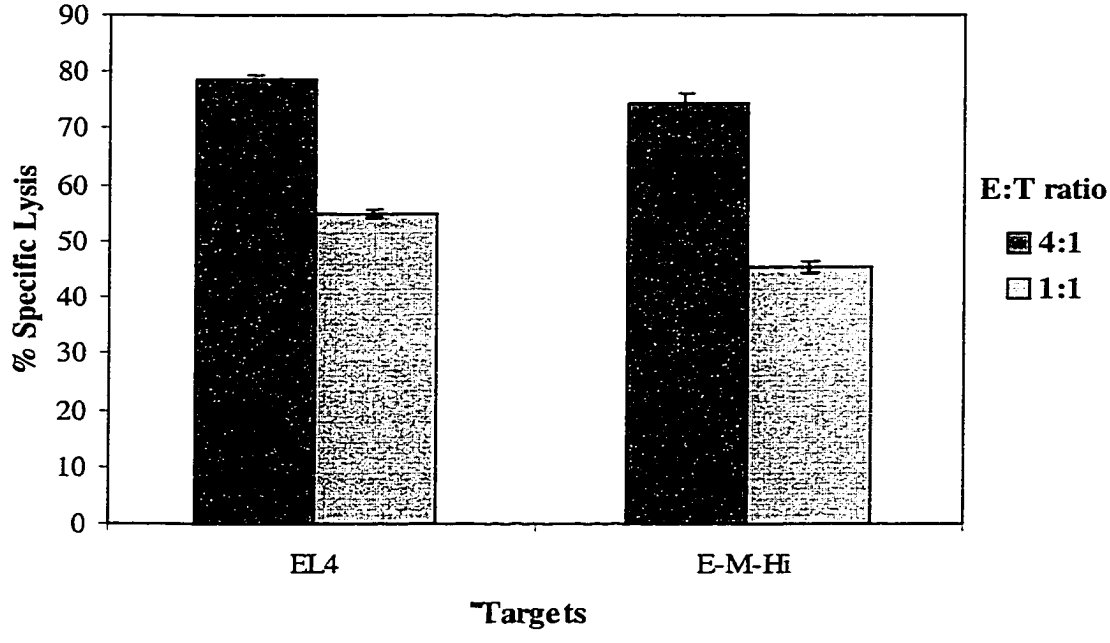


Figure 6-4: Specific Lysis of EL4 and MUC1-transfected EL4 by the CTL clone 11 is equivalent.

The CTL clone 11 was added to ^{51}Cr- labelled EL4 (positive control), A20 (negative control) and E-M-Hi target cells for 4 hours at effector:target (E:T) ratios of 4:1 and 1:1 after which time supernatants were collected and measured for amount of ^{51}Cr released. Results are shown as mean percent specific lysis ± SEM (n = 3). (The negative control cell line, A20, had a high spontaneous release of ^{51}Cr resulting in a negative % specific lysis value).

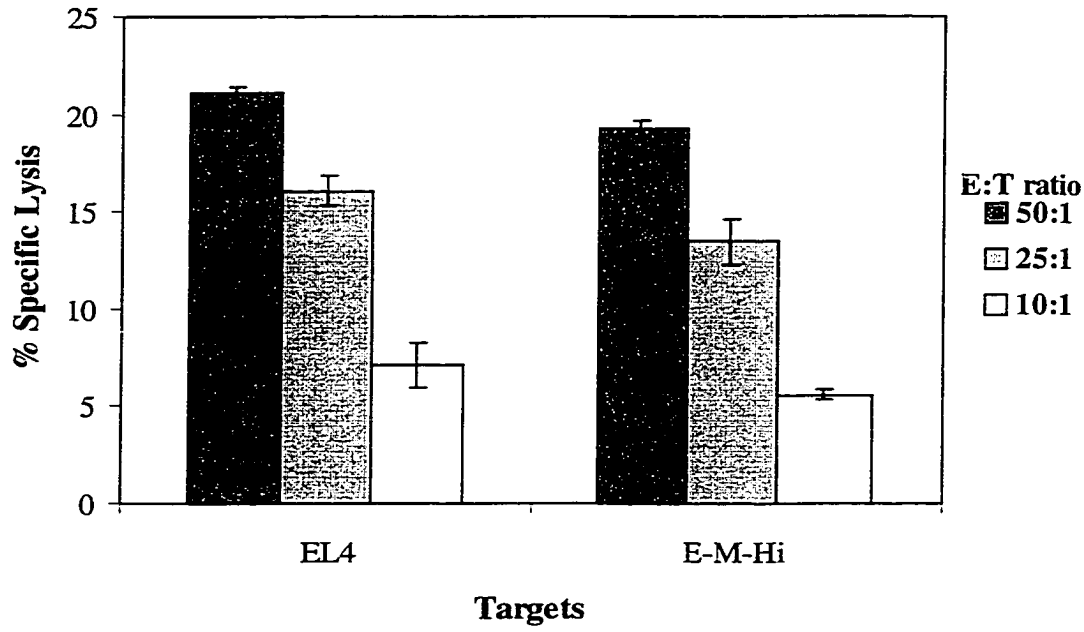


Figure 6-5: Specific lysis of EL4 and MUC1-transfected EL4 by the allo-CTL line CBAαB6 is low, but equivalent.

The allo-CTL line CBAαB6 was added to ^{51}Cr-labelled EL4 (positive control), A20 (negative control) and E-M-Hi target cells for 4 hours at effector:target (E:T) ratios of 50:1, 25:1 and 10:1 after which time supernatants were collected and measured for amount of ^{51}Cr released. Results are shown as mean percent specific lysis ± SEM (n = 3). (The negative control cell line, A20, had a high spontaneous release of ^{51}Cr resulting in a negative % specific lysis value).

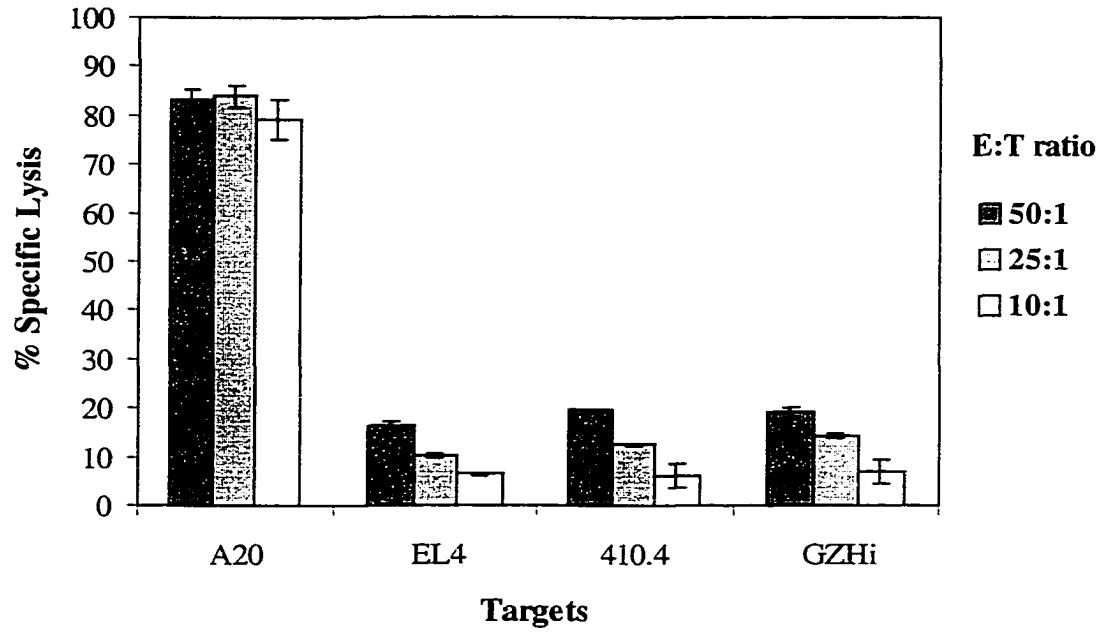


Figure 6-6: Specific lysis of 410.4 and GZHi by the allo-CTL line B6αBalb/c is undetectable above background.

The allo-CTL line B6αBalb/c was added to ^{51}Cr-labelled A20 (positive control), EL4 (negative control), GZHi and 410.4 target cells for 4 hours at effector:target (E:T) ratios of 50:1, 25:1 and 10:1 after which time supernatants were collected and measured for amount of ^{51}Cr released. Results are shown as mean percent specific lysis ± SEM (n = 3).

