Regulation of tumor cell expression of the carbohydrate epitope sialyl Lewis X in the tumor microenvironment: implications for metastasis

---

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF THE UNIVERSITY OF MINNESOTA

BY

Katherine Anne Krieser

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

Adviser: Dr. Catherine St. Hill

January 2010

Acknowledgments

I'd like to thank my advisor Dr. Catherine St. Hill for giving me the opportunity to do this research, for helping me to develop the analytical skills necessary for creative scientific thought and experimental design, and for helping me to obtain my Master's of Science degree. I'd also like to thank Dr. Mariya Farooqui for her indispensible support and assistance while working in the laboratory. Thank you to all of the other laboratory members who have provided technical assistance. Finally, I would like to thank my committee members, Drs. Al Beitz and Leslie Sharkey for their insightful input on this research.

Dedication

I'd like to dedicate this work to my extremely supportive parents, Peter Krieser and Susan Bieniek, and to my loving boyfriend Kyle Brandy.

Abstract

The association between inflammation and cancer has been recognized for almost 150 years. The processes of migration of cancer cells to metastatic sites and leukocyte recruitment to sites of inflammation have much in common. Both cell types display specialized carbohydrates modified with sialyl Lewis X (sLe^x) epitopes on their cell surfaces. These sLe^x epitopes are highly expressed on leukocytes and are ligands for selectin adhesion molecules present on activated vascular endothelium at sites of inflammation. The binding interactions between sLe^x ligands on leukocytes with endothelial selectins are well characterized and promote leukocyte trafficking to lymphoid tissues and sites of inflammation. Similarly, malignancy is associated with expression of sLe^x structures on cancer cells that aid tumor invasion and metastasis. High expression of sLe^x in many types of human carcinomas is associated with an advanced stage of disease and poor patient prognosis. Synthesis of sLe^x is dependent on activity of the glycosyltransferase enzymes α2,3-sialyltransferase and α1,3-fucosyltransferase-III (FucT-III). Exposure to the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) has been described to up-regulate FucT-III resulting in increased sLe^x in the airways of patients with respiratory disease, however, little is known about the molecular mechanisms involved in the regulation of sLe^x expression in the inflammatory lung tumor microenvironment. The overall objectives of this study are: 1) to investigate the role of cytokines and inflammatory cells in regulation of sLe^x expression on non small cell lung cancer cells, 2) to evaluate the role of FucT-III, sLe^x expression, and inflammatory cells in the acquisition of metastatic properties by lung cancer cells, and 3) to characterize the backbone protein and carbohydrate structures that display sLe^x on lung and colon cancer cells. Our major findings are presented in

Chapters 2 and 3 and the conclusions drawn from this study are highlighted in Chapter 4. Our results suggest that FucT-III activity in non small cell lung cancers recruit neutrophils in the tumor microenvironment and enhance the metastatic potential of lung tumor cells through the promotion of sLe^x expression and acquisition of invasive and anchorage independent qualities. Thus, inhibition of FucT-III enzyme activity and sLe^x expression may represent promising therapeutic targets for the control of lung cancer metastasis.

Table *of* Contents

ACKNOWLEDGMENTS ..... **i**

DEDICATION ..... **ii**

ABSTRACT ..... **iii**

TABLE *OF* CONTENTS ..... **v**

LIST *OF* TABLES ..... **viii**

LIST *OF* FIGURES ..... **ix**

LIST *OF* ABBREVIATIONS ..... **xii**

CHAPTER 1: LITERATURE REVIEW **1**

INTRODUCTION 1

PATHOGENESIS OF LUNG INFLAMMATION AND CANCER 2

THE ROLE OF INFLAMMATION IN CANCER PROGRESSION 4

LEUKOCYTE ATTACHMENT TO ENDOTHELIUM IS MEDIATED BY INTERACTIONS BETWEEN SIALYL LEWIS X (sLe^x) AND ENDOTHELIAL SELECTINS 10

CANCER CELL METASTASIS 12

ENZYMATIC SYNTHESIS OF CARBOHYDRATES CONTAINING sLe^x 14

THE SIGNIFICANCE OF FUCT-III EXPRESSION IN THE METASTATIC POTENTIAL OF CANCER CELLS 17

THE SIGNIFICANCE OF C2GNT1 EXPRESSION IN SELECTIN BINDING AND THE METASTATIC POTENTIAL OF CANCER CELLS 18

THE RELATIONSHIP BETWEEN CANCER ASSOCIATED INFLAMMATION AND CANCER CELL EXPRESSION OF METASTASIS PROMOTING SELECTIN LIGANDS 21

RESEARCH OBJECTIVES 22

CHAPTER 2: OVEREXPRESSION OF α1,3 FUCOSYLTRANSFERASE-III IN LUNG TUMOR CELLS LEADS TO INCREASED SECRETION OF NEUTROPHIL CHEMOATTRACTANTS AND ENHANCEMENT OF METASTASIS PROMOTING PROPERTIES. **23**

SUMMARY 23

INTRODUCTION 24

MATERIALS AND METHODS 26

General Reagents *26*

Cell Culture *26*

Construction of the FucT-III/pcDNA 3.1 neo(+) Plasmid *27*
Stable Transfection of Lung Cancer Cells with FucT-III *28*
RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) *29*
Flow Cytometric Analysis *30*
Cytokine Detection *31*
IL-8 Enzyme-Linked Immunosorbent Assay (ELISA) *31*
Stimulation with rTNF-α *32*
E-selectin Binding Assay *32*
Human Neutrophil Isolation *33*
Cancer Cell Treatment with Neutrophil Conditioned Media (NCM) *34*
Co-Culture of Human Neutrophils with Cancer Cells *34*
Histopathology *35*
Antibodies *35*
Immunohistochemistry *36*
Histologic Grading System for Lung Tissues *37*
TNF-α neutralization *38*
Microscopic Cell Culture Photographs *38*
Cell Invasion Assay *38*
Statistical Analysis *39*
RESULTS 39
Stable transfection of H1299 cells with FucT-III induces cell surface sLe^x expression *39*
FucT-III transfected H1299 cells secrete increased levels of pro-inflammatory chemokines *42*
Stimulation with rTNF-α up-regulates sLe^x expression and E-selectin binding ability in FucT-III transfected lung cancer cells *44*
FucT-III transfected H1299 cells cultured in neutrophil conditioned media (NCM) or with neutrophils up-regulate sLe^x expression *49*
Neutrophil infiltration is associated with sLe^x positive tissues in lung tumor samples *51*
Up-regulation of sLe^x expression in FucT-III transfected H1299 cells by NCM is independent of TNF-α *57*
Neither TNF-α nor NCM induce sLe^x up-regulation by increasing FucT-III gene transcription *59*

Neutrophils induce TNF-α dependent morphological changes in H1299 cells.....60
Overexpression of FucT-III and stimulation with TNF-α both enhance the invasive potential H1299 cells.....64
DISCUSSION 66

CHAPTER 3: IDENTIFICATION OF PROTEINS ON CARCINOMA CELLS MODIFIED BY THE HIGH AFFINITY SELECTIN LIGAND C2-O-SLE^X.....73

SUMMARY 73
INTRODUCTION 74
MATERIALS AND METHODS 76
General Reagents 76
Cell Culture 76
Construction of the FucT-III/pcDNA 3.1 neo(+) Plasmid 76
FucT-III Stable Transfection 77
Gene Silencing of C2GnT1 Using Lentiviral Short Hairpin RNAs (shRNA) 77
Preparation of Lentiviral Particles 77
Transduction of H1299/FucT-III and LS174T Cells with C2GnT1 shRNA 77
RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).78
Flow Cytometric Analysis 78
Cell Lysates.....79
E-selectin Immunoprecipitation (IP).....79
Western Blotting.....79
Glycoprotein Gel Staining.....80
RESULTS.....81
Transfection of H1299 cells with FucT-III induces C2-O-sLe^x expression 81
Expression of sLe^x and C2-O-sLe^x is decreased in cells transduced with lentiviral C2GnT1 shRNA 81
Glycosylated proteins in H1299 and LS174T cells bind to E-selectin.....83
MUC 3 is a candidate protein for C2-O-sLe^x glycosylation.....91
DISCUSSION 93

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS 96

REFERENCES.....103

List *of* Tables

TABLE 1.....30
Primer sequences used in RT-PCR (chapter 2)

TABLE 2.....53
Staining scores for reactivity of lung tissues with CSLEX-1 and Neutrophil Elastase antibodies.

TABLE 3.....78
Primer sequences used in RT-PCR (chapter 3)

List *of* Figures

FIGURE 1 6
Graphical depiction of the proposed link between inflammation and cancer progression. Adapted from Porta et. al [25].

FIGURE 2 11
Graphical depiction of leukocyte rolling and attachment on inflamed endothelium. Adapted from Immunobiology, 7th edition [63].

FIGURE 3 12
Graphical depiction of primary and secondary leukocyte capture on endothelium. Adapted from Jadhav et. al. [64].

FIGURE 4 13
Graphical depiction of the steps involved in cancer cell metastasis. Adapted from The Biology of Cancer [67].

FIGURE 5 16
Graphical depiction of the enzymatic cascade and monosaccharide linkages resulting in post- translational sLe^x and C2-O-sLe^x carbohydrate modifications of proteins in the golgi.

FIGURE 6 32
Human IL-8 ELISA standard concentration curve graph.

FIGURE 7 43
Transfection of H1299 cells with the FucT-III gene resulted in expression of FucT-III mRNA and de novo synthesis of cell surface sLe^x.

FIGURE 8 43
FucT-III transfected H1299 cells secrete increased levels of pro-inflammatory chemokines.

FIGURE 9 46
rTNF-α up-regulates sLe^x expression and E-selectin binding of FucT-III transfected lung tumor cells.

FIGURE 10 50
FucT-III transfected H1299 cells up-regulate sLe^x expression after culture in NCM or with neutrophils.

FIGURE 11 54
Lung tumor sLe^x expression and neutrophil infiltration are associated in immunohistochemically stained lung tissue samples.

FIGURE 12 58
FucT-III transfected H1299 cells up-regulate sLe^x in response to NCM independently of TNF-α.

FIGURE 13 59
FucT-III mRNA levels do not increase in response to stimulation the rTNFα- or NCM.

FIGURE 14 61
Culture of H1299 cells with rTNF-α or NCM results in morphological changes that are TNF-α dependent.

FIGURE 15 63
FucT-III transfected H1299 cells become non-adherent when co-cultured with neutrophils.

FIGURE 16 65
Stimulation with rTNF- increases the invasiveness of H1299 parent and FucT-III transfected cells.

FIGURE 17 66
The invasiveness of FucT-III transfected H1299 cells is increased by culture in NCM and is a result of TNF-α.

FIGURE 18 82
Cell surface expression of sLe^x and C2-O-sLe^x are altered by manipulation of the genes C2GnT1 and FucT-III.

FIGURE 19 85
Three glycosylated proteins are detected in LS174T and H1299 cells following IP with an E-selectin/Fc chimera and Western blotting with CHO-131 mAb.

FIGURE 20 89
Western blotting with the mAb CHO-131 identifies similar protein bands as a mouse IgM isotype control antibody.

FIGURE 21 90
An anti-MUC5AC antibody detects proteins in E-selectin IP samples although MUC5AC mRNA is absent from the cells.

FIGURE 22 91
Western blotting with an anti-GAPDH antibody detects one band of the expected molecular weight.

FIGURE 23 92
H1299 cells express MUC3 mRNA and proteins.

FIGURE 24 100
Graphical depiction of the proposed mechanism linking tumor cell FucT-III expression, chemokine secretion, inflammatory cells, and increased tumor cell metastatic potential.

List *of* Abbreviations

(in order of appearance)

- sLe^x - sialyl Lewis X
- IL - interleukin
- ROS - reactive oxygen species
- MCP-1 - monocyte chemoattractant protein-1
- mAb - monoclonal antibody
- FGFR - fibroblast growth factor receptor
- CCL2 - chemokine ligand 2
- ER - estrogen receptor
- TNF-α - tumor necrosis factor alpha
- NSAIDs - non-steroidal anti-inflammatory drugs
- N-linked - nitrogen-linked
- O-linked - oxygen-linked
- GalNAc - N-acetylgalactosamine
- C2-O-sLe^x - sLe^x on a core 2 O-linked glycan
- GlcNAc - N-acetylglucosamine
- C2GnT1 - core 2 β-1,6-N-acetylglucosaminyltransferase
- FucT-III - α1,3-fucosyltransferase-III
- PSGL-1 - P-selectin glycoprotein ligand-1
- NSCLC - non-small cell lung carcinoma
- CHO - Chinese hamster ovary
- WT - wild type
- KO - knockout
- NCM - neutrophil conditioned media
- rTNF-α - recombinant TNF-α
- DPBS - Dulbecco's phosphate buffered saline
- FBS - fetal bovine serum
- P/S - 100 U/ml penicillin/100 µg/ml streptomycin
- RT-PCR - reverse transcriptase polymerase chain reaction
- FACS - flow activated cell sorting
- PCR - polymerase chain reaction
- PE - phycoerythrin
- SFM - serum free media

- ELISA - enzyme-linked immunosorbent assay
- H&E – hematoxylin and eosin
- HRP - horseradish peroxidase
- TBS - tris buffered saline
- DAB - chromogen 3,3'-diaminobenzidine tetrachloride
- SCLC - small cell lung carcinoma
- kDa - kilo Dalton
- MUC - mucin
- MUC-1 - human mucin 1
- shRNA - short hairpin RNAs
- MUC3 - human mucin 3
- IP - immunoprecipitation
- MUC5AC - human mucin 5AC
- GAPDH - glyceraldehyde-3-phosphate dehydrogenase

Chapter 1: Literature Review

Introduction

The following review focuses largely on the pathogenesis of human lung cancer but includes research data from other types of cancer as well. It aims primarily to discuss the scientific findings on two currently distinct tumor progression and metastasis promoting processes: inflammation, in terms of microenvironmental cytokines and inflammatory cell populations, and aberrant tumor cell expression of glycosyltransferase enzymes which results in the presentation of the carbohydrate selectin ligand sialyl Lewis X (sLe^x) which facilitates metastasis through adhesion to endothelial selectins. Inflammation has been recognized to be associated with cancer development and progression for almost 150 years. However, cancer cell expression of the sLe^x epitope, which is normally expressed by leukocytes, is a more recent discovery. Expression of sLe^x has been demonstrated in most types of solid tumors and shown to predict tumor metastasis and poor patient prognosis. Here we detail the process of selectin – sLe^x mediated leukocyte and cancer cell attachment to endothelium and tissue extravasation, review the cascade of glycosyltransferase enzymes that mediates the formation of the sLe^x structure, and present the proteins known to be modified by sLe^x in leukocytes. Finally, we propose the hypothesis that tumor associated inflammation and expression of the selectin ligand sLe^x do not coincidentally promote tumor progression and metastasis but rather, are linked as the inflammatory microenvironment at a primary tumor may work to augment metastatic potential by increasing tumor cell sLe^x expression.

Pathogenesis of Lung Inflammation and Cancer

Each year in the United States, lung cancer accounts for 15% of newly diagnosed cancer cases in both men and women. Furthermore, it is responsible for 31% and 26% of cancer related deaths in men and women respectively; this is a greater proportion than the number of deaths attributed to prostate or breast, colon and rectum, and pancreatic cancers combined. The 5 year survival expectancy for people diagnosed with early stage lung cancer is 49.3%, however, only 16% of lung cancer cases are diagnosed at an early stage. The survival expectancy drops to 15.5% if the cancer has metastasized to other parts of the lung or to a regional lymph node and to only 2.1% after the development of distant metastases [1].

Patient death may be caused by the growth of the primary lung tumor which results in mechanical disruption of lung function. Shortness of breath and/or pneumonia can result from pleural effusion or collapse of part of the lung due to blockage of an airway. If the tumor grows into the mid chest, it can directly affect cardiac function by compressing the superior vena cava, interfering with blood flow through the heart, or by causing irregular heart beat rhythms. The spread of tumor cells throughout the lungs may result in reduced lung function leading to low blood oxygen levels, shortness of breath, and eventual heart failure. As lung tumor cells most commonly spread to the adrenal glands, bones, brain, liver, and spinal cord, death may also be caused by metastatic lung tumors which interfere with other vital physiological processes [2].

Cigarette smoking has been attributed to 85% of lung cancer cases and 10% of people who have smoked at some point in their life will eventually develop lung cancer. Additionally, the risk of developing lung cancer has been positively correlated to the number of years as well as the number of cigarettes a person has smoked. Non-

smokers may develop lung cancer as a result of chronic inhalation of second hand smoke or other carcinogens [2].

Because of the abundant evidence that cigarette smoking causes lung disease, researchers have extensively examined the molecular changes that occur in the lungs due to smoking. In a study by van der Vaart et. al. on the acute effects of smoking, it was found that in the 0 – 3 hour period after smoking just two cigarettes, the amount of the neutrophil chemoattractant interleukin (IL)-8 secreted by whole blood cells was significantly elevated, and after 3 – 12 hours, the number of neutrophils in sputum increased significantly as well [3]. It has also been shown that nicotine directly affects lung neutrophils in a concentration dependent manner, causing them to synthesize more reactive oxygen species (ROS) and secrete more IL-8 thus increasing neutrophil influx to the lungs resulting in neutrophilia [4]. These findings are especially interesting as increased expression of IL-8 has been implicated in the pathogenesis of many inflammatory conditions [5-11]. This is most likely due to its ability to attract and activate neutrophils causing them to secrete tissue damaging ROS [12]. Although neutrophils are of great importance for the clearance of invading pathogens by the innate immune system [13- 14], excessive neutrophil infiltration has been implicated as a causative factor in multiple chronic inflammatory disorders including those affecting the lungs, liver, digestive tract, and oral cavity [14-18].

In corroboration with the results published by van der Vaart et. al., studies investigating the molecular changes induced by chronic cigarette smoking have also found that smoking promotes a pro-inflammatory pulmonary environment. Expression of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1), as well as the number of neutrophils and monocytes in bronchoalveolar lavage fluid were all found to be significantly elevated in samples from chronic smokers. The levels

of these inflammatory markers were also seen to positively correlate with the number of cigarettes smoked per day [19]. In a study by Takizawa et. al., small airway epithelial cells excised from current smokers showed significantly higher IL-8 mRNA expression and spontaneous secretion than cells excised from healthy tissue donors. The magnitude of IL-8 mRNA expression also correlated with the estimated number of cigarettes the person had smoked over their life time [20]. These, along with numerous other studies [21-23] have shown that cigarette smoking leads to chronic pulmonary inflammation characterized by excessive neutrophil and monocyte infiltration and chemokine production which increases with the frequency of smoking.

The Role of Inflammation in Cancer Progression

As previously noted, the risk of developing lung cancer, like the severity of smoking related inflammation, increases with the frequency of smoking; this similarity is most likely not a coincidence. Although at least 50 known carcinogenic chemicals are present in cigarette smoke [24], the development and progression of lung cancer is likely to be promoted by the smoke induced inflammatory environment. The link between chronic inflammation and cancer development has been hypothesized since 1863 when it was proposed by Rudolf Virchow [25].

The acute inflammatory response is necessary for proper immune function and protection from infection and is usually a short lived event [13-14]. However, when an inflammatory response is inadequately resolved or sustained by persistent exposure to an inflammatory stimulus (such as in the case of repetitive cigarette smoking), the chronic presence of inflammatory mediators results in inflammation related tissue damage and disease that has been linked to cancer development [14, 25-27]. Cancer has been shown to arise from chronic inflammation induced by gastric infections with *Helicobacter pylori*, infections with the hepatitis B or C virus, autoimmune inflammatory

disorders such as inflammatory bowel diseases, and other inflammatory conditions such as prostatitis [25, 28-30].

Pre-existing chronic inflammation may predispose tissue to cancer development. This process is referred to as the “extrinsic inflammatory pathway”. Conversely, tumors that arise as a result of genetic alterations, in the absence of chronic inflammation, are generally found to have a “smouldering” inflammatory environment at the tumor site that promotes tumor progression. This process is referred to as the “intrinsic inflammatory pathway” [25, 28-29].

Infiltration of inflammatory cells at the tumor site has been shown to be necessary for cancer progression and metastasis. Schwetfeger et. al. used a mouse model of early mammary tumor development to show that epithelial cell hyperplasia and angiogenesis are dependent on the presence of recruited macrophages [30]. Similarly, in a study by Tazawa et. al., a non metastatic murine fibrosarcoma cell line was shown to become metastatic when tumor cells were inoculated into mice along with a gelatin sponge that caused an inflammatory response consisting of mostly infiltrated neutrophils. When neutrophils were depleted by treatment with an anti-granulocyte monoclonal antibody (mAb), the tumor cells were unable to acquire a metastatic phenotype; thus indicating neutrophils as the necessary agents for metastatic progression [31]. Tumor associated macrophages and especially neutrophils have also been implicated as crucial mediators of pancreatic islet cell tumor angiogenesis [32]. These studies highlight the importance of the presence of inflammatory cells in tumor progression.

Figure 1 graphically depicts the cancer promoting role of inflammatory cells and the above described extrinsic and intrinsic inflammatory processes.

FIGURE 1

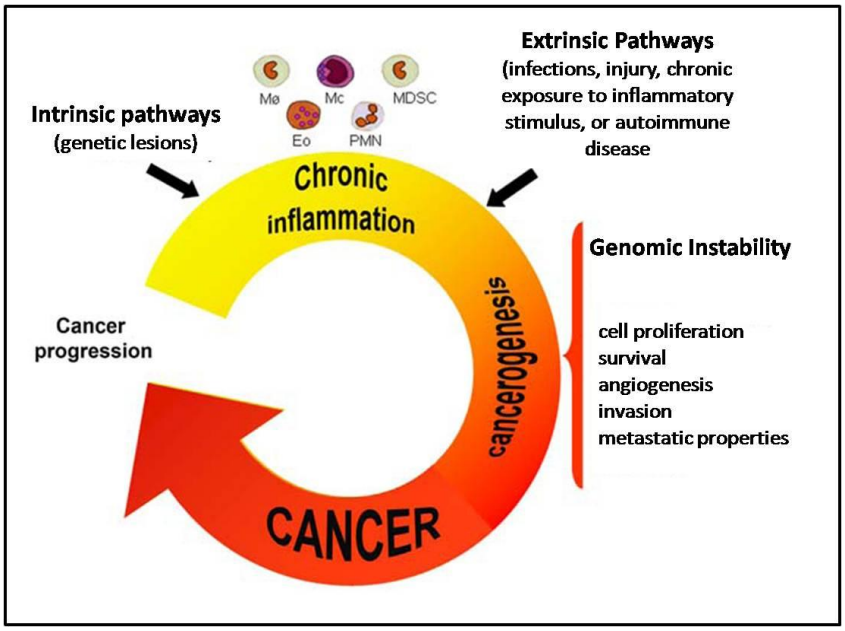


Figure 1: Graphical depiction of the proposed link between inflammation and cancer progression. Adapted from Porta et. al [25]. Genetic mutations may lead to inflammation ("intrinsic pathways") or inflammation may lead to genetic mutations ("extrinsic pathways"). In either situation, the inflammatory environment provides signals (described in greater detail below) that promote cell proliferation, evasion of apoptosis, angiogenesis, invasion, and acquisition of metastatic properties which all support the progression of tumor cells.

The stimulatory interactions between tumor cells and infiltrating leukocytes are largely a result of chemokines secreted by tumor cells and cytokines secreted by the infiltrating leukocytes. It is hypothesized that the genetic alterations present in tumor cells may lead to aberrant chemoattractive signaling [25, 29]. For example, it has been shown that induced activation of fibroblast growth factor receptor (FGFR) (a gene known to be aberrantly expressed in human breast cancer) in mouse mammary tissue leads to increased transcription of the macrophage chemoattractants chemokine ligand 5 (RANTES), chemokine ligand 2 (CCL2), and osteopontin and results in increased macrophage recruitment [30]. Additionally, progressed estrogen receptor (ER)-negative breast cancer cells show increased expression of IL-8 due to loss of ER expression

which, in normal tissue, downregulates the IL-8 promoter. Transfection with interfering RNA was used to knockdown IL-8 expression in an ER-negative breast cancer cell line, and when transplanted into mice the metastatic potential of tumors derived from transfected cells was significantly decreased. The authors hypothesized that the reduced rate of metastasis may have been attributed to the reduction in neutrophil infiltration at the primary tumor site which was due to reduced IL-8 signaling [33]. Finally, MCP-1 was found to be aberrantly expressed in approximately 55% of esophageal squamous cell carcinomas and correlated with macrophage infiltration, angiogenesis, and poor prognosis [34].

Once recruited to the tumor site by chemokines, leukocytes stimulate cancer progression through secretion of cytokines that promote angiogenesis and tumor cell growth, migration, and metastatic properties. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine normally secreted by innate immune cells in response to invading pathogens. At high doses, it induces cancer cell apoptosis; however, at low doses it promotes tumor cell growth, migration, and metastasis [35]. Interestingly, it has been reported that neutrophils isolated from human blood spontaneously secrete 25 – 50 pg/ml TNF-α (a relatively low concentration) in the absence of bacterial stimulation [36-37], as would be the case for neutrophils recruited to a tumor site. Therefore, it is likely that tumor associated neutrophils play a supportive role in tumor progression, at least in part, due to constitutive secretion of small amounts of TNF-α. Further support for the role of TNF-α in tumor progression is gained from clinical trials that have shown disease stabilization in patients with advanced stage solid tumors upon treatment with TNF-α inhibitors [29]. In a study on the efficacy of infliximab, a mAb against TNF-α, treatment resulted in disease stabilization for 7 out of 41 patients as well as decreased

blood plasma levels of another inflammatory cytokine, IL-6, and the macrophage chemoattractant CCL2 [38].

The presence of the macrophage derived inflammatory cytokine IL-1β in the tumor microenvironment has been shown to be crucial for metastasis of melanoma cells as well as for angiogenesis and growth of both melanoma and mammary cell tumors. IL-1β was also found to be partially responsible for secretion of TNF-α in macrophage – melanoma cell co-cultures [39]. The critical role IL-1β in angiogenesis was recently confirmed by Carmi et. al., and its mechanism of action was determined to be through stimulation of other infiltrated inflammatory cells which in turn produce endothelial cell growth and activating factors [40].

IL-6 is another inflammatory cytokine secreted by macrophages in response to invading pathogens and has been found to be important for tumor growth, angiogenesis, and anti-apoptotic signaling [41-43].

Further support for the role of inflammation in cancer development and progression has been found through epidemiological studies on the use of non-steroidal anti-inflammatory drugs (NSAIDs) which have demonstrated that consistent use of NSAIDs correlate with reduced cancer incidence [25,29,44]. In a long term randomized study, it was found that consumption of 300 mg or more of NSAIDs per day, for at least 5 years, prevented the development of primary colon cancer [45]. Additionally, in a study of chemically induced lung cancer in rats, in which the tumors were characterized by an inflammatory environment consisting of infiltrated neutrophils and macrophages, treatment with herbal anti-inflammatory substances or NSAIDs and antibiotics inhibited lung cancer progression [46].

The innate inflammatory response associated with tumors has also been demonstrated to inhibit anti-cancer adaptive immunity [25, 29]. As discussed earlier, genetic changes in tumor cells may lead to secretion of macrophage chemoattractants such as MCP-1 or CCL2 thus increasing macrophage presence in the tumor microenvironment. Macrophages may adopt different “polarizations” (classified as M1 or M2) causing them to function differently in terms of the cytokines they secrete and their effect on adaptive immune cells. M1 polarized macrophages exert cytotoxicity towards intracellular microbial invaders and express high levels of ROS and TNF-α, have an enhanced antigen presentation capacity, and promote a cytotoxic Th1 T-cell response. Therefore, M1 polarized macrophages tend to interfere with cancer progression. M2 polarized macrophages however, dampen M1 mediated inflammation and are active during extracellular and parasitic infections. M2 macrophages induce a Th2 T-cell response, secrete high levels of the immunosuppressive cytokine IL-10 as well as low levels of the inflammatory cytokines IL-1β, IL-6, and TNF-α [47-49], and promote angiogenesis and tissue repair resulting in promotion of tumor cell growth [25,47,49]. The signals that dictate macrophage polarization are not fully understood [25], however, macrophages associated with established tumors are generally found to exhibit the M2 polarization [49-50]. Accordingly, in a mouse model of prostate cancer growth and metastasis, tumor growth was significantly slowed when treatment with docetaxel (an anti-mitotic chemotherapy medication [51]) was supplemented with a mAb against CCL2 to reduce macrophage recruitment [52].

Additionally, it has been demonstrated that IL-6 and IL-10 present in the tumor microenvironment prevent maturation of dendritic cells, and therefore, inhibit efficient tumor antigen presentation to adaptive immune cells [53]. Other mechanisms of

suppression of anti-tumor T-cell immunity by myeloid – derived suppressor cells have also been described [54].

Leukocyte Attachment to Endothelium is mediated by Interactions between Sialyl Lewis X (sLe^x) and Endothelial Selectins

Sialyl Lewis X (sLe^x) is a terminal carbohydrate modification expressed on membrane bound proteins on the surface of predominantly neutrophils, however, it may also be expressed on monocytes/macrophages, immature dendritic cells, and T-cells found at sites of inflammation [55-56]. In recent decades sLe^x has been shown to be aberrantly expressed on a variety of different cancer cells. Expression of sLe^x guides leukocytes to sites of inflammation by adhering to endothelial expressed selectins. The selectins play a critical role in the early events of leukocyte adhesion to the walls of post-capillary venules. They are involved in the recruitment of leukocytes and lymphocytes into sites of inflammation and their emigration into lymphoid organs. Three selectins have been described: L-selectin (CD62L) is expressed on all circulating leukocytes and subpopulations of lymphocytes; P-selectin (CD62P) is expressed on activated platelets and endothelial cells; and E-selectin (CD62E) is expressed on activated endothelial cells [57]. sLe^x acts as a ligand for E- and P-selectin expressed on activated endothelial cells in the blood vessels of inflamed tissue [58-59]. The interactions between sLe^x and selectins facilitate the characteristic rolling of leukocytes and lymphocytes over endothelial cells [60] before firm, integrin mediated, arrest and extravasation into inflamed tissue [61] (Figure 2). Leukocytes may adhere to endothelium directly through interactions with P- and/or E- selectin (primary capture) or may adhere initially to primarily bound leukocytes through interactions mediated by L-selectin before directly binding to endothelium (secondary capture) (Figure 3). Both the primary and secondary

processes of endothelial attachment are common physiological events known to occur when leukocytes are being recruited to sites of inflammation [62]. These processes of leukocyte recruitment to sites of inflammation have been well established and supported as an efficacious mechanism of permitting circulating cells to extravasate into tissue.

FIGURE 2a

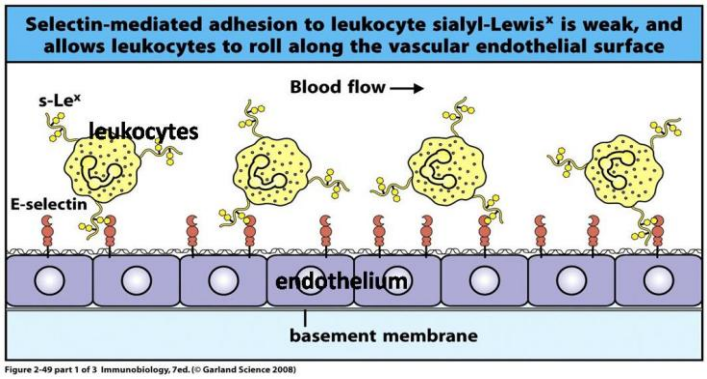


FIGURE 2b

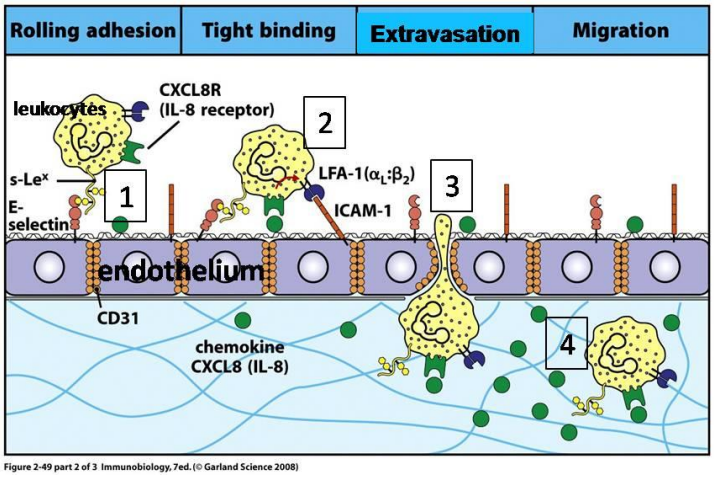


Figure 2: Graphical depiction of leukocyte rolling and attachment on inflamed endothelium. Adapted from Immunobiology, 7th edition [63]. Figure 2a: During the initial step of cell attachment to inflamed/activated endothelium, leukocytes roll along endothelium via weak, transient interactions between leukocyte expressed sLe^x and endothelial expressed E-selectin. Figure 2b: After slowing by rolling on the endothelium (figure 2a and 2b – 1), firm, static adhesion is mediated by integrins (2). Cells then extravasate through endothelial cells into the underlying tissue (3), and migrate through tissue toward a chemoattractant gradient (4).

FIGURE 3

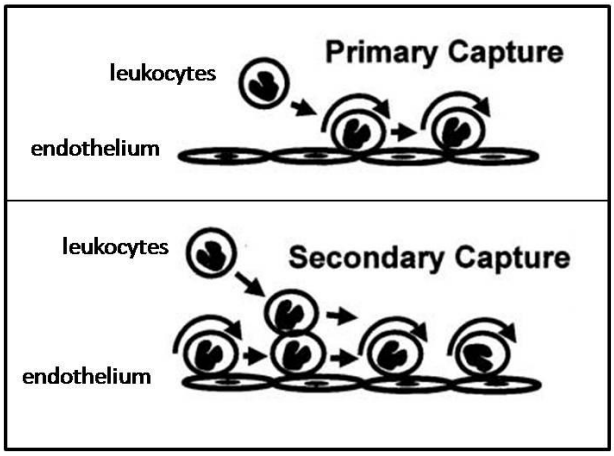


Figure 3: Graphical depiction of primary and secondary leukocyte capture on endothelium. Adapted from Jadhav et. al. [64]. Leukocytes may directly attach to endothelium (primary capture) or may secondarily attach to other leukocytes already attached to the endothelium *before* they themselves become directly attached to the endothelium (secondary capture).

Cancer Cell Metastasis

Formation of metastases requires tumor cells that have escaped into the circulation from a primary tumor, to travel from the circulation back into tissue at a distant site (Figure 4). Two hypotheses on the mechanism of metastasis predominate: First, metastasis may result from mechanical entrapment of circulating tumor cells due to their relatively large size in comparison to the small diameter of microvessels [65]. Second, a growing accumulation of experimental evidence suggests that tumor cells aberrantly express cell surface adhesion molecules and may be able to disseminate into new tissue through attachment to vascular endothelium via a series of molecular adhesion steps similar to the process used by leukocytes and lymphocytes to travel to lymphoid tissues and sites of inflammation.

sLe^x expression has been identified on numerous types of carcinoma cells and may be a crucial molecule involved in metastasis as it facilitates the initial step in primary endothelial attachment. Accordingly, primary attachment of cancer cells to endothelium has been demonstrated to be facilitated mainly through an sLe^x – E-selectin mediated mechanism [66]. Secondary interactions with endothelium bound neutrophils have also been shown to mediate metastasis via an L-selectin dependent mechanism [64].

