THE UNIVERSITY OF CALGARY

Molecular Mechanisms of Eosinophil Recruitment to Breast Carcinoma Sites

by

Shahina Ali

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

CALGARY, ALBERTA

MARCH, 2001

Abstract

Eosinophils are commonly thought to play a major role in parasitic inflammation, allergic disease and bronchial asthma, however, extensive eosinophilia is also associated with a number of different human cancers, including breast cancer. The clinical data on whether eosinophil recruitment contributes to a poor or favorable prognosis has yet to be settled. The aim of this thesis was to identify the molecular mechanisms involved in eosinophil recruitment to breast carcinoma sites and to reveal a functional role for eosinophils in breast carcinoma sites. Under the influence of TNF-α, a pro-inflammatory cytokine commonly found at breast carcinoma sites, we found that breast carcinoma cells released mediators that can activate and promote eosinophil migration. Supernatant from breast carcinoma cells also induced eosinophils to generate superoxide. Eosinophils were also found to release matrix metalloproteinase-9, a protease that could be involved in remodeling the tumor microenvironment and potentially encourage tumor progression.

Acknowledgements

I would like to thank the following people for their contributions:

To Kamala Patel for giving me a chance in her laboratory and providing supervision and guidance throughout my program.

To the Patel lab, Evelyn Lailey, Shehzad Iqbal, Bao Dang, Susan Cuvelier, Tom Wierzbicki, for making the lab an enjoyable place to work in. I will always remember you guys and your friendship!

To my parents for supporting me for so long and always motivating me to better myself.

To Seema, Saqib, Karim and Rahim for always making me laugh when I needed it.

To Bill for continually being an important part of my life and never allowing the physical distance between us affect our relationship. Thanks for always believing in me and reassuring me of my abilities.

To the Canadian Breast Cancer Foundation, Alberta Chapter and the Canadian Breast Cancer Research Initiative for their financial support.

Dedication

I would like to dedicate this thesis to the most important people in my life,

my family and my future husband, Bill,

who encouraged and supported me through my Masters.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

APMA	*p*-aminophenylmercuric acetate
CCR	CC Chemokine Receptor
CD16-PE	CD16-Phycoerythrin
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular Matrix
ECP	Eosinophil Cationic Protein
EDN	Eosinophil-derived Neurotoxin
EPO	Eosinophil Peroxidase
ELISA	Enzyme Linked Immunosorbent Assay
ENA-78	Epithelial-cell Derived Neutrophil Activating Protein-78
FACS	Fluorescent Activated Cell Sorter
FBS	Fetal Bovine Serum
fMLP	N-Formyl-Methionyl-Leucyl-Phenylalanine
Fn	Fibronectin
GAM-FITC	Goat anti-mouse-Fluorescein Isothiocyanate
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
Gro-α	Growth Related Oncogene-alpha
HBSS	Hanks' Balanced Salt Solution
ICAM-1	Intercellular Adhesion Molecule-1
IL	Interleukin
INF-γ	Interferon-gamma
IP-10	Interferon Inducible Protein-10

LLC	Lewis Lung Carcinoma
LTB_4	Leukotriene B_4
M199	Media 199
M199/A	Media 199 + albumin
mAb	Monoclonal Antibody
MACS	Magnetic Activated Cell Sorter
MAdCAM-1	Mucosal Addressin Cell Adhesion Molecule-1
MCP	Monocyte Chemotactic Factor
MIG	Monokine Induced by Interferon Gamma
MIP-1α	Macrophage Inflammatory Protein-1alpha
MMP	Matrix Metalloproteinase
MT1-MMP	Membrane Type 1-Matrix Metalloproteinase
PAF	Platelet-activating Factor
PMA	Phorbol-12-Myristate-13-Acetate
RANTES	Regulated Upon Activated Normal T Cell Expressed and Secreted
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SEM	Standard Error of the Mean
SDF-1	Stromal Cell-Derived Factor 1
SOD	Superoxide Dismutase
TALs	Tumor-associated Leukocytes
TAMs	Tumor-associated Macrophages
TILs	Tumor-infiltrating Lymphocytes
TGF-β1	Transforming Growth Factor-Beta 1

TNF-α	Tumor Necrosis Factor-alpha
TNFR	Tumor Necrosis Factor Receptor
TTBS	Tween in Tris Buffered Saline
VCAM-1	Vascular Cell Adhesion Molecule-1

Epigraph

"If we knew what it was we were doing, it would not be called research, would it?"

Albert Einstein

Introduction

During an inflammatory process, both leukocytes and soluble mediators interact with one another to remove foreign pathogens, repair wounded tissue and restore normal function (Paul, 1999). Typically, recruitment of leukocytes to a specific tissue site from the bloodstream involves four major steps: rolling, activation, firm adhesion and transmigration. Eosinophil infiltration is associated with parasitic infection, bronchial asthma as well as certain types of cancers, including breast cancer (Samoszuk, 1997). Interestingly, one study showed that as many as 88% of human breast cancer tumors showed evidence of eosinophil infiltration (Samoszuk *et al.*, 1996). This infiltrate was preferentially composed of eosinophils as neutrophils were only seen in 3 of the cases studied (Samoszuk, 1997). The role eosinophils are playing at the tumor site is unknown. Some studies have shown that eosinophil recruitment into tumor sites can be associated with a good prognosis (Bethwaite *et al.*, 1993; Ownby *et al.*, 1983; Lowe *et al.*, 1981).

Selective eosinophil recruitment has also been identified in lungs of asthmatics. With the extensive research in asthma, insight from the mechanisms identified in eosinophil recruitment in asthma may provide a starting point for investigating the mechanisms used in breast cancer. The purpose of the following study was to unveil the mechanisms eosinophils use to extravasate into tumor sites (Figure 1.1). Uncovering the mechanisms that eosinophils use to migrate into tumor sites may lead to the development of new therapeutic strategies, not only in breast cancer but also in the many other cancers that have observed eosinophilia.

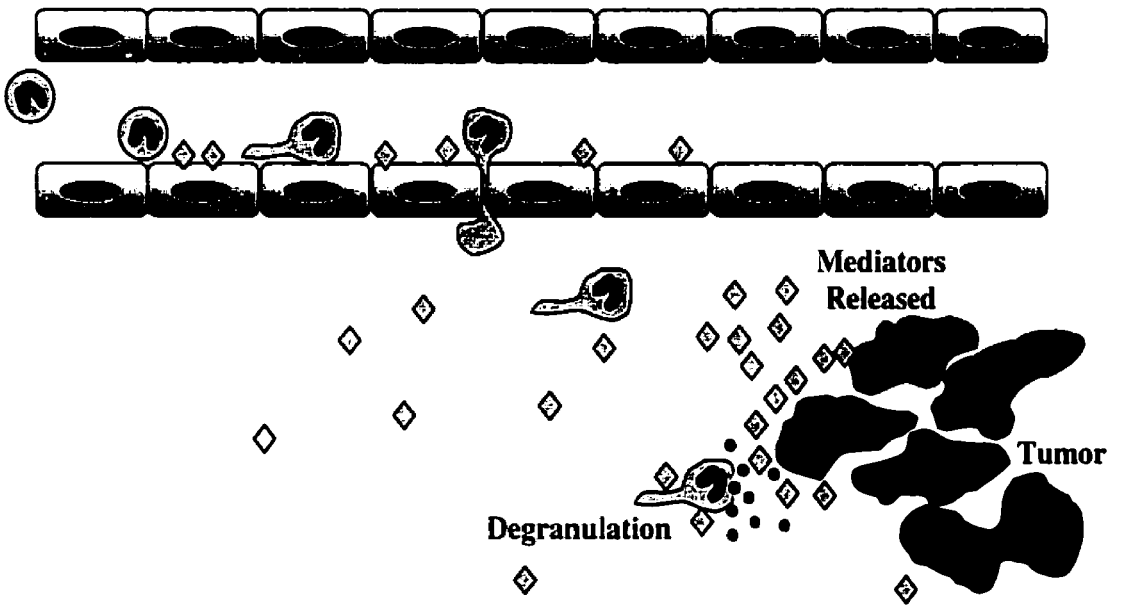


Figure 1.1. Model for eosinophil recruitment and activation to breast carcinoma sites. We hypothesize that TNF-α-stimulated breast carcinoma cells release mediators into the environment that mediate the recruitment of eosinophils from the vasculature. Once recruited, eosinophils are activated by mediators released from the breast carcinoma cells. Activation could include induction of degranulation and the generation of superoxide. ◇ = mediators released from breast carcinoma cells, such as chemokines; • = degranulation products released from eosinophils, such as MMPs.

Background and Significance

1.1 Breast Cancer Biology

Breast cancer is an ancient disease with reference to breast tumors dating as far back as 3000 BC by the Egyptians (Donegan and Spratt, 1995). Breast cancer is the uncontrolled proliferation of epithelial cells lining the ducts or lobules of the breast (Figure 1.2) (Donegan and Spratt, 1995). The skin, subcutaneous tissue and breast tissue comprise the three major structures of the breast (Harris *et al.*, 1996). The breast tissue includes connective tissue, fat, blood vessels, nerves and the lymphatics of the breast (Harris *et al.*, 1996). There are between five and ten major collecting milk ducts that open up into the nipple. Each duct drains a lobe made up of 20 to 40 lobules and each lobule is made of between 10 and 100 alveoli or tubulosaccular secretory units (Harris *et al.*, 1996). The internal mammary and lateral thoracic arteries comprise the major blood supply of the breast (Harris *et al.*, 1996). The majority (97%) of the lymph draining from the breast goes to the axillary nodes and the remaining 3% of the lymph travels to the internal mammary lymph nodes (Hultborn *et al.*, 1955).

There are two types of breast cancer: ductal carcinoma and lobular carcinoma (Harrison, 1994). Ductal carcinoma refers to tumors arising from the epithelial cells of the milk ducts where as lobular carcinoma refers to malignant cells in the lobules of the breast. Pre-cancerous lesions or in situ carcinomas refer to tumors that are malignant, but not invasive. These tumors are referred to as either ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS).

Five thousand years later the cause of breast cancer is still unknown. There are however, some factors that put some women at a greater risk for developing breast cancer

than others. In 1998, one in nine women in Canada had a lifetime probability of developing cancer and of these, one in twenty-five would die from the disease (Statistics Canada, 1998). After the age of 25 the incidence of breast cancer begins to increase and about 78% of all breast cancer cases occur in women after the age of 50 (Canadian Cancer Statistics, 1998). From these observations, it is apparent that age is a major risk factor in developing breast cancer with most of cases occurring after the age of 50. There are three time points in a womens life that can influence her chances of developing breast cancer: age of menarchy, age at full term pregnancy and age of menopause (Harrison, 1998). Women who start their menstrual cycle early in life have an increased lifetime risk of developing breast cancer. In addition, the risk of developing breast cancer in women who have had children by the age of 18 is approximately 30 to 40% the risk of women who never gave birth (Harrison, 1994). The mean age of women at the onset of menopause is 52 and women who enter it by the age of 40 decrease their lifetime risk of developing breast cancer by about 35% (Harrison, 1994). In addition to age, other risk factors include diet, alcohol consumption, radiation exposure and exogenous hormones (Harrison, 1998).

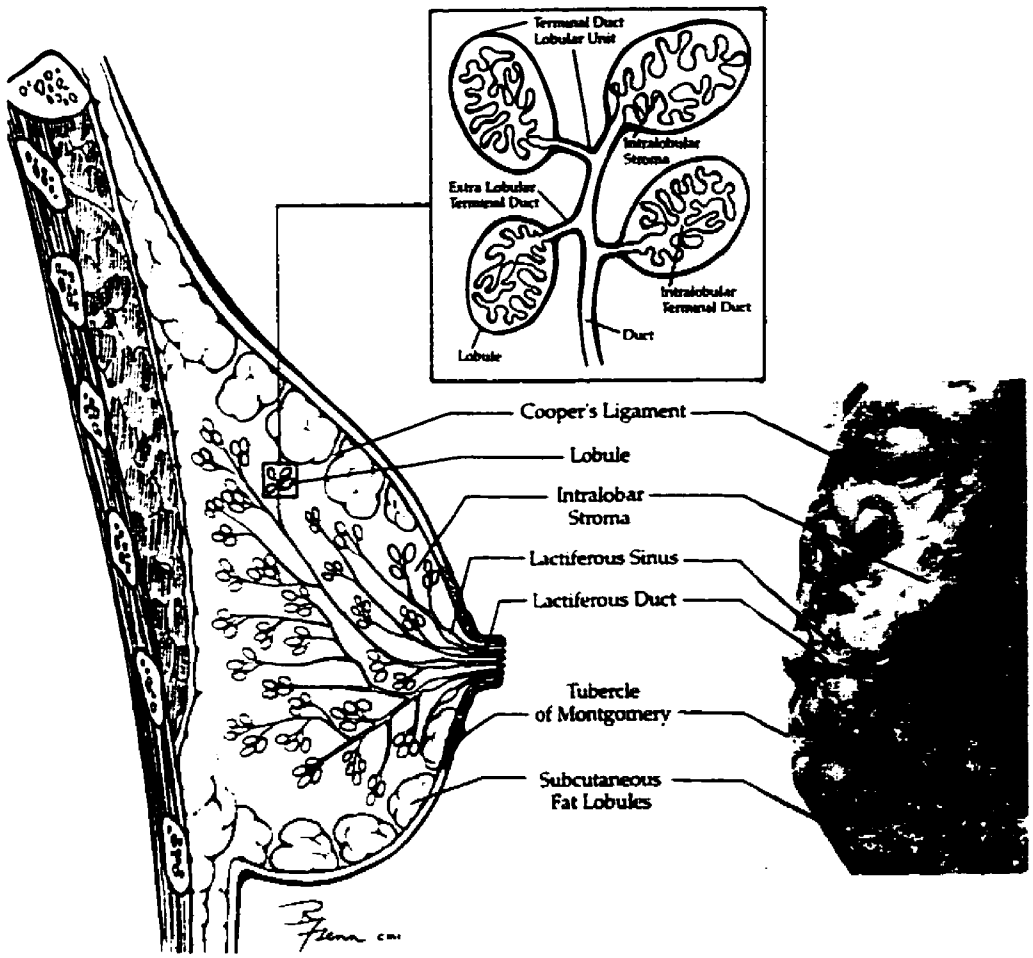


Figure 1.2. Schematic view of the milk ducts and lobules that form the inner architecture of the breast. This figure was taken from Donegan and Spratt (1995). Use of this figure was granted by the copyright owner.