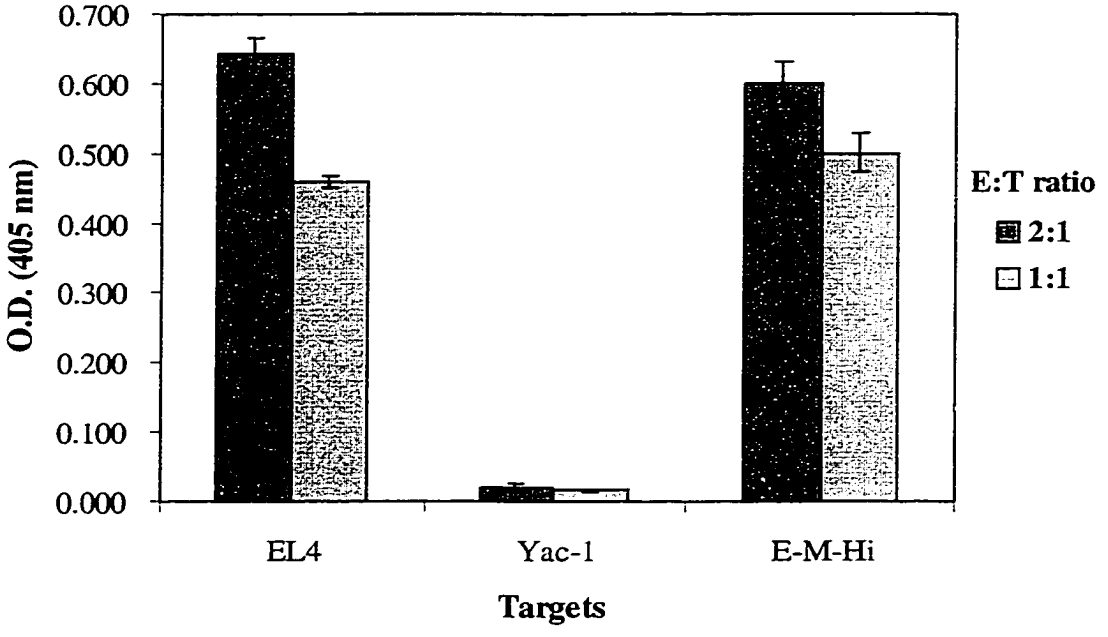


Figure 6-7: Cell surface MUC1 on target cells does not inhibit degranulation by CTL clone 11 as detected by SER.

The CTL clone 11 was added to EL4 (positive control), Yac-1 (negative control) and E-M-Hi target cells for 2.5 hours at effector:target (E:T) ratios of 2:1 and 1:1. Supernatants were collected and placed into new wells to which BLT-DNTB reaction buffer was added. Results were read, after 20 minutes, on an ELISA microplate reader and are represented as mean O.D. at 405 nm ± SEM (n = 3).

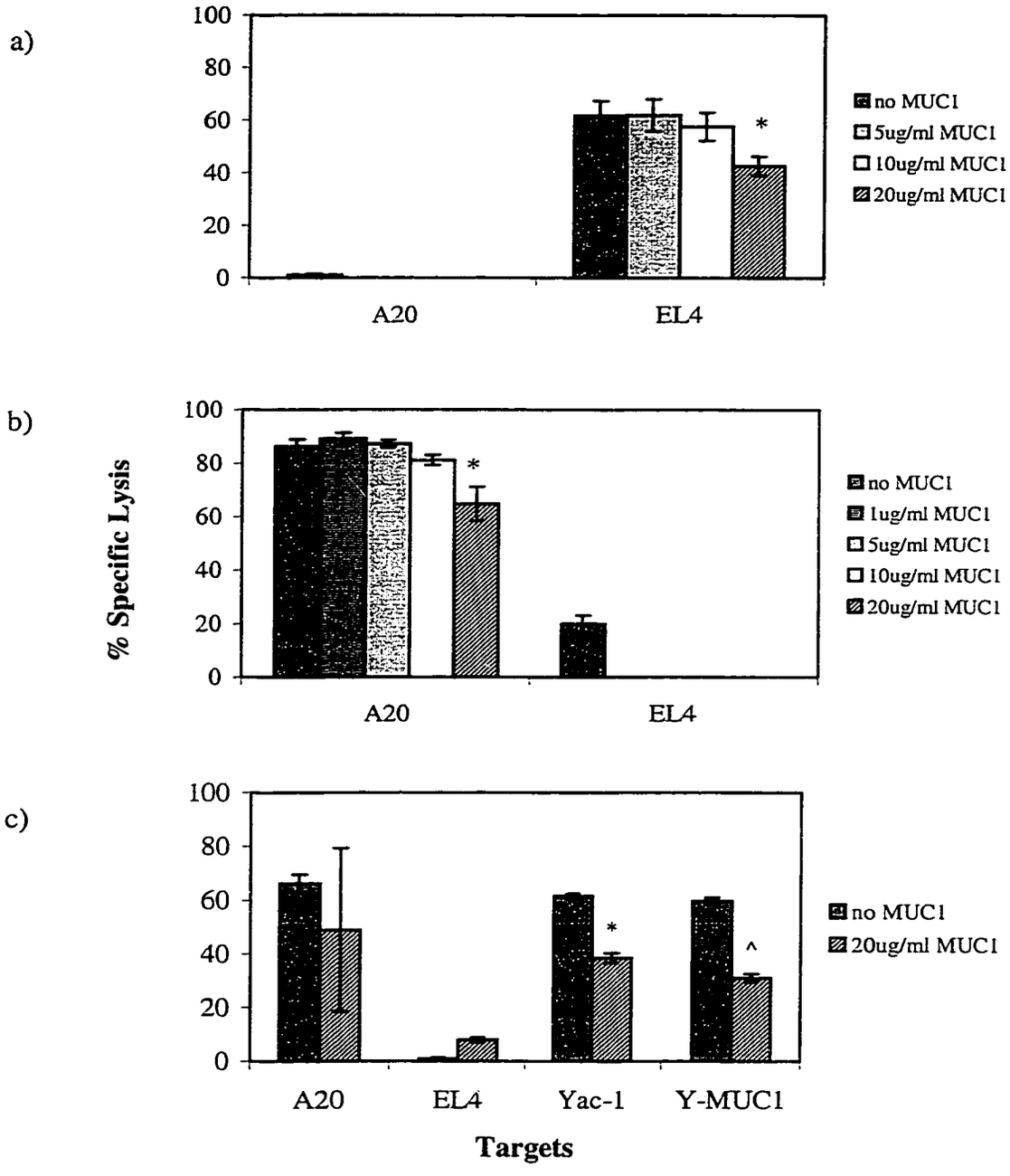


Figure 6-8: Soluble MUC1 mucin produces a small but consistent inhibition of % specific lysis by CTLs.

CTLs were added to ^{51}Cr-labelled target cells for 4 hours after which time supernatants were collected and measured for amount of ^{51}Cr released. a) Clone 11 CTLs (n = 6; *p = 0.0205 vs no MUC1), b) B6αBalb/c CTLs (n = 3; *p = 0.0613 vs no MUC1) and c) Clone 10/1 CTLs (n = 3; *p = 0.0019, ^p = 0.0002). Results are shown as mean percent specific lysis ± SEM.

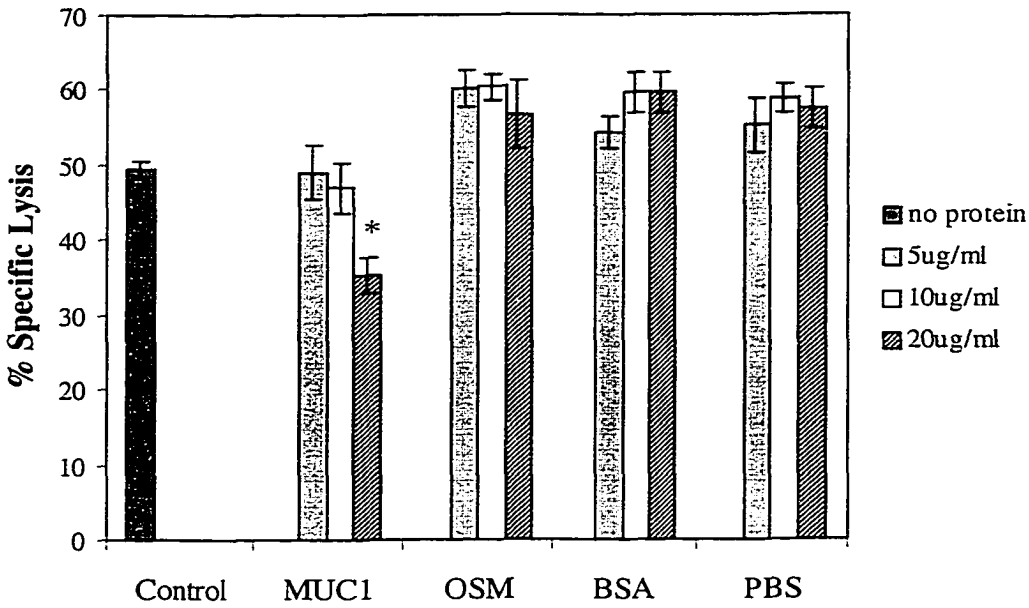


Figure 6-9: Inhibition of lysis of target cells is specific to MUC1 mucin.