FIGURE 4

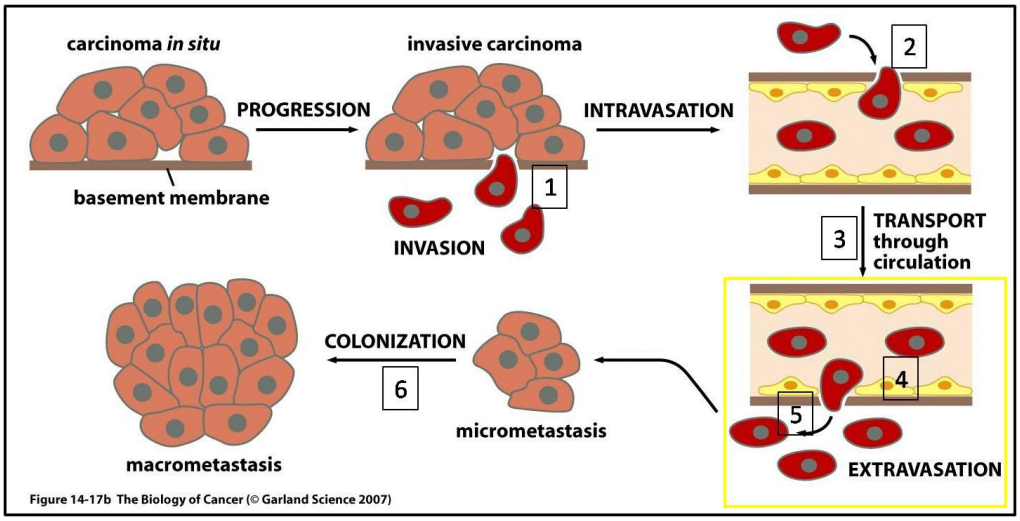


Figure 4: Graphical depiction of the steps involved in cancer cell metastasis. Adapted from The Biology of Cancer [67]. In order to form tumors at distant sites, cells must migrate away from the primary tumor mass (1), travel into the vasculature (2), circulate in the blood stream (3), adhere to endothelium (4), migrate through endothelial cells and subsequent tissue (5), and proliferate at the new site (6). The yellow box highlights a crucial step in the metastatic process as cancer cells *must* escape the circulation in order to seed new tumors in distant tissues.

Emphasizing the importance of the sLe^x epitope in general tumor biology and the metastatic process, sLe^x has been shown to be present on 55-90% of lung, gastric, liver, gallbladder, bladder, colorectal, head and neck, ovarian, breast, and prostate carcinomas as well as lymph node and other metastases of these tumors [68-78]. Strikingly, the 10 year survival rate of patients with colorectal tumors that expressed high

amounts of sLe^x versus tumors with low or no sLe^x expression was shown to be 35.1% versus 85.7% respectively [79].

Takada et. al demonstrated in 12 different epithelial cancer cell lines representing colon, digestive tract, lung, and testicular teratocarcinomas, that the majority of cellular adhesion to endothelial cells was dependent upon cancer cell expression of sLe^x and mediated by endothelial expressed E-selectin rather than other known cell adhesion molecules [66]. sLe^x dependent carcinoma cell attachment to endothelial cells has also been directly demonstrated in head and neck squamous cell and bladder carcinoma cell lines [80-81]. In a comparative study between two human gastric carcinoma cell lines, one sLe^x positive and one sLe^x negative, the sLe^x positive cell line exhibited stronger E-selectin mediated adhesion to endothelial cells, and the *in vivo* metastatic ability of sLe^x positive cells in nude mice was found to be markedly reduced upon treatment with an anti-sLe^x antibody [82]. Increased selectin dependent binding to activated endothelium by highly sLe^x positive cells has also been confirmed in colon cancer cells [83].

Clinical studies have supported E-selectin dependent cancer cell – endothelium adhesion as an important mode of metastasis. Kobayashi et. al. determined that the effectiveness of cimetidine, a drug shown to increase the survival of colorectal cancer patients, may be due to its ability to inhibit endothelial E-selectin expression. In patients with colorectal tumors expressing high levels of sLe^x, treatment with cimetidine increased the 10 year survival rate by 60.4% while treatment of patients whose tumors expressed low or no sLe^x was shown to have no effect on the survival rate [79, 84].

Enzymatic Synthesis of Carbohydrates Containing sLe^x

Expression of the carbohydrate selectin ligand sLe^x is the result of post-translational protein modification by the sequential activity of specific glycosyltransferase enzymes

located in the golgi of the cell [85]. While the sLe^x epitope may be found on nitrogen (N)-linked glycans [86-87] and glycolipids [88], high affinity binding between sLe^x and E- and P-selectin results when sLe^x is located on an oxygen (O)-linked glycan [89-90]. Formation of the O-linked, selectin ligand carbohydrate chain is initiated by the activity of N-acetyl galactosaminyltransferase, an enzyme that adds N-acetylgalactosamine (GalNAc) to an exposed serine or threonine residue in a fully folded protein. The O-glycan chain is then elongated by enzymes that synthesize the growing chain's core structure. Importantly, although 8 core structures have been observed [91], high affinity binding between sLe^x and selectins results only when sLe^x sits on a core 2 O-linked glycan (this structure referred to as C2-O-sLe^x) [92-96]. Initially, a core 1 structure is formed by the β1-3 addition of galactose to the GalNAc residue by the enzyme core 1 β-1,3 galactosyltransferase. An N-acetylglucosamine (GlcNAc) molecule is then β1-6 linked to the GalNAc by the branching enzyme core 2 β-1,6-N-acetylglucosaminyltransferase (C2GnT1), resulting in a doubly branched, core 2 structure. Next, the GlcNAc is β1-4 linked to a galactose molecule by the enzyme β1-4Gal-T. α2,3-sialtransferase then catalyzes the addition of a sialic acid group to the β1-4 linked galactose through an α2-3 linkage. Finally, formation of the terminal sLe^x epitope results when a fucose monosaccharide is α1-3 linked to the GlcNAc; this reaction is catalyzed by the last enzyme in the cascade, α1,3-fucosyltransferase [58, 94]. This enzyme has several different isotypes however, this discussion will focus only on α1,3-fucosyltransferase-III (FucT-III). The final C2-O-sLe^x structure consists of the grouped components of the core 2 branched structure, the GlcNAc – galactose – sialic acid chain, and the GlcNAc bound fucose [97] (Figure 5).

FIGURE 5a

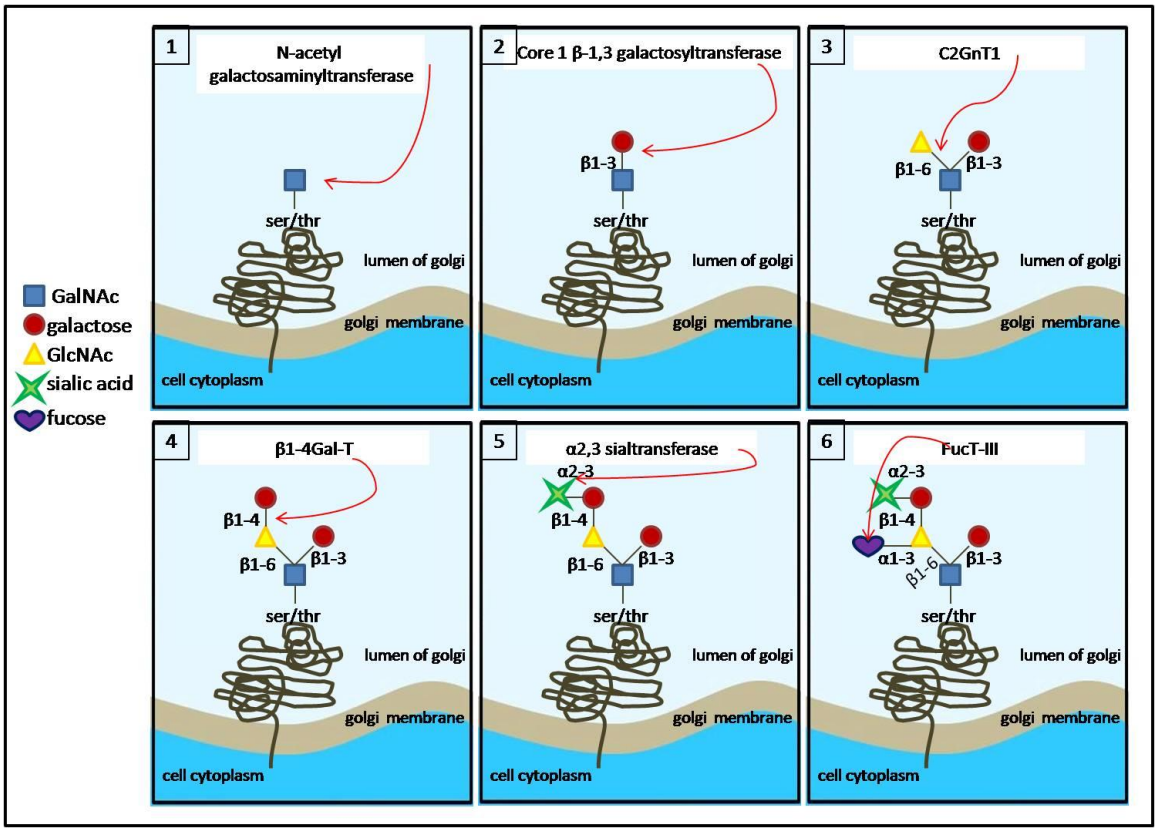


FIGURE 5b

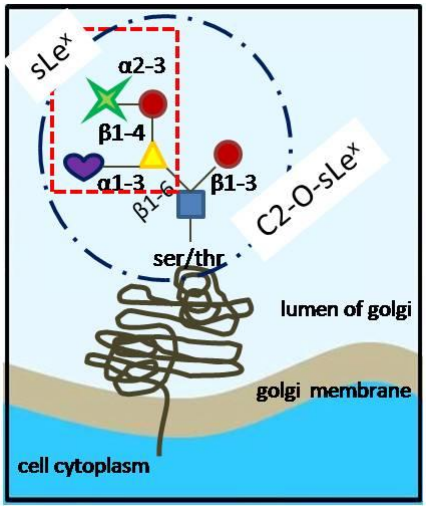


Figure 5: Graphical depiction of the enzymatic cascade and monosaccharide linkages resulting in post- translational sLe^x and C2-O-sLe^x carbohydrate modifications of proteins in the golgi. Figure 5a: O-linked glycosylation is initiated by the addition of a GalNAc to an exposed serine or threonine residue (ser/thr) (1), a core 1 structure is made with a β1-3 linkage of a galactose (2), the core 2 structure is formed by the β1-6 addition of a GlcNAc to the GalNAc (3), another galactose is then β1-4 linked to the GlcNAc (4), a sialic acid is α2-3 linked to the previously added galactose (5), finally a fucose is added to the GlcNAc with an α1-3 linkage (6). Figure 5b: Graphical distinction between the sLe^x and C2-O-sLe^x epitopes. The red dashed box indicates the sLe^x structure which can be found on O- or N-linked glycoproteins or glycolipids. The dark blue dashed circle indicates the C2-O-sle^x structure which necessarily includes all of the monosaccharide components.

The carbohydrate modification plus the protein backbone to which it is attached collectively create the complete, high affinity selectin ligand. Neutrophils, monocytes, dendritic cells, and subsets of T-cells display the C2-O-sLe^x epitope on the membrane bound, extracellular glycoprotein, P-selectin glycoprotein ligand-1 (PSGL-1) [56, 98-100]. Expression of PSGL-1 is necessary for P-selectin binding and the post-translational glycosylation of PSGL1 allows it to act as both a high affinity ligand for P-selectin as well as a lower affinity ligand for E-selectin [89, 100-101]. In contrast to P-selectin, multiple sLe^x modified protein backbones including CD43 [95], ESL-1, and CD44 [102-104] have been shown to support immune cell attachment to E-selectin.

The Significance of FucT-III Expression in the Metastatic Potential of Cancer Cells

Cancer cells have been shown to express sLe^x [68-78] and posses E-selectin binding and metastatic ability [66, 80-83]. Concurrently, aberrant expression of the glycosyltransferase enzymes responsible for the synthesis of sLe^x has been demonstrated in tumor cells and positively correlated with an increased metastatic potential. Togayachi et. al. assessed the expression of 5 different fucosyltransferases in non-small cell lung carcinomas (NSCLC) and FucT-III was the only enzyme found to be greatly increased in all of the lung tumor samples when compared to normal tissue [105].

Assessment of two lung adenocarcinoma cell lines, one known to be metastatic and one known to be non-metastatic, revealed up-regulation of FucT-III expression as well as sLe^x dependent E-selectin binding ability only in the metastatic cells [106]. Similarly, metastatic prostate cancer cells were seen to express 31 fold more FucT-III mRNA than normal prostate tissue [107].

Further supporting FucT-III's role in metastasis promotion, when FucT-III mRNA was assessed from metastatic, primary, and normal liver samples, expression was found to be the highest in tissue from the metastatic lesions [108]. This implies that high levels of sLe^x were expressed on the circulating cancer cells that were able to successfully metastasize. Additionally, expression of a metastasis suppressor gene in primary liver tumors was shown to negatively correlate with expression of FucT-III and sLe^x and metastatic potential. The authors hypothesized that one of the mechanisms by which the metastasis suppressor gene worked was by interfering with FucT-III expression [109]. Finally, in further support of the promotion of metastasis by aberrant expression of FucT-III, inhibition of FucT-III by transfection with an antisense sequence has been demonstrated to deprive previously metastatic cells of their E-selectin binding and metastatic abilities [110-111].

The Significance of C2GnT1 Expression in Selectin Binding and the Metastatic Potential of Cancer Cells

It has been well established in leukocytes that glycoprotein binding to P- and E- selectin is dependent upon the activity of C2GnT1 and the presence of the C2-O-sLe^x structure. The role of C2GnT1 and C2-O-sLe^x in cancer cell metastasis, however, is not fully elucidated and warrants further research. The importance of C2GnT1 function in selectin – ligand interactions has been notably highlighted in several studies utilizing different experimental methods.

Kumar et. al. illustrated the significance of core 2 branching in P-selectin binding by transfecting Chinese hamster ovary (CHO) cells with PSGL-1 and either FucT-III alone or FucT-III *and* C2GnT1. Although both sets of transfectants expressed equal amounts of sLe^x, only the CHO cells transfected with both enzymes bound to P-selectin. The absence of C2GnT1 had no effect on binding to E-selectin as both sets of transfectants bound equally well [90]. In contrast, when the CHO cell model was used by Li et. al., binding to P-selectin *as well as E-selectin* was completely eliminated in the absence of C2GnT1 [94]. However, these results were refuted by Snapp et. al. in a study that compared the selectin binding and rolling capabilities of neutrophils and T-cells from wild type (WT) versus C2GnT1 knockout (KO) mice. In both neutrophils and activated T-cells, P-selectin dependant rolling was abolished in KO mice while rolling on E-selectin remained unchanged. Concurrently, dual flow cytometric staining of activated T- cells showed that only cells from WT mice, expressing C2-O-sLe^x, bound to P-selectin. E-selectin was shown to bind to cells expressing both low and high amounts of C2-O-sLe^x indicating that the core 2 structure neither enhanced nor interfered with E-selectin binding [93].

The *in vivo* importance of C2-O-sLe^x in leukocyte adhesion to vascular P- and E-selectin was assessed by Sperandio et. al.. In support of other findings, leukocytes from C2GnT1 knockout mice showed an 85% reduction in P-selectin dependent rolling accompanied by a 2 fold increase in rolling velocity. Interestingly, C2-O-sLe^x expression was also shown to be important in E-selectin binding. In venules induced to express E-selectin by stimulation with TNF-α, E-selectin dependant leukocyte rolling in KO mice was decreased by 70% and significantly reduced binding to E-selectin was observed at moderate wall shear stress rates. A smaller reduction in E-selectin binding was observed at lower wall shear stress rates indicating that sLe^x attached to core 2 O-linked

glycans may confer stronger binding to E-selectin than when sLe^x is attached to other glycan structures [94]. Finally, Matsumoto et. al. demonstrated that the exclusive E-selectin ligand CD43 bound to E-selectin only when it was modified by both C2GnT1 and FucT-III, thus emphasizing the necessity of the core 2 sLe^x epitope in E-selectin binding [95].

These data taken together lead to the conclusion that C2-O-sLe^x attached to PSGL-1 is necessary for P-selectin binding and E-selectin is less restrictive in its binding preferences; however, E-selectin may bind more tightly to proteins modified with the C2-O-sLe^x epitope (although this concept is supported by conflicting data). Since binding to E-selectin has been shown to play a pivotal role in cancer cell metastasis, determination of the level of C2GnT1 expression may provide insight to a particular cancer's metastatic potential.

Even though a consensus about the importance of C2GnT1 in immune cell attachment to E-selectin has yet to be reached, there is evidence that aberrant expression of C2GnT1 and C2-O-sLe^x in cancer cells may promote E-selectin mediated metastasis. First, C2-O-sLe^x expressing colon cancer cells have been shown to bind to E-selectin under shear flow conditions [112]. Additionally, C2GnT1 mRNA was present in 63% and 73.2% of colorectal and lung tumors respectively and positively correlated with malignant transformation, the amount of vascular invasion, depth of tumor invasion, and lymph node metastasis [113-114]. C2-O-sLe^x has also been shown to be present on carcinoma tissue samples and correlated with metastasis. The mAb CHO-131 that specifically recognizes the C2-O-sLe^x epitope [115] was used for immunohistochemical analysis of the expression of C2-O-sLe^x on primary colorectal carcinomas, adenomas, metastatic liver tumors, normal colorectal tissue, and normal liver tissue. The normal tissues and adenomas showed no reactivity with CHO-131, however, 70% of primary

colorectal carcinomas and 87% liver metastases did react with CHO-131 indicating the presence of C2-O-sLe^x. C2GnT1 mRNA from colorectal carcinomas was also observed to be 15 fold higher than in normal tissue [116]. Since more metastatic tumors showed C2-O-sLe^x expression than primary tumors, the notion of C2-O-sLe^x mediated metastasis is supported and leads to the hypothesis that tumor cells 'armed' with C2-O-sLe^x are better able to adhere to endothelium, migrate into new tissues, and seed a new tumor.

The Relationship between Cancer Associated Inflammation and Cancer Cell Expression of Metastasis Promoting Selectin Ligands

The preceding discussion introduced two relatively unrelated fields of research on cancer progression: first, the relationship between cancer progression and inflammation and second, the promotion of metastasis through binding of cancer cell expressed carbohydrate selectin ligands sLe^x and/or C2-O-sLe^x to endothelial E-selectin. Although these aspects of tumor progression have yet to be definitively scientifically linked, taken together, these data indicate that the rate of metastasis is increased when either inflammation is associated with a primary tumor or when tumor cells aberrantly express glycosyltransferase enzymes and sLe^x epitopes. This common finding is most likely not a coincidence but rather, is due to cytokine mediated signaling between cancer cells and infiltrating inflammatory cells which ultimately promotes an increase in sLe^x expression and the metastatic properties of tumor cells.

Currently, investigation of the link between these two fields of research has been minimal. In an attempt to characterize the effect of surgical trauma induced inflammation on cancer cell surface markers, Kuninaka et. al. stimulated lung cancer cell lines with the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. TNF-α was the only cytokine seen to affect sLe^x expression and caused it to be up-regulated [117].

Accordingly, small biopsies of bronchial mucosa were shown to increase FucT-III mRNA upon stimulation with TNF-α [118]. TNF-α stimulation of colon cancer cells was demonstrated to result in increased FucT-III activity, sLe^x expression, and E-selectin binding ability [119]. All of these studies support the hypothesis that an inflammatory environment (at least where TNF-α is present) leads to increased cancer cell expression of sLe^x and resulting metastatic properties. However, a more physiologically realistic approach, involving more than a single cytokine, to simulate an inflammatory tumor microenvironment and determine the effect on sLe^x and tumor cell metastatic properties has yet to be described.

Research Objectives

As the existence of a mechanistic link between cancer associated inflammation and sLe^x expression is currently under explored, the following investigation aims to shed light on: the influence of lung cancer cell FucT-III expression on chemokine secretion, the dependence of TNF-α induced sLe^x up-regulation on concentration and duration of exposure, the effect of, and correlation between neutrophil presence and cancer cell sLe^x expression, and the role of neutrophil derived TNF-α in lung cancer cell metastatic potential.

Additionally, although the protein that is decorated by the high affinity selectin ligand C2-O-sLe^x has been identified as PSGL-1 in leukocytes, the cancer cell protein/s to which C2-O-sLe^x is attached is/are currently unknown. With the ultimate goal of attaining a better understanding of cancer cell – E-selectin binding properties, the following research also aims to use Western blotting techniques with the CHO-131 mAb to identify the proteins in colon and lung carcinoma cell lines to which C2-O-sLe^x is attached.

Chapter 2: Overexpression of α1,3 fucosyltransferase-III in lung tumor cells leads to increased secretion of neutrophil chemoattractants and enhancement of metastasis promoting properties.

Summary

Both tumor associated inflammation and tumor cell expression of the carbohydrate E-selectin ligand sialyl Lewis X (sLe^x) have been demonstrated to promote tumor progression and increase metastatic potential. However, the relationship between these processes has yet to be thoroughly investigated. Thus far, only the pro-inflammatory cytokine TNF-α has consistently been shown to increase cancer cell sLe^x and mRNA expression of α1,3 fucosyltransferase-III (FucT-III), the rate limiting enzyme responsible for the formation of sLe^x. In this study we stably transfected FucT-III into H1299 NSCLC cells which naturally don't express cell surface sLe^x and express very low levels of FucT-III mRNA. We assessed the influence of FucT-III expression on the secretion of tumor microenvironmental chemokines and examined the effect of culture in neutrophil conditioned media (NCM) or co-culture with freshly isolated neutrophils on tumor cell sLe^x expression. Immunohistochemical staining of a 96 sample lung tissue array, consisting of normal, inflamed, and cancerous tissue was used to assess the association between lung tumor sLe^x expression and neutrophil infiltration in a physiologically relevant system. Here we report that recombinant (r)TNF-α induced sLe^x up-regulation was concentration dependent. We also determined that FucT-III overexpression may promote tumor cell progression and metastasis through mechanisms *independent* of sLe^x. FucT-III transfected cells were shown to secrete increased amounts of the pro-inflammatory chemokines IL-8, GROα, and MCP-1 and observed to invade matrigel faster than untransfected or mock transfected cells. Culture in NCM and co-culture with

neutrophils resulted in increased sLe^x expression in FucT-III transfected cells, the magnitude of which was shown to be dependent on the neutrophil: cancer cell ratio. A positive association between sLe^x expression and neutrophil infiltration was also confirmed in 71% of sLe^x positive lung tumors. Unexpectedly, neutrophil induced sLe^x up-regulation was found to be independent of TNF-α and was not the result of increased FucT-III transcription. We also report the observation that neutrophils induced H1299 cell anchorage independence and increased invasion; this result was shown to be dependent on TNF-α and independent of FucT-III. From these results, we hypothesize that FucT-III overexpression and recruited neutrophils collectively act to increase the metastatic potential of lung tumor cells through both sLe^x dependent and independent mechanisms.

Introduction

Aberrant expression of glycosyltransferase enzymes and the resulting carbohydrate E-selectin ligand sialyl Lewis X (sLe^x), has been described in most types of carcinomas [68-78]. sLe^x expression has been demonstrated to greatly increase tumor cell metastatic ability by facilitating adhesion to endothelium through a process similar to that used by leukocytes to adhere to inflamed and activated endothelium [64, 66, 79-84]. Cancer associated inflammation has been extensively linked to the promotion of tumor progression and metastasis [25, 28-29]. So far, the influence of tumor microenvironmental inflammation on cancer cell sLe^x expression and metastatic potential has not been thoroughly investigated.

Current research on the interactions between inflammation and aberrant tumor cell expression of the glycosyltrasferase enzyme α1,3 fucosyltransferase-III (FucT-III) and its carbohydrate product sLe^x has been limited to experiments in which cancer tissue samples or cell lines were stimulated with a single pro-inflammatory cytokine and the

effects on sLe^x expression were reported. TNF-α is the pro-inflammatory cytokine most commonly demonstrated to up-regulate cancer cell sLe^x expression and has been shown to increase FucT-III mRNA expression levels and sLe^x mediated E-selectin binding ability [117-120]. These reports however, used only a single, extremely high [121-124] dose of TNF-α (20 and 40 ng/ml) and did not present data on the magnitude of sLe^x up-regulation with lower doses of TNF-α. These studies also did not investigate the interactions between inflammatory cells and cancer cells which would necessarily occur in the microenvironment of an inflamed tumor.

Genetic changes resulting from malignant transformation in breast and esophageal carcinomas have been shown to augment tumor associated inflammation by increasing the secretion of chemokines by tumor cells which results in innate immune cell infiltration and an inflammatory tumor microenvironment [30, 33-34]. Thus far, the effects of overexpression of the FucT-III gene on chemokine secretion by lung carcinoma cells have not been investigated.

We transfected the human FucT-III gene into FucT-III mRNA and sLe^x negative NSCLC cell lines (H1299 and A549) and showed that overexpression of FucT-III lead to a moderate amount of cell surface sLe^x expression which was subject to concentration dependent, rTNF-α induced up-regulation. We present the novel finding that overexpression of FucT-III lead to increased secretion of the neutrophil chemoattractants IL-8 and GROα and the monocyte chemoattractant MCP-1. Additionally, co-culture of FucT-III and sLe^x expressing cancer cells with freshly isolated neutrophils resulted in increased cancer cell sLe^x expression, the magnitude of which was dependent on the ratio of neutrophils: cancer cells. Finally, we observed that neutrophil derived TNF-α caused cancer cells to adopt an anchorage independent morphology and acquire an increased invasive ability. Importantly, these results have been associated with an

increased probability of metastasis and indicate that neutrophils increase the metastatic potential of lung carcinoma cells.

Materials and Methods

General Reagents

Dulbecco's phosphate buffered saline (DPBS) and all cell culture reagents, unless otherwise noted, were purchased from Mediatech, Manassas, VA. Sodium Bicarbonate, trypsin, EDTA, and goat serum were purchased from Invitrogen, Carlsbad, CA. Fetal bovine serum (FBS) was purchased from Atlas Biologicals, Fort Collins, CO. The mouse IgM mAb CSLEX-1 that recognizes the sLe^X carbohydrate antigen was generously provided by Dr. Bruce Walcheck, University of Minnesota.

Cell Culture

All cells were cultured under standard conditions in a humidified incubator at 37^{o}C with 5% CO_2. H1299 cells derived from a lymph node metastasis of a NSCLC and A549 primary lung carcinoma cells were obtained from American Type Culture Collection (Manassas, VA). H1299 cells were cultured in RPMI 1640 with 2 mM L-glutamine, 10% FBS, 100 U/ml penicillin/100 µg/ml streptomycin (P/S), 1 mM sodium pyruvate, 10 mM HEPES buffer, and 1.5 g/L sodium bicarbonate. A549 cells were cultured in Ham's F-12 with 2 mM L-glutamine, 10% FBS, P/S, and 1.5 g/L sodium bicarbonate. To maintain actively dividing cells, every 3 to 4 days, culture media was removed, cells were washed with DPBS with 1 mM EDTA, and trypsinized with 0.25% trypsin/EDTA. Once lifted from the flask bottom, cells were resuspended in media and pipetted up and down with a serological pipette to break up cell clumps. The cells were stained with 0.04% trypan blue (Mediatech, Inc.) and counted with a hemocytometer; dead cells were excluded

from the count. $2x10^6$ cells were added to a standard T75 tissue culture flask and placed back in the incubator for further propagation.

Construction of the FucT-III/pcDNA 3.1 neo(+) Plasmid

The human FucT-III gene cloned in the pRc/RSV vector was a generous gift from Dr. Rodger P. McEver, Oklahoma Medical Research Foundation, Oklahoma City, OK. The FucT-III gene was excised out of the pRc/RSV vector by restriction enzyme digestion with HindIII and XbaI (New England Biolabs, Ipswich, MA) and cloned into a pcDNA3.1 (+)neo expression vector (Invitrogen). DH5α E-coli cells (Invitrogen) were transformed with either the empty pcDNA3.1 (+)neo vector or the vector containing FucT-III and grown in Luria-Bertani broth with 200 µg/ml ampicillin (Fischer Scientific, Fair Lawn, NJ) to select for successfully transformed cells. Plasmids were isolated from bacterial cells with the QIAprep Spin Miniprep Kit (QIAGEN Sciences, MA) and DNA concentration was determined with a spectrophotometer (Eppendorf, Hamburg, Germany). The empty, control pcDNA 3.1 (+)neo vector and the vector containing FucT-III were linearized by digestion with the restriction enzyme Mfe I (New England BioLabs). Five µg plasmid DNA was combined with 5 µl NEB buffer 4, 40 units Mfe I, and water to a final volume of 50 µl. The mixture was incubated at 37^{o}C for 3 hours followed by 65^{o}C for 20 minutes to inactivate the enzyme. The digested sample was run on a 1.5% agarose gel (ISCBioExpress, Kaysville, UT) with 0.5 µg/ml ethidium bromide (Fischer Scientific) at 110 volts for 1.5 hours. The resulting band was visualized on an Eagle Eye II machine (Stratagene, La Jolla, CA) using Alphaimager IS-2200 software (Alpha Innotech, San Leandro, CA). The band was cut from the gel and the linearized DNA was purified with the PureLink Quick Gel Extraction Kit (Invitrogen). The DNA concentration was determined by spectrophotometry.

Stable Transfection of Lung Cancer Cells with FucT-III

H1299 or A549 cells, which endogenously express very low levels of FucT-III mRNA, were cultured in a 6 well tissue culture plate at a density of $0.35x10^6$ cells per well for 24 hours. Stable transfections with the FucT-III/pcDNA 3.1 neo(+) plasmid were performed with the Lipofectamine 2000 reagent (Invitrogen). Cells transfected with the empty pcDNA 3.1 neo(+) plasmid (mock-transfected cells) served as controls. Linearized plasmid DNA (4 µg) was diluted in opti-MEM to a final volume of 250 µl. Five µl of Lipofectamine 2000 was added to 245 µl opti-MEM and incubated at room temperature for 5 minutes. The diluted DNA and Lipofectamine mixtures were then added together for a final volume of 500 µl, gently mixed, and incubated at room temperature for 20 minutes. The culture media was removed from the cells and replaced with 2 ml opti-MEM with P/S. Five hundred µl of the DNA/Lipofectamine mixture was then added to the cells. The plate was rocked back and forth briefly to mix the contents and incubated for 24 hours. The media was then replaced with fresh media and the cells were allowed to grow to 90% confluency. The cells were then trypsinized and moved to a standard T25 tissue culture flask. After 24 hours, 1 mg/ml G418 (Sigma-Aldrich, St. Louis, MO) was added to the media to select for stably transfected clones. Transfected cells were treated with G418 for at least 2 weeks before use in experiments and treatment was maintained for all subsequent culture. The expression of FucT-III was verified by assessment of FucT-III gene mRNA expression using reverse transcriptase polymerase chain reaction (RT-PCR). Flow activated cell sorting (FACS) analysis was used to assess the reactivity of FucT-III and mock-transfected cells with the mAb CSLEX-1 that detects the sLe^x epitope. (The RT-PCR and FACS methods are described below).

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was isolated from the cells by adding 1 ml Trizol reagent (Invitrogen) per well and incubating for 5 minutes at room temperature. The solution was pipetted up and down to mix, transferred to a 1.5 ml tube, and let set for 3 more minutes. Two hundred µl of Chloroform (Sigma-Aldrich) was then added, the samples were shaken vigorously to mix, and left to incubate for 3 minutes at room temperature. The samples were centrifuged at 12,000 X g for 15 minutes at 4^{o}C. The top clear layer was placed into a new tube and 0.5 ml 100% isopropanol was added, mixed slowly, and incubated for 20 minutes at room temperature. The samples were centrifuged at 12,000 X g for 15 minutes at 4^{o}C. The supernatant was removed and the RNA pellets were washed in 70% ethanol in DEPC treated water and centrifuged at 12,000 X g for 15 minutes at 4^{o}C. The supernatant was again removed and the RNA pellets were resuspended in 15 µl DEPC treated water. The RNA concentration was measured with a spectrophotometer and samples were stored at -80^{o}C until use.

The First-Strand Synthesis System (Invitrogen) was used according to the manufacturer's instructions to prepare cDNA from 5 µg of total RNA. Twenty µl of DEPC treated water was added to the finished cDNA samples and the cDNA was stored at -20^{o}C until use. Each polymerase chain reaction (PCR) used 3 µl of the cDNA sample. Paq 5000 polymerase (Invitrogen) was used according to the manufacturer's instructions with the appropriate oligonucleotide primers shown in Table 1 to amplify the cDNA gene copies of the glycosyltransferase enzyme FucT-III and β-actin as a control gene. The cDNA was amplified in an icycler thermocycler (Bio-Rad Laboratories, Hercules, CA) with one cycle at 94^{o}C for 1 minute followed by 35 cycles of denaturation at 94^{o}C, annealing at 58^{o}C, and extension at 72^{o}C for 50 seconds each, and finishing with 72^{o}C for 10 minutes. Samples were kept at 4^{o}C until use. The PCR samples were run on a

1.5% agarose gel with 0.5 µg/ml ethidium bromide at 110 volts for 1.5 hours and the DNA bands were visualized on an Eagle Eye II machine using Alphaimager IS-2200 software.

TABLE 1: PRIMER SEQUENCES USED IN RT-PCR

Gene	GenBank Accession Number	Primer Sequences
β-actin	NM_001101	Sense: 5'-GCT CGT CGT CGA CAA CGG CT-3' Antisense: 5'-CAA ACA TGA TCT GGG TCA TCT TCT C -3'
FucT-III	NM_000149	Sense: 5'-CCT CCT GAT CCT GCT ATG GA-3' Antisense: 5'-GCG GTA GGA CAT GGT GAG AT-3'

Flow Cytometric Analysis

Cells were trypsinized and resuspended in culture media. Approximately $0.25x10^6$ cells were added to 5 ml round-bottom tubes (BD Biosciences Discovery Labware, Bedford, MA). The cells were washed with FACS wash buffer (1X DPBS, 1% goat serum, and 5 mM NaN_3) and centrifuged at 402 X g for 5 minutes at 23°C. The wash buffer was decanted from the tubes and the tube openings were blotted on paper towels to remove excess liquid. Primary antibodies CSLEX-1 or IgM isotype control (Caltag Laboratories, Burlingame, CA) were diluted to 10 µg/ml with FACS wash buffer. One hundred µl of the diluted primary antibody was added to the necessary tubes. The tubes were vortexed, incubated on ice for 20 minutes, and vortexed again. The samples were washed with FACS buffer as mentioned above. Phycoerythrin (PE) conjugated $F(ab')_2$ goat anti-mouse IgM (Jackson Immunoresearch Laboratories, West Grove, PA), was used to detect bound CSLEX-1. The secondary antibody was diluted 1:200 with FACS wash buffer and 100 µl was added to each tube. Tubes were vortexed, incubated on ice in the dark for 20 minutes, and vortexed again. The samples were washed and the labeled cells were resuspended in 200 µl FACS wash buffer. Approximately 10,000 cells were

examined per sample. Data was collected by flow cytometry on a FACSCanto instrument using BD FACSDiva software (Becton-Dickinson Biosciences, San Jose, CA).