1.2 Leukocytes and Breast Cancer

The immune system is capable of responding to tumors. In breast cancer there is evidence of systemic, regional and intratumoral leukocyte activation which has been associated with a good prognosis (Wong *et al.*, 1998). Human breast cancer sites are infiltrated by a heterogenous population of leukocytes, termed tumor-associated leukocytes (TALs) (O'Sullivan and Lewis, 1994). Variable proportions of T cells, B cells, NK cells, macrophages (O'Sullivan and Lewis, 1994), neutrophils, eosinophils (Samoszuk *et al.*, 1996), fibroblasts and endothelial cells (Lewis *et al.*, 1995) have been identified to accumulate mostly within the stromal elements surrounding tumor islands and occasionally some of these cells are found within tumor nests (reviewed in O'Sullivan and Lewis, 1994). Numerous studies have attempted to find correlations between the composition of the tumor-infiltrating leukocyte population and prognosis of the patient. Unfortunately these studies have not led to any predictable relationships (Wong *et al.*, 1998). T cells and macrophages have been identified as the major leukocyte subsets infiltrating breast tumor sites where as natural killer and B cells are seldomly found. T cells can constitute between 30% and 50% of the total TIL population and macrophages can represent as much as 80% of the total TIL population (reviewed in O'Sullivan and Lewis, 1994).

TALs can communicate with each other and with breast carcinoma cells through the release of cytokines and growth factors (O'Sullivan and Lewis, 1994). Measuring the secretion of cytokines is one method to determine the function of TALs. Using immunohistochemistry, Camp and collegues demonstrated, *in situ*, that tumor-infiltrating lymphocytes (TILs) are able to produce IL-2, TNF-α, IL-4, TGF-β1, IL-10, IFN-γ and

GM-CSF (Camp *et al.*, 1996). TILs associated with malignant breast tumors produced significantly more IL-2 and TNF-α than TILs associated with benign tumors (Camp *et al.*, 1996). Tumor-associated macrophages (TAMs) in breast cancer were shown to be the primary cell that contained immunoreactive TNF-α and 17.5% of invasive breast carcinomas investigated contained TNF-α (Lewis *et al.*, 1995). A recent study looked at the intracellular cytokine profile of three different leukocyte populations from breast cancer patients. Tumor tissue, axillary lymph nodes and peripheral blood lymphocytes were examined and shown to synthesize type 1 cytokines including TNF-α, IFN-γ and IL-2 following stimulation with PMA+I (Wong *et al.*, 1998). It is apparent that the tumor microenvironment is under the influence of many different cytokines and these cytokines can have diverse effects on residing cells.

TNF-α was originally described to be released by bacillus Calmette-Guérin (BCG)-infected mice given endotoxin and serum from these mice was able to cause necrosis of Meth A sarcoma cells *in vivo* (Carswell *et al.*, 1975). Besides inhibiting tumor cell growth, TNF has a wide range of biological activities which are largely dependent on the amount of TNF present. Low doses of TNF act locally and can cause upregulation of adhesion molecules on endothelial cells, stimulation of other leukocytes to secrete chemokines, activation of inflammatory leukocytes to destroy microbes and stimulation of tissue remodeling/angiogenesis (reviewed in Abbas *et al.*, 1997). High doses of TNF can induce fever, stimulate leukocytes and endothelial cells to secrete cytokines into the circulation, impair stem cell division, activate the coagulation system and inhibit angiogenesis (reviewed in Lewis *et al.*, 1995; Abbas *et al.*, 1997). It is

unlikely that TNF-α would reach into the microgram quantities in breast carcinoma sites, therefore local responses of TNF would be predicted (Lewis *et al.*, 1995).

The major cellular source of TNF-α is from mononuclear phagocytes, however under the appropriate stimulation T cells, B cells, NK cells, mast cells, neutrophils, eosinophils, keratinocytes, vascular endothelial cells and smooth muscle cells are also capable of producing TNF-α (reviewed in Paul, 1999). To add to the complex network of TNF-α production, it can also be upregulated by a number of different cytokines including, TNF-α itself, IFN-α, IFN-γ, IL-2, IL-1 and GM-CSF (reviewed in Paul, 1999).

1.3 Eosinophils and Breast Cancer

1.3.1 Tumor-associated eosinophilia

Tumor-associated eosinophilia has been described for many years and has been identified in a number of hematologic and non-hematologic tumors including, acute myelogenous leukemia, Hodgkin's and non-Hodgkin's lymphomas (Samoszuk *et al.*, 1986 and Samoszuk *et al.*, 1987), cervical, lung, skin, stomach, uterine (Bethwaite *et al.*, 1993), breast, large bowel, endometriosis (Blumenthal *et al.*, 2000) and nasopharynx carcinoma (reviewed in Samoszuk, 1997; Lowe *et al.*, 1981). It has been recently shown that eosinophils also play a role in the normal development of the mammary gland (Gouon-Evans *et al.*, 2000). Eosinophils can be detected through a variety of techniques including, hematoxylin and eosin staining, autofluorescence of eosinophil granules and green light epifluorescence (reviewed in Samoszuk, 1997). However, tissue eosinophils often take an ameboid or "medusa" cell morphology that makes their identification difficult in routinely stained specimens (Samoszuk, 1997; Hanker *et al.*, 1980; Hanjer *et al.*, 1981). In addition, eosinophils also go unnoticed in routinely stained tissue sections

by the simple examination of the presence of intact eosinophils. The total degree of eosinophil infiltration in a tissue can be determined through the use of a monoclonal antibody to eosinophil peroxidase (EPO) which will identify both intact and degranulated eosinophils (Samoszuk *et al.*, 1987).

Samoszuk *et al.* published an interesting paper showing occult deposition of eosinophil peroxidase in human breast cancer (Samoszuk *et al.*, 1996). The study included 25 cases of ductal adenocarcinomas, one case of lobular carcinoma of the breast and two specimens from benign breast tissue were used as controls. Eosinophil peroxidase (EPO) deposits were detected in 88% of the breast cancer specimens and 53% of the specimens had extensive deposition of EPO located in the connective tissue stroma around and within the tumor (Figure 1.3 and 1.4). The benign breast tissue had no evidence of EPO deposits. Due to the fact that routinely stained breast cancer specimens do not contain a large eosinophil infiltrate an additional 24 breast cancer specimens were examined by this group. Careful microscopic examination revealed that eosinophils were frequently present and the lack of eosinophils identified in breast cancer specimens was most likely due to the medusa cell morphology making these cells difficult to identify. In the original study, only three of the 26 cases showed extensive myeloperoxidase (MPO) activity which suggests that eosinophil specific recruitment signals were employed. The mechanism responsible for selective deposition of EPO around and within breast tumor sites still remains unclear.

Figure 1.3. Immunohistochemistry detection of EPO in stroma of breast cancers. (A) Cryostat section of breast cancer incubated with the negative control monoclonal antibody. There was no background staining. (B) EPO deposits are seen as dark blue staining at the interface between tumor and adjacent normal fatty tissue. (C) The connective tissue stroma within breast cancers contained abundant deposits of EPO in a fibrillar or ameboid pattern. Nuclear fast red counterstain and nitroblue terazolium substrate: Original magnifications, x250 (A and B) and x400 (C). Figure and legend were taken from Samoszuk (1996). Permission for use of this figure was granted by the American Society for Investigative Pathology.

Figure 1.4. Cryostat sections of breast carcinomas incubated with monoclonal antibody specific for EPO. (A) Abundant deposits of EPO nearly encircle a nest of tumor cells. (B) Densely staining EPO activity was sometimes present within ducts composed of malignant cells but was not detectable in adjacent normal tissue. (C) Granular deposits of EPO were detectable in the stroma between tumor cells, even in the absence of intact eosinophils. Original magnifications, x250 (A and C) and x100 (B). Figure and legend were taken from Samoszuk (1996). Permission for use of this figure was granted by the American Society for Investigative Pathology.

1.3.2 Possible Roles for Eosinophils in the Tissue

Many studies have tried to link tumor eosinophilia with prognosis. Von Wasielewski *et al.* (2000) showed that eosinophil infiltration into the tissue was a sign of poor prognosis in patients with nodular sclerosing Hodgkin's. However, patients with primary breast cancer who had normal or high levels of preoperative eosinophils and/or lymphocytes had significantly less risk of recurrent disease (Ownby *et al.*, 1983). Tissue-associated tissue eosinophilia (TATE) in women with stage IB cervical carcinoma has also been shown to improve survival (Bethwaite *et al.*, 1993). TATE and tumor-associated blood eosinophilia (TABE) may occur separately or together but in general, tumors with TATE alone are associated with a good prognosis and tumors with TABE are associated with a poor prognosis (Lowe *et al.*, 1981).

The identification of eosinophils at tumor sites is known, but the role they play in tumor progression is unclear. Some have speculated that eosinophils may be participating in more of a passive role such as general tissue remodeling rather than being involved in a host immune response (Blumenthal *et al.*, 2000; Samoszuk, 1997). Eosinophils, however, can respond to tumors and they have been shown to possibly have direct tumorcidal activity. This has been most convincingly shown by an experimental approach called the tumor-cytokine transplantation assay where tumor cell lines are transfected with a specific cytokine gene (Tepper *et al.*, 1989). The tumor cells are then transplanted into mice and the effect of the locally expressed cytokine on tumor development and leukocyte infiltration is compared with mock-transfected tumor cells. Tepper *et al.* transfected murine tumor cells to produce IL-4 and found a substantial reduction in tumor growth, in addition to the infiltration of eosinophils and macrophages

to the tumor site (Tepper *et al.*, 1989). Lymphocytes and neutrophils were absent in the inflammatory infiltrate. Interestingly, depletion of eosinophils in these mice restored tumorigenicity despite the continued expression of IL-4 by these tumor cells suggesting the importance of eosinophils in antitumor activity (Tepper *et al.*, 1992). Macrophages were also identified to be recruited to IL-4 producing tumors, however they provided little role in antitumor activity since animals depleted of eosinophils restored tumorigenicity. Tumor cells transfected with IL-2 or IL-7 have also shown eosinophil infiltration correlating with tumor regression (Forni *et al.*, 1987; McBride *et al.*, 1992). In addition, evidence from *in vitro* studies have shown that eosinophils are cytotoxic to tumor cells (Roberts *et al.*, 1991) and that cooperation between eosinophils and macrophages may be involved in controlling tumor growth (Spessotto *et al.*, 1995). Therefore it is possible that eosinophil recruitment into tumor sites is playing a direct role in tumor regression, however the cytokine profile of the tumor has a major role in this process.

Once in the tissue, eosinophils are becoming activated due to the high degree of degranulation seen by Samoszuk (Samoszuk *et al.*, 1996). However, the factors that are modulating this activation is unknown. One goal of this thesis was to identify mediators released by breast carcinoma cells that can cause eosinophil activation such as the release of reactive oxygen species that could potentially contribute to tumor cell lysis.