The CTL clone 11 was added to ^{51}Cr-labelled EL4 target cells for 4 hours at an effector:target ratio of 1:1 in the presence or absence of 20, 10 or 5 μg/ml MUC1, OSM, BSA or PBS after which time supernatants were collected and measured for amount of ^{51}Cr released. Results are shown as mean percent specific lysis ± SEM (n = 3); *p = 0.0168.

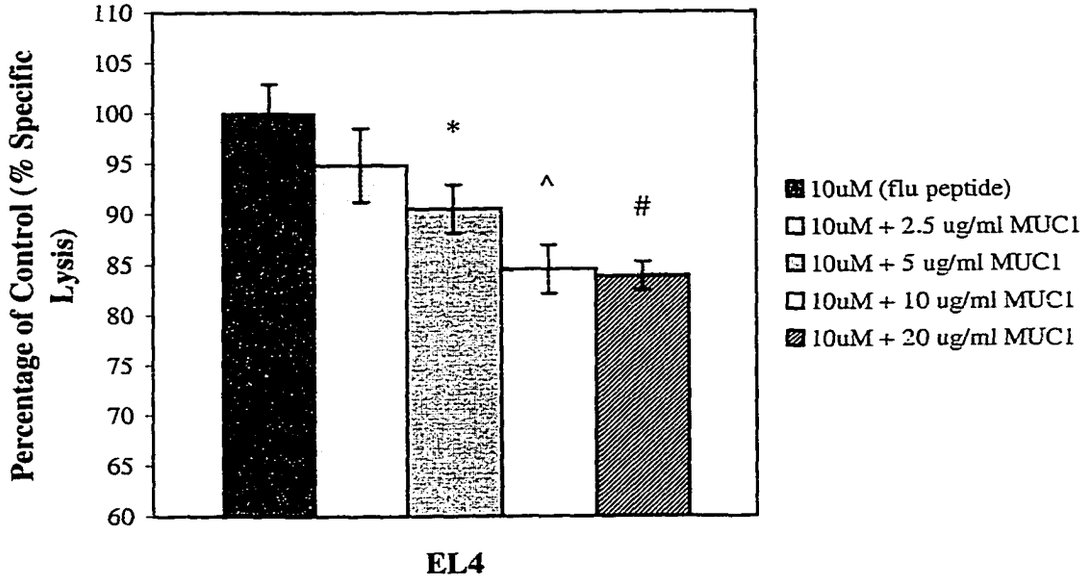


Figure 6-10: Soluble MUC1 mucin significantly inhibits killing of targets by a syngeneic, flu-specific CTL clone.

The CTL clone 3/4 was added to peptide-pulsed (flu-peptide $NP_{365-380}$), ^{51}Cr-labelled EL4 target cells in the presence or absence of 20, 10, or 5 μg/ml MUC1 mucin for 4 hours after which time supernatants were collected and measured for amount of ^{51}Cr released. Results are expressed as a percentage of the % specific lysis of the positive control in order to compare across experiments with different backgrounds. Results are shown as the mean percent of control ± SEM (n = 9); *p = 0.0254, ^p = 0.0010, $^{\#}$p = 0.0004.

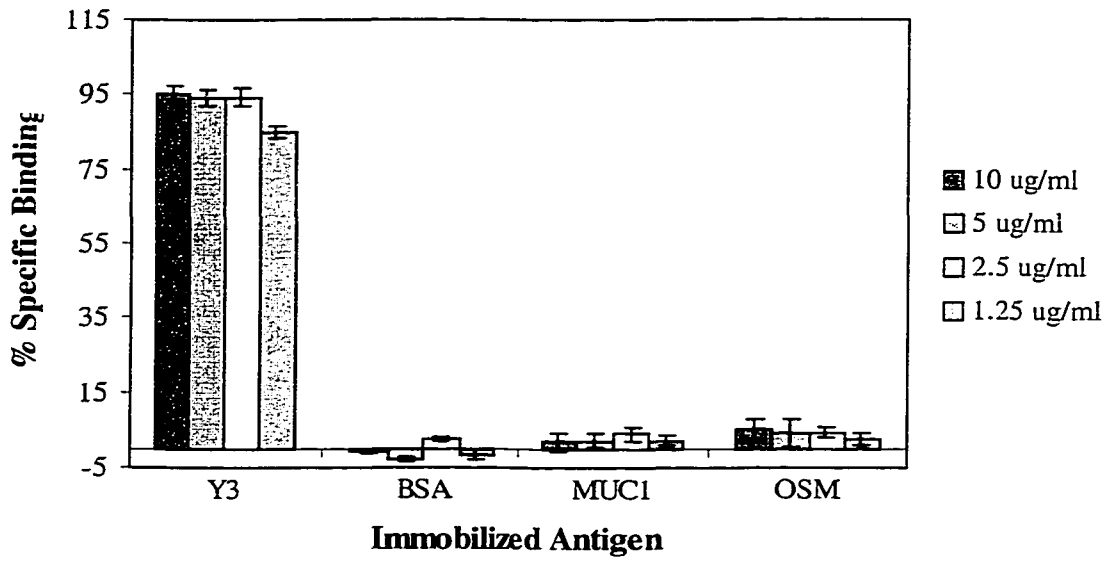


Figure 6-11: CTL clone 3/4 does not bind to immobilized MUC1 mucin.

Antigens were immobilized on plastic 96 well plates overnight (Y3 = the positive control, anti-H-2K^b antibody). ^{51}Cr-labelled clone 3/4 CTLs were added for one hour then washed gently and counted. Results are shown as mean percent specific binding ± SEM (n = 3).

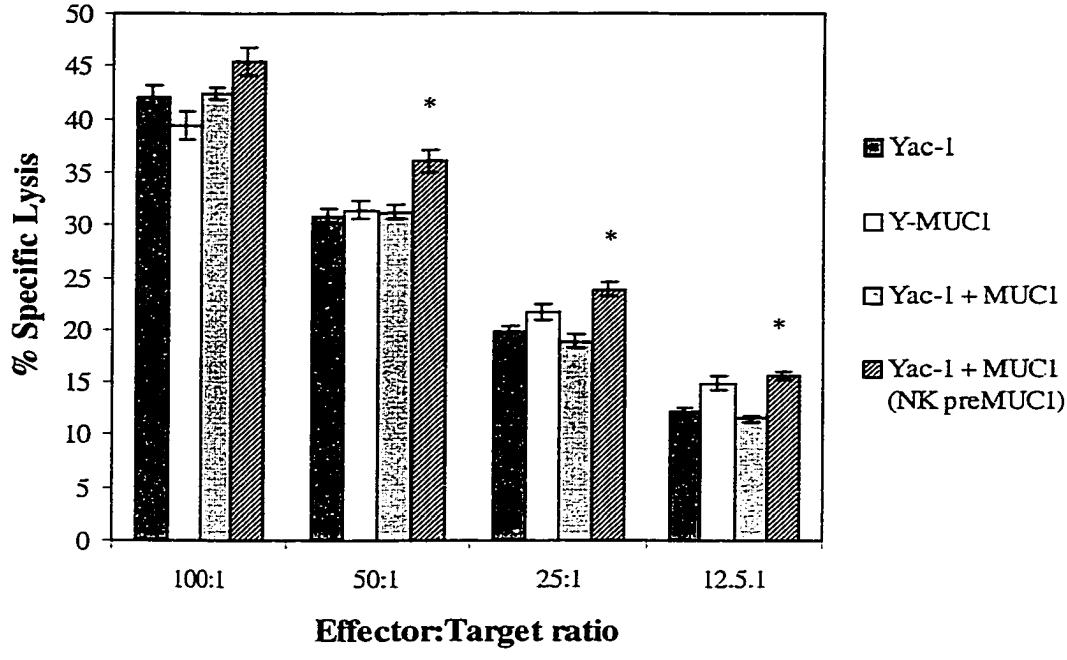


Figure 6-12: Neither cell surface MUC1 nor soluble MUC1 mucin significantly inhibit killing of target cells by NK cells.