Cytokine Detection

H1299 cells were cultured in a 6 well tissue culture plate at a density of $0.15x10^6$ cells per well for 24 hours. The media was replaced with 2 ml serum free media (SFM) and the cells were allowed to grow for an additional 72 hours. The conditioned media was collected and stored at -80^{o}C until use. Cytokines were detected with the Human Cytokine Antibody Array 1 (Ray Biotech, Inc., Norcross, GA) according to the manufacturer's instructions. After the addition of a chemiluminescent reagent, the detection membranes were exposed to Amersham Hyperfilm ECL, high performance chemiluminescence film (GE Healthcare Limited, Buckinghamshire, UK) and developed with a AGFA CP 1000 film processor (Pacesetter Medical, Inc., St. Paul, MN). The data was quantified by densitometry using Image J software (National Institutes of Health, Bethesda, MD).

IL-8 Enzyme-Linked Immunosorbent Assay (ELISA)

H1299 cells were cultured as described above for the cytokine detection assays. The conditioned media was collected and stored at -80^{o}C until use. The human IL-8 ELISA kit (Ray Biotech, Inc.) was used according to the manufacturer's instructions to detect secreted IL-8. The optical density at 450nm was assessed with a SpectraMax Plus 385 microplate reader and SoftMax Pro 3.1.1 software (Molecular Devices, Sunnyvale, CA). Each sample and standard was run in duplicate. The concentration of IL-8 in samples was determined by comparison with the standard curve (Figure 6) and the equation:

$$Y = -0.00005X^2 + 0.0205X + 0.1498$$

FIGURE 6

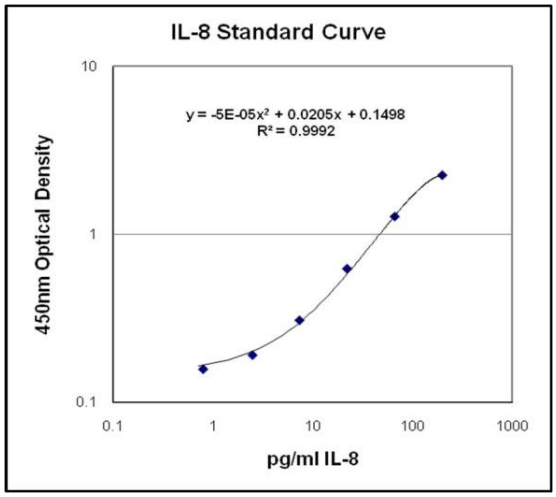


Figure 6: Human IL-8 ELISA standard concentration curve graph.

Stimulation with rTNF-α

Unmanipulated, mock, or FucT-III transfected H1299 or A549 cells were cultured in a 6 well plate at a density of $0.25x10^6$ cells per well for 24 hours. The media was replaced with 2 ml SFM and the cells were cultured for an additional 24 hours. Carrier-Free rTNF-α (R&D Systems, Minneapolis, MN) was diluted to the necessary concentration (per experiment) in 2 ml fresh SFM and added to separate wells of the cells. Control wells received fresh SFM without rTNF-α. The cells were cultured for up to 72 hours before analysis.

E-selectin Binding Assay

Cells were trypsinized and resuspended in cell culture media. Approximately $0.25x10^6$ cells were added to 5 ml round-bottom tubes and washed as described above for flow cytometric analysis. Recombinant mouse E-selectin/Fc chimera (R&D Systems) was diluted to 10 µg/ml in binding buffer (1X HBSS without Ca^{2+} and Mg^{2+}, 3% FBS, and 2 mM $CaCl_2$). To verify the specificity binding interactions, E-selectin chimera was also diluted in EDTA buffer (1X HBSS without Ca^{2+} or Mg^{2+}, 3% FBS, and 10 mM EDTA).

Each cell sample received 100 µl of either the binding or EDTA buffer containing the diluted E-selectin chimera. The tubes were vortexed, incubated on ice for 30 minutes, and vortexed again. The cells were washed as described above. PE conjugated $F(ab')_2$ goat anti-human IgG (Jackson Immunoresearch Laboratories), diluted 1:200 with FACS wash buffer, was used to detect bound E-selectin chimera. One hundred µl of the diluted secondary antibody was added to each tube. The tubes were vortexed, incubated on ice in the dark for 30 minutes, and vortexed again. To further verify the specificity binding interactions, 50 µl of each sample from the tubes containing the binding buffer was then transferred to new tubes. All tubes were washed as described above. Labeled cells in the original binding and EDTA buffers were resuspended in 200 µl FACS wash buffer and kept on ice. Five hundred µl of EDTA buffer was added to the tubes containing the transferred binding buffer samples and incubated at room temperature, in the dark, for 1 hour with vortexing every 10 minutes. The samples were then washed as described above and resuspended in 200 ul FACS wash buffer. Approximately 10,000 cells were examined per sample. Data was collected on a FACSCanto instrument using BD FACSDiva software.

Human Neutrophil Isolation

Between 40 and 60 ml of blood was drawn from a healthy donor into sodium heparin Vacutainers (Becton-Dickinson, Franklin Lakes, NJ). Approximately 20 ml of blood was then pooled in 50 ml conical tubes. An equal volume of 3% dextran (Fischer Scientific, Pittsburgh, PA) in DPBS without Ca^{2+} or Mg^{2+} was added to the blood and mixed gently. The tubes were incubated for 30 minutes at room temperature to allow erythrocytes to settle to the bottom. The clear supernatant was transferred to 50 ml conical tubes, centrifuged at 402 X g for 5 minutes at 23^{o}C, and the supernatant was discarded. Four ml of room temperature nanopure water was added to the cell pellets and swirled gently

to mix. DPBS without Ca^{2+} or Mg^{2+} was added to the tubes to a final volume of 40 ml, mixed gently, and centrifuged at 402 X g for 5 minutes at 23^{o}C. The supernatant was discarded and the cell pellets were resuspended and mixed gently in 10 ml of DPBS without Ca^{2+} or Mg^{2+}. The cells were then filtered into a single 50 ml conical tube through a 40 µm cell strainer to remove clumps. Using a serological pipette, an equal volume of Ficoll lymphocyte separation media (Mediatech, Inc.) was added beneath the cell suspension. The solution was centrifuged at 1118 X g for 10 minutes at room temperature. The supernatant was aspirated off of the resulting neutrophil pellet and the sides of the tube were carefully wiped with a sterile cotton swab to remove residual monocytes and lymphocytes. The neutrophils were then resuspended in approximately 10 ml of opti-MEM with P/S and stained with 0.04% trypan blue, counted with a hemocytometer, and assessed for viability.

Cancer Cell Treatment with Neutrophil Conditioned Media (NCM)

H1299 cells were prepared as for rTNF-α stimulation experiments. Neutrophils were cultured in opti-MEM with P/S in a 6 well plate at a density of $1.25x10^{6}$ cells per ml for 2 hours. After the incubation the resulting conditioned media was collected from the wells, combined, and centrifuged at 402 X g for 5 minutes at 23^{o}C to remove the residual neutrophils. The SFM was removed from the H1299 cells and replaced with 2 ml per well of NCM. Control wells received only opti-MEM with P/S. The treated H1299 cells were incubated for 48 hours.

Co-Culture of Human Neutrophils with Cancer Cells

H1299 cells were prepared as for rTNF-α stimulation experiments. The serum free media was removed from the H1299 cells and replaced with 2 ml opti-MEM with P/S. Neutrophils were added to the H1299 cells in the following neutrophil: cancer cell ratios:

1.25 million neutrophils for 5:1, 250,000 neutrophils for 1:1, and 50,000 neutrophils for 1:5. The cells were incubated for 48 hours.

Histopathology

Human lung tissue sections used for immunohistochemical staining were of 4 μm thickness, fixed in 10% neutral buffered formalin, and embedded in paraffin. A human lung cancer tissue array was obtained from Pantomics, Inc., San Francisco, CA., and archived samples of normal lung and lung carcinoma tissues were from the Tissue Procurement Facility, University of Minnesota. The lung tissue array consisted of duplicate samples from 48 individual specimens removed from patients undergoing surgical resection of lung tumors (a total of 96 core samples) and was composed of: 4 normal tissues (8 cores), 6 inflamed tissues (12 cores), a granuloma (2 cores), a granuloma mixed with squamous cell carcinoma (2 cores), 12 squamous cell carcinomas (24 cores), 12 adenocarcinomas (24 cores), 3 adenosquamous carcinomas (6 cores), 3 small cell carcinomas (6 cores), a bronchioloalveolar carcinoma (2 cores), a large cell carcinoma (2 cores), and 4 papillary carcinomas (8 cores). We performed immunohistochemical staining on serial sections of each tissue sample. Tissue samples were stained with hematoxylin & eosin (H&E) by the University of Minnesota's Comparative Pathology Core Resource facility.

Antibodies

The primary antibodies for the immunohistochemistry experiments were as follows: CSLEX-1 mAb (15 μg/ml) was used to detect the sLe^x epitope, rabbit polyclonal Neutrophil Elastase antibody (0.5 μg/ml) (Abcam, Cambridge, MA) was used to detect the presence of neutrophils, and Keratin, Pan Ab-3 (Ck-Lu5) mAb (1:50) (NeoMarkers, Fremont, Ca) which stains for a broad spectrum of cytokeratin molecules was used as a positive control to detect epithelial cells. Serum from the species in which the primary

antibody was derived was diluted 1:10,000 in DPBS without Ca^{2+} or Mg^{2+} and was used as a negative control. Secondary antibodies were: horseradish peroxidase (HRP)-conjugated μ chain specific goat anti-mouse IgM (Chemicon, Temecula, CA) was used at a 1:24 dilution to detect bound CSLEX-1, HRP-conjugated donkey anti-rabbit IgG (BioFX Laboratories, Owings Mills, MD) was used at a 1:500 dilution to detect bound Neutrophil Elastase antibody, and HRP-conjugated goat anti-mouse IgG (Invitrogen) was used at a 1:50 dilution to detect bound Ck-Lu5 mAb. All antibodies were diluted in antibody diluent (Dako, Denmark).

Immunohistochemistry

Tissue slides, all reagents, and diluted antibodies were warmed to 30^{o}C before use and all incubation periods were conducted in a humidifier chamber at 30^{o}C unless otherwise noted. Tissues were de-paraffinized by 3 minute incubations in three changes of 100% xylene, re-hydrated through 30 second incubations in each of 3 changes of 100% and 1 change each of 95% and 75% ethanol. Tissue slides were washed in running distilled water for 1 minute. Staining with Neutrophil Elastase and Ck-Lu5 required an antigen retrieval step. When staining with Neutrophil Elastase, slides were incubated for 10 minutes with Pepsin Solution Digest-All 3 ready to use antigen retrieval reagent (Invitrogen). When staining with Ck-Lu5, slides were incubated for 5 minutes with Protenase K enzyme (Dako) diluted 1:5 in 0.05 M Tris-HCL, pH 7.6. The slides were washed in running distilled water for 10 minutes. Staining with all primary antibodies continued with the following steps. Tissues were washed in 1X TBS buffer (1X Tris/Tween 20 Tris buffered saline (TBS), pH 7.6) for 2 minutes, incubated in 3% hydrogen peroxide (diluted from a 30% stock solution in DPBS) for 15 minutes to block endogenous peroxidase activity, and washed 3 times for 2 minutes in 1X TBS buffer. Non-specific binding sites in tissues were blocked by incubating the slides for 10 minutes

with serum-free, ready to use Protein Block (Dako). Excess protein block was removed and diluted primary antibody or serum control was applied to each tissue without washing. Tissues were incubated for 2 hours with CSLEX-1 and Neutrophil Elastase antibodies and for 1 hour with the Ck-Lu5 antibody. Tissues were washed 3 times as above and the appropriate diluted secondary antibody was added. Tissues were incubated for 1 hour to detect CSLEX-1 and for 30 minutes to detect Neutrophil Elastase and Ck-Lu5. Tissues were washed 3 times as above and bound HRP was detected with the peroxidase substrate chromogen 3,3'-diaminobenzidine tetrachloride (DAB) (Vector Laboratories, Inc., Burlingame, CA). Tissues were protected from light and incubated with the DAB reagent for 10 minutes at room temperature and washed in running distilled water for 5 minutes. Tissues were counterstained with Myer's Hematoxylin (Dako) and washed in running distilled water for 5 minutes. Tissues were dehydrated through 30 second incubations in 70%, 95%, three separate changes of 100% ethanol, and three changes of xylene. The tissue slides were cover slipped with Permount (Fischer Scientific) and evaluated by light microscopy.

Histologic Grading System for Lung Tissues

The presence of staining with CSLEX-1 and Neutrophil Elastase antibodies in lung tissues was evaluated in a semi-quantitative manner. The entire tissue core of each sample on the array was assessed at 100X magnification for distribution of sLe^x expression (as indicated by reactivity with CSLEX-1 mAb) and the number of neutrophils present (as indicated by reactivity with Neutrophil Elastase polyclonal antibody). Positive staining was defined as cells in which cytoplasmic and/or cell membrane labeling with the relevant antibody was detected, regardless of the intensity of staining. For both antibodies, the presence of staining was scored from 0 – 3. For CSLEX-1 reactivity, score 0, no visible immunostaining; score 1, less than 25% of the total tissue

area was positive; score 2, 26 – 50% of the total tissue area was positive; and score 3, greater than 50% of the total tissue area was positive. For reactivity with neutrophil elastase, score 0, cells were not visibly immunostained; score 1, 1-10 cells in the total tissue area were positive; score 2, 11-35 cells in the total tissue area were positive; score 3, more than 35 cells in the total tissue area were positive. Since duplicate samples were provided on the tissue array, redundancy was avoided by averaging and rounding up (if necessary) the individual CSLEX-1 and Neutrophil Elastase staining scores from each core of a tissue specimen to give an overall score for that tissue.

TNF-α neutralization

Ten µg/ml LEAF purified TNF-α blocking antibody (BioLegend, San Diego, CA) was added to either NCM or opti-MEM with P/S containing 5 ng/ml rTNF-α. The media with blocking antibody was incubated on a rocker at room temperature for 1 hour and used in subsequent experiments.

Microscopic Cell Culture Photographs

Cells were digitally photographed at 100X in tissue culture plates with a Zeiss Axiovert 200 inverted microscope and an AxioCam MRc camera using AxioVision 4.1 software (Carl Zeiss, Inc., Germany).

Cell Invasion Assay

Invasions assays were performed using BioCoat™ Matrigel™ Invasion Chamber system (Becton-Dickinson, Bedford, MA, USA) in 24 well tissue culture plates according to the manufacturer's instructions. To create a chemoattractant gradient, 0.5 ml H1299 cell media (for rTNF-α stimulation assays) or opti-MEM with P/S (for NCM assays) containing 10% FBS was added to a 24 well plate. Twenty five thousand H1299 cells were resuspended in 0.5 ml media containing 2% FBS and 5 ng/ml rTNF-α, NCM, NCM

that had been incubated with a TNF-α blocking antibody (described above), or simply 2% FBS or opti-MEM with P/S for control samples. Resuspended cells were added to the top side of the matrigel inserts and incubated for 24 hours. Culture media was aspirated from the chamber and non-invaded cells were wiped off of the membrane with a cotton swab. The invaded cells in the matrigel membranes were stained in Diff-Quik (Jorgensen Laboratories, Inc., CO.), mounted on Superfrost microscope slides (Fisher Scientific) with Fluoromount-G (SouthernBiotech, Birmingham, AL), and 3 or 5 fields at 50 or 100X magnification respectively were digitally photographed with a Zeiss Axiovert 200 inverted microscope and an AxioCam MRc camera using AxioVision 4.1 software. The number of invaded cells/field were counted and averaged to obtain numerical data.

Statistical Analysis

Numerical significance between arrays of experimental data, with the exception of the immunohistochemical staining, was determined by application of a 2-tailed Student's t-Test. P-values below 0.05 were considered significant.

Results

Stable transfection of H1299 cells with FucT-III induces cell surface sLe^x expression

H1299 cells, which were derived from a lymph node metastasis of a NSCLC, were used to investigate the influence of the FucT-III enzyme and sLe^x epitope on cytokines and chemokines secreted into the tumor microenvironment and tumor cell metastatic potential. H1299 cells were decidedly a good tumor cell line in which to examine the effect of FucT-III expression as they endogenously expressed very low levels of FucT-III mRNA. These cells therefore also didn't express sLe^x (the enzymatic product of FucT-III) on their surface as determined by flow cytometry. The FucT-III gene was inserted into the pcDNA 1.3 neo(+) plasmid vector and stably transfected into H1299 cells.

(Control cells transfected with only the empty vector will be further referred to as mock-transfected cells and cells transfected with the gene will be referred to as FucT-III transfected cells). H1299 cells stably transfected with the FucT-III gene displayed high expression of FucT-III mRNA compared to unmanipulated (parent) or mock-transfected cells as determined by RT-PCR. The mRNA expression of the housekeeping gene β-actin was used as an internal control (Figure 7a).

Cell surface expression of sLe^x was assessed and compared between the H1299 parent, mock-transfected, and FucT-III transfected cells by flow cytometry and reactivity with the CSLEX-1 mAb. In contrast to the parent and mock-transfected cells which did not express sLe^x, transfection with FucT-III resulted in sLe^x expression by approximately 50% of the cells (Figure 7b-7c). Staining of the FucT-III cells with an IgM isotype control antibody resulted in a negative signal (Figure 7c).

FIGURE 7a

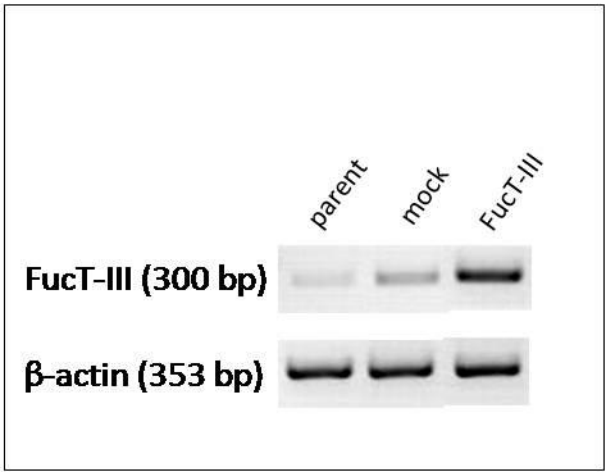


FIGURE 7b

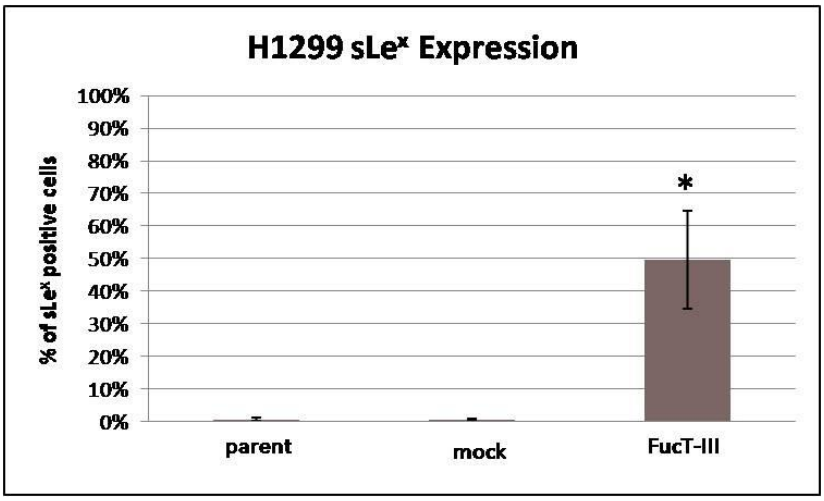


FIGURE 7c

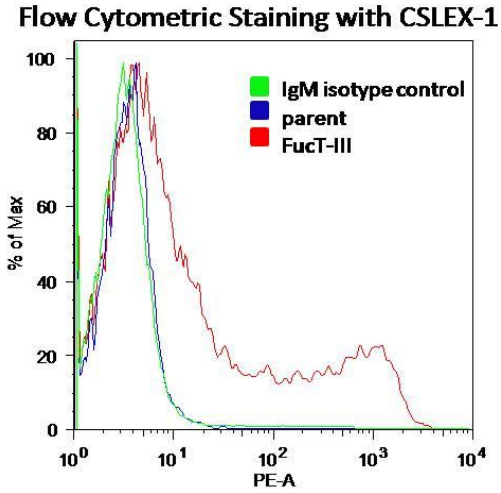


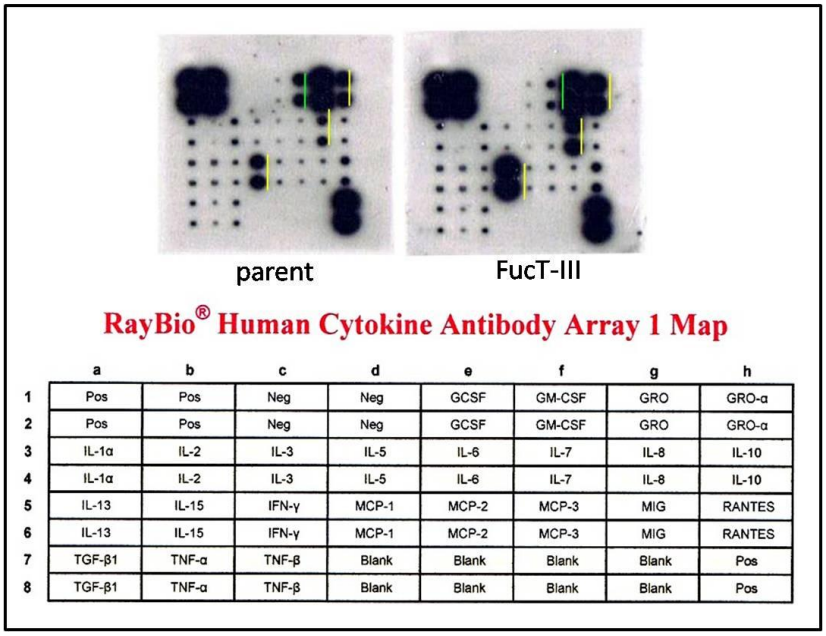
Figure 7: Transfection of H1299 cells with the FucT-III gene results in expression of FucT-III mRNA and de novo synthesis of cell surface sLe^x. (a) RT-PCR of FucT-III and β-actin mRNA isolated from parent, mock-transfected, and FucT-III transfected cells. One representative experiment of five is shown. (b) Flow cytometric analysis of cell surface sLe^x expression after transfection with FucT-III as determined by reactivity with the CSLEX-1 mAb. Results shown are the average of 14 experiments. * indicates a significant difference between parent and FucT-III transfected cells, $p < 0.0001$. (c) A representative histogram comparing the flow cytometric staining intensity with CSLEX-1 mAb of parent (blue) versus FucT-III transfected (red) cells. The result of FucT-III transfected cell staining with an IgM isotype control antibody is shown in green.

FucT-III transfected H1299 cells secrete increased levels of pro-inflammatory chemokines

Cytokines and chemokines summon cell populations, orchestrate cellular activities, and direct the inflammatory polarization of cells in a given tissue microenvironment. To determine whether or not overexpression of FucT-III in H1299 cells could alter their cytokine expression profile, we assessed the secretion of cytokines and chemokines from parent versus FucT-III transfected cells after 72 hours in culture. The presence and relative abundance of cytokines was assessed with a human anti-cytokine antibody membrane array. When compared to the unmanipulated parent cells, the FucT-III transfected cells were found to secrete significantly more GROα ($p = 0.03$) and MCP-1 ($p = 0.01$). Although not considered statistically significant, FucT-III cells also secreted increased levels of IL-8 ($p = 0.07$) and decreased levels of GM-CSF ($p = 0.07$) (Figure 8a-b).

IL-8 has been extensively described as a potent neutrophil chemoattractant and increased secretion of IL-8 has been implicated in the pathogenesis of neutrophil mediated chronic inflammatory diseases that are associated with the development of metaplasia [5-11, 14-18]. Therefore, the observed change in IL-8 secretion was verified using a human IL-8 ELISA. FucT-III transfected cells were shown to secrete 0.5 fold more IL-8 than parent cells ($p = < 0.005$). Unexpectedly, the mock-transfected cells consistently showed a 0.5 fold decrease in IL-8 secretion (Figure 8c).

FIGURE 8a



	a	b	c	d	e	f	g	h
1	Pos	Pos	Neg	Neg	GCSF	GM-CSF	GRO	GRO-α
2	Pos	Pos	Neg	Neg	GCSF	GM-CSF	GRO	GRO-α
3	IL-1α	IL-2	IL-3	IL-5	IL-6	IL-7	IL-8	IL-10
4	IL-1α	IL-2	IL-3	IL-5	IL-6	IL-7	IL-8	IL-10
5	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MIG	RANTES
6	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MIG	RANTES
7	TGF-β1	TNF-α	TNF-β	Blank	Blank	Blank	Blank	Pos
8	TGF-β1	TNF-α	TNF-β	Blank	Blank	Blank	Blank	Pos

FIGURE 8b

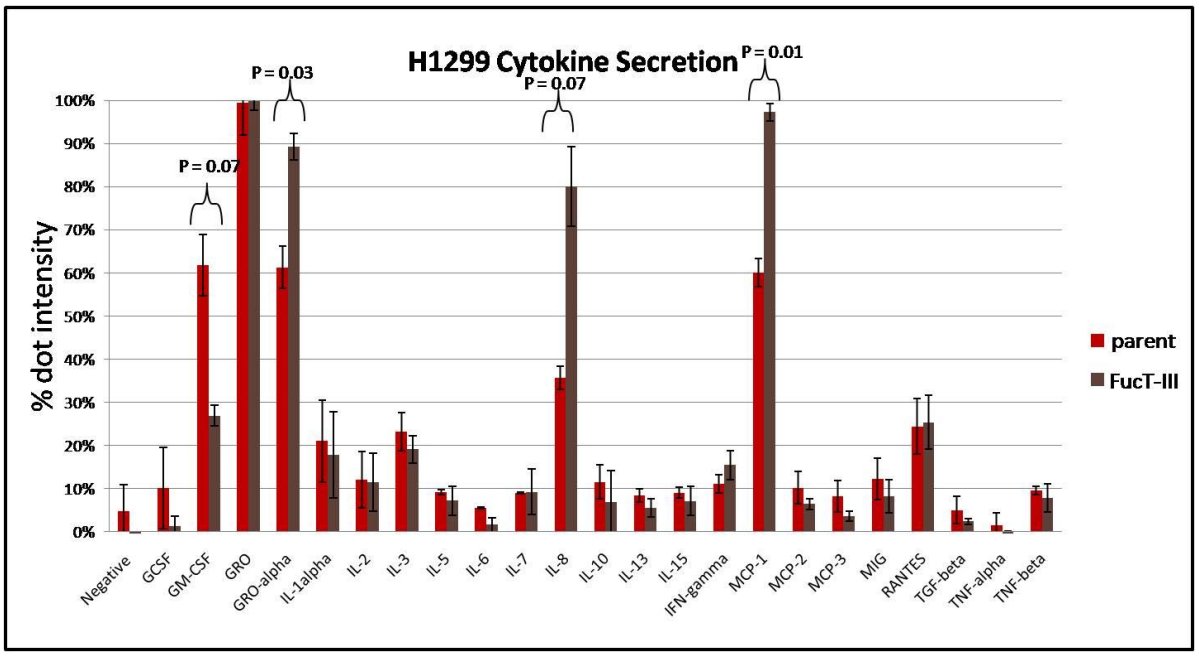


FIGURE 8C

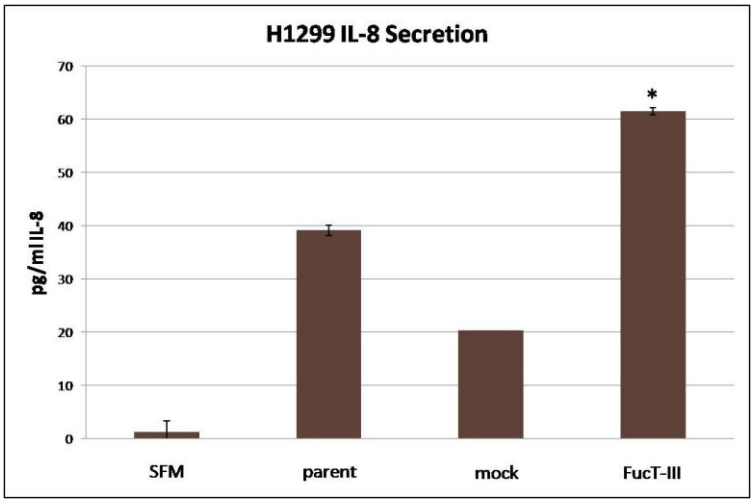


Figure 8: FucT-III transfected H1299 cells secrete increased levels of pro-inflammatory chemokines. (a) SFM that had been conditioned by parent or FucT-III transfected cells for 72 hours was tested for relative abundance of cytokines and chemokines using anti-cytokine antibody membrane arrays. In FucT-III transfected cells, spots representing decreased cytokine levels are indicated by a green bar and increased cytokine levels are indicated by a yellow bar. (b) The spot intensities for each cytokine were quantified by densitometry. Blank intensity values were subtracted from all of the dot values and cytokine dot intensity values were normalized to the positive control dot intensities. Values shown for each cytokine represent the average of the normalized intensities of both dots. P-values for cytokines shown to be significantly differentially secreted, in parent versus FucT-III transfected cells, are shown in the graph above the respective cytokines. (c) The amount of IL-8 secreted into SFM over a period of 72 hours by parent, mock-transfected, or FucT-III transfected cells was assessed by an ELISA for human IL-8. Results shown are the average of two simultaneously run samples and are representative of two experiments. * indicates significantly higher levels of IL-8 secretion by FucT-III transfected cells when compared to parent cells, $p < 0.005$.

Stimulation with rTNF-α up-regulates sLe^x expression and E-selectin binding ability in FucT-III transfected lung cancer cells

In inflamed tissue, neutrophils and monocytes are significant sources of the inflammatory cytokine TNF-α [36, 125], therefore, TNF-α is likely to be present in the microenvironment of a tumor being infiltrated by inflammatory cells. To determine if treatment with rTNF-α altered H1299 cell sLe^x expression, and to investigate the dynamics of the influence of rTNF-α on sLe^x, experimental concentration and time curves were completed. H1299 parent and FucT-III transfected cells were stimulated

with increasing concentrations of rTNF-α and tested for sLe^x expression by flow cytometry. The expression of sLe^x in parent H1299 cells was not induced by treatment with rTNF-α. However, in FucT-III transfected cells, concentrations of rTNF-α ranging from 1 – 100 ng/ml were increasingly effective in up-regulating sLe^x expression by 5 – almost 40% respectively (Figure 9a). All further experiments were conducted with 5 ng/ml rTNF-α. This concentration consistently resulted in a significant increase in sLe^x expression while not greatly exceeding the range of physiologically relevant concentrations.

To determine if the duration of stimulation with rTNF-α had an effect on the magnitude of the increase in sLe^x expression, H1299 parent and FucT-III transfected cells were stimulated with 5 ng/ml rTNF-α for 24, 48, and 72 hours and tested for sLe^x expression. When compared to unstimulated cells, the TNF-α dependent increase in sLe^x expression was similar at each time point examined (approximately 20% higher) (Figure 9b). Since no appreciable difference in the magnitude of TNF-α dependent sLe^x up-regulation was seen between 24 and 48 hours, and the cells began to grow past confluency and die off at 72 hours, subsequent experiments were conducted with a 48 hour stimulation period.

To determine whether similar results would be observed in another lung carcinoma cell line, A549 cells were stably transfected with FucT-III and submitted to stimulation with rTNF-α. Transfection with FucT-III resulted in expression of sLe^x by approximately 32% of A549 cells. Upon stimulation with rTNF-α, the fraction of cells expressing sLe^x was increased by approximately 20% in H1299 cells and 16% in A549 cells. Similarly to the H1299 cells, the parent A549 cells were not induced to express sLe^x after stimulation with rTNF-α (Figure 9c-d).

The sLe^x epitope is abundant on several different types of human carcinoma cells and is a well defined ligand for E-selectin. We observed that stimulation with rTNF-α consistently increased sLe^x expression in FucT-III transfected human lung cancer cells. We therefore, investigated whether rTNF-α stimulation influenced the E-selectin binding ability of FucT-III transfected cells. Unmanipulated and transfected cells were either left untreated or stimulated with rTNF-α under the conditions described above and their adhesiveness to an E-selectin/Fc chimeric molecule was analyzed by flow cytometry. Selectin binding is calcium dependent, and therefore, the specificity of binding was verified by conducting the assays in buffers containing either the metal chelator EDTA or Ca^{2+}. Binding specificity was additionally verified by running the experiment in the Ca2+ buffer followed by incubation with EDTA to release bound E-selectin. Parent H1299 cells did not bind to E-selectin and, as expected, rTNF-α stimulation did not result in increased binding of the parent cells to E-selectin. However, stimulation of the FucT-III transfected cells with rTNF-α resulted in a significant, 16% increase in E-selectin binding ability (9e). Similar results were obtained when the parent and FucT-III transfected A549 cells were stimulated with rTNF-α and used in E-selectin binding assays (Figure 9f).