1.3.3 Tumor Cells and Adhesion Molecules.

There are four main families of vascular cell adhesion molecules: selectins, mucin-like family, Ig superfamily and integrins (reviewed in Gonzalez-Amaro and Sanchez-Madrid, 1999). The selectins are a family of membrane glycoproteins that have

distal-like lectin domains and can bind to sialylated carbohydrate moieties presented by another family of adhesion molecules called mucins. Three proteins L-, E- and P-selectin make up the selectin family. L-selectin is usually present on leukocytes, whereas E-selectin is found on endothelial cells and P-selectin is expressed by both endothelial cells and platelets. Integrins, another adhesion molecule family, are heterodimeric proteins that are expressed by most cell types, but specific subgroups are exclusively present on leukocytes. VCAM-1 and ICAM-1 are members of the immunoglobulin superfamily of adhesion molecules and they are known to bind to integrins.

Breast carcinoma cells are also known to express adhesion molecules. Sialyl Lewis X antigen (SLe^x) (Fukushima *et al.*, 1984), E-cadherin (Gonzalez *et al.*, 1999), ICAM-1 (Budinsky *et al.*, 1997; Ogawa *et al.*, 1998), galectin-3 (Idikio, 1998), CD44 (de la Torre *et al.*, 1995), α_1-$\alpha_5(\beta_1)$-integrins (van der Pluijm *et al.*, 1997), $\alpha_v\beta_3$-integrin (van der Pluijm *et al.*, 1997; Liapis *et al.*, 1996) and β_3, β_4 and β_5 subfamilies (Gui *et al.*, 1995) have been shown to be expressed by breast carcinoma cells. It should be noted that not all breast carcinoma cells express these adhesion molecules due to the heterogeneity of tumor cells. Adhesion molecules expressed on breast carcinoma cells are involved in tumor cell-tumor cell adhesion and in the recruitment process of metastatic cells to secondary sites.

As eosinophils gain access to the tumor microenvironment it is probable that they receive an activation signal to cause the observed degranulation and release of EPO (Samoszuk *et al.*, 1996). Adhesion to breast carcinoma cells may either prime eosinophils or provide the final stop signal that they need to cause degranulation. In previous studies, eosinophil adhesion alone to VCAM-1 can cause the generation of

superoxide anion (O_2^-) (Nagata *et al.*, 1995). It has also been shown that eosinophil adhesion to ICAM-1 in the presence of GM-CSF can cause release of O_2^- and eosinophil-derived neurotoxin (Nagata *et al.*, 1998).

1.4 Selective Eosinophil Recruitment

Eosinophils are bone marrow-derived granulocytes that represent between 1-6% of the total white blood cell population in normal individuals. These relatively low numbers of eosinophils dramatically increase in the tissue of individuals with allergy and asthma (Bousquet *et al.*, 1990; Sedgwick *et al.*, 1991). It is interesting to note that recruitment of eosinophils in chronic allergic inflammation does not include the recruitment of neutrophils (Bradley *et al.*, 1991; Poston *et al.*, 1992), therefore selective recruitment mechanisms exist for eosinophils. Due to the similarity of selective eosinophil recruitment and the extensive research in asthma, insight from the mechanisms identified in eosinophil recruitment in asthma may provide a starting point for investigating the mechanisms used in breast cancer.

Selective recruitment of eosinophils into the tissue is believed to be due to selective adhesion pathways, specific chemotactic factors and enhanced survival by certain cytokines (Wardlaw *et al.*, 1995). Platelet-activating factor (PAF) and C5a are potent chemoattractants for eosinophils (Wardlaw *et al.*, 1986), however, the nonspecific effects of PAF can also cause neutrophil aggregation and chemotaxis (Shaw *et al.*, 1981). IL-3, IL-5 (Yamaguchi *et al.*, 1988) and GM-CSF not only participate in eosinophil differentiation, maturation and survival these cytokines are also chemotactic for eosinophils (reviewed in Wardlaw *et al.*, 1995). Aside from lipid mediators and

cytokines, members of the chemokine family have been shown to promote preferential migration of eosinophils over neutrophils.

1.4.1 Chemokines

Chemokines (for review see Baggiolini, 1998; Mantovani, 1999; Premack and Schall, 1996; Luster, 1998) are low molecular weight proteins, between 8 and 12 kDa in size, and have been traditionally thought to play a role in leukocyte recruitment and activation at sites of inflammation (Proost *et al.*, 1996; Taub, 1996). However the function of chemokines has recently expanded to include tissue morphogenesis, angiogenesis, proliferation and apoptosis of cells (Nickel *et al.*, 1999). Chemokines have also been shown to be capable of activating integrins on a cell, increasing avidity for its assumed ligand (Weber *et al.*, 1995; Weber *et al.*, 1996a). This was specifically seen in eosinophils when RANTES, a CC chemokine, activated VLA-4 on the eosinophil, increasing its adhesiveness to VCAM-1 (Weber *et al.*, 1996b). Chemokines have four conserved cysteine residues that form two disulfide bonds. The family of chemokines is subdivided based on the position of the first two cysteine residues. The CXC or α subfamily of chemokines have one amino acid separating the first two cysteines and the CC or β chemokine family have adjacent cysteine residues. There are chemokines that do not fit into this description. Fractalkine has three intervening amino acids between the first two conserved cysteine residues and lymphotactin contains only three conserved cysteine residues. A list of the CXC and CC chemokine members can be found in Table 1.1 and are matched according to the receptors they bind to. In addition, chemokine receptor expression on human leukocytes can be found in Table 1.2.

Table 1.1. Human Chemokine Receptors and their Ligands.

Receptors	Ligand
CCR1	RANTES, MIP-1α, MCP-2, MCP-3
CCR2	MCP-1, MCP-2, MCP-3, MCP-4
CCR3	Eotaxin, Eotaxin-2, Eotaxin-3, RANTES, MCP-2, MCP-3, MCP-4
CCR4	TARC
CCR5	RANTES, MIP-1α, MIP-1β
CCR6	MIP-3α
CCR7	MIP-3β
CCR8	I-309
CCR9	TECK
CCR10	MCP-1, MCP-3
CXCR1	IL-8, GCP-2
CXCR2	IL-8, GROα,β,γ, NAP-2, ENA78, GCP-2
CXCR3	IP10, Mig, I-TAC
CXCR4	SDF
CX_3CR	Fractalkine
C	Lymphotactin

Table 1.2. Main CC and CXC Chemokine Receptor Expression on Human Leukocytes (Baggiolini *et al.*, 1999 and Baggiolini, 1998)

Leukocyte	Receptor
Eosinophils	CCR1, CCR3, CXCR2 (minor)
Neutrophils	CXCR1, CXCR2, CXCR4
Basophils	CCR1, CCR2, CCR3, CXCR2 (minor)
Monocytes	CCR1, CCR2, CXCR4, CXCR1 (minor), CXCR2 (minor)
Activated Monocytes	CCR5
B Lymphocytes	CCR6, CXCR4
T Lymphocytes	CCR4, CXCR4
Activated T Lymphocytes	CCR1, CCR2, CCR3, CCR5, CCR8, CXCR3
Activated Eosinophils	CCR1, CCR3, CXCR1, CXCR2, CXCR3, CXCR4

A number of eosinophil specific chemokines exist. RANTES is a potent chemoattractant for eosinophils and lymphocytes, but not for neutrophils (Kameyoshi *et al.*, 1992; Alam *et al.*, 1993). RANTES can also upregulate the expression of CD11b/CD18 and cause eosinophil cationic protein (ECP) degranulation in eosinophils (Alam *et al.*, 1993). MCP-3 induces calcium changes and chemotaxis as effectively as RANTES, making these two chemokines the most potent activators of chemotaxis *in vitro* (Dahinden *et al.*, 1994). Although weaker in stimulating eosinophil migration than RANTES, MIP-1α has also been shown activate eosinophils (Rot *et al.*, 1992). MCP-4 has been shown to be a chemoattractant for eosinophils, monocytes and T lymphocytes, but not for neutrophils (Uguccioni *et al.*, 1996). Having similar efficacy as RANTES in chemotaxis, eotaxin is a potent eosinophil-selective chemoattractant (Elsner *et al.*, 1996). Eotaxin-2 and eotaxin-3 have also been shown to selectively recruit eosinophils (Forssmann *et al.*, 1997; Kitaura *et al.*, 1999). All of these chemokines have been shown to preferentially recruit eosinophils and not neutrophils and therefore could be mediating tissue eosinophilia.

1.4.2 Chemokines and Breast Carcinomas

The expression of chemokines at breast carcinoma sites has not been extensively investigated and is largely unknown. The expression of RANTES has been identified to be elevated in sections of breast carcinomas from women with stage II or stage III of the disease compared with sections from stage I patients (Luboshits *et al.*, 1999). The correlation of an increase in RANTES expression with advanced stages of disease suggests a possible role in tumor progression. Expression of MCP-1 has also been shown to be present in breast cancer and increased levels have been correlated with histological

grade, an increase in TAM accumulation and with angiogenic factors such as IL-8 (Valkovic *et al.*, 1998; Ueno *et al.*, 2000). Breast carcinomas have also been shown to express TGFβ1 (Auvinen *et al.*, 1995), TGFβ2 (Auvinen *et al.*, 1995), IL-1α (Kurtzman 1999) and IL-1β (Kurtzman *et al.*, 1999; Jin *et al.*, 1997). Effects of these cytokines and chemokines on eosinophils in the tumor environment is unknown.

1.4.3 Eosinophil Functional Responses

Eosinophils are not only responsive to a number of different mediators, they are also known to produce and secrete lipid mediators, cytokines (Moqbel *et al.*, 1994), chemokines and major granule proteins (Figure 1.5) that can activate other leukocytes, even eosinophils themselves. Major eosinophil receptors are listed in Table 1.3.

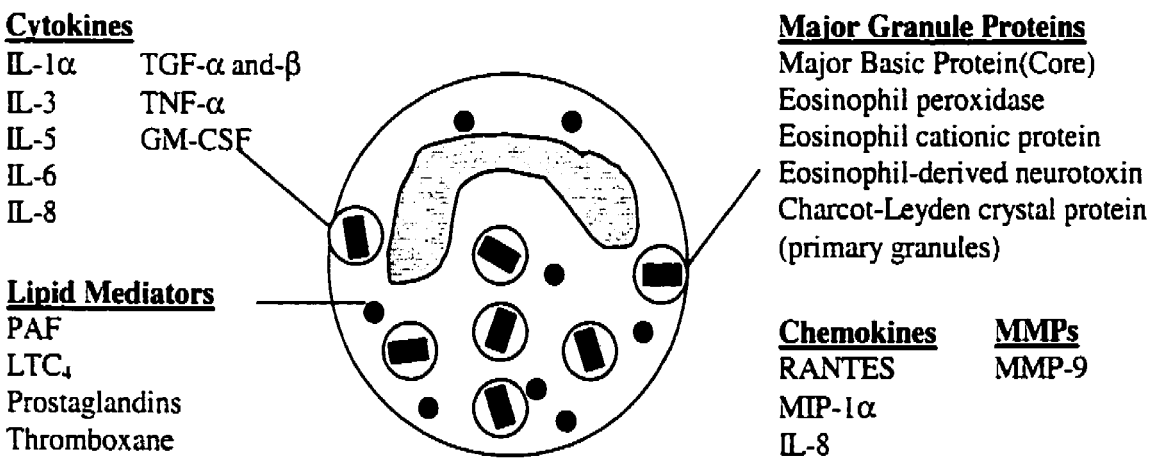


Figure 1.5. Mediators Secreted from Eosinophils

Table 1.3. Receptors found on circulating eosinophils.

Adhesion

$\alpha_4\beta_1$	PECAM
$\alpha_6\beta_1$	ICAM-1
$\alpha_4\beta_7$	ICAM-3
LFA-1 (CD11a/CD18)	L-selectin
Mac-1 (CD11b/CD18)	PSGL-1
p150,95 (CD11c/CD18)	

Chemokines

CCR1
CCR3
CXCR2 (minor)

Cytokines and Growth Factors

IL-2	GM-CSF
IL-3	IFN-γ
IL-5	TNF-α

Lipid Mediators

PAF
LTB_4

One of the most characteristic features of eosinophils are the specific granules located in the cytoplasm. There are about 20 membrane-bound granules per cell and they house four major secretory effector proteins of eosinophils. The dense core is mainly composed of major basic protein which is a cationic protein known to induce histamine release from basophils and superoxide production from neutrophils (Moy *et al.*, 1990). The less dense matrix is composed of ECP, eosinophil-derived neurotoxin (EDN) and EPO which all have some cytotoxic activity (Wardlaw *et al.*, 1995). The specific granules are also the storage site for cytokines produced by eosinophils (Figure 1.5). Upon the appropriate stimulus, activated eosinophils can release their cytotoxic granules

causing tissue damage as well as activating nearby mast cells, neutrophils and macrophages.