NK cells were added to ^{51}Cr-labelled Yac-1 and Y-MUC1 (Yac-1 transfected with MUC1) cells at effector to target ratios of 100:1, 50:1, 25:1 and 12.5:1 for 4 hours after which time supernatants were collected and measured for amount of ^{51}Cr released. Half of the NK cells were mixed with 10 μg/ml MUC1 mucin (+ MUC1) just prior to addition to target cells. (NK preMUC1 = 30 minute preincubation of NK cells with MUC1 prior to addition to target cells). Results are shown as mean percent specific lysis ± SEM (n = 5); *$p \leq 0.005$ (compared to Yac-1).

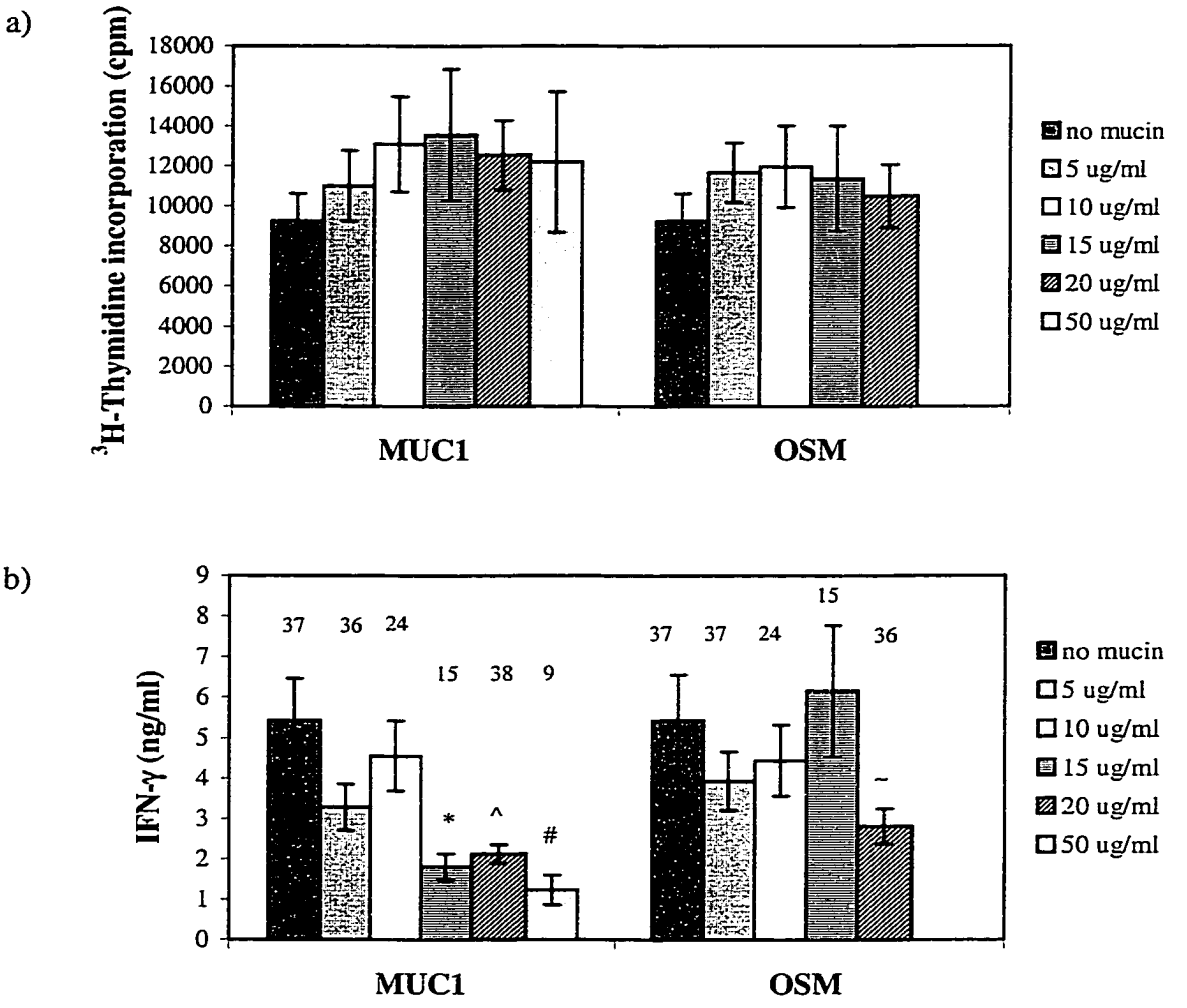
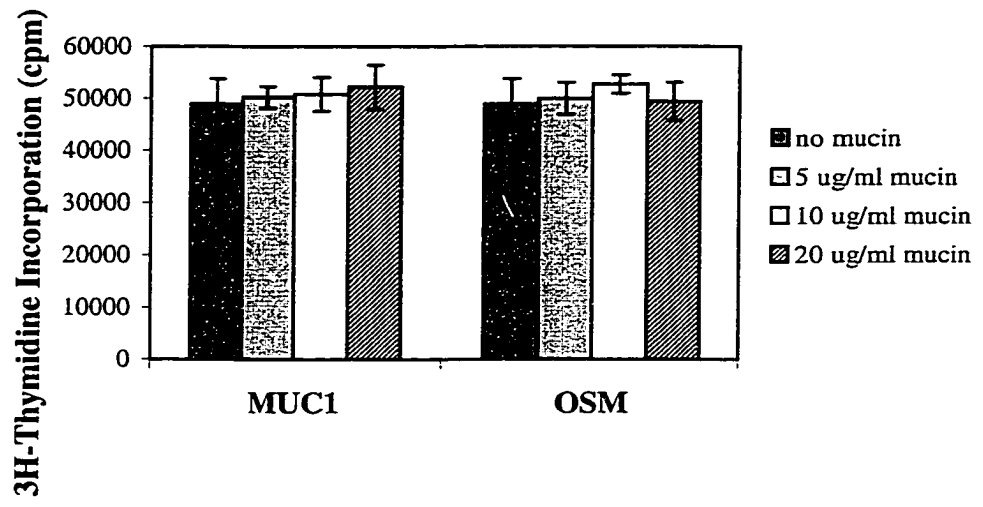


Figure 6-13: Addition of soluble MUC1 mucin to primary allo-MLRs significantly inhibits IFN-γ production while not affecting T cell proliferation.

Allo-MLR assays were set up as described in **CHAPTER II**. a) T cell proliferation measured by ^{3}H-Thymidine incorporation in cpms, and b) IFN-γ measured in ng/ml from supernatants from T cell proliferation. Results are shown as mean ± SEM (n = number above each bar – same n for a) and b)); *p = 0.0053, ^p = 0.0076, $^{\#}$p = 0.0012, ˜p = 0.0395.

a)



b)

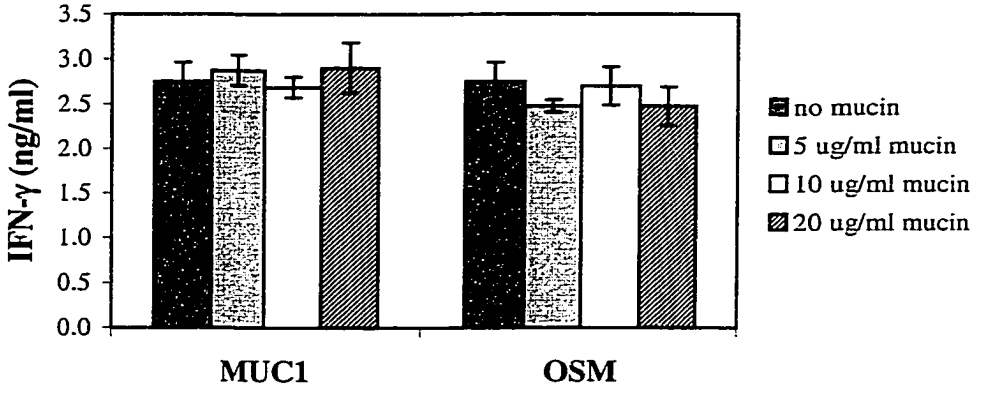


Figure 6-14: Addition of soluble MUC1 mucin to an antigen-specific response does not inhibit T cell proliferation or IFN-γ production.

Mice were immunized with the peptide BP1-148 and after 7 days their T cells were tested *in vitro* in a recall response to BP1-148 in the presence or absence of either MUC1 or OSM mucin. a) Proliferation was measured in cpms and, b) IFN-γ was measured in ng/ml from 1:5 dilutions of the supernatants from the T cell proliferation. Results are shown as mean ± SEM (n = 5).

CHAPTER VII

Discussion

Cancer is the second leading cause of death in North America after cardiovascular disease. Currently surgery, radiation and chemotherapy are the best methods of treating cancer. In an effort to find less toxic methods of treatment and possible methods of prevention, immunotherapies are being investigated. When designing and testing novel immunotherapies, it is important to have suitable animal models. Using appropriate models, mechanisms of tumor progression and lethality can be investigated as well as determining the best approach to inhibit the growth of a particular tumor.