FIGURE 9a

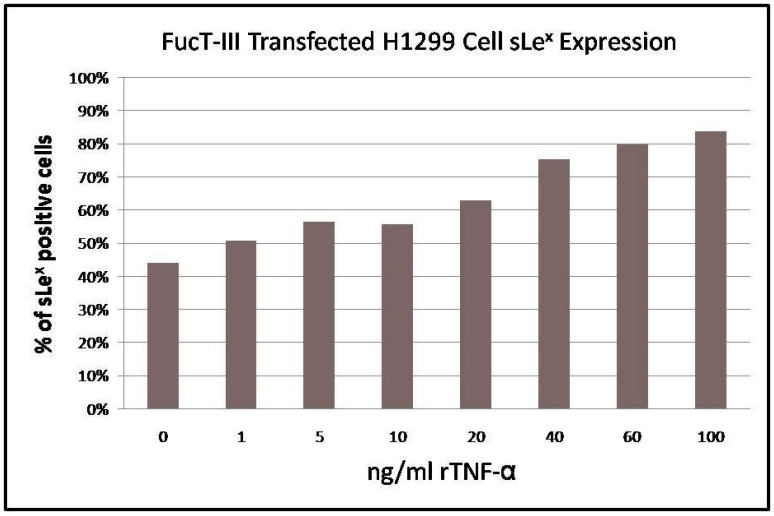


FIGURE 9b

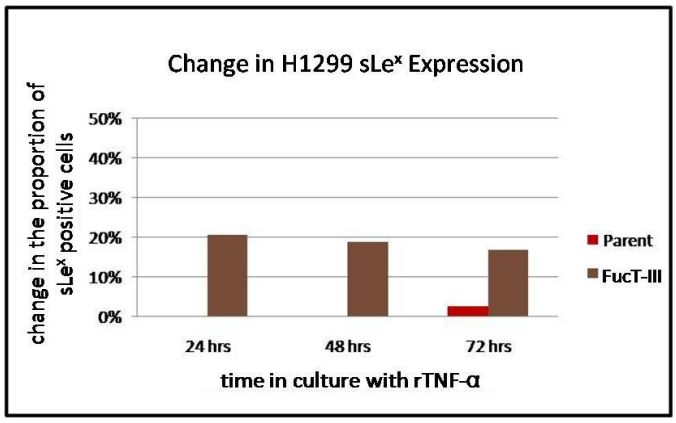


FIGURE 9C

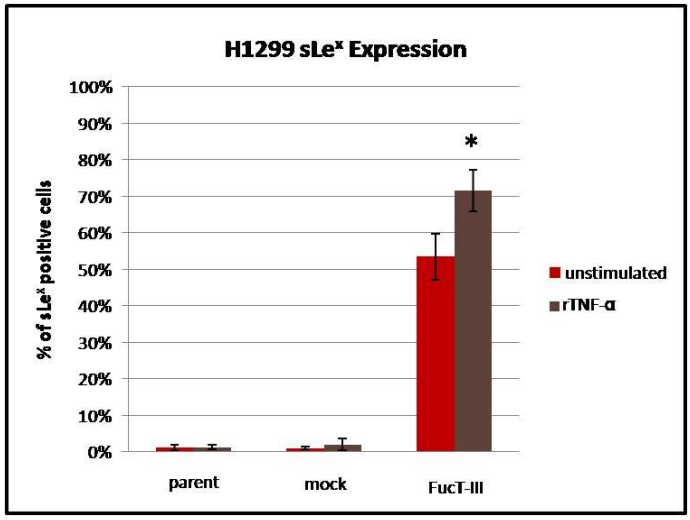


FIGURE 9d

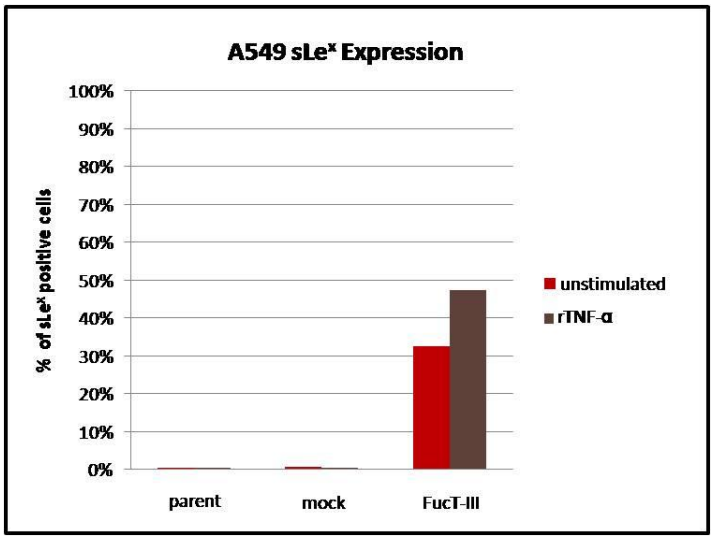


FIGURE 9e

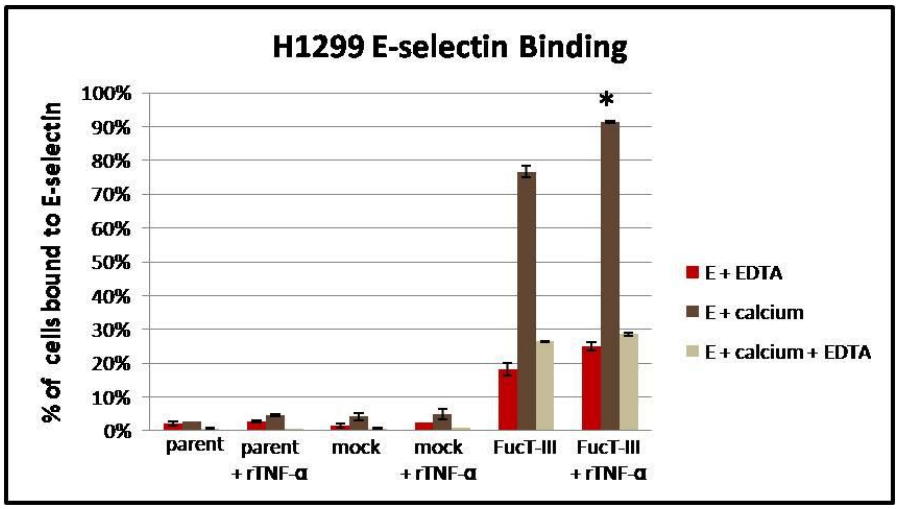


FIGURE 9f

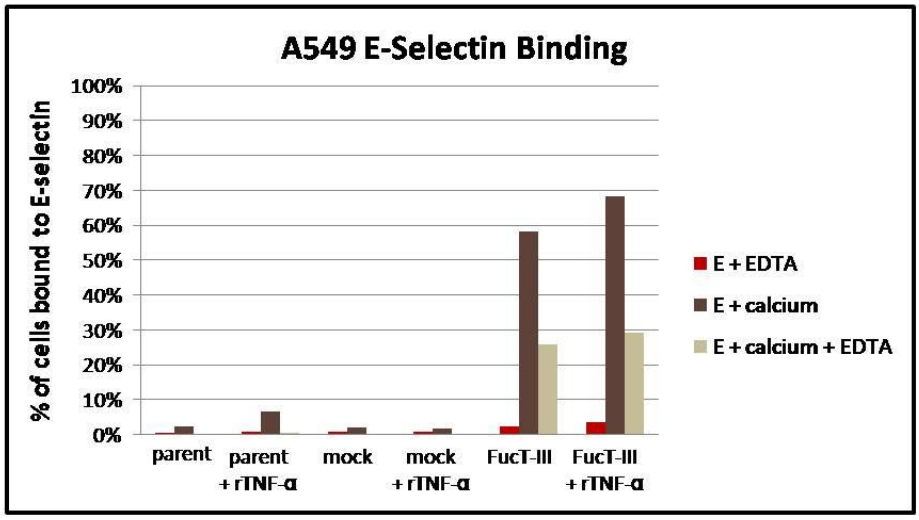


Figure 9: rTNF-α up-regulates sLe^x expression and E-selectin binding of FucT-III transfected lung tumor cells. (a) FucT-III transfected H1299 cells were stimulated with increasing concentrations of rTNF-α for 48 hours and sLe^x expression was assessed by flow cytometric analysis of reactivity with the CSLEX-1 mAb. Results of one representative experiment of two repetitions are shown. (b) H1299 parent and FucT-III transfected cells were left untreated or stimulated with 5 ng/ml rTNF-α. The levels of cell surface sLe^x expression were assessed at 24, 48, and 72 hours by flow cytometric analysis of reactivity with the CSLEX-1 mAb. Results of one representative experiment of two repetitions are shown. (c) H1299 parent, mock-transfected, and FucT-III transfected cells were left untreated or stimulated with 5 ng/ml rTNF-α for 48 hours and sLe^x expression was assessed by flow cytometric analysis of reactivity with the CSLEX-1 mAb. The mean value of 7 experiments is shown. * indicates increased sLe^x

expression in FucT-III transfected cells stimulated with rTNF-α versus unstimulated cells, $p = 0.0001$. (d) A549 parent, mock-transfected, and FucT-III transfected cells were left untreated or stimulated with 5 ng/ml rTNF-α for 48 hours and sLe^x expression was assessed by flow cytometric analysis of reactivity with the CSLEX-1 mAb. Results of one representative experiment of two repetitions are shown. (e) H1299 parent, mock-transfected, and FucT-III transfected cells were left untreated or stimulated with 5 ng/ml rTNF-α for 48 hours and binding to E-selectin was assessed by flow cytometry. Results shown are the average of two experiments. * indicates increased E-selectin binding in FucT-III transfected cells stimulated with rTNF-α versus unstimulated cells, $p < 0.05$. (f) A549 parent, mock-transfected, and FucT-III transfected cells were left untreated or stimulated with 5 ng/ml rTNF-α for 48 hours and binding to E-selectin was assessed by flow cytometry. Results of one representative experiment of two repetitions are shown.

FucT-III transfected H1299 cells cultured in neutrophil conditioned media (NCM) or with neutrophils up-regulate sLe^x expression

Since neutrophils are one of the inflammatory cell types that secrete TNF-α into inflamed tissue [36], and overexpression of FucT-III in H1299 cells resulted in a significant increase in secretion of neutrophil chemoattractants, we assessed whether factors in NCM or cell-to-cell contact resulting from co-culture of neutrophils with H1299 cells was able to alter tumor cell expressed sLe^x. Blood was drawn from a healthy donor and isolated neutrophils were cultured in opti-MEM for 2 hours. The cancer cells were then cultured in NCM for 48 hours and sLe^x expression was determined by flow cytometry. As with rTNF-α stimulation, NCM was unable to induce sLe^x expression in the parent H1299 cells, however, a significant increase in sLe^x expression, between 12 – 20%, was observed in the FucT-III transfected cells (Figure 10a).

Subsequently, FucT-III transfected H1299 cells were co-cultured with freshly isolated neutrophils at various neutrophil: cancer cell ratios ranging from 5:1 to 1:5 for 48 hours. Only the FucT-III cells were assessed in these experiments as the parent cells never showed any induction of sLe^x expression. Co-cultured FucT-III cells were assessed for sLe^x expression by flow cytometry. Although, neutrophils express high levels of sLe^x on

their cell membranes and are strongly reactive with the CSLEX-1 mAb, we were able to distinguish cancer cells from neutrophils and exclude the latter population from our analysis based on differences in size between the two cell types. As observed for culture of FucT-III transfected H1299 cells in NCM, co-culture of these cells with neutrophils resulted in a significant increase cancer cell sLe^x expression. The magnitude of sLe^x up-regulation corresponded to the number of neutrophils present with a maximal increase in sLe^x expression observed at a 5:1 ratio of neutrophils to cancer cells which resulted in an approximate 45% increase in sLe^x expression (Figure 10b).

FIGURE 10a

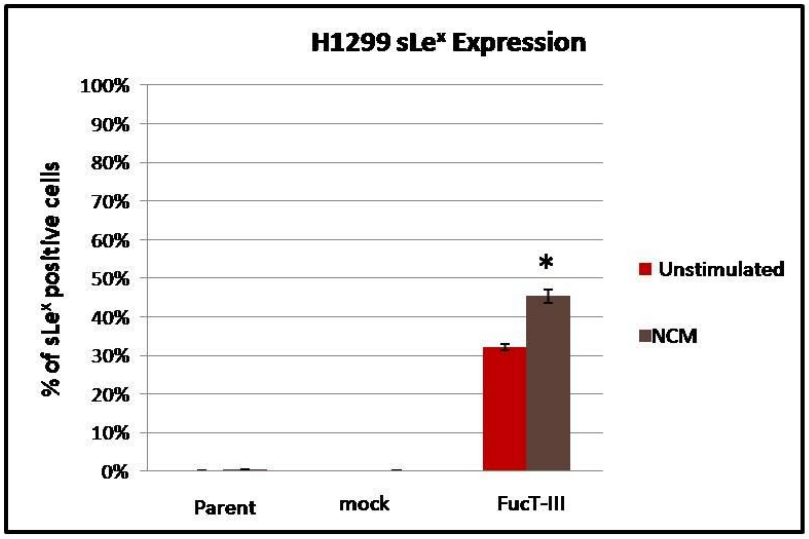


FIGURE 10b

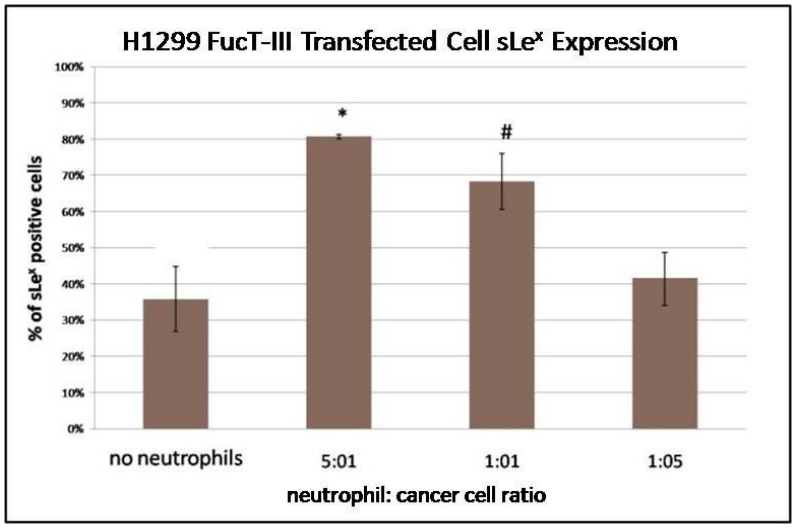


Figure 10: FucT-III transfected H1299 cells up-regulate sLe^x expression after culture in NCM or with neutrophils. (a) H1299 parent, mock-transfected, and FucT-III transfected cells were left untreated or cultured in NCM for 48 hours and sLe^x expression was assessed by flow cytometric analysis of reactivity with the CSLEX-1 mAb. Results shown are the average of two experiments. * indicates a significant increase in the proportion of sLe^x positive FucT-III transfected cells cultured in NCM versus left untreated, $p < 0.05$. (b) H1299 cells transfected FucT-III were co-cultured with neutrophils at the indicated concentrations for 48 hours and sLe^x expression was assessed by flow cytometric analysis of reactivity with the CSLEX-1 mAb. Results shown are the average of 4 experiments. * and # indicate a significant increase in the proportion of sLe^x positive cells co-cultured at a 5:1 and 1:1 neutrophil to cancer cell ratio respectively versus untreated cells, $p < 0.005$ in both instances.

Neutrophil infiltration is associated with sLe^x positive tissues in lung tumor samples

As a first step to determine if the observed FucT-III induced increase in neutrophil chemoattractant secretion and up-regulation of sLe^x in the presence of neutrophils was physiologically relevant, we compared the expression of sLe^x (confirmed by tissue reactivity of the CSLEX-1 mAb) and the presence of neutrophils (identified by staining with a polyclonal antibody against neutrophil elastase) in human lung tissue samples by immunohistochemistry as described in Materials and Methods (Table 2). As expected, all of the lung tissues examined stained strongly with the Ck-Lu5 mAb. None of the normal lung tissues reacted with the CSLEX-1 mAb, however, unexpectedly the normal

tissue showed substantial neutrophil infiltration (Table 2). As neutrophils express abundant amounts of the sLe^x epitope, they were detected by reactivity with the CSLEX-1 mAb in tissue samples that otherwise stained negatively for sLe^x (Figure 11a).

In 5 of 6 inflamed tissue samples, less than 25% of the total tissue area stained positively for sLe^x and one sample showed no expression of sLe^x. As anticipated, high numbers of neutrophils were detected in inflamed samples and they were observed to predominate around isolated areas of sLe^x positive tissue (Figure 11b). All of the squamous cell carcinomas and 10 of 12 adenocarcinomas showed at least minimal reactivity with the CSLEX-1 mAb and 5 of 12 and 8 of 12 squamous cell carcinomas and adenocarcinomas respectively showed positive staining covering more than 25% of the total tissue area. With the exception of 3 adenocarcinomas, the CSLEX-1 and Neutrophil Elastase staining scores for all of these tissues differed by no more than one scoring point (Table 2). Neutrophils were also observed to be more plentiful in areas of tissue that showed increased staining with the CSLEX-1 mAb (Figure 11c-d). Histological grades were provided for the squamous cell carcinomas and adenocarcinomas on the lung tissue array, neither the extent of reactivity with the CSLEX-1 mAb nor the number of infiltrated neutrophils was associated with tumor stage.

Three of four papillary carcinomas showed substantial reactivity with the CSLEX-1 mAb. Accordingly, high numbers of neutrophils were found in the sLe^x positive samples while only a few (less than 10) neutrophils were detected in the sLe^x negative sample (Table 2). Of interest, 3 of 3 small cell carcinomas did not react with the CSLEX-1 mAb and a small number (less than 10) of neutrophils were detected in only 2 of the samples (Table 2, Figure 11e). The two lung granuloma samples did not stain with the CSLEX-1 mAb but, as expected, neutrophils were detected in these tissues. The bronchioloalveolar and large cell carcinoma samples (n = 1 each) both exhibited minimal reactivity with the

CSLEX-1 mAb however, low (less than 10) and high (greater than 35) numbers of neutrophils were detected in these tissues respectively (Table 2). Only one of the adenosquamous cell carcinomas showed reactivity with the CSLEX-1 mAb and the magnitude of neutrophil infiltration in these tissues did not correspond to sLe^x expression.

Overall, 28 of 36 lung tumors stained positively with the CSLEX-1 mAb and 17 of these samples showed staining covering more than 25% of the total tissue area. Moderate to high numbers of neutrophils were detected in 12 (71%) of the 17 highly sLe^x positive tumor tissue samples. In contrast, only 6 (32%) of the 19 samples found to react minimally or not at all with the CSLEX-1 mAb showed moderate to high numbers of infiltrating neutrophils.

TABLE 2

normal	normal	normal	normal	inflamed	inflamed	inflamed	inflamed	inflamed	inflamed	GRAN	GRAN
0 (3)	0 (3)	0 (3)	0 (2)	1 (1)	1 (3)	1 (3)	0 (2)	1 (3)	1 (3)	0 (2)	0 (3)
SQCC	SQCC	SQCC	SQCC	SQCC	SQCC	SQCC	SQCC	SQCC	SQCC	SQCC	SQCC
2 (3)	1 (2)	2 (3)	1 (2)	2 (2)	1 (0)	3 (2)	1 (1)	1 (1)	3 (3)	1 (1)	1 (0)
AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC
0 (3)	2 (2)	3 (1)	3 (2)	2 (1)	3 (1)	2 (1)	3 (3)	1 (2)	1 (1)	0 (1)	2 (2)
ASQCC	ASQCC	ASQCC	PAPC	PAPC	PAPC	PAPC	SCC	SCC	SCC	LCC	BRALC
0 (1)	0 (3)	2 (0)	3 (3)	3 (3)	3 (2)	0 (1)	0 (1)	0 (0)	0 (1)	1 (3)	1(1)

Table 2: **Staining scores for reactivity of lung tissues with CSLEX-1 and Neutrophil Elastase antibodies.** Serial sections of tissues were stained with CSLEX-1 and Neutrophil Elastase antibodies. The numbers in the body of the table indicate the average distribution of staining from two separate cores of an individual tissue. The bracketed numbers indicate the average score for the number of neutrophils detected in two separate cores of the same tissue as was stained with the CSLEX-1 mAb. GRAN: granuloma, SQCC: squamous cell carcinoma, AC: adenocarcinoma, ASQCC: adenosquamous cell carcinoma, PAPC: papillary carcinoma, SCC: small cell carcinoma, LCC: large cell carcinoma, BRALC: bronchioloalveolar carcinoma.

FIGURE 11a

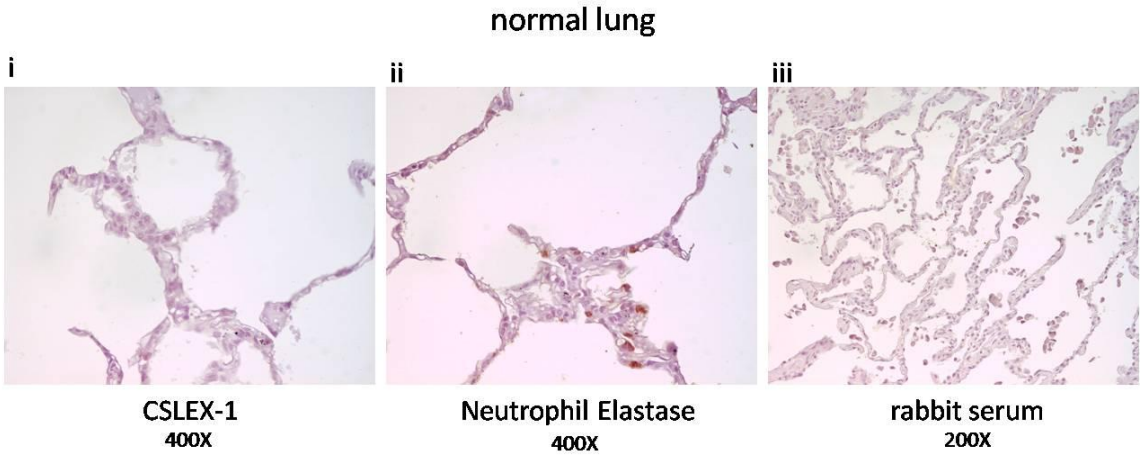


FIGURE 11b

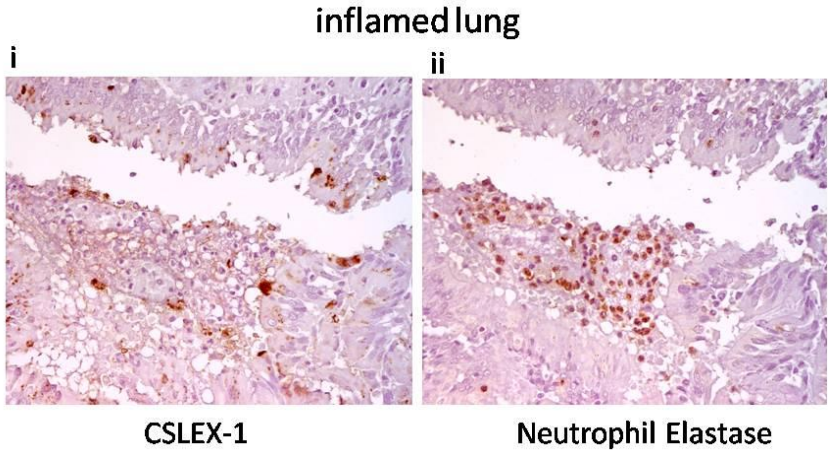


FIGURE 11c

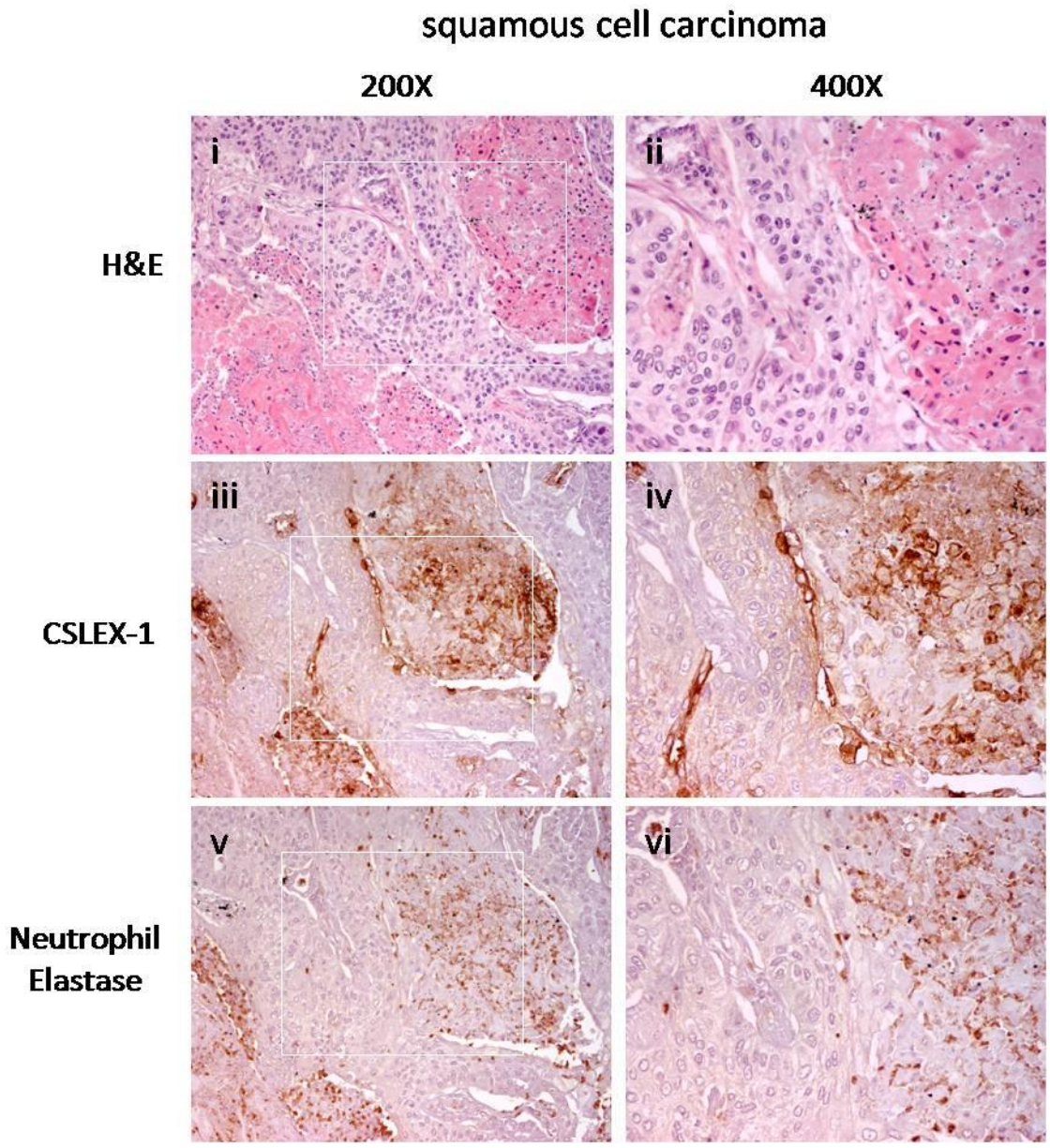

squamous cell carcinoma
200X
400X
H&E
CSLEX-1
Neutrophil Elastase
i
ii
iii
iv
v
vi

FIGURE 11d

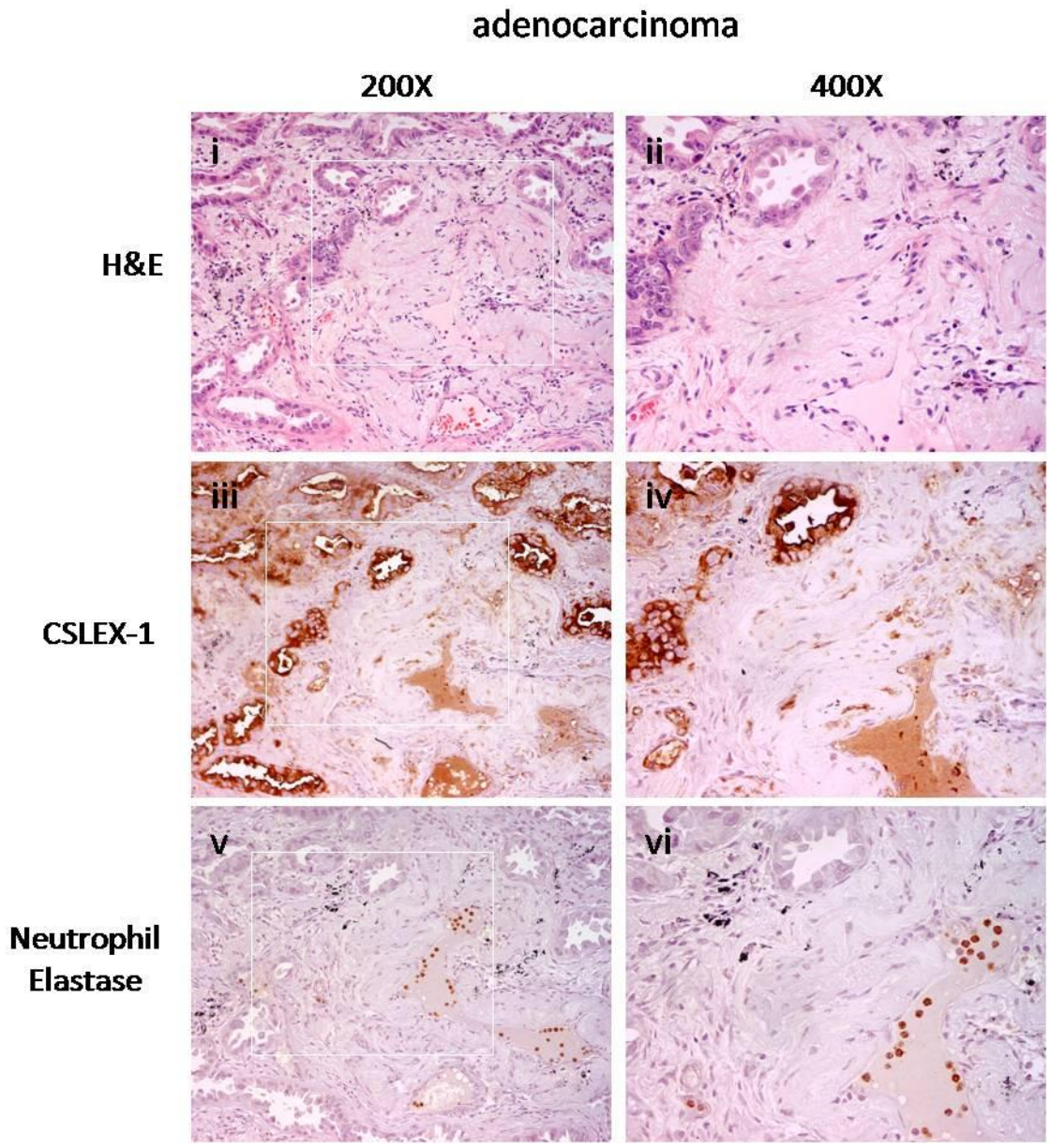

adenocarcinoma
200X
400X
H&E
CSLEX-1
Neutrophil Elastase
i
ii
iii
iv
v
vi

FIGURE 11e

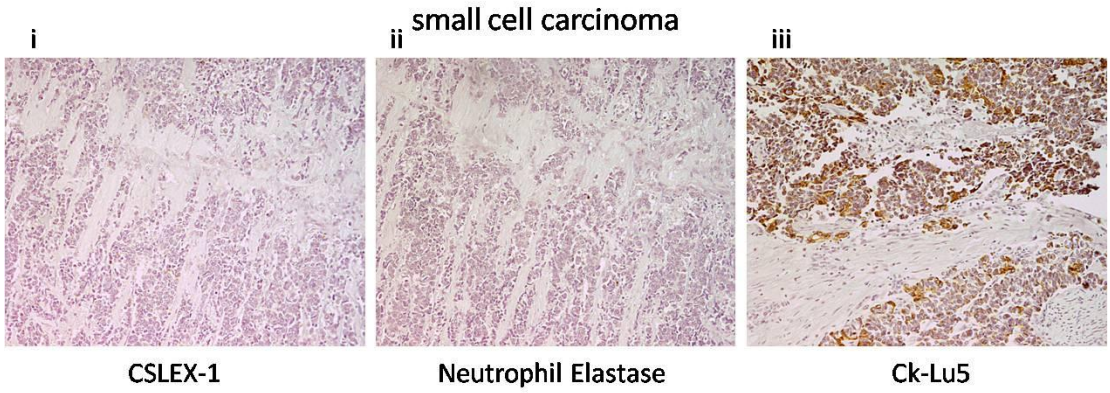


Figure 11: Lung tumor sLe^x expression and neutrophil infiltration are associated in immunohistochemically stained lung tissue samples. In figures 10a-e, all images are of tissues from the human lung cancer tissue array unless otherwise noted. The hematoxylin counter stain appears purple, positive specific antibody staining is shown in brown. (a) Representative images of normal lung (magnification indicated in figure). i: CSLEX-1 mAb staining, ii: Neutrophil Elastase staining, iii: rabbit serum control staining (this tissue was normal lung obtained from the University of Minnesota Tissue Procurement Facility). (b) Representative images of inflamed lung (400X magnification). i: CSLEX-1 mAb staining, ii: Neutrophil Elastase staining. (c) Representative images of an sLe^x positive squamous cell carcinoma. i, iii, and v were taken at 200X magnification, ii, iv, and vi were taken at 400X magnification, the white box in i, iii, and v indicates the magnified field in ii, iv, and vi. i-ii: H&E staining, iii-iv: CSLEX-1 mAb staining, v-vi: Neutrophil Elastase staining. (d) Representative images of an sLe^x positive adenocarcinoma. i, iii, and v were taken at 200X magnification, ii, iv, and vi were taken at 400X magnification, the white box in i, iii, and v indicates the magnified field in ii, iv, and vi. i-ii: H&E staining, iii-iv: CSLEX-1 mAb staining, v-vi: Neutrophil Elastase staining. (e) Representative images of a small cell carcinoma (200X magnification). i: CSLEX-1 mAb staining, ii: Neutrophil Elastase staining, iii: Ck-Lu5 mAb staining.

Up-regulation of sLe^x expression in FucT-III transfected H1299 cells by NCM is independent of TNF-α

Freshly isolated, resting neutrophils secrete 25 – 50 pg/ml TNF-α into their media during a 24 hour time period [36-37]. In order to determine the contribution of TNF-α in the up-regulation of sLe^x observed after culture of FucT-III transfected H1299 cells in NCM, we divided the FucT-III transfected cells into separate groups and cultured them in NCM for 48 hours in the presence or absence of 10 μg/ml of a TNF-α neutralizing antibody. As a control, FucT-III transfected cells were also cultured with 5 ng/ml rTNF-α for 48 hours in

the presence or absence of 10 µg/ml of a TNF-α neutralizing antibody. Treatment of cancer cells cultured in basal media with the TNF-α neutralizing antibody showed no effect on sLe^x expression. Exposure of cells stimulated with rTNF-α to the TNF-α neutralizing antibody interfered with cancer cell sLe^x up-regulation, reducing sLe^x expression from approximately 70% to 45%. In contrast, the TNF-α neutralizing antibody did not interfere with the up-regulation of cancer cell expressed sLe^x observed when the cells were cultured in NCM (Figure 12). Increased concentrations of the TNF-α neutralizing antibody (up to 20 µg/ml) and extended incubation times (up to 2 hours) were tested, however, sLe^x up-regulation by NCM was never inhibited (data not shown).