Eosinophils can also generate reactive oxygen species, adding to the destructive capabilities of these cells. Although neutrophils are better phagocytes than eosinophils, eosinophils have between three to six times more NADPH oxidase activity (DeChatelet *et al.*, 1977) and two fold more cytochrome b_{558} than neutrophils (Segal *et al.*, 1981). Activated eosinophils can responded by producing superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Once an eosinophil is activated there is an increase in oxygen consumption and superoxide is formed by the donation of an electron from NADPH to molecular oxygen through NADPH oxidase forming a free radical. Hydrogen peroxide can be formed by the dismutation of $O_2^{\cdot}$ and when combined with EPO and a halide can have toxic properties to tumor cells (Jong and Klebanoff, 1980). Secretory IgA (Abu-Ghazaleh *et al.*, 1989) and PAF (Kroegel *et al.*, 1989) have been identified to be strong secretagogues for eosinophils in inducing superoxide. Chemokines have also been shown to induce superoxide generation from eosinophils, such as eotaxin (Elsner *et al.*, 1996), eotaxin-2 (Elsner *et al.*, 1998) and MCP-3 (Elsner *et al.*, 1996).

1.5 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of 17 structurally and functionally related zinc endopeptidases that are capable of degrading extracellular components (reviewed in Westermarck and Kahari, 1999). Members of the MMP family can be further divided into subgroups based on substrate specificity and structure. There are five subgroups consisting of: collagenases, gelatinases, stromelysins, membrane-type-like, and other MMPs. The expression of MMPs can be induced at the transcriptional

level by stimulation from cytokines, growth factors or cell contact to the ECM (reviewed in Kugler, 1999). The activity of MMPs are also tightly regulated based on the fact that most MMPs are released in an inactive form and require the presence of other proteinases for activation. Tissue inhibitors of metalloproteases (TIMPs), naturally occurring inhibitors of MMPs, can also inhibit the actions of MMPs in a 1:1 stoichiometric ratio. MMP activity prevails when the balance of MMPs and TIMPs is disrupted and MMPs are in molar excess to their inhibitors.

Structurally, MMPs contain a propeptide, a catalytic domain and a C-terminal hemopexin domain. The gelatinases, MMP-2 and MMP-9, contain a fibronectin-like domain within the catalytic domain and unique to MMP-9, a collagen V-like domain is present in between the catalytic and hemopexin domain. The fibronectin-like domains can bind gelatin and loss of this domain results in no gelatinolytic activity. Loss of the collagen V-like domain seems to have no consequence on substrate specificity or enzyme activity. Most MMPs are released in an inactive state which is due to the interaction of a cysteine residue in the prodomain with a zinc molecule in the catalytic domain. This conformation renders the enzyme's active site inaccessible. Activation can occur by either disrupting the cysteine-zinc interaction or by cleavage of the prodomain.

MMPs are important proteinases that are involved in many normal physiological processes including, uterine implantation, menstruation, embryonic growth, tissue morphogenesis and tissue repair (Shapiro, 1998). Inappropriate or overexpression of certain MMPs has contributed to the disease progression of rhematoid arthritis, osteoarthritis, multiple sclerosis, cardiovascular disease and to the progression of tumors

(Shapiro, 1998). Tumor invasion, metastasis and angiogenesis have all shown to involve the action of MMPs.

1.5.1 MMPs and Breast Cancer

The correlation between increased MMP activity with tumor progression was first described by Liotta *et al.* when they showed tumor cells with higher metastatic efficiency had the greatest level of type IV collagen-degrading activity (Liotta *et al.*, 1980). Since tumor progression has been shown to correlate with an increase in MMP expression, a number of studies have investigated the profile of MMP expression in breast cancer. Stromal cells surrounding malignant epithelial cells have been shown to express stromelysin-3 in which expression was absent in *in situ* lesions or in stromal cells surrounding normal ducts (Basset *et al.*, 1990). This suggests that a possible role for stromelysin-3 in tumor progression may exist. The expression of MMP-3 (stromelysin-1) in normal mammary epithelial cells has been shown to transform these cells into invasive mesenchymal-like tumors (Sternlicht *et al.*, 1999) MMP-2 and MMP-9 levels have been shown to co-upregulate with endothelial growth factors in human breast carcinomas, indicating a possible role in neovascularization (Kurizaki *et al.*, 1998). Davies *et al.* (1993) also identified MMP-2 and MMP-9 in malignant breast tissue and demonstrated that MMP-9 levels increased with tumor stage. Absent in normal breast tissue, collagenase-3 (MMP-13) expression was found to be localized in stromal cells immediately adjacent to epithelial tumor cells (Freije *et al.*, 1994; Uria *et al.*, 1997). MT1-MMP has been shown to be expressed by tumor cells taken from invasive breast carcinomas (Ueno *et al.*, 1997; Dalberg *et al.*, 2000). It is interesting to note that the major sources of MMPs in breast cancer arise from the stromal cells such as macrophages

and fibroblasts and not from the tumor cells themselves (Kurizaki *et al.*, 1998). Growth factors released from breast carcinoma cells have been shown to increase MMP gene expression in stromal cells (Basset *et al.*, 1990), supporting the idea that tumor cells can communicate with stromal cells and modulate expression of MMPs.

Eosinophils stimulated with IL-5 and PAF are known to release MMP-9 (Okada *et al.*, 1997) and recently it has been shown that TNF-α can also increase *de novo* MMP-9 synthesis and release from eosinophils (Schwingshackl *et al.*, 1999). It is therefore possible for eosinophils to be influenced by mediators present at breast carcinoma sites and contribute to tissue remodeling through the release of MMPs.

1.5.2 Stromal Cells and Tumor Progression

Metastasis is the spread of cancer cells from a primary tumor to distant sites in the body. Tumor cells must detach from the primary tumor, move through the ECM, enter the circulation, adhere to endothelium, extravasate into the tissue and grow to form a new tumor at a new site. In order for this process to occur, tumor cells themselves must have the capability to produce proteases to transverse through the ECM. This observation has lead to skepticism on the role stromal cells have on tumor progression. There is however growing evidence that stromal cells do play a role in tumor progression.

One example of the role stromal cells play in tumor progression was demonstrated by using MMP-2 deficient mice (Itoh *et al.*, 1998). B16-BL6 melanoma and Lewis lung carcinoma (LLC) cells were implanted into wild type and MMP-2 deficient mice. Angiogenesis and tumor growth rates were found to be significantly lower in mutant mice than wild type mice. Gelatin zymograms were carried out on soluble fractions from the tumors. MMP-2 activity was seen in extracts from wild type mice, but not from

mutant mice, that received B16-BL6 melanoma cells, suggesting that MMP-2 was derived from host cells and aiding in tumor progression. Extracts taken from wild type and mutant mice injected with LLC both contained MMP-2 suggesting that LLC cells secrete MMP-2 *in vivo*. However LLC-derived MMP-2 had little effect on tumor progression since MMP-2 deficient mice had slower growing tumors than wild type mice. These studies not only show that MMP-2 plays an important role in tumor progression, but also that host derived, not tumor derived, MMP-2 significantly enhanced tumor progression. As mentioned earlier, stromelysin-3 is only found in stromal cells surrounding epithelial tumor cells (Basset *et al.*, 1990) and has been associated with malignant process. Masson *et al.* (1998) showed that stromelysin-3 deficient mice had lower tumor incidence, smaller tumor size and lost the capacity to promote implantation of MCF-7 cells, a breast cancer cell line. These data suggest that stromal derived stromelysin-3 contributes to tumor progression and supports the idea that stromal cells play a role in tumor progression.

Taken together, stromal cells have the potential of influencing tumor progression. Therefore, it could be hypothesized that extensive eosinophil recruitment could have an impact on tumor progression through the release of MMPs.

Hypothesis & Specific Aims

Hypothesis 1: Breast carcinoma cells stimulated with cytokines will increase the expression of adhesion molecules on their surface.

Objectives:

1. To determine if TNF-α can upregulate the expression of adhesion molecules.
2. To identify if eosinophils adhere to TNF-α stimulated breast carcinoma cells.
3. Which adhesion molecules are responsible for eosinophil adhesion on breast carcinoma cells?

Hypothesis 2: Breast carcinoma cells stimulated with TNF-α will release eosinophil specific chemoattractants and activators.

Objectives:

1. Do TNF-α-stimulated breast carcinoma cells release eosinophil activators and/or chemoattractants?
2. Do breast carcinoma cells release mediator(s) that can stimulate an increase in eosinophil adhesion to these cells?
3. Can these mediator(s) cause a superoxide burst in eosinophils?
4. Characterize and identify the activators and/or chemoattractants released by TNF-α-stimulated breast carcinoma cells.
5. Can breast carcinoma supernatants induce the expression of chemokine receptors not normally found on eosinophils?

Hypothesis 3: Eosinophils found in breast tumor sites are changing the tumor microenvironment through the release and/or activation of MMPs.

Objectives:

1. Can eosinophils release matrix metalloproteinases?
2. Can breast carcinoma supernatants induce MMP-9 release from eosinophils?
3. Can TNF-α alone induce MMP-9 release from eosinophils?
4. Which TNF receptor is responsible for inducing MMP-9 release from eosinophils?
5. Can MMP-9 release from eosinophils stimulated with breast carcinoma cell supernatant be blocked with a TNF receptor antibody?
6. Can RANTES, MCP-1 or IL-8 induce MMP-9 release from eosinophils?
7. Can eosinophil adhesion on breast carcinoma cells stimulate MMP-9 release?

Chapter Two: Materials and Methods

2.1 Reagents and Antibodies

Recombinant human tumor necrosis factor-α (TNF-α) was purchased from R & D Systems (Minneapolis, MN). Recombinant human eotaxin and RANTES were both purchased from Serotec (Oxford, England). Cytochrome c, superoxide dismutase (SOD) and *p*-aminophenylmercuric acetate (APMA) were all from Sigma (Oakville, ON). Hanks' balanced salt solution with Ca^{2+} and Mg^{2+} (HBSS) and Media 199 (M199) were both purchased from Gibco BRL, Life Technologies (Grand Island, NY). All plasticware was from Becton Dickinson (Franklin Lakes, NJ).

Dr. Roy Lobb (Biogen, Boston, MA) kindly provided the anti-human VCAM-1 monoclonal antibody (mAb) 4B9, which was prepared and characterized as described (Carlos *et al.*, 1990). Antibodies against VCAM-1 (1.G11B1), CD49d, eotaxin and RANTES were purchased from Serotec (Oxford, England). Both the anti-human P-selectin G1 and anti-human E-selectin ES1 mAbs were kindly provided by Dr. Rodger McEver (University of Oklahoma, Oklahoma City, OK) and were prepared and characterized as described (Geng *et al.*, 1990; Patel *et al.*, 1995a). The anti-human CD18 mAb was from Coulter-Immunotech (Burlington, ON) and the anti-human MCP-4 mAb was purchased from ID Labs (London, ON). All other antibodies used were purchased from R & D Systems (Minneapolis, MN).

All ELISA kits were purchased from R&D Systems (Minneapolis, MN) with the exception of the MCP-3 ELISA kit which was purchased from Anogen (Mississauga, Ontario, Canada). The gelatinase activity assay kit was from Chemicon International (Temecula, CA).

2.2 Experimental Methods

2.2.1 Eosinophil Isolation

In order to determine possible interactions between breast cancer cells and eosinophils, peripheral blood eosinophils were isolated. The donors used in our experiments were chosen from the general population and were not selected for particular characteristics such as allergy or asthma. Approximately 20 different donors were used throughout the course of this study. Venous blood was obtained from blood donors and sedimented on dextran for one hour. Red blood cells were removed by hypotonic lysis and granulocytes were isolated by density centrifugation on lymphoprep 1077 (Life Technologies, Grand Island, NY). Isolated granulocytes were then labeled with anti-CD3 and anti-CD16 MicroBeads (Miltenyi, Auburn, CA) for 30 minutes. After this time $CD3^+$ (T cells) and $CD16^+$ (mostly neutrophils) cells were magnetically labeled. A type CS Magnetic Activated Cell Separator (MACS) depletion column (Miltenyi, Auburn, CA) was placed in the magnetic field of a MACS separator and positively labeled cells were retained in the column and therefore separated from the desired, unlabeled eosinophils. Greater than 95% eosinophil purity can be achieved with this method. Freshly isolated eosinophils were used in all experiments performed.

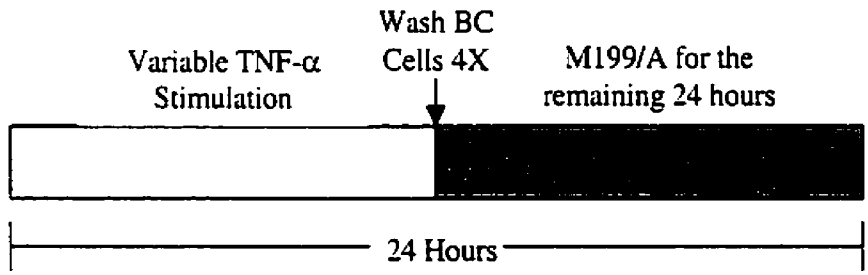
2.2.2 Cell Culture

Isolating and culturing human breast cancer cells from primary tumor cells has been extremely unsuccessful (Ethier *et al.*, 1996). Breast carcinoma cell lines were used in the study as our approach to answering the specific aims. MDA-MB-435S, BT-20, SK-BR-3 and MDA-MB-486 breast carcinoma cells lines were used and were kindly provided by Drs. Patrick Lee and Karl Riabowol (University of Calgary, Calgary, Ab).