A) MUC1 animal model

i) Tumor cell line

In order for an immunotherapy to be effective it must elicit an appropriate immune response against the tumor. Cancer-associated mucins contain epitopes that can be potentially recognized as foreign by the immune system and they have been the focus of many forms of immunotherapy. There are several reports in the literature which show that MUC1 mucin is immunogenic in mice [Apostolopoulos et al, 1994, 1995; Lalani et al, 1991; Ding et al, 1993; Acres et al, 1993]. However, these models relied on the rejection of MUC1 positive tumors and did not investigate the role of MUC1 in tumor

progression. My work involved developing a mouse mammary adenocarcinoma cell line, containing full-length human MUC1 cDNA, that would be stable *in vivo* and that could metastasize and kill mice. While challenge with GZHi cells could kill mice, the survival rate of the mice was similar to parental 410.4 challenged mice. Examination of the tumors from GZHi challenged mice revealed $MUC1^-$ tumor cells similar to results reported by other groups [Lalani et al, 1991; Apostolopolous et al, 1994; Rowse et al, 1998]. Surprisingly, GZLo cells were more lethal than the parental cell line in both normal as well as immune deficient mice at cell doses less than 10^6. GZLo remains stable for surface MUC1 expression for over two months both *in vitro* and *in vivo* and secretes MUC1 mucin detectable in the serum of tumor bearing mice. Previous models have not been able to demonstrate cell surface MUC1 stability on a murine adenocarcinoma cell line for this length of time [Lalani et al, 1991; Smorodinsky et al, 1994] and have not reported the presence of MUC1 mucin in the serum of tumor bearing mice, which happens in human cancer patients. These cells have already been used to demonstrate the binding of ICAM-1 to MUC1 [Regimbald et al, 1996] and as a tumor model for testing liposomal MUC1 peptide-based vaccines [Samuel et al, 1998].

ii) Mouse model

In normal C.B-17 mice, the presence of human MUC1 mucin on the surface of the GZLo cell line was associated with decreased survival compared to the WILD type tumor cells (Figure 4-18). There was no correlation between tumorigenicity and lethality. In addition, there was no difference in the number of grossly detectable metastases between

the two groups at the time of death. However, the mice injected with WILD type cells had longer to develop metastases since the mice lived longer, consistent with the possibility that GZLo tumor cells might metastasize earlier than WILD type cells.

Decreased survival following challenge with GZLo tumor cells compared to mice challenged with GZHi tumor cells was also seen in CB6F1 mice although at a high challenge dose (10^6 tumor cells) survival of mice injected with GZLo tumor cells was comparable to the survival of mice injected with WILD type tumor cells. A lower challenge dose of GZLo tumor cells may be preferred over a higher dose for studying effects of MUC1 since low dose GZLo challenged mice showed a significant correlation between rising serum MUC1 and decreased survival. The level of serum MUC1 rather than primary tumor burden appears to be a better prognostic indicator in human cancer patients [Maclean et al, 1997; Reddish et al, 1996]. These data are consistent with a possible immunosuppressive role for MUC1 secreted by GZLo cells.

There was no dose effect seen for the number of tumor cells injected in the pilot study but in the repeat study there was a dose effect. This may have been due to the small group numbers used in the pilot study and the fact that the pilot study was terminated at day 63 with the majority of mice still alive. The repeat study used larger groups of mice and the experiment was carried out over a longer time frame, to see if a dose effect could be determined. In the repeat study, at the time point of 60 days the mice were just beginning to die as can be seen in Figure 4-6 providing a possible explanation as to why a dose effect was not observed in the pilot study. This could also account for a lack of difference in tumor growth or size in the pilot study.

There was a small but insignificant dose effect with the parental 410.4 cells in the pilot study. The transfected cell lines grew more slowly as a subcutaneous tumor compared to 410.4 cells. A lower tumor area and slower growth for the transfected cell lines was not unexpected since they carry a foreign protein and this type of rejection has been reported [Lalani et al, 1991]. This indicated that there was likely an immune response to the MUC1 on transfected cells. However, despite the presence of a foreign tumor antigen, GZLo challenged mice surprisingly had shorter survival than parental 410.4 challenged mice. GZHi challenged mice in the pilot study, also with lower tumor area than parental cells, did not differ in survival from 410.4 challenged mice. Previous reports using human MUC1 transfected murine cells have indicated that human MUC1 mucin is immunogenic in normal mice resulting in rejection of the transfected tumors [Apostolopoulos et al, 1994, 1995; Lalani et al, 1991; Ding et al, 1993; Acres et al, 1993]. The results using the GZHi cell line are in agreement with this hypothesis. However, shorter survival using the GZLo cell line in mice was unexpected and contrary to previous results using human MUC1 transfectants in mice [Apostolopoulos et al, 1994, 1995; Lalani et al, 1991; Ding et al, 1993; Acres et al, 1993].

It has been suggested that overexpression of MUC1 mucin may be an important factor in tumor progression [Hilkens et al, 1992]. *In vitro* studies have shown that cell-surface mucins can interfere with host immune responses [Sherblom and Moody, 1986; Bharathan et al, 1990; van de Wiel-van Kemenade et al, 1993]. In addition, cell-surface mucins have been implicated in the masking of cell-surface recognition molecules, such as MHC molecules [Codington et al, 1975; Jentoft, 1990; van de Wiel-van Kemenade et al, 1993]. These studies together suggest that cell-surface MUC1 is important to tumor

progression and immune evasion. Upon examining the tumor cells from each group of normal mice, it was noted that the majority of GZHi tumors had a high percentage of cells negative for cell-surface MUC1 expression. This was consistent with previous studies which showed that MUC1-transfected tumors that were not rejected *in vivo* in normal mice had lost cell surface expression of MUC1 [Lalani et al, 1991; Apostolopolous et al, 1994; Rowse et al, 1998]. It was possible that the higher levels of MUC1 on GZHi cells induced a greater immune response against $MUC1^+$ cells than the MUC1 on GZLo cells, which may have contributed to immune evasion instead.

Furthermore, in the pilot study the mice that died earliest, which consisted mainly of GZLo challenged mice, had higher levels of serum MUC1 than those mice that survived to day 63. The higher levels of MUC1 mucin in the serum were not the result of larger subcutaneous tumors since those mice that died sooner had smaller subcutaneous tumors. This indicated that in this model the presence of serum MUC1 and not increased tumorigenicity was correlated to decreased survival. This was consistent with human studies where high levels of serum MUC1 and not tumor burden correlated with poor prognosis [Maclean et al, 1997; Reddish et al, 1996].

The repeat study revealed similar results: GZHi challenged mice survived longer than GZLo challenged mice, GZHi tumors lost cell surface MUC1 expression, while GZLo tumors did not and GZLo challenged mice had high levels of serum MUC1 which correlated to decreased survival, while GZHi challenged mice had negligible levels of serum MUC1. These results are compatible with the hypothesis that the GZHi cell line induced an anti-MUC1 immune response in these mice. Early processing and presentation of MUC1 peptides by APCs to T cells may result in an immune response to

the $MUC1^+$ GZHi cells, allowing only negative revertants to grow out. On the other hand, GZLo cells may express low enough MUC1 levels to allow efficient metastasis and prevent immune stimulation. The amount of MUC1 mucin present could be important in determining the outcome of the immune response.

Both GZHi and GZLo challenged mice produced anti-MUC1 antibodies although the role of these antibodies is unclear. Previous reports have indicated that an antibody response does not correlate with tumor rejection, but rather the presence of antibody most likely indicates a bias towards a humoral rather than a cellular response and therefore tumor progression [Acres et al, 1993; Apostolopolous et al, 1994; Graham et al, 1996]. An IgG_1 response has been shown to directly correlate with the growth of subcutaneous tumors, as well as the appearance and growth of tumor foci in the lungs of intravenously challenged, MUC1-vaccinated mice [Samuel et al, 1998]. Interestingly, in GZLo challenged mice, those with low serum MUC1 levels had a significant correlation between high anti-MUC1 antibodies and decreased survival (Figure 4-13). GZLo challenged mice with rising serum MUC1 levels (ie. those that died earliest) had no significant correlation between anti-MUC1 antibody level and survival. Rising serum MUC1 levels have been postulated in this model to predict poor prognosis in mice. However, in the remaining mice (those with low serum MUC1), high antibody levels could predict poor prognosis. High antibody levels could be indicative of a primarily humoral response in these mice resulting in tumor progression and death [Acres et al, 1993; Apostolopolous et al, 1994; Graham et al, 1996]. In addition, high antibody levels could contribute to low levels of detectable serum MUC1 through incorporation into immune complexes. The incorporation of soluble MUC1 into immune complexes affects

the detection of circulating MUC1 as well as anti-MUC1 antibodies [Gourevitch et al, 1995]. Therefore, in the absence of rising serum MUC1 levels, high antibody titres could also predict poor prognosis, in GZLo-challenged mice.