FIGURE 12

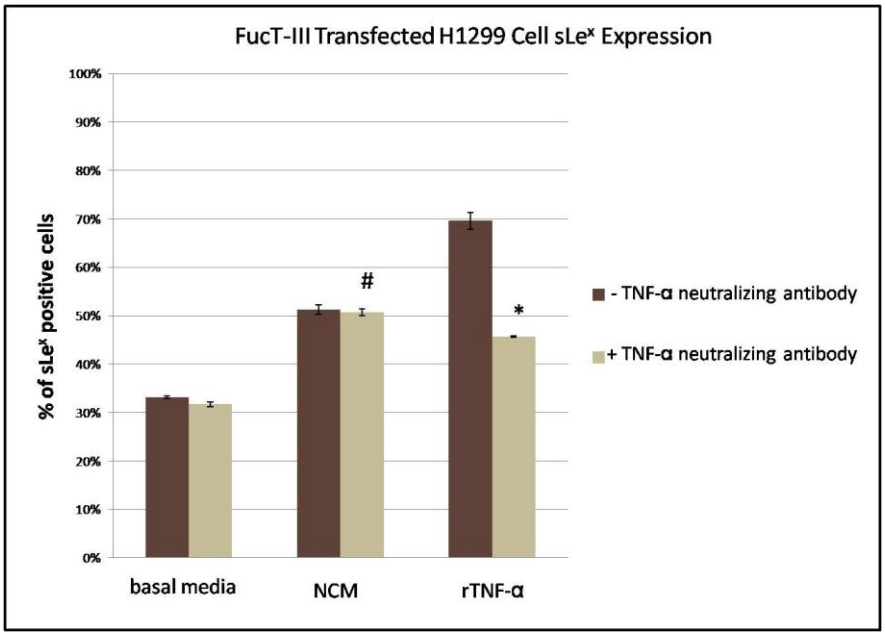


Figure 12: FucT-III transfected H1299 cells up-regulate sLe^x in response to NCM independently of TNF-α. FucT-III transfected H1299 cells were left untreated, cultured with 5 ng/ml rTNF-α, or cultured with NCM for 48 hours in the presence or absence of 10 µg/ml TNF-α neutralizing antibody. The expression of sLe^x by cancer cells was assessed by flow cytometric analysis of reactivity with the CSLEX-1 mAb. Mean values of two experiments are shown. * indicates a significant decrease in the proportion of sLe^x positive cells cultured with 5 ng/ml rTNF-α in the presence versus absence of 10 µg/ml TNF-α neutralizing antibody, $p < 0.005$. #

indicates a non-significant decrease in the proportion of sLe^x positive cells cultured in NCM in the presence versus absence of 10 µg/ml TNF-α neutralizing antibody, p = 0.74.

Neither TNF-α nor NCM induce sLe^x up-regulation by increasing FucT-III gene transcription

To determine whether the up-regulation of cell surface expressed sLe^x observed after stimulation of FucT-III transfected cells with rTNF-α or NCM was due to increased transcription of the FucT-III gene, we compared mRNA levels of FucT-III in unstimulated cells versus cells stimulated with 5ng/ml rTNF-α or NCM for 24 and 48 hours. The mRNA levels of FucT-III detected by RT-PCR remained similar between unstimulated cells and cells stimulated with rTNF-α or NCM for 24 and 48 hours (Figure 13 and data not shown).

FIGURE 13

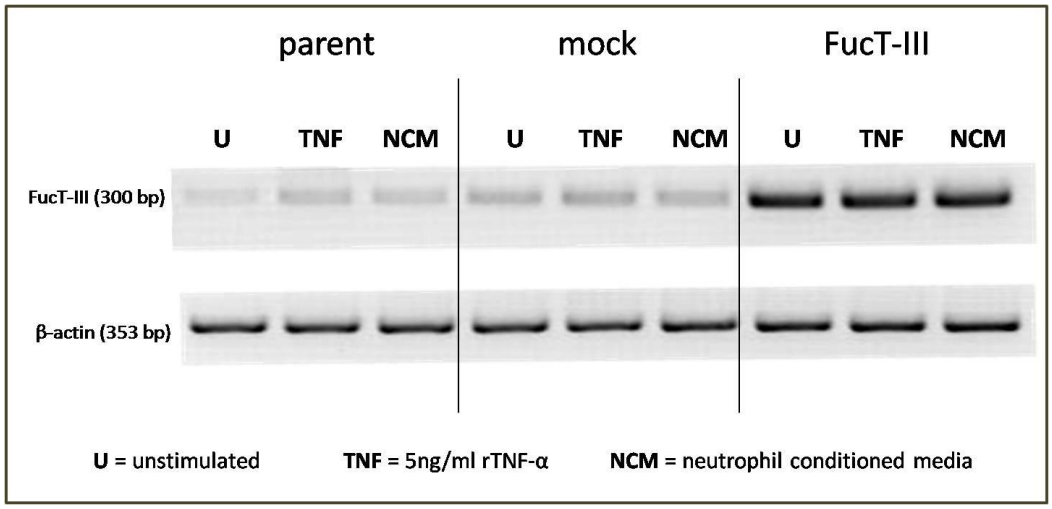


Figure 13: FucT-III mRNA levels do not increase in response to stimulation the rTNFα- or NCM. RT-PCR was performed to detect FucT-III mRNA in unstimulated H1299 parent, mock-transfected, and FucT-III transfected cells and in the three groups of cells after 24 hour stimulation with 5 ng/ml rTNF-α or NCM. Detection of β–actin mRNA was used as a control. Results of one representative experiment of two are shown.

Neutrophils induce TNF-α dependent morphological changes in H1299 cells

Stimulation of H1299 parent, mock-transfected, and FucT-III transfected cells with 5 ng/ml rTNF-α or NCM for 48 hours was observed to result in alteration of their morphology compared to unstimulated cells. Cells cultured in the presence of rTNF-α adopted a dendritic-like and elongated morphology. Cells cultured in NCM also adopted a similar, however more extreme, version of this morphology and tended to grow on top of one another rather than in a monolayer on the plate bottom. Addition of 10 µg/ml of a TNF-α neutralizing antibody consistently reversed this change in cells stimulated with either rTNF-α or NCM (Figure 14). Of note, these morphological changes were observed in both untransfected and transfected H1299 cells and therefore, occurred independently of the FucT-III gene, however, results from only the FucT-III transfected cells are shown. Treatment of unstimulated cells in basal SFM with the TNF-α neutralizing antibody showed no effect on cell morphology (data not shown).

Co-culture of neutrophils with FucT-III transfected cells at a neutrophil to cancer cell ratio of 1:5 resulted in morphologic changes resembling the changes observed for culture in NCM (Figure 15a). Co-culture at a ratio of 5:1 and 1:1 resulted in non-adherent growth of the cancer cells (data not shown and Figure 15b). The Photomicrographs presented here were take 48 hours after the addition of neutrophils to the cancer cells, however, the cells began to grow in a non-adherent fashion after approximately 12 and 24 hours of co-culture respectively. The non-adherent cells were not dead as dividing cells were evident and, if a single cell suspension of these cells was created and the cells were re-plated, the non-adherent cells would regain their adherent phenotype. These experiments were only completed with the FucT-III transfected cells and therefore, the effects of co-culture of parent H1299 cells with neutrophils on cellular morphology has yet to be investigated.

FIGURE 14

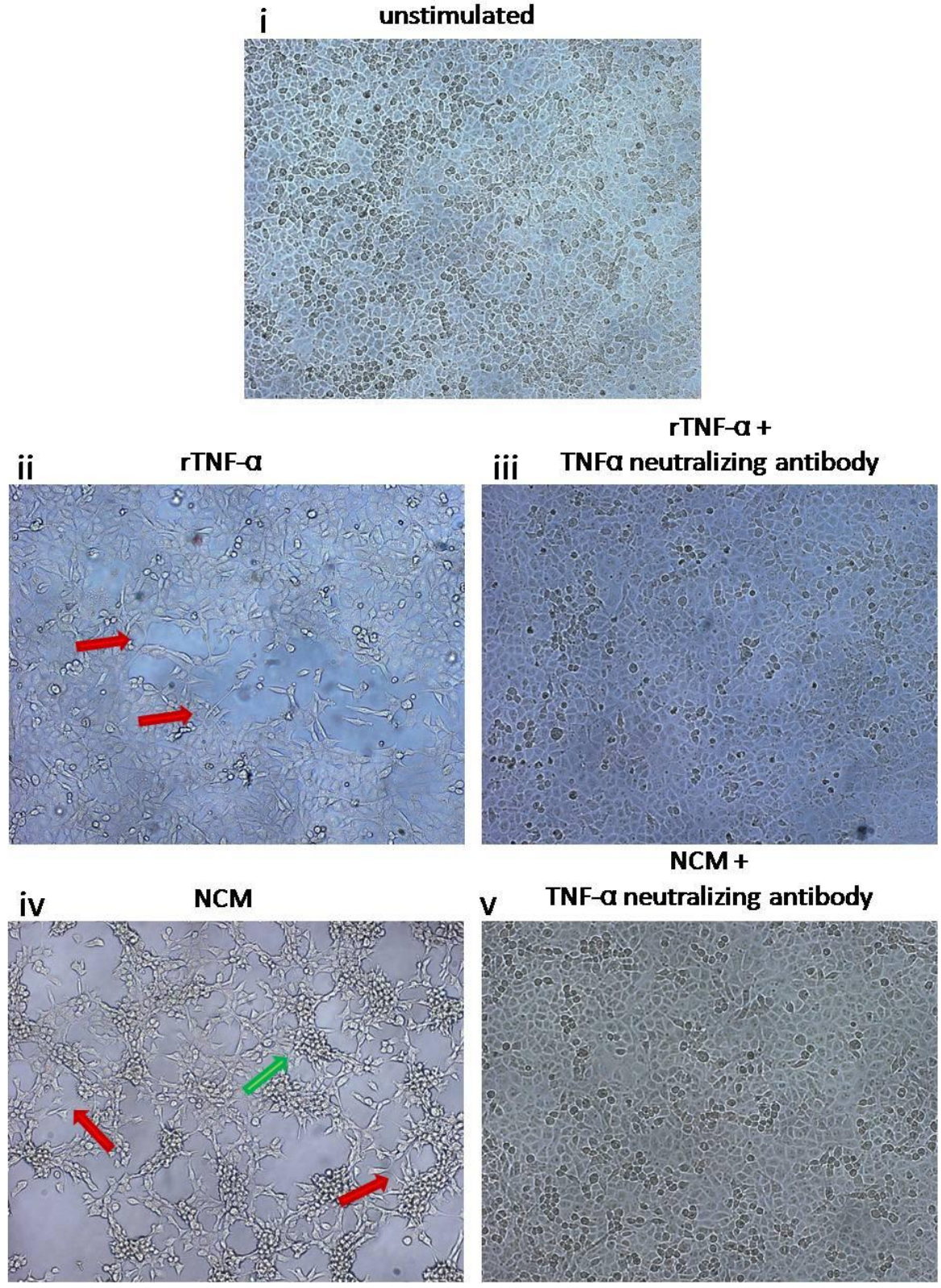

i
unstimulated
ii
rTNF-α
iii
rTNF-α +
TNFα neutralizing antibody
iv
NCM
v
NCM +
TNF-α neutralizing antibody

Figure 14: Culture of H1299 cells with rTNF-α or NCM results in morphological changes that are TNF-α dependent. All photomicrographs are of FucT-III transfected H1299 cells and were taken 48 hours after the addition of stimulus at 100X magnification. i: unstimulated cells demonstrating their common morphological appearance. ii: cells stimulated with 5 ng/ml rTNF-α, note the dendritic-like processes indicated by the red arrows. iii: cells stimulated with 5 ng/ml rTNF-α in the presence of 10 µg/ml TNF-α neutralizing antibody, the morphology is similar to that of the unstimulated cells. iv: cells cultured in NCM, note the dendritic-like processes, similar to the cells stimulated with rTNF-α, indicated by the red arrows and cells growing on top of one another indicated by the green arrow. v: cells cultured in NCM in the presence of 10 µg/ml TNF-α neutralizing antibody, the morphology is similar to that of the unstimulated cells.

FIGURE 15a

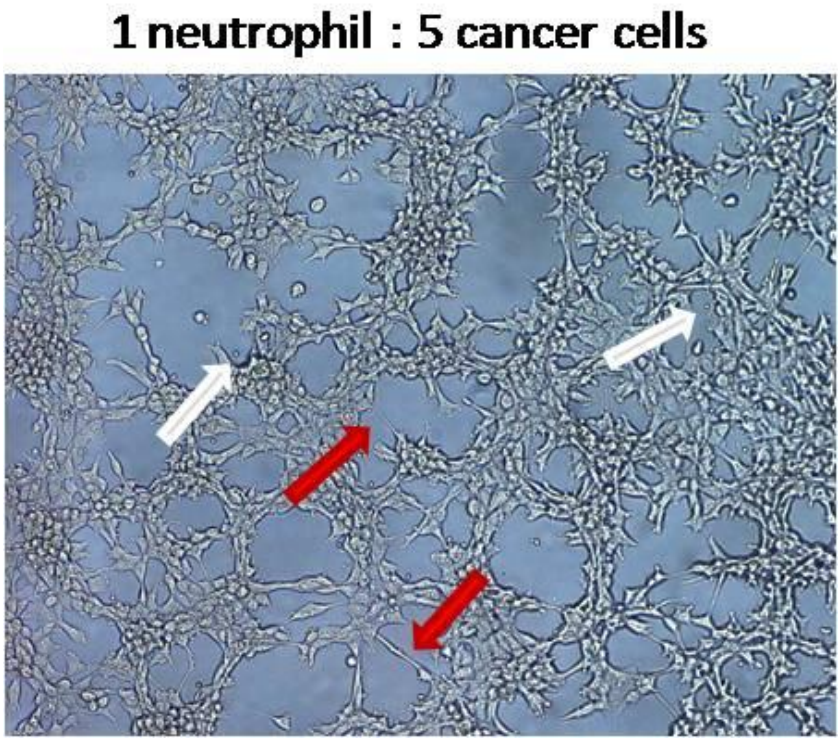


FIGURE 15b

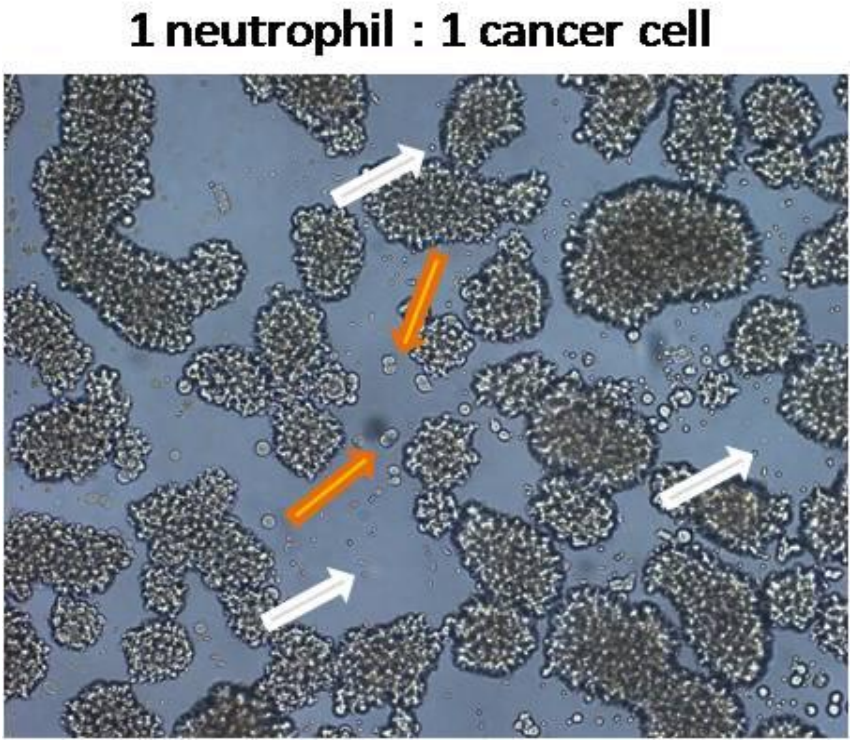


Figure 15: FucT-III transfected H1299 cells become non-adherent when co-cultured with neutrophils. Both photomicrographs were taken 48 hours after the addition of neutrophils at 100X magnification. (a) Neutrophils were cultured with cancer cells at a ratio of 1:5, note the dendritic-like processes exhibited by the cancer cells, similar to those presented in Figure 14 ii and iv, indicated by the red arrows. Neutrophils are indicated with white arrows. (b) Neutrophils were cultured with cancer cells at a ratio of 1:1, note the non-adherent cells growing in suspended clumps. Dividing cells are indicated with orange arrows and neutrophils are indicated with white arrows.

Overexpression of FucT-III and stimulation with TNF-α both enhance the invasive potential H1299 cells

To investigate whether the observed TNF-α and neutrophil mediated changes in cancer cell morphology translated into functionally significant metastasis promoting properties, a Matrigel invasion assay was used to evaluate the invasive ability of H1299 cells in the presence or absence of 5 ng/ml rTNF-α or NCM. Interestingly, irrespective of rTNF-α stimulation, the FucT-III transfected cells were significantly more invasive than the parent cells ($p = 0.02$). Addition of rTNF-α significantly increased the invasiveness of both the parent and FucT-III transfected cells ($p = 0.03$ and 0.005 respectively). Treatment with rTNF-α did not however, increase the invasiveness of the mock-transfected cells (Figure 16). Consistent with previous results, FucT-III transfected cells cultured NCM showed increased invasiveness, and treatment with 10 µg/ml of a TNF-α neutralizing antibody decreased invasiveness back to the level observed for unstimulated cells (Figure 17).

FIGURE 16a

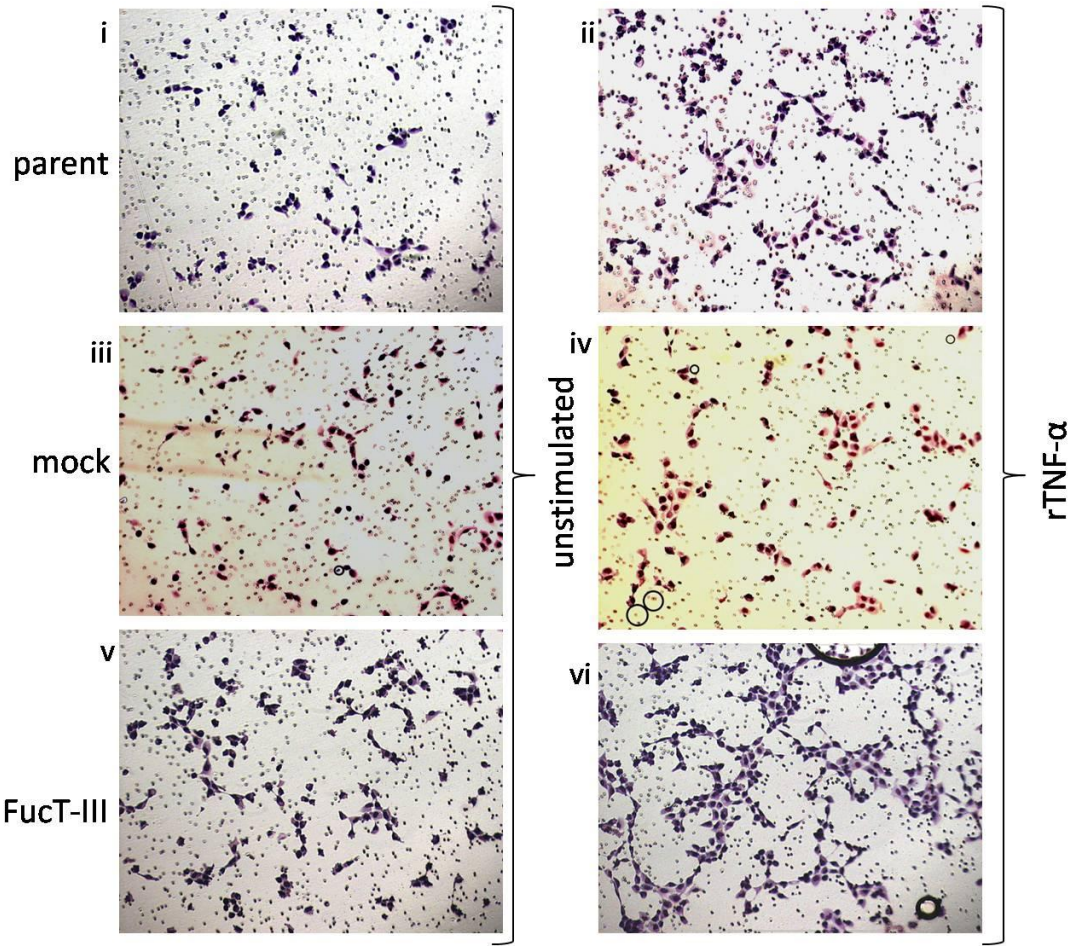


FIGURE 16b

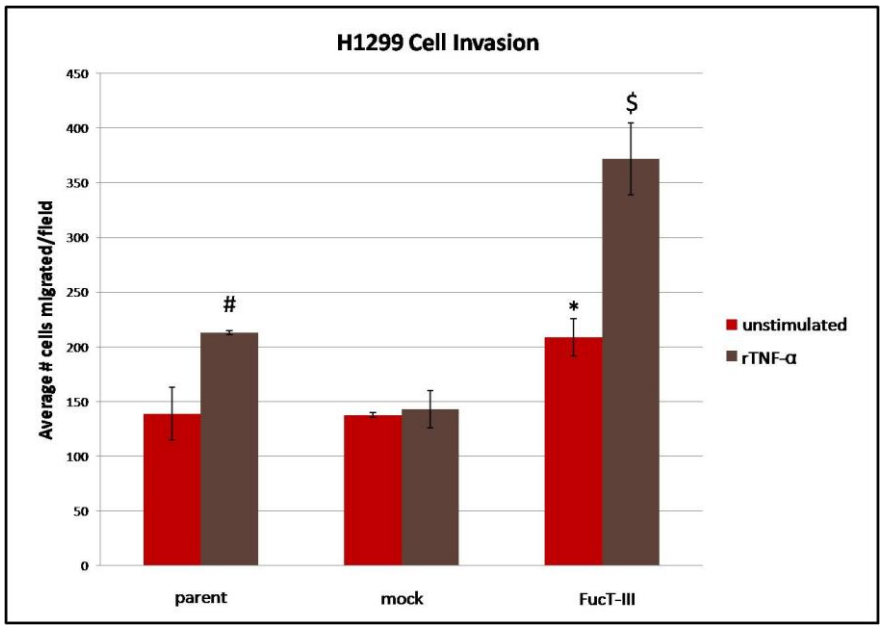


Figure 16: Stimulation with rTNF- increases the invasiveness of H1299 parent and FucT-III transfected cells. H1299 parent, mock-transfected, and FucT-III transfected cells were assessed for invasive ability in the presence or absence of 5 ng/ml rTNF-α. (a) Representative photomicrographs of invasion assay membranes taken at 100X magnification. i: parent cells, ii: parent cells stimulated with rTNF-α, iii: mock-transfected cells, iv: mock-transfected cells stimulated with rTNF-α, v: FucT-III transfected cells, vi: FucT-III transfected cells stimulated with rTNF-α. (b) The number of invading cells per field in 5 separate fields were counted and the 3 most similar values were averaged. One representative experiment of 3 repetitions is shown. * indicates a significant increase in the number of invaded FucT-III transfected cells versus parent cells, p = 0.02. # and $ indicate significantly increased invasion in rTNF-α stimulated parent and FucT-III transfected cells respectively versus unstimulated cells, p = 0.03 and 0.005 respectively.

FIGURE 17

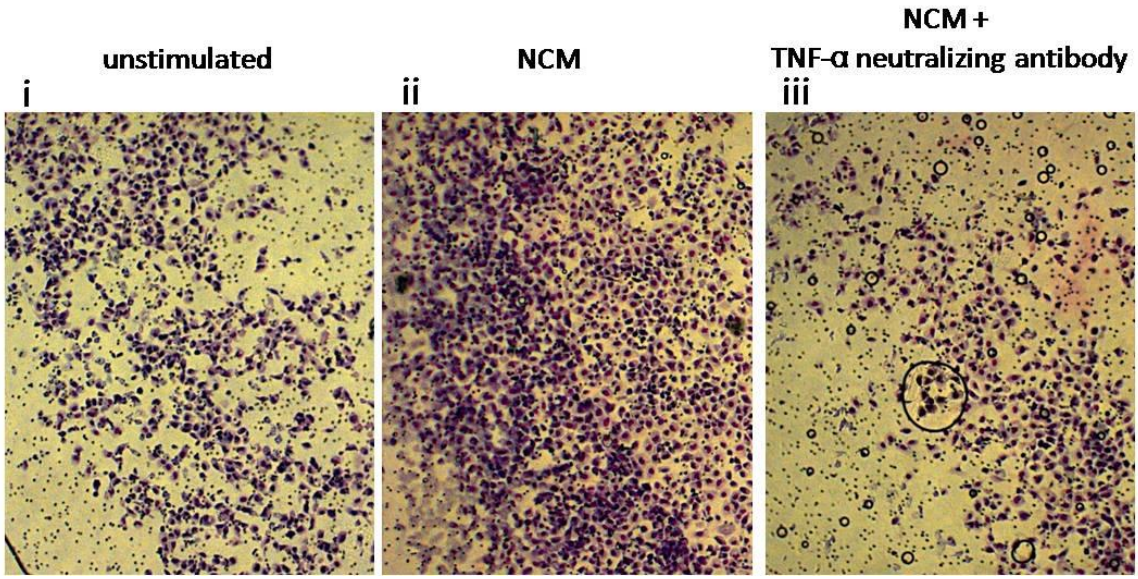


Figure 17: The invasiveness of FucT-III transfected H1299 cells is increased by culture in NCM and is a result of TNF-α. Photomicrographs of invasion assay membranes taken at 50X magnification. i: unstimulated FucT-III transfected cells, ii: FucT-III transfected cells cultured in NCM, iii: FucT-III transfected cells cultured in NCM in the presence of 10 ug/ml TNF-α neutralizing antibody, note that the quantity of invaded cells is similar to that of the unstimulated cells. Images shown are one representative field from two independent experiments.

Discussion

In this study, we present a novel cancer promoting inflammatory cycle in which increased expression of FucT-III in lung tumor cells leads to increased secretion of neutrophil chemoattractants which consequently results in increased neutrophil

infiltration, enhanced tumor cell sLe^x expression, and increased invasive ability and thus, a heightened metastatic potential. In contrast to other studies that use the influence of only individual cytokines to investigate the relationship between inflammation and sLe^x expression on tumor cells, we used culture in NCM and neutrophil: cancer cell co-cultures to more closely approximate the physiological interactions that occur in an inflamed tumor microenvironment. With this model we showed that neutrophils secrete a component, other than TNF-α, capable of increasing sLe^x expression on FucT-III transfected lung cancer cells.

Our findings support and extend previous reports that sLe^x is highly expressed on human lung squamous cell carcinomas and adenocarcinomas [126-128] and is less frequently expressed by small cell carcinomas. We demonstrated that 71% of highly sLe^x positive lung carcinomas were also infiltrated with elevated numbers of neutrophils which were generally associated with clusters of highly sLe^x positive tumor cells. Additionally, substantial neutrophil infiltration was only detected in 32% of carcinomas that didn't express or expressed only low levels sLe^x. Unexpectedly, considerable numbers of neutrophils were detected in samples of normal lung tissue. We did not have access to patient histories, however, we speculate that this finding may be explained by the fact that these lung tissues consisted of adjacent normal lung epithelium removed from patients undergoing surgical resection of a lung tumor. Since cigarette smoking is attributed to 85% of lung cancer cases [2] and has been shown to induce a largely neutrophil mediated inflammatory pulmonary environment [3-4, 19-23] inflammation due to cigarette smoking could have been present in seemingly normal tissue. (Please refer to chapter 1, The Pathogenesis of Lung Inflammation and Cancer for a more detailed description).

Interestingly, despite the consistently observed up-regulation in sLe^x expression upon stimulation with rTNF-α or NCM, we saw that neither mode of stimulation resulted in increased FucT-III mRNA expression. This result is in opposition to observations reported in bronchial mucosa samples and an airway carcinoma cell line in which stimulation with rTNF-α resulted in a significant increase in FucT-III transcription [118, 120]. The discrepancy in findings may be due to differences in the amount of TNF-α used: the referenced studies stimulated cells with 20 and 40 ng/ml rTNF-α which were respectively 4- and 8-fold higher than the concentration used in our study. The rTNF-α concentration of 5 ng/ml that we used is closer to the range of physiological relevant concentrations *in vivo*. In humans, TNF-α concentrations in inflammatory conditions are generally in the pg/ml range but concentrations of up to 3 ng/ml have been shown to be secreted by properly activated immune cells [36-37,121-124]. Our results suggest that lower doses of rTNF-α and neutrophil-derived soluble factors may increase sLe^x expression by post transcriptional or post translational control of the FucT-III enzyme instead of increasing FucT-III gene transcription.

Aberrant expression of FucT-III has been shown to correlate with tumor progression and metastasis [105, 107-109, 111]. This result has thus far been attributed to FucT-III's crucial role as the rate limiting enzyme in the formation of the E-selectin ligand sLe^x [106, 109]. However, our observation that overexpression of FucT-III conferred an enhanced invasive ability and resulted in increased secretion of IL-8, GROα, and MCP-1, implicates a more complex role for FucT-III expression in progressing lung tumor cells. Notably, while a 0.5 fold increase in IL-8 secretion was observed in FucT-III transfected lung carcinoma cells, a 0.5 fold decrease in IL-8 secretion was observed in mock-transfected cells. From this result, we may rule out a non-specific vector dependent increase in IL-8 secretion.

IL-8 has been shown to act as an autocrine growth factor for NSCLC cell lines [129-130]. In support of this finding, we observed that FucT-III transfected H1299 NSCLC cells were significantly more proliferative than mock- transfected cells (data not shown). Furthermore, when a panel of lung cancer cell lines was assessed for IL-8 mRNA and secretion, all of the NSCLC cells showed moderate to high expression of IL-8 while all of the small cell lung carcinoma (SCLC) cells expressed very low or undetectable amounts of IL-8 [129]. Accordingly, 85% of the NSCLC that we examined by immunohistochemistry were positive for sLe^x expression, 57% of which were infiltrated by greater than 10 to greater than 35 neutrophils per total tissue sample area. None of the SCLC expressed sLe^x, nor were more than a fewer than 10 neutrophils detected in each sample. These results suggest that FucT-III may play a role in IL-8 secretion *in vivo*. IL-8 has also been shown to positively correlate with NSCLC angiogenesis, tumor progression, and patient survival [131-132], as well as with the metastatic potential of melanomas [133], and has been implicated in the promotion of neutrophil mediated angiogenesis and metastasis [31-33].

Secretion of MCP-1 was also determined to be augmented by overexpression of FucT-III in lung carcinoma cells. In breast cancer, tumor environmental MCP-1 concentrations were shown to positively correlate with IL-8 concentrations, as well as with the amount of tumor associated macrophage infiltration, angiogenic factors, and the probability of early disease relapse [124]. Macrophages are a significant source of TNF-α in the tumor environment [48-49]. Although neutrophil derived TNF-α was found not to be responsible for up-regulation of tumor cell sLe^x, enhanced secretion of MCP-1, resulting from FucT-III gene activity, may recruit macrophages to lung tumors which may in turn up-regulate tumor cell sLe^x via secretion of macrophage-derived TNF-α.

Further supporting FucT-III's metastasis promoting function, lung carcinoma cells transfected with FucT-III, irrespective of stimulation with recombinant or neutrophil derived TNF-α, were more invasive than untransfected cells, indicating that the presence and activity of the FucT-III gene conferred an increased invasive potential to lung carcinoma cells. Invasiveness of the parent and FucT-III transfected cells was further enhanced by stimulation with rTNF-α. Surprisingly, rTNF-α did not increase the invasiveness of mock-transfected lung carcinoma cells. Therefore, the transfection vector may have played a role in suppressing the rTNF-α mediated increase in invasiveness and a non-specific, vector dependent increase in migration may be ruled out for the FucT-III transfected cells. In support of the validity of this *in vitro* assay in determining *in vivo* tumor cell metastatic potential, a study on an unrelated tumor metastasis suppressor gene transfected into H1299 cells, reported that the cells with a greater *in vitro* invasive ability were also able to form a greater number of metastases *in vivo* [134]; thus, directly linking *in vitro* invasive ability to functional, *in vivo* metastatic potential.

We observed that culture of lung carcinoma cells in NCM or co-culture with neutrophils resulted in changes in cancer cell morphology that were independent of FucT-III. This result is in agreement with similar observations reported by Katayma et. al.. Co-culture of neutrophils with a squamous cell carcinoma cell line resulted in a neutrophil: cancer cell ratio dependent loss of substratum adhesion accompanied by very little cell death [135]. This investigation however, found neutrophil elastase to be the causative agent and did not investigate the influence of TNF-α on loss of substratum adhesion. Here we show that treatment of NCM with a TNF-α neutralizing antibody reversed the morphological change and confirmed TNF-α as the soluble factor secreted by neutrophils responsible for induction of phenotypic changes. Of note, the TNF-α

neutralizing antibody was not tested in co-culture experiments because two cell types were involved. Blockade of TNF-α may have interfered with other unidentified interactions between neutrophils and cancer cells resulting in an experimental observation lacking the necessary controls. However, it can be deduced from the striking similarity between the morphological changes elicited by NCM or co-culture at a 1:5 neutrophil to cancer cell ratio that TNF-α is also involved in the induction of the morphologic changes observed in the co-culture experiments.

A defined set of pathogenic consequences from loss of tumor cell anchorage dependence is not fully established, however, detachment from substratum by tumor cells has been indicated to promote metastasis. Loss of anchorage dependence and individual cell sloughing off of primary tumors has been extensively described as a mode of metastasis of ovarian cancer [136-137]. Conversely, when the metastatic abilities of adherent vs. non-adherent rat tumor cell lines were tested, no difference in metastatic ability was seen. The authors concluded that, although non-adherent properties "may facilitate metastatic spread" they alone, are not sufficient to initiate metastatic tumors [138]. However, a lung tumor in which FucT-III has become active and sLe^x is expressed, may be endowed with enhanced metastatic properties through increased recruitment of neutrophils which induce a non-adherent and invasive phenotype which subsequently allows cancer cells to detach from the primary tumor and enter the circulation where they may adhere to endothelium via interactions between E-selectin and sLe^x.

Other glycosyltransferase enzymes contribute to the synthesis and expression of sLe^x, including α2,3-sialtransferase [97] and α1,3 fucosyltransferase-VII (FucT-VII) [139-140] and the influence of these enzymes on the expression of sLe^x in our studies cannot be ruled out. In spite of this limitation, our study supports the notion that, in addition to

synthesizing sLe^x which has been extensively demonstrated to promote metastasis, FucT-III may mediate tumor progression and metastasis by increasing tumor cell invasiveness and secretion of inflammatory chemokines by a mechanism that is independent of the expression of cell surface sLe^x. Thus, regulation of FucT-III gene expression and activity may represent an important, new genetic target for the prevention of cancer progression.

Chapter 3: Identification of proteins on carcinoma cells modified by the high affinity selectin ligand C2-O-sLe^x.

Summary

The carbohydrate selectin ligand sialyl Lewis X (sLe^x) is expressed on leukocytes as a terminal structure of extracellular, membrane bound proteins. The sLe^x epitope is crucial for selectin mediated leukocyte rolling on inflamed endothelium. On leukocytes, sLe^x attachment to a core 2 O-linked glycan (C2-O-sLe^x) has been shown to confer a higher selectin binding affinity than when sLe^x is attached to other core glycan structures. In contrast to the lack of knowledge of the role of C2-O-sLex in the growth, adhesion, invasion, and metastasis of cancer cells, high expression of sLe^x has been extensively described to be associated with advanced stages of several cancers and poor prognosis and survival of patients. On cancer cells, sLe^x binds to endothelial selectins by a mechanism similar to that used by leukocytes to migrate to sites of inflammation. Multiple glycoprotein and glycolipid backbones have been shown to be modified by sLe^x in cancer cells, however, the protein(s) modified by the C2-O-sLe^x structure are currently unknown. We used Western blotting techniques with the CHO-131 mAb, which specifically recognizes the C2-O-sLe^x structure, to determine the C2-O-sLe^x modified proteins found in LS174T colon carcinoma cells and in H1299 NSCLC cells genetically manipulated to express C2-O-sLe^x. Although we did not specifically characterize the protein to which C2-O-sLe^x is attached in colon and lung carcinoma cells due to technical limitations of our system, in agreement with previous reports, we determined that C2-O-sLe^x is present on a 150 kilo Dalton (kDa) protein.