MDA-MB-435S, BT-20, SK-BR-3 and MDA-MB-486 cell lines were maintained in DMEM (Dulbecco's Modified Eagle Medium, Life Technologies, Grand Island, NY) with 10% FBS (fetal bovine serum) and antibiotics.

2.2.3 Stimulation of Breast Carcinoma Cells

Confluent monolayers of breast carcinoma cells were washed with M199 once. M199 containing 0.5% Human Serum Albumin (M199/A) or M199 containing 20 ng/mL TNF-α was added to cells and allowed to incubate at 37°C for the indicated times. In time course assays, cells were stimulated with TNF-α for the indicated time, supernatants were harvested, cells were washed four times with M199 and finally M199/A was added for the remaining 24 hour stimulation. Therefore, for the 24 hour time point the TNF-α used to stimulate the cells is still present in the supernatant.



2.2.4 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was used to determine mRNA expression of various adhesion molecules and chemokines in breast carcinoma cells. Confluent monolayers of breast carcinoma cell lines were stimulated with either M199/A alone or M199/A containing 20 ng/mL TNF-α for 4 or 24 hours. Supernatants were removed and cells were washed once with M199 and immediately lysed in TRIzol reagent (Life Technologies, Grand Island, NY).

Total RNA was extracted according to manufacturer's instructions. RNA concentrations and purity were determined by measuring the absorbance at 280 and 260 nm by using either a GeneQuant spectrophotometer (Pharmacia, Pascataway, NJ) or a SPECTRAmax PLUS spectrophotometer (Molecular Devices, Sunnyvale, CA). Reverse transcription (RT) of the isolated RNA using Superscript II (Life Technologies) was performed according to manufacturer's instructions. PCR was performed with Qiagen Master Mix using 2 μl of the RT reaction as template cDNA and the appropriate primer pairs. Primers for P- and E-selectin, VCAM-1, ICAM-1, RANTES, MCP-1, MCP-3, MCP-4, Eotaxin, Eotaxin-2, Eotaxin-3, MIP-1α, IL-8, Gro-α, PF-4, SDF-1, ENA-78, IP-10 and MIG were used to assay for the presence of mRNA in the breast carcinoma cell lines. β-actin is a housekeeping gene and was used as a positive control to ensure that the PCR reaction was successful. All CXC chemokine primers were designed and kindly provided by Steve Kerfoot (University of Calgary, Calgary, AB). Table 1.4 summarizes the primer pairs used.

Table 1.4. Sequences of primer pairs for adhesion molecules, CC and CXC chemokines.

Primer	Sense (5' to 3')	Anti-Sense (5' to 3')	Band Size (bp)
β-Actin	CATGGATGATGATATCGCCG	ACAGCCTGGATAGCAACGTA	417
P-Selectin	TGAAGAAAAAGCACGCATTG	AGCGGCTCACACGAAATAG	714
E-Selectin	AACGAGAAGCCAACGTGTAA	CTTTTGGTAGCTTCCGTCTG	935
ICAM-1	GGCAAGAACCTTACCCTACG	GAGACCTCTGGCTTCGTCAG	586
VCAM-1	AGGGGACCACATCTACGCT	ACAGAGCTCCCATTCACGA	1043
RANTES	TCATTGCTACTGCCCTCTGC	GCTCGTCGTGGTCAGAATCT	336
MCP-1	ATAGCAGCCACTTCATTCC	TTCCCCAAGTCTCTGTATCT	466
MCP-3	ATGCTTGTCCCTTTTGATCG	GGGGACAGTGGCTACTGG	325
MCP-4	TGAAATCAAGCTGGAGTACG	ACCCCTGGGAACCGAATAC	300
Eotaxin	CCAAGGATATCTGTGCCGA	GTGACAGGGGATCGAATTG	350
Eotaxin-2	ACATCATCCCTACGGGCTC	GTTTGGTTGCCAGGATATCT	300
Eotaxin-3	AGTCTCCACCTTGGAACTG	AGTCACAATTGTTTCGGAGTT	199
MIP-1α	ACTGCTGCCCTTGCTGTC	GCAACAACCAGTCCATAGAA	350
IL-8	AGGAAGAAACCACCGGAAG	TTCTCCCGTGCAATATCTAG	625
Gro-α	CCTGCATCCCCCATAGTTA	TCCCCTGCCTTCACAATGA	502
PF-4	TCCTGCCACTTGTGGTCG	GCAAATGCACACACGTAGG	273
SDF-1	AGACAAGTGTGCATTGACCCG	GGCCTTAGTCTAAGCTGCTACGTGT	320
ENA-78	CGGATCCTCCAATCTTCG	GCTGAAAGCTTAAGCGCC	635
IP-10	GCACCATGAATCAAACTGCGA	GGAAGCACTGCATCGATTTTG	334
MIG	CGAAAATCTCAACGTTCTCG	CTCTGGAGGCTGCAGTACG	399

2.2.5 Western Blots

Determination of protein in breast carcinoma cells was accomplished by western blot as previously described (Patel *et al.*, 1995b). Briefly, breast carcinoma cells were grown to confluence in 6-well plates and then stimulated with either M199/A or M199/A containing 20 ng/mL of TNF-α for 4 or 24 hours. Cells were harvested by treatment with Versene (Life Technologies, Grand Island, NY) and pelleted for 5 minutes at 310xg. Cells were then lysed in lysis buffer containing 1% Triton X-100, 5 mM EDTA for 30 minutes on ice and then spun to 20,000xg for 10 minutes at 4°C. Supernatants were carefully transferred to a new tube and resuspended in 1X laemeli's sample buffer. Proteins were separated on a 7.5% SDS-PAGE for 1 hour at 200V and later transferred to polyvinylidene difluoride membrane for 1 hour at 200 milliamps. Membranes were blocked in 5% skim milk powder in 0.05% Tween in Tris Base Saline (TBS) (TTBS) anywhere from 2 hours to overnight. Primary antibody was used at optimal concentrations of 2 μg/ml in 5% skim milk powder in TTBS and incubation times varied from 2 hours to overnight. All overnight incubations occurred at 4°C. Multiple VCAM-1 antibodies (4B9, 4B2, 1.G11B1) were used for western blot analysis, however the only antibody that recognized VCAM-1 from breast carcinoma cells was clone 1.G11B1. Both 4B9 and 4B2 antibodies block binding to domain 1 on VCAM-1, however the binding site for 1.G11B1 is unknown. Unbound primary antibody was removed by three 10 minute washes in TTBS. The secondary antibody, horseradish peroxidase-conjugated anti-mouse IgG antibody (GAM-HRP) (Amersham, Buckinghamshire, England), was added in a 1:2000 dilution for 1 hour at room temperature. Membranes were washed

again in TTBS, developed by the addition of ECL reagent (Amersham, Life Science, Buckinghamshire, UK) and visualized by chemiluminescence on film.

2.2.6 Surface Expression ELISA

Surface expression of adhesion molecules on control and TNF-α breast carcinoma cells were determined by a modified ELISA. Confluent monolayers of breast carcinoma cells were washed with M199 and stimulated for 24 hours with M199/A alone or M199/A containing 20ng/mL of TNF-α. After the cells were washed gently with HBSS, they were fixed with 1% paraformaldehyde for 30 mins at 4°C. The cells were washed again with HBSS and nonspecific binding sites were blocked with a 1% solution of Human Serum Albumin (HSA) in HBSS (HBSS/A) for at least 30 mins at 37 °C. Primary antibody for VCAM-1 (1.G11B1), ICAM-1, P-Selectin and E-Selectin were diluted to 2 μg/ml in 1% HBSS/A and incubated for 30 mins at 37 °C. The breast carcinoma cells were carefully washed three times with HBSS to remove any unbound antibody. A 1:1000 dilution of GAM-HRP antibody was added and incubated for 30 mins at 37 °C. Cells were washed once more and antibody binding was determined using a trimethyl-benzadine one-step substrate and quantified by reading absorbance at 450 nm. An isotype matched non-immune IgG was used to determine background antibody binding and was subtracted from all absorbance readings.

2.2.7 Static Adhesion to Breast Carcinoma Cells

Static adhesion assays on breast carcinoma cells were used to determine if eosinophils were capable of binding to breast carcinoma cells. Breast carcinoma cells were grown in 24-well plates and treated with M199/A alone or TNF-α for 24 hours. Supernatants were either removed or they were left on the cells. The first experimental

approach would identify whether eosinophils can bind to breast carcinoma cells and the second approach would determine if breast carcinoma cells release mediator(s) that can stimulate an increase in eosinophil adhesion on these cells.

In experiments where the supernatant was removed, breast carcinoma cells were washed once with warm HBSS and 250 µl eosinophils ($2x10^6$ eos/ml) were added to each well and incubated at 37°C. After 5, 10 or 15 minutes, each well was washed twice with warm HBSS to remove both non-adherent and loosely adherent eosinophils. Adherent eosinophils were counted using a microscope and NIH image. For each well, three random fields were counted and averaged.

To determine whether breast carcinoma cells release mediators to increase eosinophil adhesion, cell lines were grown to confluence in 24-well plates and stimulated with 250 µl of either M199/A alone or 20 ng/ml TNF-α for 24 hours. Supernatants from half of the control and TNF-α stimulated wells were removed, washed once and 250 µl of 0.5% HBSS/A was added to each well. The other half of the control and TNF-α stimulated wells still contained the stimulation buffer. To each well, 250 µl eosinophils ($2x10^6$ eos/ml) were added and incubated for 37°C. After either 5, 10 or 15 minutes, each well was washed twice with warm HBSS to remove both non-adherent and loosely adherent eosinophils. Quantification of adherent eosinophils was performed in the same manner as above.

To determine which adhesion molecules were playing a role in the adhesion between eosinophils and breast carcinoma cells, functional blocking antibodies were used against VCAM-1 (1.G11B1), ICAM-1, α_4- and β_2-integrins. All antibodies were used at a final concentration of 5 µg/ml. Breast carcinoma cells were pre-treated with VCAM-1

and ICAM-1 mAbs for 15 minutes at 37°C. At the same time eosinophils ($2x10^6$ eos/ml) were pre-treated with either α_4 or β_2 mAbs for 15 minutes at room temperature. Eosinophils were added in the same manner as above and incubated for 15 minutes at 37°C. The remainder of the assay was carried out in the same manner as outlined above.

2.2.8 ELISA kits

Mediators released by TNF-α-stimulated breast carcinoma cells were identified by ELISA. Supernatants were harvested from breast carcinoma cells stimulated with M199/A or TNF-α for 24 hours and were then assayed by ELISA. Quantification of the mediator in the supernatant was determined by monitoring the conversion of a colormetric substrate. All manufacturer's instructions were followed.

2.2.9 Eosinophil Static Adhesion Assay to Matrix Proteins

This assay was used to determine if there are mediator(s) present in the supernatants from breast carcinoma cells that could stimulate eosinophil adhesion to either gelatin or fibronectin. Either 24- or 48-well plates were coated with 0.2% gelatin or 25 µg/ml fibronectin for 2 hours at 37 °C. Wells were washed once with warm HBSS and 225 µl eosinophils ($1-2x10^6$ eos/ml) were added to each well. 25 µl of either control or TNF-α stimulated breast carcinoma supernatants were added to the appropriate wells and plates were incubated for 10 mins at 37 °C. Wells were washed twice with warm HBSS and adherent eosinophils were quantified by assaying for eosinophil peroxidase activity (Hakansson *et al.*, 1994). Background adhesion was quantified by using HBSS/A alone and 10^{-7} M fMLP served as a positive control.

Various mAbs were used to pre-treat either eosinophils or breast carcinoma supernatants to determine if these receptors or mediators play a major role in eosinophil

adhesion on fibronectin. Supernatants were treated with 50 μg/ml of antibodies against MCP-1, MCP-3, RANTES or eotaxin. These supernatants were diluted 1:10 into the eosinophils bringing the final concentration to 5 μg/ml. Eosinophils were treated with 50 μg/ml of antibodies against α_4- and β_2-integrins, CCR3 and CXCR3.

2.2.10 Transmigration Assay

A transmigration assay was used to establish if breast carcinoma cells release chemotactic mediators for eosinophils. The membrane and plate of a 3 μm pore transwell dish (Costar) was coated with 25 μg/ml fibronectin for 2 hours at 37 °C. Either control or TNF-α stimulated breast carcinoma supernatants was added to the plate in a 1:10 dilution in HBSS/A and eosinophils ($4x10^6$ eos/ml) were added to the pre-coated transwell. Cells were allowed to transmigrate for 90 minutes at 37 °C. Eosinophils that migrated through the membrane into the plate were counted manually with a hemocytometer. HBSS/A was used as a negative control, 10^{-7} M fMLP served as the positive control and TNF-α alone was the internal control.