Poor prognosis was not observed with GZHi challenged mice, which also had low levels of serum MUC1, possibly due to "early" rejection of $MUC1^+$ GZHi tumor cells. However, anti-MUC1 antibody could be detected in GZHi challenged mice. High levels of antigen, such as that found on GZHi cells initially, typically induce a humoral response. Anti-MUC1 antibody in GZHi-challenged mice could result in increased ADCC against $MUC1^+$ GZHi cells. An anti-MUC1 humoral response coupled with or followed by an anti-MUC1 cellular response could result in the outgrowth of only $MUC1^-$ variants. A similar combination of humoral and cell-mediated MUC1 specific immune responses has been detected following MUC1 immunization [Graham et al, 1996].

In the absence of an immune response, as in SCID-BEIGE mice, it appears that GZHi cells retain cell surface MUC1 expression and produce high levels of serum MUC1 *in vivo*, further supporting the hypothesis that GZHi cells induced an anti-MUC1 immune response in normal mice. In addition, the serum MUC1 levels in SCID-BEIGE mice indicate that the amount of MUC1 secreted by tumor cells *in vivo* is proportional to the amount of MUC1 secreted by tumor cells *in vitro* as previously reported for human tumor cells [McGuckin et al, 1995]. However, GZLo cells with a lower level of both cell surface and serum MUC1 remain more lethal than GZHi under the same conditions in SCID-BEIGE mice indicating that the amount of MUC1 expressed by a cell may be important in contributing to its ability to metastasize and kill mice. Alternately, it is

possible that there are inherent differences in these two cell lines and that the GZLo cell line is more aggressive due to other factors, such as the selection process or the integration site of the transfected gene.

There was little or no difference in tumor area between GZHi and GZLo challenged CB6F1 mice. In these mice, at a tumor cell dose of 1 x 10^6, there was no significant difference in tumor area between the transfectants (GZHi and GZLo) and the parental cell line. While previous studies have shown a decrease in tumor growth rate for MUC1-transfected cells compared to parental cells [**CHAPTER IV, Section A**); Lalani et al, 1991], the results of the repeat experiment [**CHAPTER IV, Section B**)] would indicate that the MUC1-transfected cells were able to overcome any additional immune response, due to MUC1, and grow at the same rate as the parental 410.4 cells. All mice had extensive metastases at the time of death, which were difficult to quantify. However, GZLo cells may have been able to metastasize earlier as mice challenged with these cells died earlier with similar metastatic extent as GZHi and 410.4 challenged mice which died at a later date and therefore had longer to develop metastases. This would need to be investigated systematically. No differences in metastases were distinguishable between the groups at day 63 in the pilot study.

Survival of mice, in the repeat study, challenged with 410.4 did not differ significantly from survival of mice challenged with GZLo at a tumor challenge dose of 1 x 10^6 cells. Both groups of mice had extensive metastases at the time of death. The parental cell line, 410.4, does not express human MUC1, but nevertheless is still a metastatic tumor cell line that can kill mice. The lack of difference in survival at this dose may indicate that the MUC1 on GZLo cells is either not immunogenic or is weakly

immunogenic, but the GZLo cells are able to overcome this immune response and kill the mice. If this is the case, then GZLo is in fact more lethal than 410.4, as GZLo would have to prevail against a specific immune attack to maintain its growth. In C.B-17 mice at a lower tumor cell dose, challenge with GZLo cells resulted in significantly shorter survival than challenge with 410.4 cells, supporting the hypothesis that MUC1 contributed to decreased survival.

The fact that SCID mice challenged with GZLo cells, but not 410.4 cells, had significantly longer median survival than similarly challenged SCID-BEIGE mice suggests that MUC1 expressed by GZLo cells may be interacting with NK cells *in vivo* resulting in the inhibition of the growth of $MUC1^+$ tumors to some degree. However, the statistical difference may not be biologically relevant, as the difference in median survival is only one day. Previous results have indicated that cell surface mucins inhibit NK cell killing although the mechanism is unknown [Sherblom and Moody, 1986]. However, cell surface MUC1 was not able to inhibit NK killing of MUC1 transfected targets (Figure 6-12). The present results in SCID and SCID-BEIGE mice suggest that NK cells are able to eliminate some GZLo cells, possibly during metastasis. Preincubation of NK cells with soluble MUC1 prior to addition to target cells was able to increase target cell lysis (Figure 6-12). Perhaps soluble MUC1 contributed to the slight difference seen in survival of SCID versus SCID-BEIGE mice challenged with GZLo cells.

The fact that the CB6F1 mice challenged with the GZHi cell line survived longer than GZLo challenged mice and subsequently lost surface MUC1 expression is compatible with the hypothesis that the GZHi cell line induced an anti-MUC1 immune

response in these mice. The possibility that GZHi spontaneously reverted to a negative phenotype shortly after injection is unlikely since GZHi cells were able to retain cell surface MUC1 and secrete high levels of soluble MUC1 in SCID-BEIGE mice. These results are compatible with the hypothesis that the higher initial concentration of MUC1 on GZHi cells was able to induce an effective anti-MUC1 immune response in CB6F1 mice, while the lower surface concentrations of MUC1 on the GZLo cell line allowed for "sneaking through" of GZLo tumors. In addition, it is possible that secreted or shed MUC1 mucin from GZLo tumors could induce immunosuppression, allowing tumors to grow faster in "normal" mice.

B) Soluble MUC1 contribution to immune modulation

In clinical studies it has been shown that while there is no correlation between serum MUC1 mucin levels and tumor burden in a population of cancer patients, serum MUC1 mucin level was a predictor of poor survival in metastatic colorectal, pancreatic, breast and ovarian cancer patients [Maclean et al, 1997: Reddish et al, 1996]. In breast cancer, rising levels of serum MUC1 mucin in individual patients was a highly significant and independent predictor of recurrent breast cancer [Chan et al, 1997]. A major component of the carbohydrate on MUC1, STn has also been associated with poor prognosis in number of patients with colorectal, ovarian and gastric cancers [Itzkowitz et al, 1990; Kobayashi et al, 1992; Werther et al, 1996]. In a previous animal model it was shown that epiglycanin, a murine mucin-like molecule, could induce a state of immunosuppression when injected i.v., leading to shorter survival times following tumor challenge [Fung and Longenecker, 1991].

i) In vivo soluble MUC1

In C.B-17 and CB6F1 mice there was no correlation between serum MUC1 levels and primary tumor area. However, there was MUC1 mucin present in the serum of GZLo challenged mice and rising serum MUC1 levels in these mice could be used as a predictor of poor prognosis, similar to humans. Although rising serum MUC1 correlates to poor prognosis, it is possible that circulating MUC1 does not directly contribute to tumor progression and death. Increased levels of serum MUC1 may be due to a loss of liver function or some other metabolic defect in the mice that is secondary to the cancer and not directly related to the effects of MUC1.

In mice exposed to circulating, soluble MUC1 mucin *in vivo*, immunization with a MUC1 specific peptide resulted in an antigen-specific inhibitory effect on IFN-γ production, while immunization with an unrelated peptide, HIV-V3, did not affect IFN-γ production or proliferation as measured *in vitro*.