Introduction

Metastasizing cancer cells bind to endothelium through a mechanism very similar to the one used by leukocytes to migrate to sites of inflammation [64, 66]. The adhesion of leukocytes and cancer cells to endothelium is initiated by binding of sialyl Lewis X (sLe^x) present on these cells to endothelial selectins which allows cells to roll over endothelium followed by firm, integrin-dependent adhesion and subsequent migration into tissue. The carbohydrate epitope sLe^x acts as a ligand for selectins [58-59]. It is a terminal structure attached to glycan chains and may be found on N- [86-87] or O-linked glycans [89-90] or glycolipids [88]. When expressed on leukocytes, sLe^x attached to a core 2 O-linked glycan structure (C2-O-sLe^x) allows much higher affinity binding to endothelial P-selectin than sLe^x on other structures [90, 93]. C2-O-sLe^x has been shown to confer a higher binding affinity to E-selectin [94, 103].

Synthesis of sLe^x type carbohydrate epitopes occurs in the golgi of the cell through the actions of glycosyltransferase enzymes that post-translationally modify fully folded proteins [85]. Formation of the core 2, O-linked glycan structure requires the activity of the enzyme core 2 β-1,6-N-acetylglucosaminyltransferase (C2GnT1). Although many other enzymes participate in the creation of the entire C2-O-sLe^x structure, the full epitope may not be completed without the activity of the enzyme α1,3 fucosyltransferase-III (FucT-III), the rate limiting enzyme in the formation of the terminal sLe^x structure.

The carbohydrate modification plus the protein backbone to which it is attached collectively create the complete selectin ligand. In immune cells, multiple sLe^x modified proteins including CD43 [94], CD44, and ESL-1 [102-104] have been shown to support E-selectin binding. The carbohydrate-modified protein backbone has been shown to play an important role in selectin binding affinity. For example, it is necessary for C2-O-

sLe^x to be attached to a PSGL-1 backbone to function as a high affinity P-selectin ligand. C2-O-sLe^x-modified PSGL-1 also acts as a lower affinity ligand for E-selectin [89, 100-101]. C2-O-sLe^x-modified CD43 can also function as an E-selectin ligand [95].

Similar to immune cells, multiple protein backbones display the sLe^x epitope on cancer cells that exhibit selectin binding ability. CD24 and variant CD44 are modified with sLe^x and act as ligands for P-selectin in adenocarcinoma and colon carcinoma cell lines respectively [141-144]. Colon carcinoma cells expressing sLe^x-modified podocalyxin-like protein bind to both E- and L-selectin [145]. Mucins compose another family of proteins susceptible to modification with carbohydrate selectin ligands in cancer cells. Mucins are heavily O-glycosylated proteins produced by epithelial cells and are critical for mucosal defense, immune responses, and cell adhesion. They have been demonstrated to be overexpressed and aberrantly glycosylated in many types of cancer [146]. For instance, human mucin 1 (MUC-1) is a mucin present on colon carcinoma cells that is modified with sLe^x and possesses E-selectin binding ability [147].

Using the novel mAb CHO-131 which specifically recognizes the C2-O-sLex structure [115], we demonstrate that the high affinity selectin ligand C2-O-sLe^x is expressed in colorectal adenocarcinomas [116] and multiple carcinoma cell lines [112, and unpublished data]. The CHO-131 mAb requires the functional activity of α2,3-sialtransferase, FucT-III, and C2GnT1 for its reactivity. Although several proteins present on cancer cells have been determined to be modified by sLe^x, the proteins modified by C2-O-sLe^x are currently unknown. Beum et. al., used pancreatic cancer cells to indirectly determine that MUC-1 could be modified by C2-O-sLe^x. Overexpression of C2GnT1 resulted in an increase in CSLEX-1 mAb reactivity [148]. (CSLEX-1 mAb detects the sLe^x epitope on several types of glycan structures).

However, the significance of these findings is unclear because C2GnT1 expressing cells were unable to bind to selectins.

In our study, we performed Western blotting techniques using the CHO-131 mAb to characterize the proteins that are modified with C2-O-sLe^x on LS174T colon carcinoma cells and H1299 NSCLC cells.

Materials and Methods

General Reagents

The mouse IgM mAb CHO-131 that recognizes the C2-O-sLe^X structure was generously provided by Dr. Bruce Walcheck, University of Minnesota. All other reagents are described in chapter 2, Materials and Methods.

Cell Culture

Standard cell culture conditions were used to maintain the cell lines. See chapter 2, Materials and Methods, for a more detailed description of these procedures. LS174T colorectal adenocarcinoma cells and H1299 cells derived from a lymph node metastasis of a NSCLC were obtained from American Type Culture Collection. LS174T cells were cultured in MEM with 2 mM L-glutamine, 10% FBS, P/S, 1 mM sodium pyruvate, 1 mM non-essential amino acids, and 1.5 g/L sodium bicarbonate. After trypsinization, LS174T cells were run through a 22-gauge needle to create single cell suspensions. H1299 cells were cultured in RPMI 1640 with 2 mM L-glutamine, 10% FBS, P/S, 1 mM sodium pyruvate, 10 mM HEPES buffer, and 1.5 g/L sodium bicarbonate.

Construction of the FucT-III/pcDNA 3.1 neo(+) Plasmid

The FucT-III plasmid was generated as described in chapter 2, Materials and Methods.

FucT-III Stable Transfection

H1299 cells were stably transfected with FucT-III to generate H1299/FucT-III cells as described in chapter 2, Materials and Methods.

Gene Silencing of C2GnT1 Using Lentiviral Short Hairpin RNAs (shRNA)

Preparation of Lentiviral Particles

A lentiviral vector pGIPZ-C2GnT1-shRNAmir construct engineered to silence human C2GnT1 gene expression, and a control pGIPZ vector expressing non-silencing shRNA, were purchased from Open Biosystems (Huntsville, AL). Lentiviral supernatants were prepared by stable transfection of $2.5x10^6$ 293T cells with 2 µg each of the C2GnT1 shRNA vector, pCMVΔ8.91R expressing Gag and pol genes, and pMDG-VSV-G expressing the envelope protein required for viral packaging, using the ExGen 500 reagent. Viral supernatants were collected after 48 hours, filtered through 0.45 µM filters, aliquoted, and stored at -80^{o}C until use.

Transduction of H1299/FucT-III and LS174T Cells with C2GnT1 shRNA

H1299 cells transfected with FucT-III and LS174T cells were plated at a density of $0.25x10^6$ cells per well in a 6 well tissue culture plate. After 24 hours of cell growth under standard conditions, cells were treated with 8 µg/ml of polybrene and incubated for 4 hours at 37^{o}C. The media was replaced with a total volume of 2 ml fresh media containing 1 ml of viral supernatant and 8 µg/ml polybrene. The plate was centrifuged at 1847 X g for 1 hour at room temperature and the cells were allowed to grow overnight under standard culture conditions. The media was replaced with fresh media and growth continued until 90% confluency. The C2GnT1 shRNA expressing clones were selected using 2.5 µg/ml of puromycin (Invitrogen). The positive clones were analyzed

for mRNA expression levels of C2GnT1 by RT-PCR and cell surface expression of C2-O-sLe^X by flow cytometric analysis of cells with the CHO-131 mAb.

RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA isolation and RT-PCR was performed as described in chapter 2, Materials and Methods. The primer sequences shown in Table 3 were used to amplify the cDNA gene copies of the glycosyltransferase genes C2GnT1 and FucT-III (shown in Table 1), mucin (MUC) genes, and β-actin (shown in Table 1) as a control gene.

TABLE 3: PRIMER SEQUENCES USED IN RT-PCR

Gene	GenBank Accession Number	Primer Sequences
C2GnT1	NM_001490.4	Sense: 5'-GAT GTC ACC TGG AAT CAG CA-3' Antisense: 5'-GCA GCA ACG TCC TCA GCA T-3'
MUC1	NM_001018017.1	Sense: 5'-GTG CCC CCT AGC AGC AGT ACC G-3' Antisense: 5'-GAC GTG CCC CTA CAA GTT GG-3'
MUC2	NM_002457.2	Sense: 5'-CCG TCC TCC TAC CAC ATC AT-3' Antisense: 5'-CTC TCC AGG CCG TTG AAG T-3'
MUC3	XM_001125753.2	Sense: 5'-GTC GTG GGC ACT TTT TC-3' Antisense: 5'-GCA ATG CAG ACC CTT GT-3'
MUC4	NM_018406.4	Sense: 5'-ATG GTC ATC TCG GAG TTC CAG-3' Antisense: 5'-GTA GAC CAG GTC GTA GCC CTT-3'
MUC5ac	XM_002344536.1	Sense: 5'-TCC GGC CTC ATC TTC TCC-3' Antisense: 5'-ACT TGG GCA CTG GTG CTG-3'
MUC13	NM_033049.2	Sense: 5'-ACA ATG GTT CCT TCT GAA AC-3' Antisense: 5'-ACC CTT CTA AAC ACA GGC AA-3'
MUC16	NM_024690.2	Sense: 5'-GCC TCT ACC TTA ACG GTT ACA ATG AA-3' Antisense: 5'-GGT ACC CCA TGG CTG TTG TG-3'

- All MUC primer sequences except MUC5ac were originally published by Kerschner et. al., [147]

Flow Cytometric Analysis

Flow cytometry was performed as described in chapter 2, Materials and Methods. Primary antibodies CHO-131 mAb, M3.1 mAb that detects human mucin 3 (MUC3) (Abcam, Cambridge, MA), MUC1 mAb that detects human mucin 1 (Cell Signaling Technology, Inc., Danvers, MA), and IgG2a isotype control (Caltag Laboratories) were diluted to 10 µg/ml. PE conjugated F(ab')$_2$ goat anti-mouse IgM and PE conjugated

$F(ab')_2$ goat anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA) were diluted 1:200 and used as a secondary antibody to detect CHO-131 mAb, and M3.1 and MUC1 mAbs respectively.

Cell Lysates

Approximately $25x10^6$ cells were lysed in 1 ml co-immunoprecipitation (IP) buffer (50 mM tris, pH 7.4, 1% Triton X100, 150 mM NaCl, 1 mM CaCl2, 1 mM PMSF, Protease inhibitors (Roche Diagnostics Corporation, Indianapolis, IN)) and incubated with agitation for 30 minutes at 4^oC. The Lysates were then centrifuged at 8150 X *g* for 25 minutes. The supernatant was aliquoted and stored at -80^oC until use. Protein concentration was measured with a spectrophotometer using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Inc.).

E-selectin Immunoprecipitation (IP)

To isolate the E-Selectin binding proteins, 500 µg of cell lysate was incubated with 4 µg recombinant mouse E-Selectin/Fc Chimera for a final volume of 200 µl of cell lysis buffer with 0.2 µM of $CaCl_2$. The samples were incubated with agitation overnight at 4^oC. Forty µl of recombinant Protein G-Sepharose 4B Fast Flow beads (Sigma-Aldrich) prepared as a 50% suspension in co-IP buffer was added to the samples and then incubated for 3 hours with agitation at 4^oC. Unbound proteins were removed by washing the protein G beads with co-IP buffer 5 times. The beads were then resuspended in 1X Laemmli Sample Buffer (Bio-Rad Laboratories, Inc.) and used for SDS-PAGE immediately or stored at -80^oC until use.

Western Blotting

Fifty µg of the total cell lysates or IP samples were loaded on to an 8% SDS-PAGE gel and electrophoresed at 120 volts for 1.5 hours. The separated protein was transferred to

an Immobilon-P Transfer Membrane (Millipore Corporation, Medford, MA) at 40 volts overnight at 4^{o}C. The membrane was blocked with 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad Laboratories, Inc.) and probed with CHO-131 mAb (5 µg/ml), IgM isotype control (0.5 µg/ml) (Caltag Laboratories), 1-13M1 mAb (to detect human mucin 5AC (MUC5AC)) (0.031 µg/ml) (Abcam, Inc.), or anti-GAPDH mAb (to detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) (0.05 µg/ml) (Sigma-Aldrich, Inc.) for one hour at room temperature. HRP-conjugated µ chain specific goat anti-mouse IgM (Chemicon, Temecula, CA) at a 1:1,000 dilution was used to detect bound CHO-131 mAb and IgM isotype control. HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) at a 1:20,000 dilution was used to detect bound 1-13M1 mAb. HRP-conjugated donkey anti-rabbit IgG (BioFX Laboratories, Owings Mills, MD) at a 1:2,000 dilution was used to detect bound anti-GAPDH mAb. Secondary antibodies were detected with Amersham ECL Western Blotting Analysis System (GE Healthcare Limited) and exposed to Amersham Hyperfilm ECL, high performance chemiluminescence film until bands were visible on the developed film. Films were developed with a AGFA CP 1000 film processor. Equal loading of protein was determined by direct staining of the membrane with Ponceau S solution (Sigma-Aldrich).

Glycoprotein Gel Staining

Fifty µg of the total cell lysates or IP samples were loaded on to an 8% SDS-PAGE gel and electrophoresed at 110 volts for 1.5 hours. The gel was stained with the Pro-Q Emerald 300 glycoprotein staining kit (Molecular Probes, Inc., Eugene, OR) according to the manufacturer's instructions and bands were imaged using an Eagle Eye II machine and Alphaimager IS-2200 software. The gel was counterstained with SYPRO ruby

protein gel stain (Molecular Probes, Inc.) according to the manufacturer's instructions and image analysis was repeated.

Results

Transfection of H1299 cells with FucT-III induces C2-O-sLe^x expression

We determined that H1299 cells actively transcribed the C2GnT1 gene as indicated by RT-PCR analysis; however, they transcribed very little of the FucT-III gene and didn't naturally express the sLe^x epitope on their surface. After stable transfection with the FucT-III gene the resulting H1299/FucT-III cells were approximately 50% positive for sLe^x, and half of these positive cells expressed sLe^x attached to a core 2 O-linked glycan as indicated by staining with the CSLEX-1 and CHO-131 mAbs respectively (Figure 18a-b). Unlike parent H1299 cells which did not bind to E-selectin, approximately 75% of H1299/FucT-III cells bound to E-selectin (Figure 9e of chapter 2).

Expression of sLe^x and C2-O-sLe^x is decreased in cells transduced with lentiviral C2GnT1 shRNA

The expression of C2GnT1 was knocked down in both the H1299/FucT-III and LS174T cells by lentiviral infection with C2GnT1 targeted shRNA. Successful transduction with C2GnT1 shRNA was verified by RT-PCR analysis of C2GnT1 mRNA. Levels of C2GnT mRNA were decreased in transduced H1299/FucT-III and LS174T cells (H1299/FucT-III/shRNA and LS174T/shRNA cells) as indicated by bands of reduced intensity shown in Figure 18a. Cells were stained with CSLEX-1 and CHO-131 mAbs and flow cytometric analysis was performed. We determined that, in contrast to H1299 cells, LS174T cells naturally expressed high amounts of sLe^x on their surface and about 90% of these positive cells expressed sLe^x attached to a core 2 O-linked glycan. After transduction with C2GnT1 shRNA we observed a 20% reduction in sLe^x and 17% reduction in C2-O-

sLe^x expression in H1299/FucT-III/shRNA cells and a 15% reduction in sLe^x and 25% reduction in C2-O-sLe^x expression in LS174T/shRNA cells (Figure 18b).

FIGURE 18a

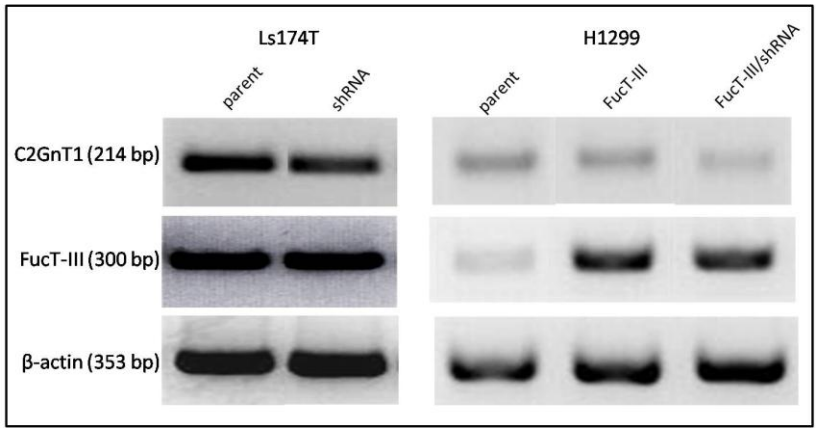


FIGURE 18b

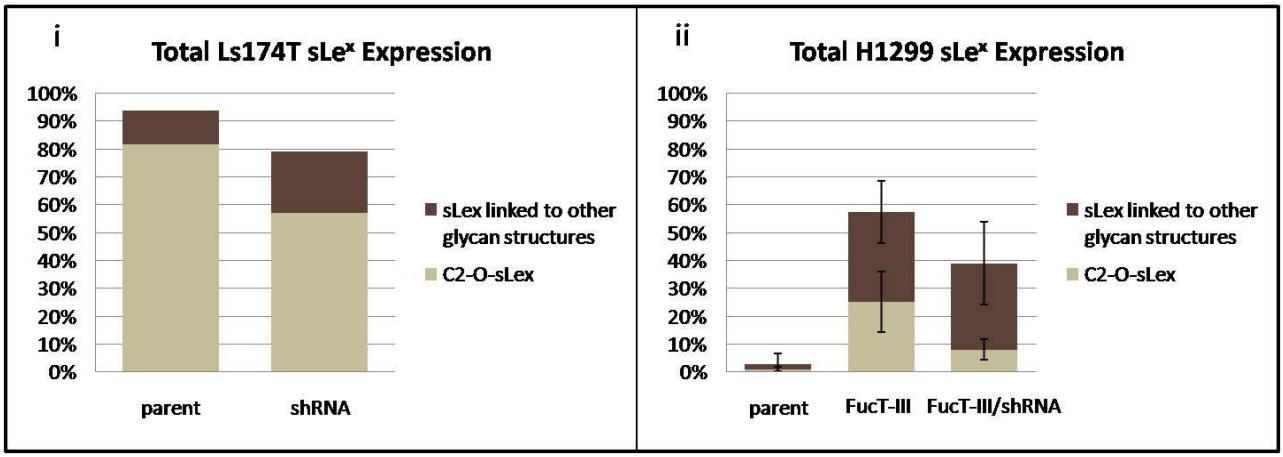


Figure 18: Cell surface expression of sLe^x and C2-O-sLe^x are altered by manipulation of the genes C2GnT1 and FucT-III. (a): RT-PCR of C2GnT1, FucT-III, and β-actin mRNAs isolated from LS174T parent and C2GnT1 shRNA transduced cells and H1299 parent, H1299/FucT-III, and H1299/FucT-III/shRNA transduced cells. One representative experiment three repetitions are shown. (b): Flow cytometric analysis of cell staining with CHO-131 and CSLEX-1 mAbs was used respectively to determine the percentage of C2-O-sLe^x positive cells as a subset of the total percentage of sLe^x positive cells. i: LS174T parent and shRNA transduced cells, ii: H1299 parent, H1299/FucT-III, and H1299/FucT-III/shRNA transduced cells. Data shown for LS174T cells is representative of three individual experiments. Data shown for H1299 cells is the average of 6 individual experiments.

Glycosylated proteins in H1299 and LS174T cells bind to E-selectin

In order to characterize the proteins that were glycosylated with the C2-O-sLe^x structure in H1299 and LS174T cells, Western blotting was performed on whole cell lysates using the CHO-131 mAb. We detected multiple protein bands of molecular weights ranging from greater than 250 to less than 37 kDa that were present in a similar pattern for both cell types (Figure 19a).

In order to increase the specificity of the assay and reduce the number of proteins being exposed to the CHO-131 mAb during the Western blotting procedure, whole cell lysate samples were Immunoprecipitated with a recombinant mouse E-Selectin/Fc chimera. Western blotting of the Immunoprecipitated samples using CHO-131 mAb revealed a marked reduction in the number of bands that were detected. Three bands of molecular weights ranging from less than 250 to greater than 100 kDa were identified in both H1299 and LS174T cells. Of these bands, the ~180 kDa band was absent in H1299 parent cells, expressed in LS174T parent and H1299/FucT-III cells, and reduced in intensity in both H1299/FucT-III/shRNA and LS174T/shRNA cells. The ~130 kDa band was reduced in intensity in LS174T/shRNA cells compared to that detected in LS174T parent cells. In contrast, unexpectedly, a ~130 kDa band was detected in H1299 parent cells and differences were not observed in band intensities when H1299 parent and H1299/FucT-III/shRNA cells were compared nor were differences detected when band intensities of H1299 parent and H1299/FucT-III cells were compared (Figure 19b).

To verify that glycosylated proteins were detected in the assays, whole cell lysates and E-selectin IP samples were electrophoresed on an SDS-PAGE gel, specifically stained for glycoproteins, and subsequently stained for total protein. Glycoprotein staining of the whole cell lysates revealed a pattern of bands remarkably similar to that obtained with CHO-131 mAb Western blotting. Total protein staining resulted in the detections of

additional bands not seen when compared with the glycoprotein stain or Western blotting with CHO-131 mAb. This result indicated that CHO-131 mAb was reactive with glycosylated proteins (Figure 19c).

Glycoprotein staining of E-selectin IP samples revealed the same three bands that were detected with Western blotting with CHO-131 mAb. Additionally, total protein staining also indicated the presence of only the same three bands confirming that IP with E-selectin effectively removed most proteins from the lysate samples and enriched only a few glycosylated proteins of high molecular weight, presumably only those that could act as E-selectin ligands (Figure 19d). We focused only on proteins in LS174T parent cells and did not examine proteins in LS174T/shRNA cells by glycoprotein staining because LS174T parent cells expressed high amounts of C2-O-sLe^x and our goal was to isolate maximal amounts of this glycan in from these cells for further characterization. Of note, a band of ~180 kDa was detected in the H1299 parent and H1299/FucT-III/shRNA cells although it was absent from the Western Blot using CHO-131.

FIGURE 19a

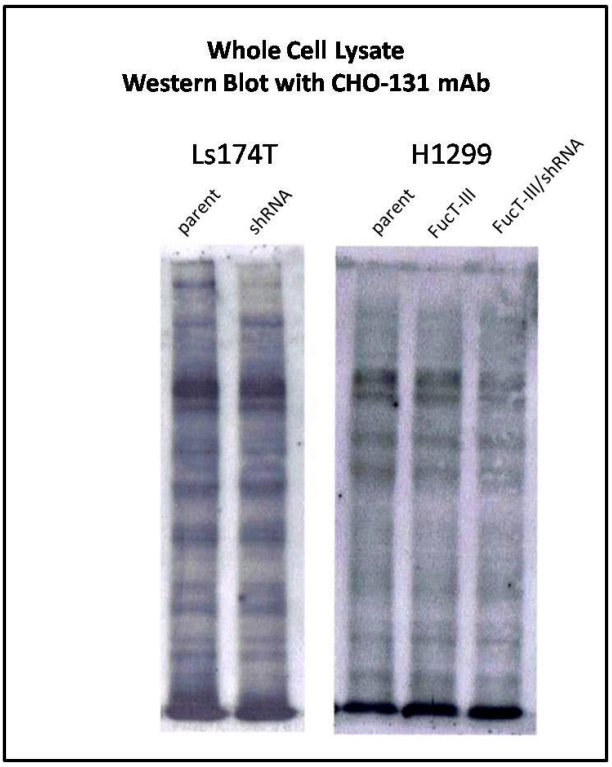


FIGURE 19b

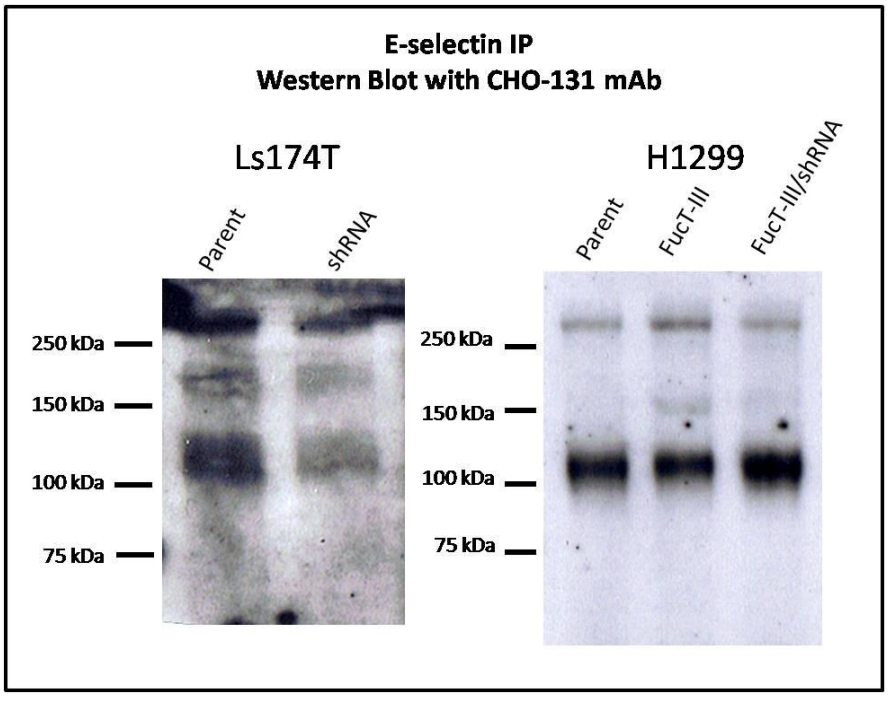


FIGURE 19c

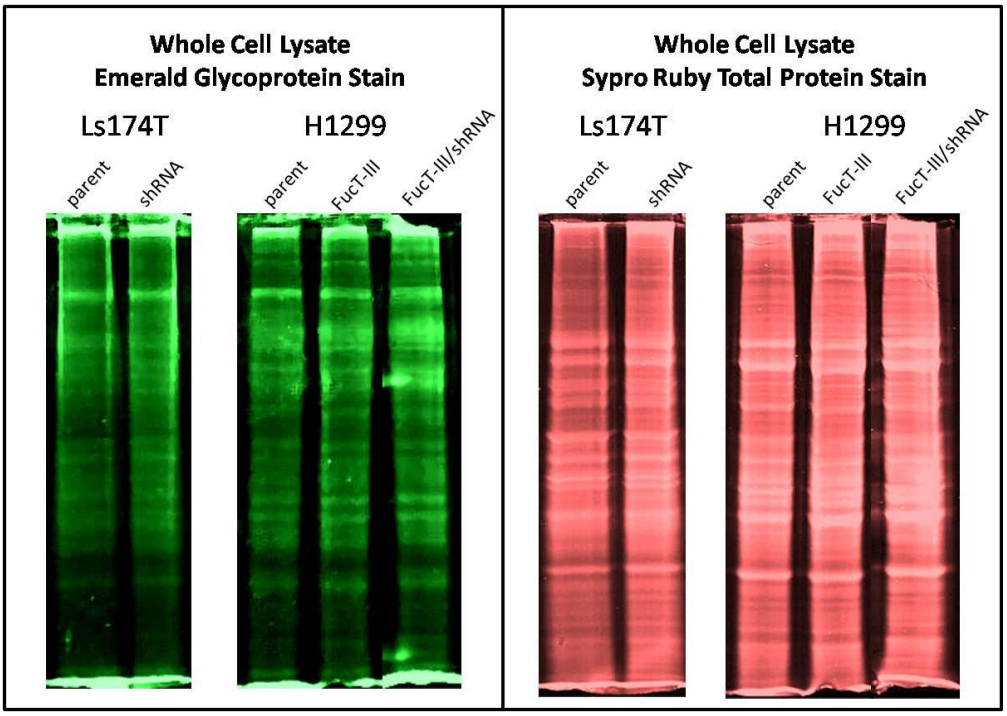


FIGURE 19d

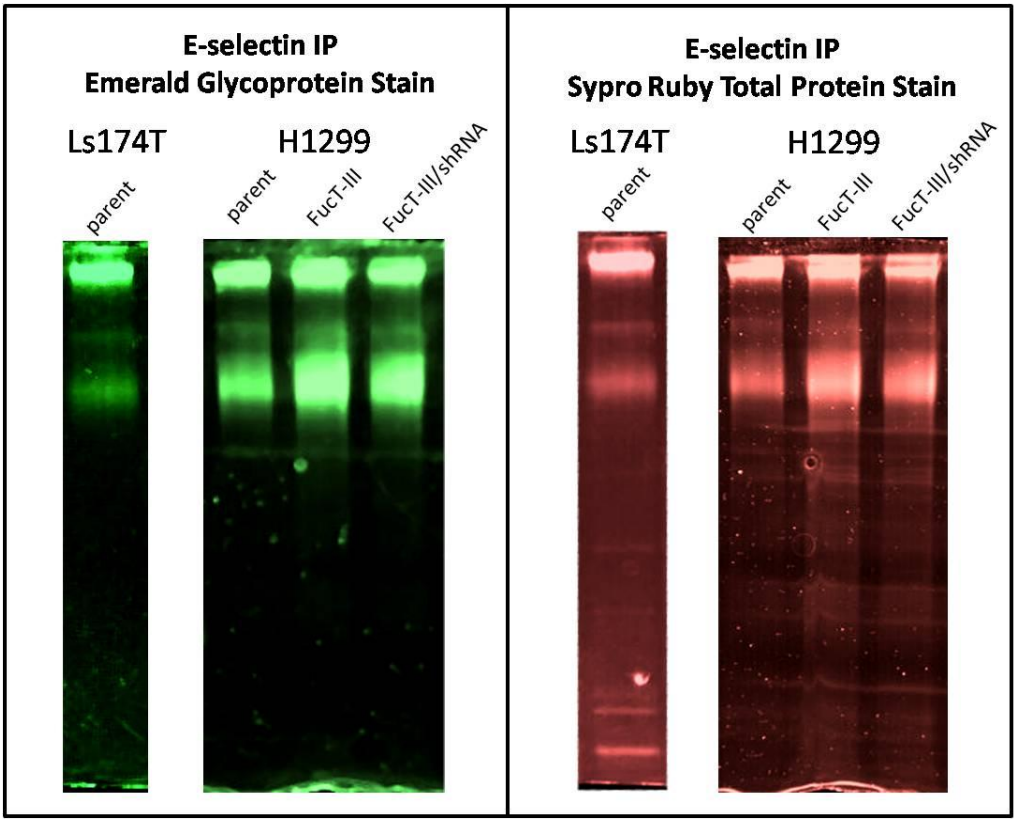


Figure 19: Three glycosylated proteins are detected in LS174T and H1299 cells following IP with an E-selectin/Fc chimera and Western blotting with CHO-131 mAb. (a): Whole cell lysates from LS174T parent and LS174T/shRNA and H1299 parent, H1299/FucT-III, and H1299/FucT-III/shRNA cells were separated on an SDS-PAGE gel, transferred to a membrane, and Western blotting was performed with CHO-131 mAb to detect proteins modified C2-O-sLe^x. (b): E-selectin binding proteins were Immunoprecipitated from LS174T parent and LS174T/shRNA and H1299 parent, H1299/FucT-III, and H1299/FucT-III/shRNA cell lysates, electrophoresed on an SDS-PAGE gel, transferred to a membrane, and Western blotting was performed with CHO-131 mAb. (c): Whole cell lysates from LS174T parent and LS174T/shRNA and H1299 parent, H1299/FucT-III, and H1299/FucT-III/shRNA cells were separated on an SDS-PAGE gel and glycosylated proteins were identified by staining the gel with Pro-Q Emerald 300 Glycoprotein Stain (gels shown on the left). The same gels were then counterstained with SYPRO Ruby Protein Gel Stain to identify total proteins that were present on the gel (gels shown on the right). (d): E-selectin binding proteins were Immunoprecipitated from LS174T parent and H1299 parent, H1299/FucT-III, and H1299/FucT-III/shRNA cell lysates and separated by SDS page gel electrophoresis. Glycosylated proteins were identified by staining the gel with Pro-Q Emerald 300 Glycoprotein Stain (gels shown on the left). The same gels were then counterstained with SYPRO Ruby Protein Gel Stain to identify total proteins that were present on the gel (shown on the right). All images are representative results of three individual experiments.

In light of unexpected results in band intensities obtained from Western blotting LS174T and H1299 cells with CHO-131 mAb, a mouse IgM isotype control antibody was tested in parallel with CHO-131 mAb in the Western blotting procedure. Simultaneously conducted Western blots of LS174T whole cell lysate samples with CHO-131 and a mouse IgM isotype control antibody resulted in an extremely similar pattern of detected bands (Figure 20). An IgM isotype control antibody obtained from a different company was also tested and showed similar results (data not shown).

To further understand our observations, a readily available unrelated antibody was tested in the Western blotting procedure. LS174T and H1299 cells were submitted to an E-selectin IP followed by Western blotting with 1-13M1, a mAb against human mucin 5AC (MUC5AC). Mucins are known to be heavily glycosylated [149]. MUC5AC is a secreted airway glycoprotein [150] known to carry the abundant sLe^x moieties [151], however, MUC5ac has not been described to participate in E-selectin binding. The 1-

13M1 antibody detects a single 130 kDa band in Western blotting procedures. However, in our hands, 1-13M1 mAb detected three bands of similar molecular weights as were detected in our previous experiments (Figure 21a).

To investigate these results further, we indirectly verified presence of MUC5AC in LS174T and H1299 cells by evaluating mRNA levels of MUC5AC in these cells by RT-PCR. The presence of MUC5AC mRNA in LS174T and H1299 cells was assessed by RT-PCR. MUC5AC mRNA was detected in the LS174T cells. However, in contrast to the observation that H1299 cells contained abundant amounts of MUC5AC protein detected by Western blot, MUC5AC mRNA was absent from H1299 cells by RT-PCR (Figure 21b).

To determine whether non-specific antibody binding was occurring in our Western blotting procedure, samples of whole cell lysates from both cell lines were evaluated for reactivity with an antibody that detects the housekeeping gene GAPDH. We observed a single band with the predicted molecular weight of 36 kDa, indicating that, using this antibody, the Western blotting procedure successfully detected the expected protein (Figure 22). E-selectin IP samples of both cell lines were also blotted with the anti-GAPDH antibody. As expected, no protein bands were detected (data not shown).