2.2.11 Superoxide Burst

Supernatants from control and TNF-α-treated breast carcinoma supernatants were used to stimulated eosinophils for varying times. Respiratory burst was determined by the ability of these activated eosinophils to reduce ferric (Fe^{+3}) cytochrome c to the ferrous form (Fe^{+2}). Ninety-six well plates were coated with 1% HBSS/A for a minimum of 2 hours at 37°C. A 2.4 mg/ml solution of cytochrome c was made in a solution of HBSS supplemented with 10 mM HEPES. Eosinophils were washed twice with warm HBSS and diluted to $0.5x10^6$ eos/ml in the cytochrome c solution. Half of this solution was treated with 40 μg/ml of superoxide dismutase (SOD) which inhibits the superoxide

anion from reducing cytochrome c. Each stimulus was paired with a SOD control. One hundred microliters of eosinophils + cytochrome c were added to each well followed by 100 µl of stimulus. Plates were read at 550 nm for 2 hours at 30 minute intervals. Plates were maintained at 37°C in the dark for the entire time course. Media alone served as the negative control and 10^{-6} M fMLP served as the positive control. The SpectraMax Plus (Molecular Devices) plate reader was used for all readings. The following equation was used to determine the nanomoles of cytochrome c reduced/10^6 cells.

Beer-Lambert Law: $A = \varepsilon cb$

Where: A = Absorbance

ε = Extinction Coefficent

c = Concentration

b = Pathlength

For cytochrome c: $\varepsilon = 21.1 \times 10^3$ $Lmol^{-1}cm^{-1}$

b for 200 µl = 0.497 cm

$\Delta A = A_T - A_{T=0}$

Final calculation: nmoles of cytochrome c reduced/10^6 cells = $[19.1(A_T - A_{T=0})] / 0.05$

Experiments were also performed to determine if TNFR1 or CCR3 receptors were involved in the activation of eosinophils to release superoxide. Eosinophils were pre-treated with either an anti-human TNFR1 blocking mAb (50 µg/ml), anti-human CCR3 mAb (5 µg/ml) or an non-immune IgG_1 mAb (50 µg/ml) for 15 minutes. Pre-treated eosinophils were used in the same manner as described above.

2.2.12 Gelatin Zymography

The presence of gelatinases, MMP-2 and MMP-9, were determined using zymography. Briefly, eosinophils ($1x10^6$ eosinophils) were stimulated with breast carcinoma supernatant for 30 minutes at room temperature in a 1:10 dilution. Cells were centrifuged for 5 mins at 4 °C. The supernatant was transferred to a new tube and non-reducing Laemmli's buffer was added to cell-free supernatants and the proteins were immediately separated on a 7.5% SDS-PAGE containing 1 mg/ml of gelatin. Following electrophoresis, the gel was washed once in rinse buffer (50 mM Tris, 10 mM $CaCl_2$, 1μM $ZnCl_2$ and 2.5% Triton X-100) for 15 minutes and further incubated overnight in rinse buffer at room temperature. The gel was then rinsed with dH_2O and incubated in reaction buffer (50 mM Tris, 10 mM $CaCl_2$ and 1μM $ZnCl_2$) for 24 hours at 37°C. Finally, the gel was stained with Coomassie blue R-250 (2.5 mg/ml in 30% isopropanol and 10% acetic acid) for 4 hours and then destained in 10% isopropanol and 10% acetic acid until clear bands were visible. Presence of gelatinases was determined by areas of the gel that were digested and therefore not stained with Coomassie blue. A Fluor-S-MAX™ MultiImager (Bio-Rad Laboratories, Hercules, CA) was used to visualize the gels and the images were then inverted to show dark bands on a clear background. Band intensities were analyzed by densitometry.

2.2.13 Gelatinase Activity Assay

A gelatinase activity assay kit was used to determine if eosinophils could secrete active MMP-2 and/or MMP-9 upon stimulation with breast carcinoma supernatants. This assay can accurately detect gelatinase activity to less than 5 ng/ml. All manufacture's

instructions were followed. Briefly, the sample was mixed with a biotinylated gelatinase substrate and any active MMP-2 and/or MMP-9 would cleave and shorten the biotinylated gelatin molecules. A portion of this mixture was added to a biotin binding plate and allowed to bind. A streptavidin-enzyme conjugate was added which would bind to the biotinylated gelatin molecules. Samples with no gelatinase activity would produce an intense color indicating that there was no cleavage of the biotinylated gelatinase substrate.

2.2.14 Activation of MMP-9

MMP's can be activated by a number of different mechanisms. *p*-aminophenylmercuric acetate (APMA) was used to activate the pro-MMP-9 released from eosinophils. Eosinophils ($1x10^6$) were stimulated for 30 mins with 2 ng/mL TNF-α. Cells were pelleted and the supernatant transferred to a new tube. Half of this supernatant was activated by 0.5 mM APMA for 1 hour at 37°C. Samples were resuspended in 1X laemeli's sample buffer and gelatin zymography was performed.

2.2.15 MMP-9 Release from Adherent Eosinophils

Eosinophils ($1x10^6$) were added to confluent monolayers of breast carcinoma cells stimulated with M199/A or TNF-α for 24 hours. Adhesion was performed in the presence and absence of supernatant. In experiments where the supernatant was removed, breast carcinoma cells were washed 4 times with HBSS and an equivalent amount of HBSS/A was added to each well. Eosinophils were allowed to adhere for 2 hours at 37°C. Cells were collected and pelleted. Supernatant was transferred to a new tube and gelatin zymography was performed. To determine whether adhesion molecule interaction was necessary for the MMP-9 release, blocking antibodies to α_4- and/or β_2-

integrins were used. Eosinophils were pre-treated with 50 μg/ml anti-α_4, anti-β_2 or both for 15 minutes prior to the static adhesion assay. Antibodies were used in 10-fold excess. Samples were collected in the same manner as above.

2.3 Statistics

All experiments were performed at least three times, unless otherwise specified, and the data are presented as the mean ± SEM of those replicates. Statistical differences between experimental groups were evaluated using either paired or unpaired Student's t-test. P values < 0.05 were considered significant.

Chapter Three: Specific Aim 1

Hypothesis: Breast carcinoma cells stimulated with cytokines will increase the expression of adhesion molecules on their surface.

Objectives:

1. To determine if TNF-α can upregulate the expression of adhesion molecules.
2. To identify if eosinophils adhere to TNF-α-stimulated breast carcinoma cells.
3. Which adhesion molecules are responsible for eosinophil adhesion on breast carcinoma cells?

3.1 Results

Breast carcinoma cell lines express ICAM-1 and VCAM-1 mRNA. In this study, four human breast carcinoma cell lines were examined under baseline conditions or after stimulation with TNF-α, since this cytokine has been previously shown to be found at breast tumor sites (Camp *et al.*, 1996, Lewis *et al.*, 1995; Wong *et al.*, 1998). Treatment of breast carcinoma cells with TNF-α did not cause a loss in cell viability, as assessed by trypan blue exclusion (data not shown). We first used RT-PCR to determine if these cells synthesized mRNA for various adhesion molecules. Stimulated cells were washed once and immediately lysed in TRIzol reagent. Total RNA was extracted and reverse transcription was performed. The cDNA was subsequently used in a PCR reaction where appropriate primer pairs were used. MDA-MB-435S and MDA-MB-468 cells both constitutively expressed high levels of mRNA for ICAM-1 (Figure 3.1). Stimulation with TNF-α did not increase this expression (Figure 3.1). In contrast, BT-20 and SK-BR-3 cells expressed only nominal levels of ICAM-1 under baseline conditions,

but stimulation with TNF-α for 24 hours dramatically increased mRNA expression (Figure 3.1). MDA-MB-468 cells also expressed high constitutive levels of mRNA for VCAM-1; whereas, all of the other cell lines had only low levels of VCAM-1 message with TNF-α stimulation dramatically increasing expression (Figure 3.1). None of the cell lines examined showed clear mRNA expression for either P-selectin or E-selectin (data not shown).

ICAM-1 and VCAM-1 proteins are present in breast carcinoma cell lines. mRNA expression of these adhesion molecules led us to examine protein expression. In these experiments, cell lines were treated with buffer alone or with buffer containing TNF-α for either 4 or 24 hours. The cells were then scraped and the Triton X-100 soluble proteins were separated by SDS-PAGE and Western blotted using the appropriate mAbs. There was robust, constitutive expression of both ICAM-1 and VCAM-1 in MDA-MB-435S and MDA-MB-468 cells but not on the other cell lines examined (Figure 3.2). Treatment of MDA-MB-435S and MDA-MB-468 cells with TNF-α led to a slight increase in the expression of both ICAM-1 and VCAM-1 (Figure 3.2). Although BT-20 and SK-BR-3 cells did show low level constitutive expression of mRNA for ICAM-1 and VCAM-1, neither protein was detected in these cell lines under control conditions (Figure 3.2). Stimulation of these cells with TNF-α for 24 hours dramatically induced the expression of both ICAM-1 and VCAM-1 by BT-20 and SK-BR-3 cells (Figure 3.2). These data represent the first description of VCAM-1 expression by tumor cell lines of epithelial origin.

VCAM-1 and ICAM-1 are present on the surface of breast carcinoma cells. Figure 3.2 shows that breast carcinoma cells can express constitutive or induced protein

for ICAM-1 and VCAM-1; however, Western blots cannot assess whether these proteins are present on the cell surface. This issue was addressed by using a modified ELISA technique to evaluate adhesion molecule surface expression. Similar to the Western blotting, both ICAM-1 and VCAM-1 were constitutively expressed on the surface of the MDA-MB-435S cells (Figure 3.3A). Although Western blotting showed an increase in ICAM-1 and VCAM-1 expression with TNF-α treatment, no significant change in the surface expression of these proteins was observed after TNF-α stimulation (Figure 3.3A). Neither ICAM-1 or VCAM-1 were constitutively expressed on the surface of BT-20 cells (Figure 3.3B). Instead there was a dramatic increase in the surface expression of both of these proteins when cells were stimulated with TNF-α (Figure 3.3B). These data were consistent with the Western blotting results.

Many investigators have shown increased levels of soluble adhesion proteins present in the serum of patients with certain types of cancer as well as during other inflammatory diseases (Liu *et al.*, 1998; Velikova *et al.*, 1997). This suggests that adhesion proteins can be released or shed, as opposed to exclusively being expressed on the cell surface after synthesis. The supernatants from these breast carcinoma cells were assayed by using either ELISA techniques or Western blotting. There was no evidence that either VCAM-1 or ICAM-1 was being released/shed from these cells (data not shown).

Eosinophils bind to breast carcinoma cells. We next wanted to determine if these breast carcinoma cell lines were able to support eosinophil adhesion. Freshly isolated eosinophils were added to either control or TNF-α-treated carcinoma cells. After 15 minutes the nonadherent and loosely adherent eosinophils were removed and the

adherent eosinophils were counted using microscopy. Eosinophils constitutively bound both to MDA-MB-435S cells and to the surrounding matrix. Stimulation with TNF-α resulted in a modest, but significant increase in eosinophil adhesion (Figure 3.4A). In contrast, few eosinophils bound to unstimulated BT-20 cells (Figure 3.4B); however, there was a significant increase in eosinophil adhesion to BT-20 cells stimulated with TNF-α with virtually all adhesion occurring directly on the cells (Figure 3.4B). Overall, more eosinophils bound to MDA-MB-435S than BT-20 cells which could be due to the higher expression of ICAM-1 and VCAM-1 on MDA-MB-435S cells. Thus eosinophils can bind directly to breast carcinoma cells.

Eosinophil adhesion to MDA-MB-435S cells requires α_4-integrins. We next used a series of blocking mAbs to identify the adhesion molecules involved in eosinophil adhesion to these cell lines. MDA-MB-435S cells were treated with buffer alone or TNF-α for 24 hours and were then pretreated for 15 minutes at 37°C with 5 μg/ml of the specified mAbs directed against ICAM-1 or VCAM-1. Alternatively, eosinophils were incubated with α_4-integrin mAb or β_2-integrin mAb for 15 minutes before addition to the cell monolayers. Adherent eosinophils were counted using microscopy. The anti-α_4-integrin mAb was the only antibody that alone reduced constitutive eosinophil adhesion to MDA-MB-435S cells (Figure 3.5A). Furthermore, an anti-α_4-integrin mAb also blocked most eosinophil adhesion to TNF-α-stimulated MDA-MB-435S cells (Figure 3.5B). A β_2-integrin mAb decreased adhesion to TNF-α-stimulated cells; however, neither ICAM-1 nor VCAM-1 mAbs alone had any effect on eosinophil adhesion (Figure 3.5B). The lack of an effect of the anti-ICAM-1 and anti-VCAM-1 mAbs may reflect the observation that eosinophil adhesion was occurring both to the breast carcinoma cells as

well as to the exposed matrix. Alternatively, both molecules may be required for optimal adhesion and blocking either one alone would have no effect on adhesion.