Exposure of unprimed T cells to MUC1 mucin *in vivo* did not appear to affect subsequent activation or differentiation of these T cells *in vitro*. However, exposure of APCs to MUC1 mucin *in vivo* appeared to affect the APCs ability to later induce IFN-γ production from primary T cells. Interestingly, APCs exposed to OSM *in vivo* were able to decrease IFN-γ production by normal T cells to a greater extent than MUC1 exposed APCs. These results suggest that not only MUC1 mucin, but also a control mucin, OSM, may have an effect on APCs *in vivo* resulting in decreased production of IFN-γ from T cells. Furthermore, in *in vitro* allo-MLR cultures, addition of OSM at a concentration of 20 μg/ml was also able to decrease IFN-γ production significantly. In those assays,

addition of MUC1 had a greater inhibitory effect than OSM (Figure 6-13b). OSM and MUC1 share the STn antigen as a major component of their carbohydrates [Hill et al, 1977]. STn has been associated with poor prognosis in colorectal, ovarian and gastric cancer patients [Itzkowitz et al, 1990; Kobayashi et al, 1992; Werther et al, 1996] and may well have functional significance in metastasis [Ogawa et al, 1992; Bresalier et al, 1996]. Immunization with a synthetic STn vaccine resulted in a specific IgG response in both mice and human breast cancer patients and these vaccine-induced, anti-STn antibodies correlated with survival of metastatic adenocarcinoma patients [Longenecker et al, 1994; MacLean et al, 1996]. It is possible that the STn moiety is contributing to or is responsible for the decreased IFN-γ production from T cells in the presence of MUC1 or OSM in the T cell proliferation assays performed.

ii) In vitro soluble MUC1

Recently it has been shown that soluble MUC1 mucin can inhibit human T-cell proliferation *in vitro* [Agrawal et al, 1998; Chan et al, 1999]. While there was no significant difference in proliferation of murine T cells in the presence or absence of MUC1 mucin in primary allo-MLRs (except experiments #3 & 4 in Table 6-2), some preliminary experiments indicated that there may be a significant decrease in IFN-γ production in the presence of MUC1 mucin but not OSM. Further experiments in 96 well plates have shown that IFN-γ production from allo-MLRs is significantly inhibited in a dose dependent fashion by soluble MUC1.

When low levels of IFN-γ were observed, no differences could be seen between any of the groups, regardless of the addition of MUC1 or OSM to allo-MLR cultures. In the experiments where IFN-γ levels were higher, a significant decrease in IFN-γ production was observed in the presence of MUC1 compared to OSM. OSM appears to inhibit IFN-γ production by somehow affecting APCs and/or their subsequent interaction with T cells *in vivo* and not on T cell proliferation cultures *in vitro*, except perhaps at higher concentrations.

Barnd et al [1989] have previously shown that a purified pancreatic tumor mucin could inhibit CTL lysis of a pancreatic tumor target, but not a control target. In the present studies, soluble MUC1 but not cell-surface MUC1 was able to inhibit, in a dose-dependent manner, the killing of target cells by different CTLs. The soluble form may interact more easily with CTLs. However, CTLs did not bind appreciably to solid phase MUC1 mucin according to the binding experiment performed. It is possible that MUC1 mucin binds to CTLs transiently and in solution thereby downregulating their function. In the assay format used, weak binding of the cells to immobilized MUC1 would not likely be detected. Recently, activated human T cells have been shown to express MUC1 [Agrawal et al, 1998]. It was postulated that MUC1 may have an important immune regulatory role and may provide a negative feedback mechanism to return the immune system to its basal resting state following antigenic stimulation [Agrawal et al, 1998b].

Although soluble MUC1 was able to inhibit CTL lysis in a dose dependent fashion, it was rarely seen to be more than 15% inhibition. The biological significance of this level of inhibition may not be relevant since there were a significant number of CTLs still capable of killing the targets. However, in the microenvironment of the tumor there

may be sufficient levels of MUC1 present to inhibit any CTLs that are encountered. It is also possible that the subtle effects seen were due to experimental procedure and that a low level of contamination of the purified mucin was causing the inhibition. Yet experiments using NK cells or splenic T cells did not show inhibition and therefore it is less likely that contaminants were responsible for the inhibition seen with CTLs, unless the CTLs used were more susceptible than other effector cells.

Neither soluble nor cell-surface MUC1 was able to inhibit NK cell killing of target cells. The experiments with SCID mice, in addition to the NK killing assays using NK cells preincubated with MUC1 suggested that the MUC1 produced on or by GZLo cells could possibly induce a limited NK response *in vivo*. This could account for the small difference in median survival observed between SCID and SCID-BEIGE mice challenged with GZLo cells. Previous results have suggested that MUC1 secreted by a colon carcinoma cell line could inhibit NK lysis of target cells [Zhang et al, 1997]. However, the concentration of MUC1 necessary for inhibition in those experiments was 50-100 μg/ml, which even the authors suggested might be considered high.

C) Cell-surface MUC1 and tumor progression

The fact that cell surface MUC1 could not inhibit killing of a variety of $MUC1^+$-target cells by a number of CTLs is contrary to the belief that the rigid structure and size of MUC1 mucin on the cell surface blocks recognition factors on the surface of the target cell [van de Wiel-van Kemenade et al, 1993]. It is likely that the number of MHC molecules and adhesion molecules on the surface of the target cell play a significant role

in the ability of the target cell to be killed and not the presence of cell-surface MUC1. In fact, in previous studies, the $MUC1^+$ target cells which showed decreased killing by CTLs also had reduced levels of ICAM-1 and HLA class I compared to control, $MUC1^-$, target cells [van de Wiel-van Kemenade et al, 1993]. In addition, activation of β1 integrins was able to restore adhesion of $MUC1^+$ cells [Wesseling et al, 1995]. In the current model it appears that soluble MUC1 has more of an impact on CTL inhibition than cell-surface MUC1. Furthermore, cell-surface MUC1 mucin was unable to inhibit killing of murine target cells by murine NK cells. Cell-surface sialomucins on target cells have previously been reported to impart resistance to NK cell killing [Sherblom and Moody, 1986; Bharathan et al, 1990].

Glycosylation contributes, in part, to the extended, rigid structure of mucins, therefore, the amount of carbohydrate is important in the generation of steric bulk [Jentoft, 1990]. It is not known to what extent human MUC1 mucin is glycosylated in murine adenocarcinoma cells. In addition, different cell types/lines have the potential to glycosylate differently [Dahiya et al, 1993]. Therefore, it is possible that a difference in glycosylation could account for the difference seen between the results presented here and those by van de Wiel-van Kemenade et al [1993], who used human MUC1-transfected human melanoma cells. In addition, glycosylation differences could account for the difference in NK killing, as previous groups examined sialomucins expressed by rat mammary tumor ascites cells [Sherblom and Moody, 1986; Bharathan et al, 1990].

D) Alternate role for MUC1 mucin in tumor progression

In view of the evidence for an immunosuppressive role for MUC1, it was surprising that in SCID-BEIGE mice, the survival of mice injected with GZLo cells is still shorter than survival of mice injected with either GZHi or WILD type cells. This suggests that a relatively low MUC1 mucin concentration may play a role in enhancing the aggressiveness of GZLo tumors by other, non-immune mechanisms.

The presence of cell-surface MUC1 on a tumor cell can contribute to the cell's ability to metastasize. Increased expression of MUC1 mucin on tumor cells contributes to decreased cellular aggregation [Ligtenberg et al, 1992] which could allow cells to "break away" from the primary tumor. In addition, overexpression of MUC1 has been shown to reduce integrin-mediated cell adhesion to various extracellular matrix (ECM) components [Wesseling et al, 1995] which could allow $MUC1^+$ tumor cells to migrate through the ECM more effectively during metastasis. It has also been shown that MUC1 mucin can bind to ICAM-1 and E-selectin [Regimbald et al, 1996; Sawada et al, 1994]. These molecules are found on endothelial cells and may allow the intravasation and extravasation of $MUC1^+$ tumor cells during metastasis. MUC1 transfectants have been shown to exhibit increased *in vitro* invasiveness, decreased binding to laminin, fibronectin, type I and type IV collagen and increased motility [Suwa et al, 1998]. In addition, the depolarized cellular distribution of MUC1 has been associated with nodal metastasis and advanced pathological stage in human non-small cell lung adenocarcinoma [Guddo et al, 1998]. The presence of O-linked glycans may also be important to $MUC1^+$ cells ability to metastasize as inhibition of O-glycan biosynthesis

was able to abolish the effect of MUC1 on increased invasiveness and motility [Suwa et al, 1998].

However, since challenge with GZLo cells also resulted in shorter survival than challenge with GZHi cells in SCID-BEIGE mice, there may be other factors affecting the lethality of GZLo versus GZHi. The exact insertion point of the MUC1 cDNA into the parental cell DNA is not known for any of the transfectants and the insertion point may affect the lethality of these cells, depending on location of integration and the genes disrupted, if any. However, it may simply be a function of the amount of MUC1 present. GZHi secretes a significantly higher amount of MUC1 both *in vitro* and *in vivo* in SCID-BEIGE mice. Excessive MUC1 mucin secreted by the cells could bind to and block the adhesion molecules necessary for the cells to migrate, thereby slowing metastasis, whereas the amount of mucin secreted by GZLo cells may not interefere with the binding of cell surface mucin to a significant extent, allowing these cells to move through the extracellular matrix and endothelium more efficiently.