FIGURE 20

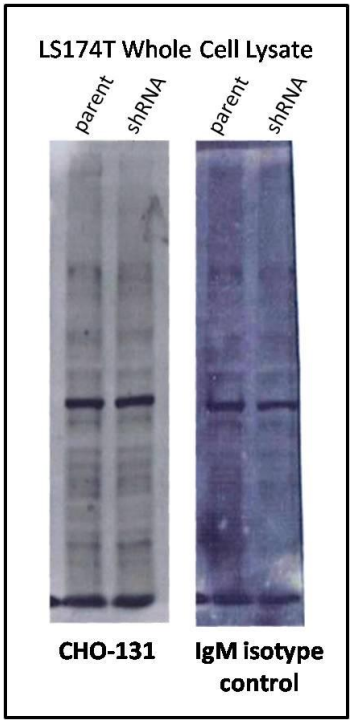


Figure 20: Western blotting with the mAb CHO-131 identifies similar protein bands as a mouse IgM isotype control antibody. LS174T parent and LS174T/shRNA whole cell lysates were separated on an SDS-PAGE gel, transferred to a membrane and blotted with CHO-131 (shown on the left) or a mouse IgM isotype control antibody (shown on the right). One representative image of 4 individual experiments is shown.

FIGURE 21a

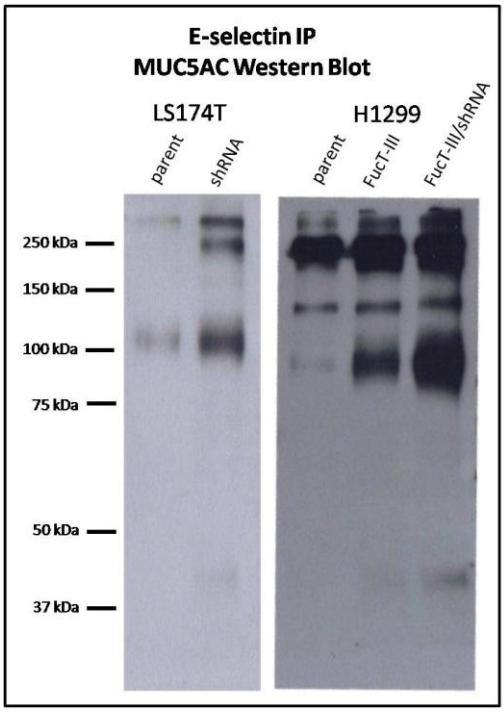


FIGURE 21b

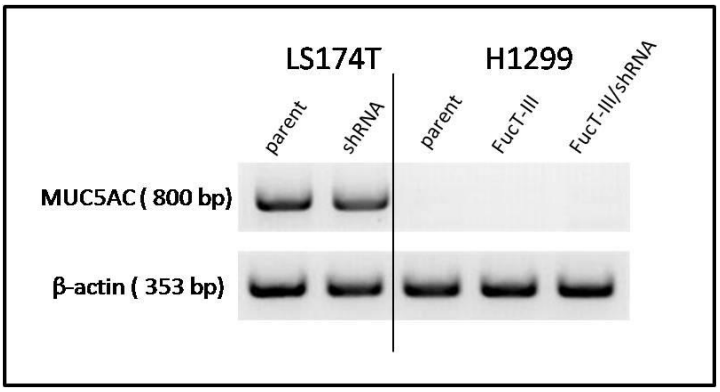


Figure 21: An anti-MUC5AC antibody detects proteins in E-selectin IP samples although MUC5AC mRNA is absent from the cells. (a): E-selectin binding proteins were Immunoprecipitated from LS174T parent and LS174T/shRNA and H1299 parent, H1299/FucT-III, and H1299/FucT-III/shRNA cell lysates, electrophoresed on an SDS-PAGE gel, transferred to a membrane, and Western blotted with 1-13M1 mAb against MUC5AC. One representative image of three individual experiments is shown. (b): RT-PCR of MUC5AC and β-actin mRNA isolated from LS174T parent and LS174T/shRNA and H1299 parent, H1299/FucT-III, and H1299/FucT-III/shRNA cells. One representative experiment of three repetitions is shown.

FIGURE 22

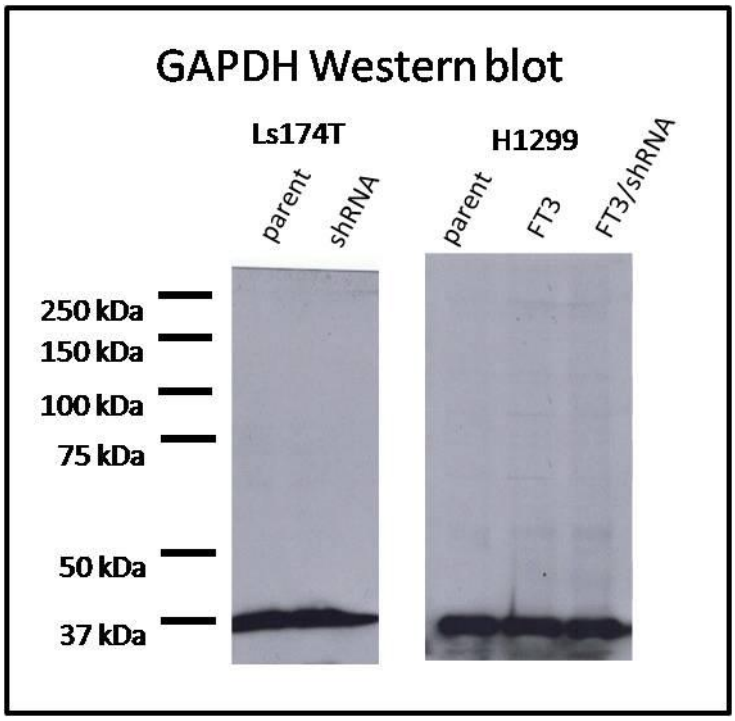


Figure 22: Western blotting with an anti-GAPDH antibody detects one band of the expected molecular weight. Whole cell lysates of LS174T parent and LS174T/shRNA and H1299 parent, H1299/FucT-III, and H1299/FucT-III/shRNA cells were separated on an SDS-PAGE gel, transferred to a membrane and blotted with anti-GAPDH mAb to identify the protein band corresponding to the housekeeping gene GAPDH. One representative image of two individual experiments is shown.

MUC 3 is a candidate protein for C2-O-sLe^x glycosylation

The nature of the scaffold to which C2-O-sLex is attached in cancer cells is unknown but mucins represent likely candidates because they are the major glycoproteins produced by epithelial tissues of the gastrointestinal tract and lungs. MUC1 has been shown to carry the sLe^x epitope and participate in E-selectin binding in cancer cells [146-147]. As a first step to determine whether C2-O-sLe^x decorates a mucin, we tested H1299 parent, H1299/FucT-III, and H1299/FucT-III/shRNA cells for the presence of a panel of mucin mRNAs by RT-PCR. All of the mucins that were tested (with the exception of MUC2)

are known to contain a transmembrane domain which could allow them to function as the protein backbone to which cell surface C2-O-sLe^x is attached. MUC3 and MUC13 were the only mucin mRNAs present in H1299 cells and manipulation of cells by transfection of FucT-III or gene knockdown of C2GnT1 had no effect on their levels of transcription (Figure 23a). Although low levels of MUC1 mRNA was detected, all groups of H1299 cells were negative for cell surface MUC1 expression as assessed by flow cytometry (data not shown).

To determine if the MUC3 mRNA was translated into protein and expressed on the cell surface, H1299 cells were stained with the M3.1 mAb that is specific for MUC3 and assessed for positive reactivity by flow cytometry. Approximately 10% of all groups of H1299 cells stained positively with M3.1 mAb. Staining of the parent cells with a mouse IgG isotype control antibody resulted in a negative signal (Figure 23b). These results indicate that H1299 cells expressed the cell surface bound mucin MUC3 which may be considered as a candidate for C2-O-sLe^x glycosylation in lung carcinoma cells.

FIGURE 23a

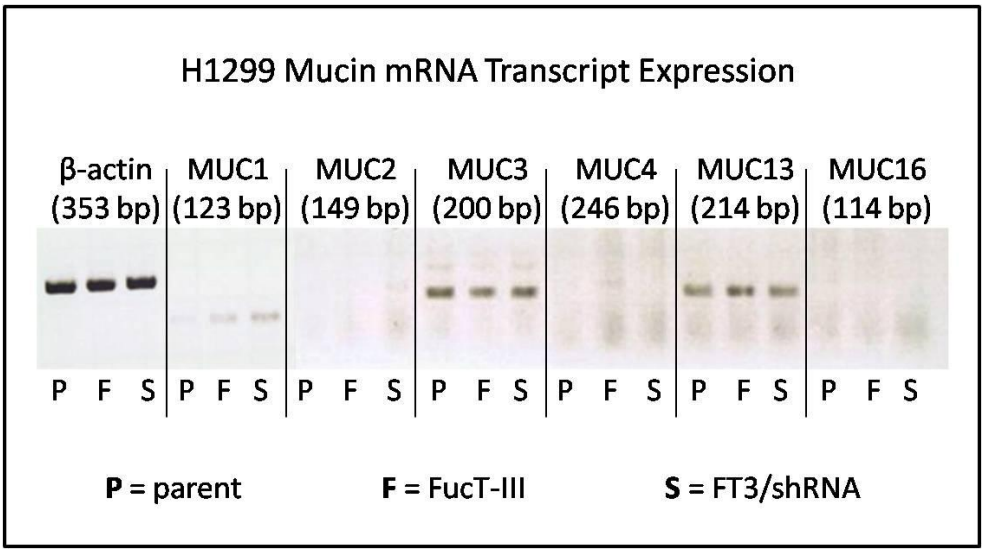


FIGURE 23b

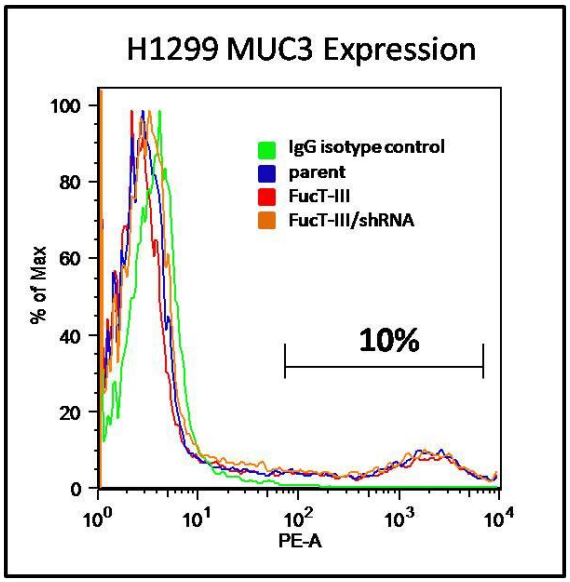


Figure 23: H1299 cells express MUC3 mRNA and protein. (a): RNA was isolated from H1299 parent, H199/FucT-III, and H1299/FucT-III/shRNA cells and RT-PCR was performed to detect mucin mRNAs. One representative experiment of two is shown. (b): The reactivity of H1299 parent, H1299/FucT-III, and H1299/FucT-III/shRNA cells with m3.1 mAb that detects MUC 3 protein was determined by flow cytometric analysis. Results of one representative experiment of two are shown.

Discussion

We made considerable progress in identification of the backbone structure attached to C2-O-sLe^x in lung (H1299) and colon (LS174T) carcinoma cell lines. We successfully isolated three C2-O-sLe^x expressing proteins in these cells by IP of cell lysates with E-selectin followed by Western blotting with CHO-131 mAb. However, several limitations of our assays prevented us from specifically characterizing the nature of the backbone structure. Unexpectedly, the three protein bands, ranging in molecular weights from less than 250 to greater than 100 kDa, were present in a similar pattern for both cell types. Additionally, we noted a discrepancy in our data for H1299 cells. We have demonstrated by flow cytometry that H1299 parent cells do not express the C2-O-sLe^x

epitope on cell membranes but we detected protein bands in H1299 parent cell lysates after immunoblotting with CHO-131 mAb, indicating that C2-O-sLe^x, possibly in an immature form, may have been present in these cells but was not transported to the cell surface. Furthermore, the intensities of the ~130 kDa band in H1299 and LS174T cells was not reduced after gene knockdown of C2GnT1, the enzyme necessary for C2-O-sLe^x synthesis. A possibility is that our gene knockdown was not complete. Alternatively, CHO-131 mAb binding may not be specific for C2-O-sLe^x in the lung and colon carcinoma cell lines tested.

In published reports, CHO-131 mAb has been successfully used to detect proteins in T-cells by Western blotting [152-153]. Descheny et. al. but not Ni et. al. showed an IgM isotype control antibody in their report. Our laboratory has recently successfully used IP with a PSGL-1 antibody and Western blotting with CHO-131 mAb to detect a single specific glycoprotein band corresponding to C2-O-sLe^x in neutrophils (unpublished results). We have also used CHO-131 mAb to detect C2-O-sLe^x on colon carcinoma cells by immunohistochemistry [116]. Therefore, we surmise that expression of C2-O-sLe^x differs in cancer cells compared to immune cells and that CHO-131 mAb reactivity with lysates of cancer cells is not as specific as demonstrated for immune cells in Western blotting procedures.

The HECA-452 mAb is specific for the sLe^x epitope and has been successfully used in Western blotting of whole cell lysates of LS174T colon carcinoma cells by Napier et. al.. This antibody is less restrictive in its binding reactivity than CHO-131 mAb because it recognizes sLe^x on several types of backbone structures. Napier et. al. detected only a single blurred band between approximately 250 – 100 kDa [143].

Interestingly, when Western blotting was performed with HECA-452 mAb using whole cell lysates of AML-139 acute myeloid leukemia cells, three high molecular weight bands were observed that appeared to be very similar in molecular weights to the bands that we detected by Western blotting and glycoprotein staining of gels after IP with E-selectin [154]. Thomas et. al. have demonstrated that IP of LS174T cells with HECA-452 mAb and subsequent protein detection with Pro-Q Emerald 300 Glycoprotein Stain resulted in a banding pattern that was similar to our findings when we performed IPs with E-selectin and glycoprotein staining using LS174T and H1299 cell lysates [145].

Importantly, in H1299 lung carcinoma cells, we detected MUC 3 mRNA and MUC3 protein expressed on the cell membrane. Expression of the FucT-III and C2GnT1 genes did not play a role in MUC3 expression. We observed that 25% of transfected H1299 cells expressed C2-O-sLe^x, however, only approximately 10% of transfected cells expressed MUC3. Therefore, MUC3 may be a candidate glycoprotein in H1299 cells that is decorated with C2-O-sLe^x but other proteins in these cells must also be modified with C2-O-sLe^x.

Despite the limitations of our study, we have provided evidence that a mucin, particularly MUC3, may act as a scaffold for C2-O-sLe^x. Further investigation is required to determine the biological significance of this potential association in cancer cells. Future studies include the use of dual fluorescent staining with CHO-131 mAb and an anti-MUC3 or other mucin antibody, and confocal microscopy, to investigate whether C2-O-sLe^x is spatially associated with a mucin on the cancer cell membrane.

Chapter 4: Conclusions and Future Directions

Much evidence supports an association between inflammation and cancer promotion and progression [25-26, 28-29, 155]. Leukocytes are key mediators of the inflammatory tumor microenvironment and tumor progression and metastasis. Classically, investigation of tumor associated leukocytes has been focused on macrophages which have been shown to support tissue hyperplasia and remodeling, tumor growth, angiogenesis, and suppression of anti-tumor adaptive immune responses [25, 28, 30, 32, 47, 49]. More recently, however, the presence of neutrophils in the tumor microenvironment has been widely demonstrated to promote angiogenesis and confer metastatic properties to tumor cells [31-33, 156-159].

Aberrant secretion of chemokines by tumor cells regulates the infiltration of leukocytes to the tumor microenvironment [28]. Changes cell signaling or genetic alterations in tumor cells contribute to the mis-regulation of chemoattractant signaling [30, 33,155]. Further, the secretion of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, and IL-8 may be increased in cancer cells and act via autocrine or paracrine signaling pathways to promote carcinogenesis [155]. The pro-inflammatory cytokine TNF-α is also secreted into the tumor microenvironment by recruited leukocytes and is involved in cellular transformation, proliferation and survival, invasion, migration, angiogenesis, and metastasis [35, 155]. TNF-α is thought to be important in mediating the pathogenesis and metastatic properties of human lung cancer by up-regulating the expression of genes encoding glycosyltransferase type enzymes. Stimulation of lung cancer cell lines or explanted bronchial mucosa with rTNF-α resulted in increased expression of the glycosyltransferase enzyme FucT-III and cell surface sLe^x, both of which have been extensively associated with promotion of metastasis [117-118]. sLe^x is a carbohydrate

epitope resulting from the activity of FucT-III and promotes metastasis of lung and other types of carcinoma cells by facilitating tumor cell attachment to endothelial E-selectin [66, 79, 80-84, 106].

Despite these observations, further investigation is required to determine the precise regulation of sLe^x expression on lung tumor cells by tumor-associated leukocytes and their secreted cytokines and the resulting effect on tumor progression and metastasis. Additionally, information is lacking on the influence of glycosyltransferase gene expression on chemokine secretion and leukocyte recruitment by lung tumor cells.

The results of our study suggest that rTNF-α up-regulates sLe^x on FucT-III expressing lung carcinoma cells and TNF-α derived from neutrophils enhances the metastatic potential of lung carcinoma cells through promotion of a non-adherent phenotype and enhancement of invasiveness. Factors secreted by neutrophils also up-regulate lung cancer cell sLe^x expression, however, this result was shown to be independent of TNF-α. Our findings are in agreement with other studies [117-118], that demonstrated that stimulation with rTNF-α results in increased cell surface expression of sLe^x in QG-95 lung cancer cells and explanted samples of bronchial mucosa. However, in contrast to other studies [118-119], we found that the rTNF-α induced increase in sLe^x was not the result of up-regulation of FucT-III mRNA and therefore, may have been due to regulation of post-transcriptional modification of the FucT-III enzyme. Notably, although Groux-Degroote et. al. demonstrated that stimulation with either IL-6 or IL-8 resulted in increased sLe^x expression in samples of healthy bronchial mucosa, only IL-8 was shown to increase FucT-III mRNA expression [160]. This indicates that TNF-α and IL-6 may be involved in similar mechanisms that do not change transcription of FucT-III, but nevertheless are responsible for up-regulation of sLe^x.

We observed that overexpression of FucT-III in lung carcinoma cells resulted in increased secretion of neutrophil chemoattractants (IL-8 and GRO-α), therefore, we focused our investigation on the effect of neutrophils and their secreted factors on lung tumor cells and found that neutrophils promote metastatic qualities. Others have also demonstrated the importance of factors secreted by neutrophils in the promotion of metastatic properties of tumor cells. In agreement with our observations, Remedi et. al. found that NCM or co-culture with neutrophils conferred anchorage independence and an invasive phenotype to rat sarcoma cells. Additionally, it was determined that neutrophils caused up-regulation of matrix metalloprotease-2 and down-regulation of ICAM-1 [158]. Wislez et. al. demonstrated that neutrophils induced the detachment of A549 cells from substratum and confirmed that TNF-α mediated this process. ICAM-1, IL-1-α, and neutrophil elastase were additionally identified as playing a role in A549 detachment mediated by neutrophils. It was also concluded that intrapulmonary tumor cell shedding was induced by tumor associated neutrophils *in vivo* and was a significant factor contributing to shorter survival time of patients [159].

We focused on the implications of interactions between IL-8 secreting tumor cells with recruited neutrophils at the primary tumor site and found that neutrophils endowed properties to tumor cells which would promote metastasis upon detachment from the primary tumor. However, neutrophils have recently been demonstrated to interact with IL-8 expressing melanoma cells in the vasculature to directly promote tumor cell attachment to endothelium and tissue extravasation [161]. Additionally, high systemic neutrophil counts have been shown to be associated with shorter survival times in patients suffering from NSCLC [162].

In summary, our findings complement and expand the body of previously published reports that indicate that neutrophils play a role on the role in the acquisition of

metastatic properties by tumor cells. Importantly, we are the first to report that overexpression of the glycosyltransferase enzyme FucT-III by lung carcinoma cells may augment the recruitment of neutrophils which in turn increase sLe^x expression on the membranes of lung carcinoma cells and promote tumor invasiveness.

Our results are exciting as they provide important new insight into the relationship between tumor-associated inflammatory cells and sLe^x expression on lung cancer cells. Based on our accumulated data and published findings, we propose the following model of the interactions in the lung tumor microenvironment that promote metastasis (Figure 24): A stimulus, possibly the carcinogenic components of cigarette smoke or a chronic state of inflammation (see chapter 1, Pathogenesis of Lung Inflammation and Cancer and The Role of Inflammation in Cancer Progression) may induce cellular genetic mutations, a loss of proliferative regulation, and the initiation of carciongenesis. These genetic changes may cause heightened activity of the FucT-III gene. This results in cell surface sLe^x presentation, increased secretion of inflammatory chemokines, and an enhanced invasive ability. The secreted chemokines may recruit an increased number of neutrophils and macrophages to the tumor site. The presence of chemokines and leukocytes may collectively promote tumor angiogenesis, proliferation, and metastatic properties including loss of anchorage dependence, an increased invasive ability, and an increase in the amount of cell surface sLe^x. Collectively, these changes may allow tumor cells to more readily detach from the primary tumor and enter the circulation, and subsequently, allow circulating tumor cells to more efficiently adhere to endothelium and migrate into new tissues to form metastases.

FIGURE 24

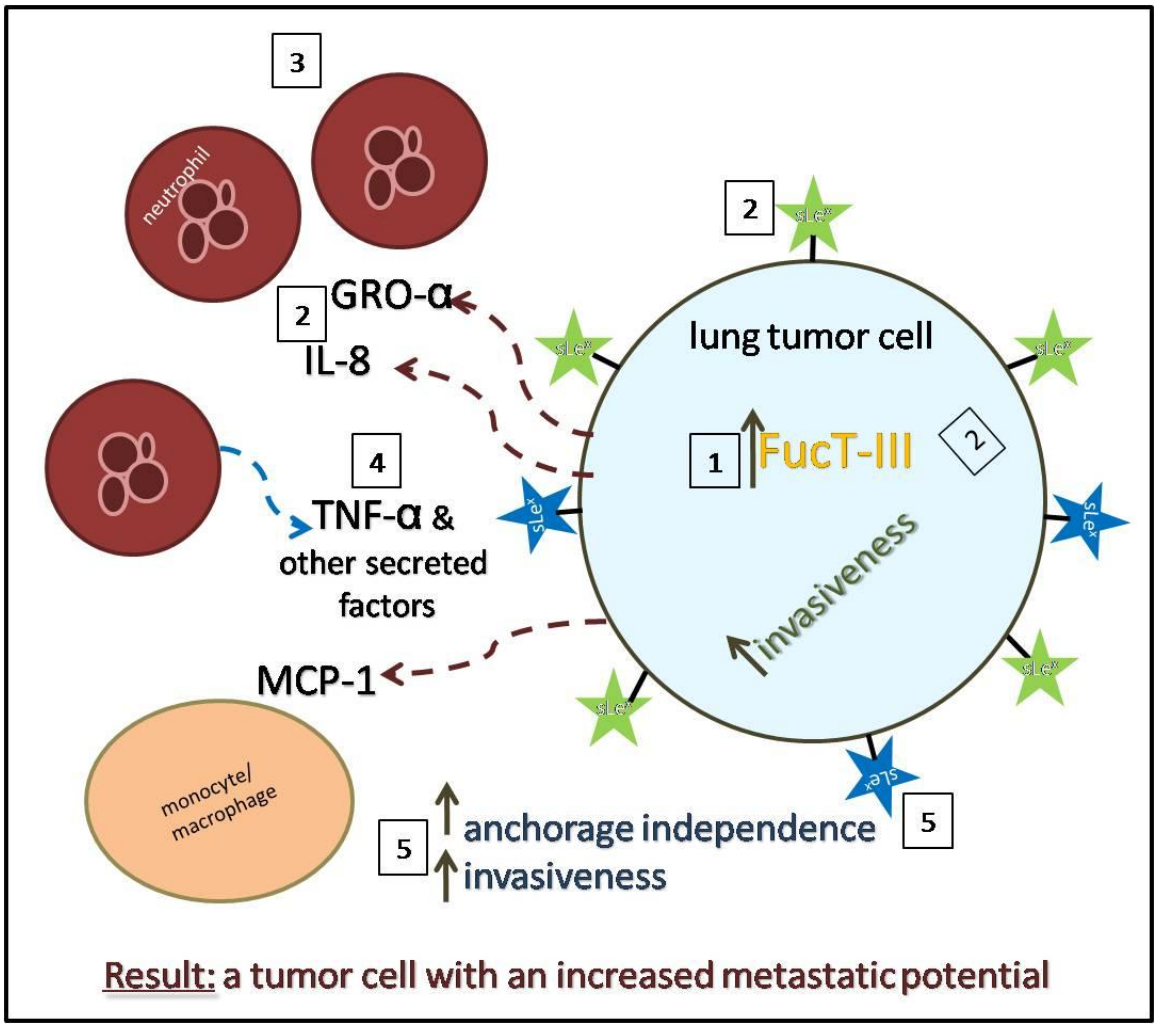


Figure 24: Graphical depiction of the proposed mechanism linking tumor cell FucT-III expression, chemokine secretion, inflammatory cells, and increased tumor cell metastatic potential. Genetic alterations and malignant transformation may lead to aberrant expression of FucT-III (1) which results in cell surface sLe^x expression (green stars), secretion of chemokines, and increased invasiveness (2). Chemokines lead to the recruitment of leukocytes (3) which secrete TNF-α and other factors (4) that result in increased sLe^x expression (blue stars), anchorage independent growth, and further increased invasive ability (5). As a result of these interactions, the tumor cell is armed with properties shown to increase metastatic potential.

Targeting tumor microenvironmental molecules has been suggested as a future potential cancer therapy [163]. We believe that our findings will be instrumental in providing crucial information for the development of therapies aimed at preventing the spread of tumor cells, including the use of chemokine inhibitors or other inflammation-reducing adjuvant therapies that may be effective in conjunction with established lung cancer treatment protocols.

These findings pave the way for additional experiments aimed at determining the molecular mechanisms involved in the interactions between cancer cells and inflammatory cells that may regulate cancer growth, invasion, and metastasis. Possible future experiments include:

1) Determination of the processes in malignant transformation responsible for aberrant FucT-III expression in cancer cells.

2) Investigation of the cellular signaling mechanisms involved in FucT-III induced up-regulation of chemokine secretion and enhancement of invasion. How and why is FucT-III influencing seemingly unrelated molecules?

3) Identification the molecular mechanisms involved in the observed FucT-III, TNF-α, and NCM induced increase in invasion. What is the role of matrix metalloproteases in invasion in our system and are signaling pathways activated?

4) Identification of the factor/s in NCM responsible for sLe^x up-regulation. Is it possible that TNF-α acts as the primary factor influencing sLe^x however, when TNF-α is blocked, does a different molecule act in its place?

5) Investigation of the mechanisms responsible for TNF-α and NCM induced sLe^x up-regulation. If sLe^x up-regulation is not due to increased FucT-III transcription, how do these stimulatory factors cause an increase in sLe^x expression?

6) Determination of the influence of other immune cell populations on tumor cell sLe^x expression. For example, MCP-1 secretion was observed to be increased

by FucT-III gene expression. What effect does monocyte conditioned media or co-culture with monocytes have on sLe^x expression and other metastatic properties?

7) Assessment of the influence of neutrophils and other immune cells on expression of the high affinity selectin ligand C2-O-sLe^x by cancer cells. Does TNF-α or other factors secreted by neutrophils or other immune cells effect the transcription, activity, or localization of the C2GnT1 gene and/or enzyme?

8) Investigation of the significance of the morphologic change observed after co-culture of cancer cells with neutrophils and the implications for *in vivo* metastasis.

9) Assessment of the interactions that may regulate FucT-III overexpression in cancer cells, the presence of neutrophils, sLe^x expression, and tumor progression and metastasis *in vivo*. Does treatment with anti-inflammatory drugs result in reduced sLe^x expression on cancer cells and what is the influence on metastasis? What are the effects of specific chemokine inhibitors? What role does expression of FucT-III play in tumor progression and tumor-associated inflammation?

References

1) American Cancer Society. Cancer Facts and Figures 2007. http://www.cancer.org/docroot/STT/stt_0_2007.asp?sitearea=STT&level=1 Visited on 11-15-2009.

2) The Merck Manuals Online Medical Library. http://www.merck.com/mmhe/sec04/ch057/ch057a.html Visited on 11-15-2009.

3) van der Vaart H, Postma DS, Timens W, Hylkema MN, Willemse BW, Boezen HM, Vonk JM, de Reus DM, Kauffman HF, ten Hacken NH. Acute effects of cigarette smoking on inflammation in healthy intermittent smokers. Respir Res. 2005 Mar 1;6:22.

4) Iho S, Tanaka Y, Takauji R, Kobayashi C, Muramatsu I, Iwasaki H, Nakamura K, Sasaki Y, Nakao K, Takahashi T. Nicotine induces human neutrophils to produce IL-8 through the generation of peroxynitrite and subsequent activation of NF-kappaB. J Leukoc Biol. 2003 Nov;74(5):942-51.

5) Nomura I, Gao B, Boguniewicz M, Darst MA, Travers JB, Leung DY. Distinct patterns of gene expression in the skin lesions of atopic dermatitis and psoriasis: a gene microarray analysis. J Allergy Clin Immunol. 2003 Dec;112(6):1195-202.

6) Damen GM, Hol J, de Ruiter L, Bouquet J, Sinaasappel M, van der Woude J, Laman JD, Hop WC, Büller HA, Escher JC, Nieuwenhuis EE. Chemokine production by buccal epithelium as a distinctive feature of pediatric Crohn disease. J Pediatr Gastroenterol Nutr. 2006 Feb;42(2):142-9.

7) Fodor M, Facskó A, Rajnavölgyi E, Hársfalvi J, Bessenyei E, Kardos L, Berta A. Enhanced release of IL-6 and IL-8 into tears in various anterior segment eye diseases. Ophthalmic Res. 2006;38(4):182-8.

8) McCormack G, Moriarty D, O'Donoghue DP, McCormick PA, Sheahan K, Baird AW. Tissue cytokine and chemokine expression in inflammatory bowel disease. Inflamm Res. 2001 Oct;50(10):491-5.

9) Nielsen OH, Rüdiger N, Gaustadnes M, Horn T. Intestinal interleukin-8 concentration and gene expression in inflammatory bowel disease. Scand J Gastroenterol. 1997 Oct;32(10):1028-34.

10) Anezaki K, Asakura H, Honma T, Ishizuka K, Funakoshi K, Tsukada Y, Narisawa R. Correlations between interleukin-8, and myeloperoxidase or luminol-dependent chemiluminescence in inflamed mucosa of ulcerative colitis. Intern Med. 1998 Mar;37(3):253-8.

11) Matsuda R, Koide T, Tokoro C, Yamamoto T, Godai T, Morohashi T, Fujita Y, Takahashi D, Kawana I, Suzuki S, Umemura S. Quantitive cytokine mRNA expression profiles in the colonic mucosa of patients with steroid naïve ulcerative colitis during active and quiescent disease. Inflamm Bowel Dis. 2009 Mar;15(3):328-34.

12) Atta-ur-Rahman, Harvey K, Siddiqui RA. Interleukin-8: An autocrine inflammatory mediator. Curr Pharm Des. 1999 Apr;5(4):241-53.

13) Hickey MJ, Kubes P. Intravascular immunity: the host–pathogen encounter in blood vessels. Nat Rev Immunol. 2009 May;9(5):364-75.

14) Chaudhuri N, Sabroe I. Basic science of the innate immune system and the lung. Paediatr Respir Rev. 2008 Dec;9(4):236-42.

15) Ohbayashi H. Novel neutrophil elastase inhibitors as a treatment for neutrophil-predominant inflammatory lung diseases. IDrugs. 2002 Sep;5(9):910-23.

16) Jaeschke H, Hasegawa T. Role of neutrophils in acute inflammatory liver injury. Liver Int. 2006 Oct;26(8):912-9.

17) Lobos EA, Sharon P, Stenson WF. Chemotactic activity in inflammatory bowel disease. Role of leukotriene B4. Dig Dis Sci. 1987 Dec;32(12):1380-8.

18) Canakçi CF, Ciçek Y, Canakçi V. Reactive oxygen species and human inflammatory periodontal diseases. Biochemistry (Mosc). 2005 Jun;70(6):619-28.

19) Kuschner WG, D'Alessandro A, Wong H, Blanc PD. Dose-dependent cigarette smoking-related inflammatory responses in healthy adults. Eur Respir J. 1996 Oct;9(10):1989-94.

20) Takizawa H, Tanaka M, Takami K, Ohtoshi T, Ito K, Satoh M, Okada Y, Yamasawa F, Umeda A. Increased expression of inflammatory mediators in small-airway epithelium from tobacco smokers. Am J Physiol Lung Cell Mol Physiol. 2000 May;278(5):L906-13.

21) Dwyer TM. Cigarette smoke-induced airway inflammation as sampled by the expired breath condensate. Am J Med Sci. 2003 Oct;326(4):174-8.

22) Hunninghake GW, Crystal RG. Cigarette smoking and lung destruction. Accumulation of neutrophils in the lungs of cigarette smokers. Am Rev Respir Dis. 1983 Nov;128(5):833-8.

23) Rylander R. Pulmonary cell responses to inhaled cigarette smoke. Arch Environ Health. 1974 Dec;29(6):329-33.

24) Quit Smoking Stop, Harmful Chemicals in Cigarettes and Tobacco Smoke. http://quit-smoking-stop.com/harmful-chemicals-in-cigarettes.html Visited 11-16-2009.

25) Porta C, Larghi P, Rimoldi M, Grazia Totaro M, Allavena P, Mantovani A, Sica A. Cellular and molecular pathways linking inflammation and cancer. Immunobiology. 2009;214(9-10):761-77.

26) Lu H, Ouyang W, Huang C. Inflammation, a key event in cancer development. Mol Cancer Res. 2006 Apr;4(4):221-33.

27) McConnell BB, Yang VW. The Role of Inflammation in the Pathogenesis of Colorectal Cancer. Curr Colorectal Cancer Rep. 2009 Apr 1;5(2):69-74.

28) Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008 Jul 24;454(7203):436-44.

29) Germano G, Allavena P, Mantovani A. Cytokines as a key component of cancer-related inflammation. Cytokine. 2008 Sep;43(3):374-9.

30) Schwertfeger KL, Xian W, Kaplan AM, Burnett SH, Cohen DA, Rosen JM. A critical role for the inflammatory response in a mouse model of preneoplastic progression. Cancer Res. 2006 Jun 1;66(11):5676-85.

31) Tazawa H, Okada F, Kobayashi T, Tada M, Mori Y, Une Y, Sendo F, Kobayashi M, Hosokawa M. Infiltration of neutrophils is required for acquisition of metastatic phenotype of benign murine fibrosarcoma cells: implication of inflammation-associated carcinogenesis and tumor progression. Am J Pathol. 2003 Dec;163(6):2221-32.

32) Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proc Natl Acad Sci U S A. 2006 Aug 15;103(33):12493-8.

33) Yao C, Lin Y, Ye CS, Bi J, Zhu YF, Wang SM. Role of interleukin-8 in the progression of estrogen receptor-negative breast cancer. Chin Med J (Engl). 2007 Oct 20;120(20):1766-72.