Eosinophil adhesion to BT-20 cells requires α_4-integrins. We next examined the effect of these mAbs on eosinophil adhesion to BT-20 cells. Pretreatment of BT-20 cells and eosinophils was carried out in the same manner as described above. Eosinophil adhesion on unstimulated BT-20 cells was not effected with antibody treatment (Figure 3.6A). mAbs directed against either VCAM-1 or α_4-integrins both attenuated eosinophil adhesion to TNF-α-stimulated BT-20 cells, although inhibition was greater with the anti-α_4-integrin mAb (Figure 3.6B). ICAM-1 mAb had no effect on adhesion, despite high expression of ICAM-1 on these cells (Figures 3.6B and 3.3B), yet a β_2-integrin mAb did decrease eosinophil adhesion however it was not significant. These data suggest that eosinophils could be binding to β_2-integrin ligands other than ICAM-1 or that eosinophils may need to be further activated, possibly by a chemokine, to effectively bind to ICAM-1 expressed on the BT-20 cells.

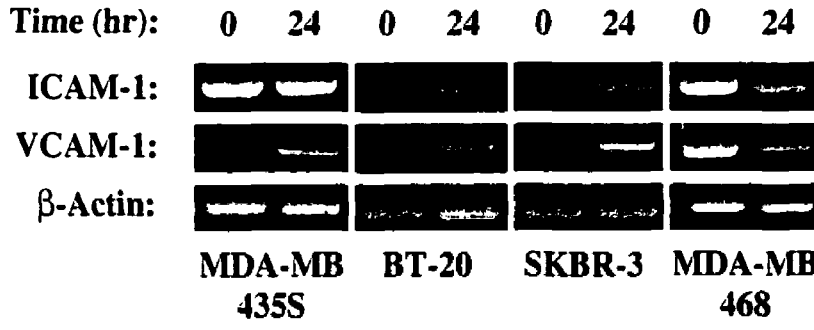


Figure 3.1. mRNA expression of adhesion molecules by breast carcinoma cells stimulated with TNF-α. Cell lines were treated with M199/A alone (Time: 0) or M199/A containing 20 ng/ml TNF-α for 24 hours (Time: 24). After the incubation, cells were lysed in TRIzol and total RNA was isolated. RT-PCR was performed as described in the methods. Experiments were performed at least three times with equivalent data. Representative data is shown.

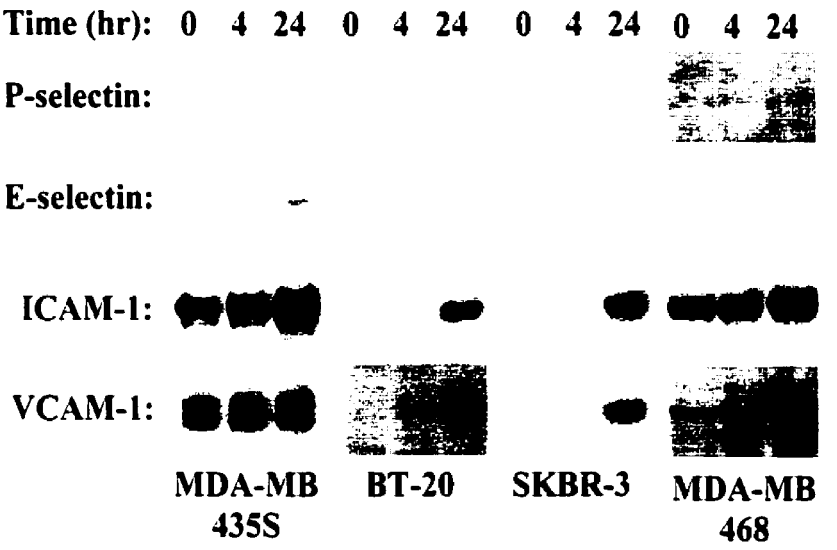


Figure 3.2. Adhesion molecule expression by breast carcinoma cells. Cell lines were treated with M199/A alone (Time: 0) or M199/A containing 20 ng/ml TNF-α for 4 or 24 hours (Time: 4, 24). After the incubation, cells were lysed in triton X-100 containing buffer and the soluble proteins were separated on SDS-PAGE. Western blotting was performed using 2 μg/ml of the specified mAbs directed against P-selectin (G1), E-selectin (ES-1), ICAM-1 (BBA 4) or VCAM-1 (1.G11B1). Experiments were performed at least three times with equivalent data. Representative data is shown.

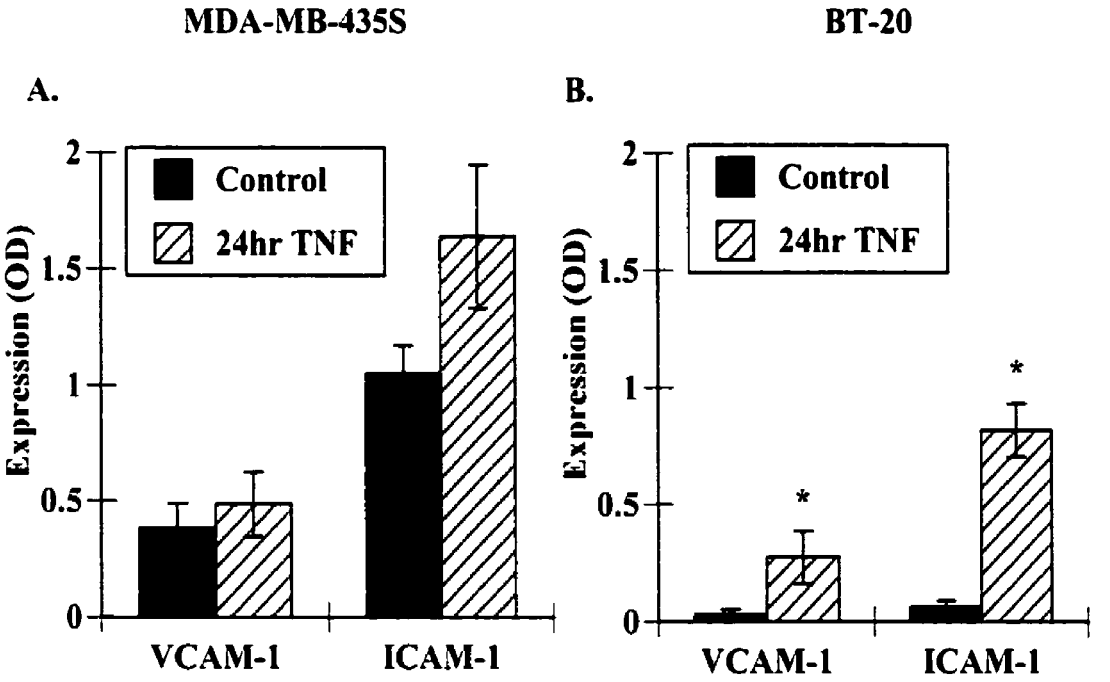


Figure 3.3. VCAM-1 and ICAM-1 are expressed on the surface of breast carcinoma cells. (A) MDA-MB-435S or (B) BT-20 cells were treated with TNF-α as described in figure 1. Cell surface expression of VCAM-1 or ICAM-1 was determined using a modified ELISA as described in the methods. Data represent the mean ± SEM of at least three experiments. * $p < 0.05$ as compared to control.

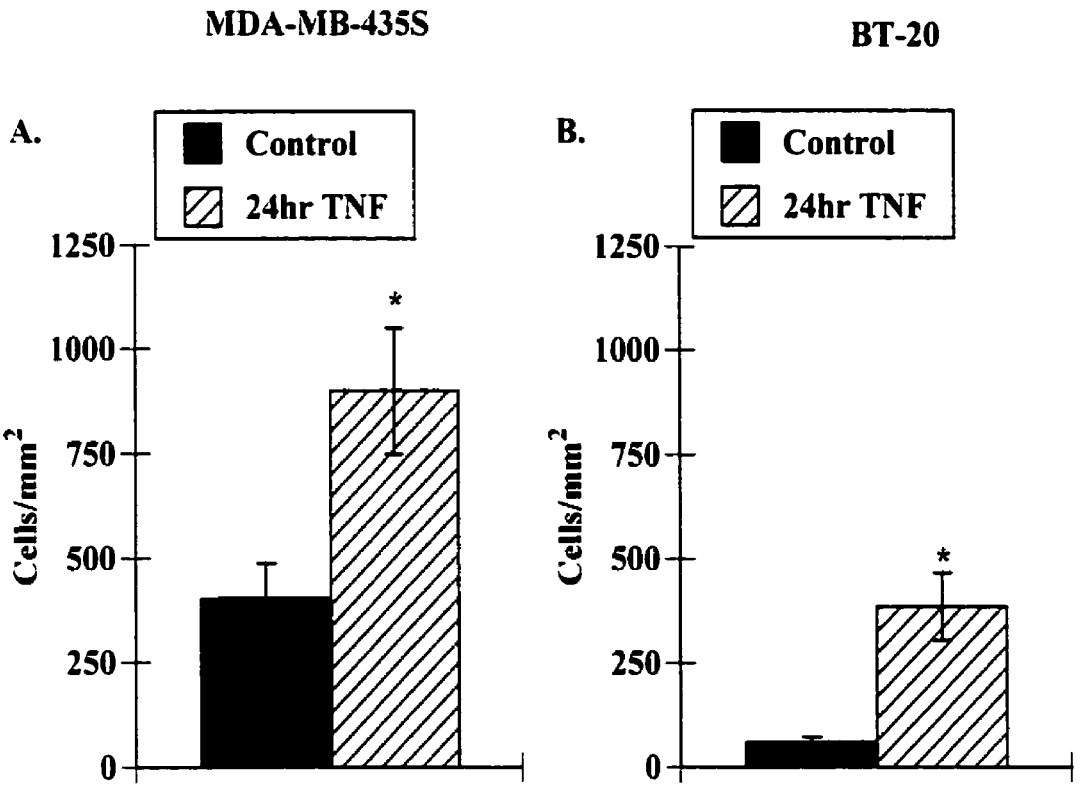


Figure 3.4. Eosinophils bind to breast carcinoma cells. Freshly isolated eosinophils ($2x10^6$/ml) were added to (A) MDA-MB-435S or (B) BT-20 cells treated with buffer alone or TNF-α as described in figure 1. After 15 minutes, non-adherent and loosely adherent cells were removed and eosinophil adhesion was determined as described in the methods. Data represent the mean ± SEM of at least three experiments. * $p< 0.05$ as compared to control.

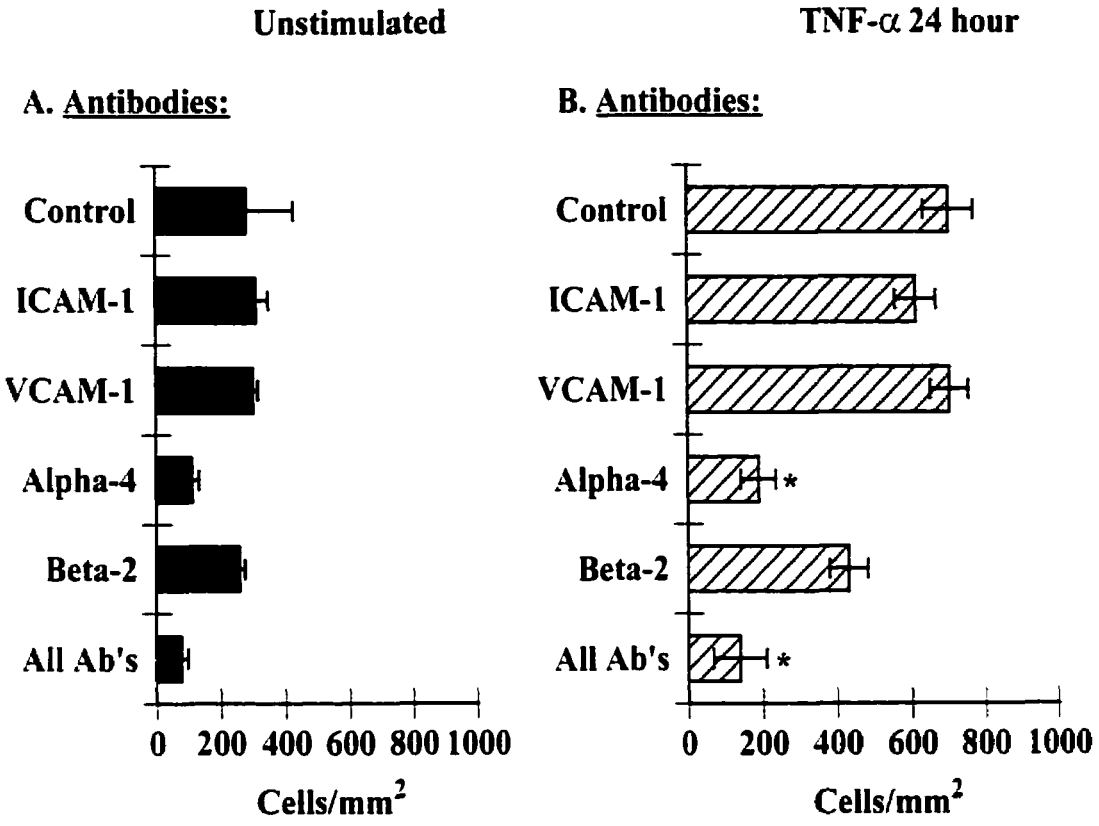


Figure 3.5. Eosinophil adhesion to MDA-MB-435S requires α4-integrins. MDA-MB-435S cells were treated with (A) buffer alone or (B) TNF-α for 24 hours as described in figure 1. MDA-MB-435S cells were pre-treated for 15 min. at 37°C with 5 μg/ml of the specified mAbs directed against ICAM-1 (BBA4) or VCAM-1 (1.G11B1). Alternatively, eosinophils were pre-incubated with α4-integrin mAb (H2/1) or β2-integrin mAb (7E4) for 15 min. prior to addition to the cell monolayers. Control describes eosinophil adhesion on MDA-MB-435S cells in the absence of antibody. Eosinophil adhesion was determined in the continued presence of the specified mAbs as described in the Methods. Data are mean ± SEM of at least three experiments. ANOVA with a Bonferroni correction was used to analyze the data. * $p < 0.05$ as compared to control.