On the other hand, it may not be cell-surface MUC1 that directly contributes to the cells ability to metastasize, but signal transduction events mediated by the cytoplasmic tail of MUC1. Association of MUC1 with β-catenin and/or other cytoskeletal components, such as actin, may influence cell-cell and cell-substratum interactions resulting in increased motility and therefore metastasis [Parry et al, 1990; Yamamoto et al, 1997]. In addition, tyrosine phosphorylation of the cytoplasmic tail and associations with molecules such Grb2 and the Sos/Ras exhange protein may affect intracellular signalling pathways and thereby impart a metastatic phenotype on these cells [Zrihan-Licht et al, 1994; Pandey et al, 1995]. The level of expression of MUC1 may

influence the number of binding sites available to these molecules and the high levels associated with GZHi may result in increased competition with normal molecules that may be necessary in facilitating cell motility and adhesion. Alternately, the high concentration of binding sites in GZHi may result in increased on-off kinetics such that a sufficient signal is not transduced or generated in GZHi cells compared to GZLo cells.

E) Conclusion

There are many advantages of the present model. The GZLo breast adenocarcinoma transfectant metastasizes following subcutaneous challenge, is stable with respect to cell surface MUC1 expression both *in vitro* and *in vivo*, and grows as a more aggressive tumor compared to the parental cell line. Most importantly, growing GZLo derived tumors appear to secrete MUC1 mucin which, like in human cancer patients [Agrawal et al, 1998; Maclean et al, 1997; Reddish et al, 1996], is associated with a poor prognosis. This model is currently being used to evaluate novel immunotherapies designed to overcome MUC1 mucin induced immunosuppression and, used in conjunction with MUC1 based cancer vaccines, to induce effective tumor rejection.

There appears to be a role for soluble MUC1 mucin in inhibiting CTL killing of target cells. In this case secreted mucin may be important in blocking CTL killing although the mechanism is as yet unknown. Likewise, there appears to be a role for soluble MUC1 in inhibiting production of IFN-γ, but not T cell proliferation of murine cells. Interestingly, other mucins, such as OSM may also have an effect on the

generation of an immune response as was seen with the decrease in production of IFN-γ from T cells stimulated with APCs previously exposed to OSM.

The MUC1 mucin appears to be contradictory. On the one hand it is immunogenic and is able to elicit an anti-tumor immune response, and on the other hand it is capable of eluding the immune system by promoting tumor progression resulting in death of the host. Further dichotomy results from the biochemical features of mucins that allow them to be both adhesive and anti-adhesive. It appears that there is a delicate balance between induction versus inhibition of the immune system by MUC1 and the amount of MUC1 mucin may play a role. These features need to be considered when designing and testing novel immunotherapies for $MUC1^+$ cancers.

CHAPTER VIII

Future Directions

From the data revealed in this thesis, there are many questions that remain to be answered with regards to MUC1 and its role in tumor progression and immune modulation. Some of these questions may unveil important information that will contribute to the design and testing of novel immunotherapies for cancer.

A) Importance of high MUC1 expression versus low MUC1 expression

There are several interesting differences between GZHi and GZLo. GZHi loses MUC1 expression in the face of immune pressure, but can retain expression in the absence of immunity. However, GZLo is still more lethal than GZHi in immune deficient mice suggesting that the amount of MUC1 may play a role in metastasis and/or lethality of the cell line or it could simply be that the two cell lines are inherently different. In order to verify some of these differences, it would be worthwhile to establish additional cell lines expressing either high or low levels of MUC1. The survival experiments could then be repeated in both normal and immune deficient mice with these new cell lines in comparison to GZHi and GZLo as well as parental 410.4 cells. Also further characterization of GZHi and GZLo could be done, such as determining copy number, to ascertain possible differences in these as well as any other transfectants.

To further delineate contributions of high versus low levels of cell-surface MUC1 expression, *in vitro* experiments could be utilized to establish differences in motility and

adhesion between GZHi versus GZLo. It has already been shown that MUC1 transfectants exhibit increased invasiveness *in vitro* and decreased binding to several ECM components over control cells [Suwa et al, 1998; Hudson et al, 1996; Wesseling et al, 1995]. It would be interesting to see if the level of expression of MUC1 on the cell surface could contribute to the extent of increased invasiveness or decreased adhesion of the cell. In addition, concurrent experiments examining the intracellular signalling events during adhesion and invasion of high and low MUC1 expressing cells could distinguish possible differences in signal transduction between the two.

Suwa et al [1998] indicated that O-linked glycosylation contributes to increased invasiveness and decreased adhesion by MUC1 since treatment with an inhibitor of O-glycan biosynthesis abolished these effects. Another possibility to account for differences between GZHi and GZLo may be differences in glycosylation. It has been suggested that overexpression of a protein, such as MUC1, with multiple acceptor sites for O-glycosylation may actually saturate the post-translational modification system resulting in novel, truncated structures [Burdick et al, 1997]. Since GZHi is producing more protein than GZLo it is possible that this may affect the glycosylation of MUC1 from these two cells, which may ultimately affect adhesion and invasiveness [Burdick et al, 1997; Suwa et al, 1998].

The size of the MUC1 mucin has been postulated as being important in determining the degree of cell-ECM interaction [Hudson et al, 1996]. However, it is possible that the amount of MUC1 has a greater influence than the actual length. In the experiments by Hudson et al [1996], while the MUC1 on the transfectant with 30 tandem repeats (TR) is surely smaller than the transfectant with 42 tandem repeats, the 30TR

transfectant appears to express more MUC1 protein by western blot. This could account for the fact that the 30TR transfectant was able to contract collagen more than the 42TR transfectant. Therefore, the amount of MUC1 expressed may be important in cell adhesion and motility and this could be addressed using the GZHi and GZLo cell lines.

B) Immune response to MUC1

The GZHi cell line appears to induce an effective anti-MUC1 immune response in normal mice. To investigate the possible effector cells or other factors involved in this immunity, antibody depletion studies or gene knockout mice could be utilized whereby groups of mice deficient in either CD4, CD8, IFN-γ, IL-10, TCRβ, CD28, etc, are monitored for survival and cell surface expression of MUC1 following challenge with GZHi cells. In addition, adoptive transfer of $CD4^+$ or $CD8^+$ T cells from mice challenged with GZHi cells to normal mice that would subsequently be challenged with GZHi could identify if the cells involved could confer immunity to other mice. Furthermore, human MUC1 transgenic mice could be utilized to evaluate the immunogenicity of GZHi cells in a system that is tolerant to MUC1.

C) Immune evasion by MUC1

GZLo cells are more lethal than parental 410.4 cells at certain doses and this may be due to an increased ability of GZLo cells to metastasize due to MUC1 and/or possible immune modulating properties of MUC1 secreted by GZLo cells.

To investigate differences in metastatic potential by GZLo versus 410.4 cells, mice would have to be systematically compared by autopsy at different time points following challenge with either GZLo or 410.4 cells. The previous experiments outlined in section A) would also indicate if GZLo cells are more invasive than 410.4 cells.

In the *in vivo* MUC1 model where mice are injected daily with MUC1 mucin i.p. for several days it was shown that soluble MUC1 could somehow inhibit the ability of APCs to induce IFN-γ production from T cells while not affecting T cell proliferation. IL-10 has been shown to have a similar effect in decreasing dendritic cell-induced INF-γ production while not affecting primary T cell proliferation [Macatonia et al, 1993]. Splenic APCs from IL-10 transgenice mice have been shown to have a modified surface phenotype, specifically showing decreases in the level of CD11c, CD40, B7.1, B7.2 and MHC class I [Sharma et al, 1999]. To determine what effect MUC1 may be having on APCs, APCs removed from mice exposed to high levels of circulating MUC1 could be examined for cell surface expression of CD11c, CD40, B7.1, B7.2 and MHC class I/II. In addition, these APCs could be examined for their ability to produce cytokines such as IL-12 [Sharma et al, 1999]. IL-12 production is downregulated in macrophages from mice with progressive tumor growth and this has been shown to be due to various tumor derived factors [Handel-Fernandez et al, 1997]. Furthermore, these *in vivo* MUC1 experiments could be used to identify what type of APC is most likely affected. IL-10 is able to inhibit the ability of macrophages and dendritic cells, but not B cells, to induce IFN-γ production from T cells [Fiorentino et al, 1991; Macatonia et al, 1993].

Soluble MUC1 appears to inhibit specific lysis of targets by CTL clones *in vitro* in a dose dependent manner. CTL from IL-10 transgenic mice have been shown to have

a reduced capacity to lyse 3LL tumors [Sharma et al, 1999]. Using the *in vivo* MUC1 model it may be possible to see if CTL from mice exposed to high levels of circulating MUC1 have a reduced capacity to lyse tumor targets. This would indicate if MUC1 is able to downregulate or inhibit the induction of CTLs rather than just blocking their interaction with targets. Similar experiments could be utilized to investigate the effect of MUC1 on NK cell killing.

CHAPTER IX

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