34) Koide N, Nishio A, Sato T, Sugiyama A, Miyagawa S. Significance of macrophage chemoattractant protein-1 expression and macrophage infiltration in squamous cell carcinoma of the esophagus. Am J Gastroenterol. 2004 Sep;99(9):1667-74.

35) McConnell BB, Yang VW. The Role of Inflammation in the Pathogenesis of Colorectal Cancer. Curr Colorectal Cancer Rep. 2009 Apr 1;5(2):69-74.

36) Dubravec DB, Spriggs DR, Mannick JA, Rodrick ML. Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha. Proc Natl Acad Sci U S A. 1990 Sep;87(17):6758-61.

37) Nikolaus S, Bauditz J, Gionchetti P, Witt C, Lochs H, Schreiber S. Increased secretion of pro-inflammatory cytokines by circulating polymorphonuclear neutrophils and regulation by interleukin 10 during intestinal inflammation. Gut. 1998 Apr;42(4):470-6.

38) Brown ER, Charles KA, Hoare SA, Rye RL, Jodrell DI, Aird RE, Vora R, Prabhakar U, Nakada M, Corringham RE, DeWitte M, Sturgeon C, Propper D, Balkwill FR, Smyth JF. A clinical study assessing the tolerability and biological effects of infliximab, a TNF--alpha inhibitor, in patients with advanced cancer. Ann Oncol. 2008 Jul;19(7):1340-6.

39) Voronov E, Shouval DS, Krelin Y, Cagnano E, Benharroch D, Iwakura Y, Dinarello CA, Apte RN. IL-1 is required for tumor invasiveness and angiogenesis. Proc Natl Acad Sci U S A. 2003 Mar 4;100(5):2645-50.

40) Carmi Y, Voronov E, Dotan S, Lahat N, Rahat MA, Fogel M, Huszar M, White MR, Dinarello CA, Apte RN. The role of macrophage-derived IL-1 in induction and maintenance of angiogenesis. J Immunol. 2009 Oct 1;183(7):4705-14.

41) Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest. 2007 May;117(5):1175-83.

42) Ishihara K, Hirano T. IL-6 in autoimmune disease and chronic inflammatory proliferative disease. Cytokine Growth Factor Rev. 2002 Aug-Oct;13(4-5):357-68.

43) Shinriki S, Jono H, Ota K, Ueda M, Kudo M, Ota T, Oike Y, Endo M, Ibusuki M, Hiraki A, Nakayama H, Yoshitake Y, Shinohara M, Ando Y. Humanized anti-interleukin-6 receptor antibody suppresses tumor angiogenesis and in vivo growth of human oral squamous cell carcinoma. Clin Cancer Res. 2009 Sep 1;15(17):5426-34.

44) Chan AT, Giovannucci EL, Schernhammer ES, Colditz GA, Hunter DJ, Willett WC, Fuchs CS. A prospective study of aspirin use and the risk for colorectal adenoma. Ann Intern Med. 2004 Feb 3;140(3):157-66.

45) Flossmann E, Rothwell PM; British Doctors Aspirin Trial and the UK-TIA Aspirin Trial. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. Lancet. 2007 May 12;369(9573):1603-13.

46) Tsutsumi M, Kitada H, Shiraiwa K, Takahama M, Tsujiuchi T, Sakitani H, Sasaki Y, Murakawa K, Yoshimoto M, Konishi Y. Inhibitory effects of combined administration of antibiotics and anti-inflammatory drugs on lung tumor development initiated by N-nitrosobis(2-hydroxypropyl)amine in rats. Carcinogenesis. 2000 Feb;21(2):251-6.

47) Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol. 2005 Dec;5(12):953-64.

48) Biswas SK, Gangi L, Paul S, Schioppa T, Saccani A, Sironi M, Bottazzi B, Doni A, Vincenzo B, Pasqualini F, Vago L, Nebuloni M, Mantovani A, Sica A. A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). Blood. 2006 Mar 1;107(5):2112-22.

49) Mantovani A, Allavena P, Sica A. Tumour-associated macrophages as a prototypic type II polarised phagocyte population: role in tumour progression. Eur J Cancer. 2004 Jul;40(11):1660-7.

50) Sica A, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. Eur J Cancer. 2006 Apr;42(6):717-27.

51) Wikipedia, the free encyclopedia,Docetaxel.http://en.wikipedia.org/wiki/Docetaxel Visited on 11-20-2009.

52) Rozel S, Galbán CJ, Nicolay K, Lee KC, Sud S, Neeley C, Snyder LA, Chenevert TL, Rehemtulla A, Ross BD, Pienta KJ. Synergy between anti-CCL2 and docetaxel as determined by DW-MRI in a metastatic bone cancer model. J Cell Biochem. 2009 May 1;107(1):58-64.

53) Allavena P, Sica A, Vecchi A, Locati M, Sozzani S, Mantovani A. The chemokine receptor switch paradigm and dendritic cell migration: its significance in tumor tissues. Immunol Rev. 2000 Oct;177:141-9.

54) Sica A, Bronte V. Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest. 2007 May;117(5):1155-66.

55) Munro JM, Lo SK, Corless C, Robertson MJ, Lee NC, Barnhill RL, Weinberg DS, Bevilacqua MP. Expression of sialyl-Lewis X, an E-selectin ligand, in inflammation, immune processes, and lymphoid tissues. Am J Pathol. 1992 Dec;141(6):1397-408.

56) Julien S, Grimshaw MJ, Sutton-Smith M, Coleman J, Morris HR, Dell A, Taylor-Papadimitriou J, Burchell JM. Sialyl-Lewis^{x} on P-Selectin Glycoprotein Ligand-1 Is Regulated during Differentiation and Maturation of Dendritic Cells: A Mechanism Involving the Glycosyltransferases C2GnT1 and ST3Gal I. J Immunol. 2007 Nov 1;179(9):5701-10.

57) Rosen SD, Bertozzi CR. The selectins and their ligands. Curr Opin Cell Biol. 1994 Oct;6(5):663-73.

58) Phillips ML, Nudelman E, Gaeta FC, Perez M, Singhal AK, Hakomori S, Paulson JC. ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-Lex. Science. 1990 Nov 23;250(4984):1130-2.

59) Zhou Q, Moore KL, Smith DF, Varki A, McEver RP, Cummings RD.The selectin GMP-140 binds to sialylated, fucosylated lactosaminoglycans on both myeloid and nonmyeloid cells. J Cell Biol. 1991 Oct;115(2):557-64.

60) Abbassi O, Kishimoto TK, McIntire LV, Anderson DC, Smith CW. E-selectin supports neutrophil rolling in vitro under conditions of flow. J Clin Invest. 1993 Dec;92(6):2719-30.

61) von Andrian UH, Chambers JD, McEvoy LM, Bargatze RF, Arfors KE, Butcher EC. Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins in vivo. Proc Natl Acad Sci U S A. 1991 Sep 1;88(17):7538-42.

62) Eriksson EE, Xie X, Werr J, Thoren P, Lindbom L. Importance of primary capture and L-selectin-dependent secondary capture in leukocyte accumulation in inflammation and atherosclerosis in vivo. J Exp Med. 2001 Jul 16;194(2):205-18.

63) Janeway's Immunobiology, 7th Edition. Kenneth Murphy, Paul Travers, Mark Walport, Michael Ehrenstein, Claudia Mauri, Allan Mowat, Andrey Shaw. Garland Science, Taylor& Francis Group, LLC. 270 Madison Ave, New York, NY 11016. 2008.

64) Jadhav S, Bochner BS, Konstantopoulos K. Hydrodynamic shear regulates the kinetics and receptor specificity of polymorphonuclear leukocyte-colon carcinoma cell adhesive interactions. J Immunol. 2001 Nov 15;167(10):5986-93.

65) Thorlacius H, Prieto J, Raud J, Gautam N, Patarroyo M, Hedqvist P, Lindbom L. Tumor cell arrest in the microcirculation: lack of evidence for a leukocyte-like rolling adhesive interaction with vascular endothelium in vivo. Clin Immunol Immunopathol. 1997 Apr;83(1):68-76.

66) Takada A, Ohmori K, Yoneda T, Tsuyuoka K, Hasegawa A, Kiso M, Kannagi R.Contribution of carbohydrate antigens sialyl Lewis A and sialyl Lewis X to adhesion of human cancer cells to vascular endothelium. Cancer Res. 1993 Jan 15;53(2):354-61.

67) The Biology of Cancer. Robert A. Weinberg. Garland Science, Taylor& Francis Group, LLC. 270 Madison Ave, New York, NY 11016. 2007.

68) Wang QY, Wu SL, Chen JH, Liu F, Chen HL. Expressions of Lewis antigens in human non-small cell pulmonary cancer and primary liver cancer with different pathological conditions. J Exp Clin Cancer Res. 2003 Sep;22(3):431-40.

69) Yamada N, Chung YS, Maeda K, Sawada T, Ikehara T, Nishino H, Okuno M, Sowa M. Increased expression of sialyl Lewis A and sialyl Lewis X in liver metastases of human colorectal carcinoma. Invasion Metastasis. 1995;15(3-4):95-102.

70) Farmer RW, Richtsmeier WJ, Scher RL. Identification of sialyl Lewis-x in squamous cell carcinoma of the head and neck. Head Neck. 1998 Dec;20(8):726-31.

71) Davidson B, Gotlieb WH, Ben-Baruch G, Kopolovic J, Goldberg I, Nesland JM, Berner A, Bjåmer A, Bryne M. Expression of carbohydrate antigens in advanced-stage ovarian carcinomas and their metastases-A clinicopathologic study. Gynecol Oncol. 2000 Apr;77(1):35-43.

72) Nakagoe T, Fukushima K, Sawai T, Tsuji T, Jibiki M, Nanashima A, Tanaka K, Yamaguchi H, Yasutake T, Ayabe H, Ishikawa H. Increased expression of sialyl Lewis(x) antigen in penetrating growth type A early gastric cancer. J Exp Clin Cancer Res. 2002 Sep;21(3):363-9.

73) Sumikura S, Ishigami S, Natsugoe S, Miyazono F, Tokuda K, Nakajo A, Okumura H, Matsumoto M, Hokita S, Aikou T. Disseminated cancer cells in the blood and expression of sialylated antigen in gastric cancer. Cancer Lett. 2003 Oct 8;200(1):77-83.

74) Kashiwagi H, Kijima H, Dowaki S, Ohtani Y, Tobita K, Yamazaki H, Nakamura M, Ueyama Y, Tanaka M, Inokuchi S, Imaizumi T, Makuuchi H. Clinicopathological significance of sialyl Lex expression in human gallbladder carcinoma. Oncol Rep. 2004 Jun;11(6):1139-43.

75) Renkonen J, Paavonen T, Renkonen R. Endothelial and epithelial expression of sialyl Lewis(x) and sialyl Lewis(a) in lesions of breast carcinoma. Int J Cancer. 1997 Jun 20;74(3):296-300.

76) Wei J, Cui L, Liu F, Fan Y, Lang R, Gu F, Guo X, Tang P, Fu L. E-selectin and Sialyl Lewis X Expression Is Associated With Lymph Node Metastasis of Invasive Micropapillary Carcinoma of the Breast. Int J Surg Pathol. 2008 Jul 8.

77) Kajiwara H, Yasuda M, Kumaki N, Shibayama T, Osamura Y. Expression of carbohydrate antigens (SSEA-1, sialyl-Lewis X, DU-PAN-2 and CA19-9) and E-selectin in urothelial carcinoma of the renal pelvis, ureter, and urinary bladder. Tokai J Exp Clin Med. 2005 Sep;30(3):177-82.

78) Jørgensen T, Berner A, Kaalhus O, Tveter KJ, Danielsen HE, Bryne M. Up-regulation of the oligosaccharide sialyl LewisX: a new prognostic parameter in metastatic prostate cancer. Cancer Res. 1995 May 1;55(9):1817-9.

79) Matsumoto S, Imaeda Y, Umemoto S, Kobayashi K, Suzuki H, Okamoto T. Cimetidine increases survival of colorectal cancer patients with high levels of sialyl Lewis-X and sialyl Lewis-A epitope expression on tumour cells. Br J Cancer. 2002 Jan 21;86(2):161-7.

80) Wenzel CT, Scher RL, Richtsmeier WJ. Adhesion of head and neck squamous cell carcinoma to endothelial cells. The missing links. Arch Otolaryngol Head Neck Surg. 1995 Nov;121(11):1279-86.

81) Fujii Y, Yoshida M, Chien LJ, Kihara K, Kageyama Y, Yasukochi Y, Oshima H. Significance of carbohydrate antigen sialyl-Lewis X, sialyl-Lewis A, and possible unknown ligands to adhesion of human urothelial cancer cells to activated endothelium. Urol Int. 2000;64(3):129-33.

82) Nakashio T, Narita T, Sato M, Akiyama S, Kasai Y, Fujiwara M, Ito K, Takagi H, Kannagi R. The association of metastasis with the expression of adhesion molecules in cell lines derived from human gastric cancer. Anticancer Res. 1997 Jan-Feb;17(1A):293-9.

83) Matsushita Y, Kitajima S, Goto M, Tezuka Y, Sagara M, Imamura H, Tanabe G, Tanaka S, Aikou T, Sato E. Selectins induced by interleukin-1beta on the human liver endothelial cells act as ligands for sialyl Lewis X-expressing human colon cancer cell metastasis. Cancer Lett. 1998 Nov 27;133(2):151-60.

84) Kobayashi K, Matsumoto S, Morishima T, Kawabe T, Okamoto T. Cimetidine inhibits cancer cell adhesion to endothelial cells and prevents metastasis by blocking E-selectin expression. Cancer Res. 2000 Jul 15;60(14):3978-84.

85) Roth J, Taatjes DJ, Weinstein J, Paulson JC, Greenwell P, Watkins WM. Differential subcompartmentation of terminal glycosylation in the Golgi apparatus of intestinal absorptive and goblet cells. J Biol Chem. 1986 Oct 25;261(30):14307-12.

86) van Rooijen JJ, Jeschke U, Kamerling JP, Vliegenthart JF. Expression of N-linked sialyl Le(x) determinants and O-glycans in the carbohydrate moiety of human amniotic fluid transferrin during pregnancy. Glycobiology. 1998 Nov;8(11):1053-64.

87) Mitoma J, Bao X, Petryanik B, Schaerli P, Gauguet JM, Yu SY, Kawashima H, Saito H, Ohtsubo K, Marth JD, Khoo KH, von Andrian UH, Lowe JB, Fukuda M. Critical functions of N-glycans in L-selectin-mediated lymphocyte homing and recruitment. Nat Immunol. 2007 Apr;8(4):409-18.

88) Stroud MR, Handa K, Salyan ME, Ito K, Levery SB, Hakomori S, Reinhold BB, Reinhold VN. Monosialogangliosides of human myelogenous leukemia HL60 cells and normal human leukocytes. 2. Characterization of E-selectin binding fractions, and structural requirements for physiological binding to E-selectin. Biochemistry. 1996 Jan 23;35(3):770-8.

89) Moore KL, Eaton SF, Lyons DE, Lichenstein HS, Cummings RD, McEver RP. The P-selectin glycoprotein ligand from human neutrophils displays sialylated, fucosylated, O-linked poly-N-acetyllactosamine. J Biol Chem. 1994 Sep 16;269(37):23318-27.

90) Larsen GR, Sako D, Ahern TJ, Shaffer M, Erban J, Sajer SA, Gibson RM, Wagner DD, Furie BC, Furie B. P-selectin and E-selectin. Distinct but overlapping leukocyte ligand specificities. J Biol Chem. 1992 Jun 5;267(16):11104-10.

91) Hounsell EF, Davies MJ, Renouf DV. O-linked protein glycosylation structure and function. Glycoconj J. 1996 Feb;13(1):19-26.

92) Kumar R, Camphausen RT, Sullivan FX, Cumming DA. Core2 beta-1,6-N-acetylglucosaminyltransferase enzyme activity is critical for P-selectin glycoprotein ligand-1 binding to P-selectin. Blood. 1996 Nov 15;88(10):3872-9.

93) Snapp KR, Heitzig CE, Ellies LG, Marth JD, Kansas GS. Differential requirements for the O-linked branching enzyme core 2 beta1-6-N-glucosaminyltransferase in biosynthesis of ligands for E-selectin and P-selectin. Blood. 2001 Jun 15;97(12):3806-11.

94) Li F, Wilkins PP, Crawley S, Weinstein J, Cummings RD, McEver RP. Post-translational modifications of recombinant P-selectin glycoprotein ligand-1 required for binding to P- and E-selectin. J Biol Chem. 1996 Feb 9;271(6):3255-64.

95) Matsumoto M, Atarashi K, Umemoto E, Furukawa Y, Shigeta A, Miyasaka M, Hirata T. CD43 functions as a ligand for E-Selectin on activated T cells. J Immunol. 2005 Dec 15;175(12):8042-50.

96) Sperandio M, Thatte A, Foy D, Ellies LG, Marth JD, Ley K. Severe impairment of leukocyte rolling in venules of core 2 glucosaminyltransferase-deficient mice. Blood. 2001 Jun 15;97(12):3812-9.

97) Van den Steen P, Rudd PM, Dwek RA, Opdenakker G. Concepts and principles of O-linked glycosylation. Crit Rev Biochem Mol Biol. 1998;33(3):151-208.

98) Kieffer JD, Fuhlbrigge RC, Armerding D, Robert C, Ferenczi K, Camphausen RT, Kupper TS.Neutrophils, monocytes, and dendritic cells express the same specialized form of PSGL-1 as do skin-homing memory T cells: cutaneous lymphocyte antigen. Biochem Biophys Res Commun. 2001 Jul 20;285(3):577-87.

99) Borges E, Tietz W, Steegmaier M, Moll T, Hallmann R, Hamann A, Vestweber D. P-selectin glycoprotein ligand-1 (PSGL-1) on T helper 1 but not on T helper 2 cells binds to P-selectin and supports migration into inflamed skin. J Exp Med. 1997 Feb 3;185(3):573-8.

100) Asa D, Raycroft L, Ma L, Aeed PA, Kaytes PS, Elhammer AP, Geng JG. The P-selectin glycoprotein ligand functions as a common human leukocyte ligand for P- and E-selectins. J Biol Chem. 1995 May 12;270(19):11662-70.

101) Sako D, Chang XJ, Barone KM, Vachino G, White HM, Shaw G, Veldman GM, Bean KM, Ahern TJ, Furie B. Expression cloning of a functional glycoprotein ligand for P-selectin. Cell. 1993 Dec 17;75(6):1179-86.

102) Lenter M, Levinovitz A, Isenmann S, Vestweber D. Monospecific and common glycoprotein ligands for E- and P-selectin on myeloid cells. J Cell Biol. 1994 Apr;125(2):471-81.

103) Katayama Y, Hidalgo A, Chang J, Peired A, Frenette PS. CD44 is a physiological E- selectin ligand on neutrophils. J Exp Med. 2005 Apr 18;201(8):1183-9.

104) Hidalgo A, Peired AJ, Wild MK, Vestweber D, Frenette PS. Complete identification of E-selectin ligands on neutrophils reveals distinct functions of PSGL-1, ESL-1, and CD44. Immunity. 2007 Apr;26(4):477-89.

105) Togayachi A, Kudo T, Ikehara Y, Iwasaki H, Nishihara S, Andoh T, Higashiyama M, Kodama K, Nakamori S, Narimatsu H. Up-regulation of Lewis enzyme (Fuc-TIII) and plasma-type alpha1,3fucosyltransferase (Fuc-TVI) expression determines the augmented expression of sialyl Lewis x antigen in non-small cell lung cancer. Int J Cancer. 1999 Sep 24;83(1):70-9.

106) Martín-Satué M, Marrugat R, Cancelas JA, Blanco J. Enhanced expression of alpha(1,3)-fucosyltransferase genes correlates with E-selectin-mediated adhesion and metastatic potential of human lung adenocarcinoma cells. Cancer Res. 1998 Apr 1;58(7):1544-50.

107) Barthel SR, Gavino JD, Wiese GK, Jaynes JM, Siddiqui J, Dimitroff CJ. Analysis of glycosyltransferase expression in metastatic prostate cancer cells capable of rolling activity on microvascular endothelial (E)-selectin. Glycobiology. 2008 Oct;18(10):806-17.

108) Fan J, Xie TP, Liu YJ, Hu Y, Gu TJ. The Expression of Human alpha(1,3) Fucosyltransferase in Human Liver Cancer by in situ Hybridization. Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai). 1996;28(5):568-571.

109) Liu F, Chen JH, Zhao JH, Fan J, Chen HL. Lewis Antigens, alpha1,3 Fucosyltransferses and the Metastatic Potential of Human Primary Liver Cancer. Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao (Shanghai). 2000;32(2):115-120.

110) Weston BW, Hiller KM, Mayben JP, Manousos GA, Bendt KM, Liu R, Cusack JC Jr. Expression of human alpha(1,3)fucosyltransferase antisense sequences inhibits selectin-mediated adhesion and liver metastasis of colon carcinoma cells. Cancer Res. 1999 May 1;59(9):2127-35.

111) Aubert M, Panicot-Dubois L, Crotte C, Sbarra V, Lombardo D, Sadoulet MO, Mas E. Peritoneal colonization by human pancreatic cancer cells is inhibited by antisense FUT3 sequence. Int J Cancer. 2000 Nov 15;88(4):558-65.

112) St Hill CA, Bullard KM, Walcheck B. Expression of the high-affinity selectin glycan ligand C2-O-sLeX by colon carcinoma cells. Cancer Lett. 2005 Jan 10;217(1):105-13.

113) Shimodaira K, Nakayama J, Nakamura N, Hasebe O, Katsuyama T, Fukuda M. Carcinoma-associated expression of core 2 beta-1,6-N-acetylglucosaminyltransferase gene in human colorectal cancer: role of O-glycans in tumor progression. Cancer Res. 1997 Dec 1;57(23):5201-6.

114) Machida E, Nakayama J, Amano J, Fukuda M. Clinicopathological significance of core 2 beta1,6-N-acetylglucosaminyltransferase messenger RNA expressed in the pulmonary adenocarcinoma determined by in situ hybridization. Cancer Res. 2001 Mar 1;61(5):2226-31.

115) Walcheck B, Leppanen A, Cummings RD, Knibbs RN, Stoolman LM, Alexander SR, Mattila PE, McEver RP. The monoclonal antibody CHO-131 binds to a core 2 O-glycan terminated with sialyl-Lewis x, which is a functional glycan ligand for P-selectin. Blood. 2002 Jun 1;99(11):4063-9.

116) St Hill CA, Farooqui M, Mitcheltree G, Gulbahce HE, Jessurun J, Cao Q, Walcheck B. The high affinity selectin glycan ligand C2-O-sLex and mRNA transcripts of the core 2 beta-1,6-N-acetylglucosaminyltransferase (C2GnT1) gene are highly expressed in human colorectal adenocarcinomas. BMC Cancer. 2009 Mar 6;9:79.

117) Kuninaka S, Yano T, Yokoyama H, Fukuyama Y, Terazaki Y, Uehara T, Kanematsu T, Asoh H, Ichinose Y. Direct influences of pro-inflammatory cytokines (IL-1beta, TNF-alpha, IL-6) on the proliferation and cell-surface antigen expression of cancer cells. Cytokine. 2000 Jan;12(1):8-11.

118) Delmotte P, Degroote S, Lafitte JJ, Lamblin G, Perini JM, Roussel P. Tumor necrosis factor alpha increases the expression of glycosyltransferases and sulfotransferases responsible for the biosynthesis of sialylated and/or sulfated Lewis x epitopes in the human bronchial mucosa. J Biol Chem. 2002 Jan 4;277(1):424-31.

119) Majuri ML, Niemelä R, Tiisala S, Renkonen O, Renkonen R. Expression and function of alpha 2,3-sialyl- and alpha 1,3/1,4-fucosyltransferases in colon adenocarcinoma cell lines: role in synthesis of E-selectin counter-receptors. Int J Cancer. 1995 Nov 15;63(4):551-9.

120) Ishibashi Y, Inouye Y, Okano T, Taniguchi A. Regulation of sialyl-Lewis x epitope expression by TNF--alpha and EGF in an airway carcinoma cell line. Glycoconj J. 2005 Feb;22(1-2):53-62.

121) Jurisic V, Terzic T, Colic S, Jurisic M. The concentration of TNF-alpha correlate with number of inflammatory cells and degree of vascularization in radicular cysts. Oral Dis. 2008 Oct;14(7):600-5.

122) Whittall T, Wang Y, Kelly CG, Thompson R, Sanderson J, Lomer M, Soon SY, Bergmeier LA, Singh M, Lehner T. Tumour necrosis factor-alpha production stimulated by heat shock protein 70 and its inhibition in circulating dendritic cells and cells eluted from mucosal tissues in Crohn's disease. Clin Exp Immunol. 2006 Mar;143(3):550-9.

123) Nedrebø T, Reed RK, Jonsson R, Berg A, Wiig H. Differential cytokine response in interstitial fluid in skin and serum during experimental inflammation in rats. J Physiol. 2004 Apr 1;556(Pt 1):193-202.

124) Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, Koike M, Inadera H, Matsushima K. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. Clin Cancer Res. 2000 Aug;6(8):3282-9.

125) Evans HG, Gullick NJ, Kelly S, Pitzalis C, Lord GM, Kirkham BW, Taams LS. In vivo activated monocytes from the site of inflammation in humans specifically promote Th17 responses. Proc Natl Acad Sci U S A. 2009 Apr 14;106(15):6232-7.

126) Koukoulis GK, Radosevich JA, Warren WH, Rosen ST, Gould VE. Immunohistochemical analysis of pulmonary and pleural neoplasms with monoclonal antibodies B72.3 and CSLEX-1. Virchows Arch B Cell Pathol Incl Mol Pathol. 1990;58(6):427-33.

127) Kasai K, Kameya T, Okuda T, Terasaki PI, Iwaki Y. Immunohistochemical examination of lung cancers using monoclonal antibodies reacting with sialosylated Lewisx and sialosylated Lewisa. Virchows Arch A Pathol Anat Histopathol. 1986;410(3):253-61.

128) Fukushima K. Expression of Lewis(x), sialylated Lewis(x), Lewis(a), and sialylated Lewis(a) antigens in human lung carcinoma. Tohoku J Exp Med. 1991 Jan;163(1):17-30.

129) Zhu YM, Webster SJ, Flower D, Woll PJ. Interleukin-8/CXCL8 is a growth factor for human lung cancer cells. Br J Cancer. 2004 Nov 29;91(11):1970-6.

130) Luppi F, Longo AM, de Boer WI, Rabe KF, Hiemstra PS. Interleukin-8 stimulates cell proliferation in non-small cell lung cancer through epidermal growth factor receptor transactivation. Lung Cancer. 2007 Apr;56(1):25-33.

131) Masuya D, Huang C, Liu D, Kameyama K, Hayashi E, Yamauchi A, Kobayashi S, Haba R, Yokomise H. The intratumoral expression of vascular endothelial growth factor and interleukin-8 associated with angiogenesis in nonsmall cell lung carcinoma patients. Cancer. 2001 Nov 15;92(10):2628-38.

132) Yuan A, Yang PC, Yu CJ, Chen WJ, Lin FY, Kuo SH, Luh KT. Interleukin-8 messenger ribonucleic acid expression correlates with tumor progression, tumor angiogenesis, patient survival, and timing of relapse in non-small-cell lung cancer. Am J Respir Crit Care Med. 2000 Nov;162(5):1957-63.

133) Singh RK, Gutman M, Radinsky R, Bucana CD, Fidler IJ. Expression of interleukin 8 correlates with the metastatic potential of human melanoma cells in nude mice. Cancer Res. 1994 Jun 15;54(12):3242-7.

134) Smith PW, Liu Y, Siefert SA, Moskaluk CA, Petroni GR, Jones DR. Breast cancer metastasis suppressor 1 (BRMS1) suppresses metastasis and correlates with improved patient survival in non-small cell lung cancer. Cancer Lett. 2009 Apr 18;276(2):196-203.

135) Katayama H, Kitagawa S, Masuyama J, Yaoita H. Polymorphonuclear leukocyte-induced detachment of cultured epidermal carcinoma cells from the substratum. J Invest Dermatol. 1991 Nov;97(5):949-52.

136) Moss NM, Barbolina MV, Liu Y, Sun L, Munshi HG, Stack MS. Ovarian cancer cell detachment and multicellular aggregate formation are regulated by membrane type 1 matrix metalloproteinase: a potential role in I.p. metastatic dissemination. Cancer Res. 2009 Sep 1;69(17):7121-9.

137) Naora H, Montell DJ. Ovarian cancer metastasis: integrating insights from disparate model organisms. Nat Rev Cancer. 2005 May;5(5):355-66.

138) Zöller M, Matzku S. Changes in adhesive properties of tumor cells do not necessarily influence metastasizing capacity. Clin Exp Metastasis. 1989 Mar-Apr;7(2):227-42.

139) Hiraiwa N, Hiraiwa M, Kannagi R. Human T-cell leukemia virus-1 encoded Tax protein transactivates alpha 1->3 fucosyltransferase Fuc-T VII, which synthesizes sialyl Lewis X, a selectin ligand expressed on adult T-cell leukemia cells. Biochem Biophys Res Commun. 1997 Feb 3;231(1):183-6.

140) Koike T, Kimura N, Miyazaki K, Yabuta T, Kumamoto K, Takenoshita S, Chen J, Kobayashi M, Hosokawa M, Taniguchi A, Kojima T, Ishida N, Kawakita M, Yamamoto H, Takematsu H, Suzuki A, Kozutsumi Y, Kannagi R. Hypoxia induces adhesion molecules on cancer cells: A missing link between Warburg effect and induction of selectin-ligand carbohydrates. Proc Natl Acad Sci U S A. 2004 May 25;101(21):8132-7.

141) Aigner S, Sthoeger ZM, Fogel M, Weber E, Zarn J, Ruppert M, Zeller Y, Vestweber D, Stahel R, Sammar M, Altevogt P. CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells. Blood. 1997 May 1;89(9):3385-95.

142) Friederichs J, Zeller Y, Hafezi-Moghadam A, Gröne HJ, Ley K, Altevogt P. The CD24/P-selectin binding pathway initiates lung arrest of human A125 adenocarcinoma cells. Cancer Res. 2000 Dec 1;60(23):6714-22.

143) Napier SL, Healy ZR, Schnaar RL, Konstantopoulos K. Selectin ligand expression regulates the initial vascular interactions of colon carcinoma cells: the roles of CD44v and alternative sialofucosylated selectin ligands. J Biol Chem. 2007 Feb 9;282(6):3433-41.

144) Lim KT, Miyazaki K, Kimura N, Izawa M, Kannagi R. Clinical application of functional glycoproteomics - dissection of glycotopes carried by soluble CD44 variants in sera of patients with cancers. Proteomics. 2008 Aug;8(16):3263-73.

145) Thomas SN, Schnaar RL, Konstantopoulos K. Podocalyxin-like protein is an E-/L-selectin ligand on colon carcinoma cells: comparative biochemical properties of selectin ligands in host and tumor cells. Am J Physiol Cell Physiol. 2009 Mar;296(3):C505-13.

146) Rachagani S, Torres MP, Moniaux N, Batra SK.Current status of mucins in the diagnosis and therapy of cancer. Biofactors. 2009 Nov-Dec;35(6):509-27.

147) Fernandez-Rodriguez J, Dwir O, Alon R, Hansson GC. Tumor cell MUC1 and CD43 are glycosylated differently with sialyl-Lewis a and x epitopes and show variable interactions with E-selectin under physiological flow conditions. Glycoconj J. 2001 Nov-Dec;18(11-12):925-30.

148) Beum PV, Singh J, Burdick M, Hollingsworth MA, Cheng PW. Expression of core 2 beta-1,6-N-acetylglucosaminyltransferase in a human pancreatic cancer cell line results in altered expression of MUC1 tumor-associated epitopes. J Biol Chem. 1999 Aug 27;274(35):24641-8.

149) Kerschner JE. Mucin gene expression in human middle ear epithelium. Laryngoscope. 2007 Sep;117(9):1666-76.

150) Kim YS, Gum J Jr, Brockhausen I. Mucin glycoproteins in neoplasia. Glycoconj J. 1996 Oct;13(5):693-707.

151) Evans CM, Kim K, Tuvim MJ, Dickey BF. Mucus hypersecretion in asthma: causes and effects. Curr Opin Pulm Med. 2009 Jan;15(1):4-11.

152) Descheny L, Gainers ME, Walcheck B, Dimitroff CJ. Ameliorating skin-homing receptors on malignant T cells with a fluorosugar analog of N-acetylglucosamine: P-selectin ligand is a more sensitive target than E-selectin ligand. J Invest Dermatol. 2006 Sep;126(9):2065-73.

153) Ni Z, Walcheck B. Cutaneous lymphocyte-associated antigen (CLA) T cells up-regulate P-selectin ligand expression upon their activation. Clin Immunol. 2009 Nov;133(2):257-64

154) Santa Cruz Biotechnology, Inc. CTAGE1(HECA-452): sc-53514 product information web page. http://www.scbt.com/datasheet-53514-ctage1-heca-452-antibody.html Visited on 11-30-2009.

155) Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G. Inflammation and cancer: how hot is the link? Biochem Pharmacol. 2006 Nov 30;72(11):1605-21.

156) Tazzyman S, Lewis CE, Murdoch C. Neutrophils: key mediators of tumour angiogenesis. Int J Exp Pathol. 2009 Jun;90(3):222-31.

157) Mantovani A. The yin-yang of tumor-associated neutrophils. Cancer Cell. 2009 Sep 8;16(3):173-4.

158) Remedi MM, Donadio AC, Chiabrando GA. Polymorphonuclear cells stimulate the migration and metastatic potential of rat sarcoma cells. Int J Exp Pathol. 2009 Feb;90(1):44-51.

159) Wislez M, Antoine M, Rabbe N, Gounant V, Poulot V, Lavolé A, Fleury-Feith J, Cadranel J. Neutrophils promote aerogenous spread of lung adenocarcinoma with bronchioloalveolar carcinoma features. Clin Cancer Res. 2007 Jun 15;13(12):3518-27.

160) Groux-Degroote S, Krzewinski-Recchi MA, Cazet A, Vincent A, Lehoux S, Lafitte JJ, Van Seuningen I, Delannoy P. IL-6 and IL-8 increase the expression of glycosyltransferases and sulfotransferases involved in the biosynthesis of sialylated and/or sulfated Lewisx epitopes in the human bronchial mucosa. Biochem J. 2008 Feb 15;410(1):213-23.

161) Dong C, Robertson GP. Immunoediting of leukocyte functions within the tumor microenvironment promotes cancer metastasis development. Biorheology. 2009;46(4):265-79.

162) Paesmans M, Sculier JP, Libert P, Bureau G, Dabouis G, Thiriaux J, Michel J, Van Cutsem O, Sergysels R, Mommen P. Prognostic factors for survival in advanced non-small-cell lung cancer: univariate and multivariate analyses including recursive partitioning and amalgamation algorithms in 1,052 patients. The European Lung Cancer Working Party. J Clin Oncol. 1995 May;13(5):1221-30.

163) Chung LW, Baseman A, Assikis V, Zhau HE. Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. J Urol. 2005 Jan;173(1):10-20.