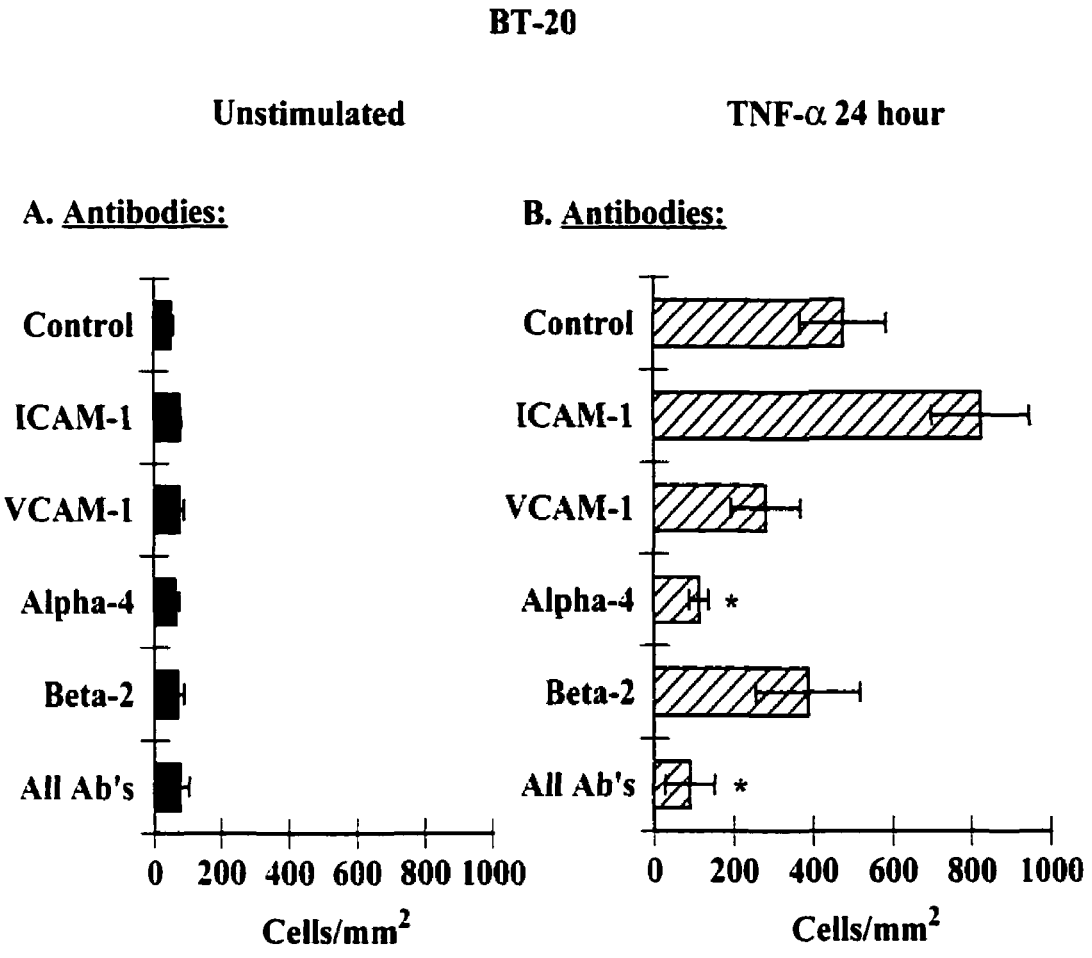


Figure 3.6. Eosinophil adhesion to BT-20 cells requires α4-integrins. BT-20 cells were treated with (A) buffer alone or (B) TNF-α for 24 hours as described in figure 1. BT-20 cells were pre-treated for 15 min. at 37°C with 5 μg/ml of the specified mAbs directed against ICAM-1 (BBA4) or VCAM-1 (1.G11B1). Alternatively, eosinophils were pre-incubated with α4-integrin mAb (H2/1) or b2-integrin mAb (7E4) for 15 min. prior to addition to the cell monolayers. Control describes eosinophil adhesion on BT-20 cells in the absence of antibody. Eosinophil adhesion was determined in the continued presence of the specified mAbs as described in the Methods. Data are mean ± SEM of at least three experiments. ANOVA with a Bonferroni correction was used to analyze the data. * $p < 0.05$ as compared to control.

3.2 Discussion

Eosinophils represent only a few percent of the peripheral blood leukocytes; however they are the predominant cell type found at sites of parasitic and allergic inflammation (Wardlaw *et al.*, 1995). It is now clear that eosinophils are also found in and around many types of tumors including breast, colon, gastric small cell and cervical carcinomas (Samoszuk, 1997). *In vitro*, eosinophils can enhance the tumoricidal activity of macrophages (Spessotto *et al.*, 1995) and *in vivo*, eosinophil infiltration into some tumors is associated with improved prognosis (Goldsmith *et al.*, 1992; Bethwaite *et al.*, 1993; Ownby *et al.*, 1983; Iwasaki *et al.*, 1986). Despite the overwhelming data showing that eosinophils are associated with numerous types of cancer, little is known about the molecular mechanisms required for localization of eosinophils to these sites.

Several families of adhesion molecules are involved in the localization of eosinophils into sites of inflammation. Endothelial cells stimulated with either TNF-α (Kitayama *et al.*, 1997) or IL-4 (Patel, 1998) have increased expression of both selectins and Ig superfamily proteins that act to tether and firmly bind eosinophils to these surfaces. Once eosinophils are bound to the endothelium, directed localization of eosinophils into tumor sites likely involves the combined actions of chemoattractants and adhesion molecules present on tumor cells themselves. Increased expression of selectins has been observed at tumor sites as compared to normal tissue samples; however, this expression is associated with the tumor endothelium (Fox *et al.*, 1995; Charpin *et al.*, 1998). In contrast, ICAM-1 is expressed both by the tumor endothelium as well as by tumor cells themselves (Regidor *et al.*, 1998; Mayer *et al.*, 1995; Dippold *et al.*, 1993). Increased expression of ICAM-1 has been seen in tissue samples of breast carcinomas

and is associated with an improved prognosis (Ogawa *et al.*, 1998). The mechanisms by which ICAM-1 expression leads to decreased tumor size are unknown, but may involve localization of leukocytes to the tumor.

In this study we used several human breast carcinoma cell lines including MDA-MB-435S, BT-20, SK-BR-3 and MDA-MB-468 to determine whether the expression of known adhesion molecules may result in eosinophil adhesion. All of the cell lines used in this study are adenocarcinomas of the breast, meaning that the tumors arose from glandular epithelial cells. The major difference between the four cell lines lies in their tumorgenicity. With MDA-MB-435S as the exception, all the cell lines used are capable of forming tumors in nude mice. Morphologically, MDA-MB-435S also differ by growing in spindle shape, whereas the other cell lines form uniform monolayers. Finally, MDA-MB-435S cells were derived from a metastatic ductal adenocarcinoma, whereas the other cell lines were derived from malignant tumors. All of these cell lines are extensively used in breast cancer research and concentrating on the MDA-MB-435S and BT-20 cell lines allowed us to identify the differences in adhesion molecule expression, cell activation and chemokine expression between these two characteristically different cell lines.

The immune system is capable of responding to tumors. In breast cancer there is evidence of systemic, regional and intratumoral leukocyte activation which has been associated with a good prognosis (Wong *et al.*, 1998). Human breast cancer sites are infiltrated by a heterogenous population of leukocytes, termed tumor-associated leukocytes (TALs) (O'Sullivan and Lewis, 1994). Variable proportions of T cells, B cells, NK cells, macrophages (O'Sullivan and Lewis, 1994), neutrophils, eosinophils

(Samozuk *et al.*, 1996), fibroblasts and endothelial cells (Lewis *et al.*, 1995) have been identified to accumulate mostly within the stromal elements surrounding tumor islands and occasionally some of these cells are found within tumor nests (reviewed in O'Sullivan and Lewis, 1994). Numerous studies have attempted to find correlations between the composition of the tumor-infiltrating leukocyte population and prognosis of the patient which has unfortunately not led to any predictable relationships (Wong *et al.*, 1998). T cells and macrophages have been identified as the major leukocyte subsets infiltrating breast tumor sites whereas natural killer and B cells are seldomly found. T cells can constitute between 30% and 50% of the total TAL population and macrophages can represent as much as 80% of the total TAL population (reviewed in O'Sullivan and Lewis, 1994).

TALs can communicate with each other and with breast carcinoma cells through the release of cytokines and growth factors (O'Sullivan and Lewis, 1994). Measuring the secretion of cytokines is one method to determine the function of TALs. Using immunohistochemistry, Camp and collegues demonstrated, *in situ*, that tumor-infiltrating lymphocytes (TILs) are able to produce IL-2, TNF-α, IL-4, TGF-β1, IL-10, IFN-γ and GM-CSF (Camp *et al.*, 1996). TILs associated with malignant breast tumors produced significantly more IL-2 and TNF-α than TILs associated with benign tumors (Camp *et al.*, 1996). Tumor-associated macrophages (TAMs) in breast cancer were shown to be the primary cell that contained immunoreactive TNF-α and 17.5% of invasive breast carcinomas investigated contained TNF-α (Lewis *et al.*, 1995). A recent study looked at the intracellular cytokine profile of three different leukocyte populations from breast cancer patients. Tumor tissue, axillary lymph nodes and peripheral blood lymphocytes

were examined and shown to synthesize type 1 cytokines including TNF-α, IFN-γ and IL-2 following stimulation with PMA + calcium ionophore (Wong *et al.*, 1998). It is apparent that the tumor microenvironment is under the influence of many different cytokines and these cytokines can have diverse effects on residing cells.

We examined adhesion molecule expression before and after stimulation with TNF–α. TNF–α has many effects on tumors cells *in vitro* including the induction of either necrotic or apoptotic cell death in some types of tumors. TNF–α, along with IL-1 and IL-6, has also been demonstrated to induce ICAM-1 expression on several types of epithelial tumors including breast carcinomas (Fox *et al.*, 1995; Budinsky *et al.*, 1997, Hutchins and Steel, 1994; Bajaj *et al.*, 1993). In parallel experiments, VCAM-1 was not detected on these cells (Fox *et al.*, 1995).

RT-PCR, western blotting and cell surface ELISAs were used to examine adhesion molecules expression on several breast carcinoma cell lines. Two of the cell lines examined, MDA-MB-435S and MDA-MB-468, constitutively expressed ICAM-1 and VCAM-1 (Figure 3.2). This expression was enhanced following exposure to TNF–α. The other cell lines examined only expressed these adhesion molecules following stimulation with TNF–α. Although ICAM-1 expression was expected based on previous studies, the expression of VCAM-1 was not expected. Using RT-PCR we amplified a band for VCAM-1 at the expected size (1043 bp). Since mRNA levels do not always correlate with protein expression, we also performed western blots and detected a protein with a molecular weight of ≈110kD. One explanation for the differences between our data and others who have examined VCAM-1 protein expression may lie in the

immunogenicity of the VCAM-1 expressed by breast carcinoma cells. This may be due to either differential splicing or post-translational modifications of VCAM-1. VCAM-1 can be alternatively spliced resulting in either a 6 domain or 7 domain form of the molecule in endothelial cells (Osborn *et al.*, 1992). We found evidence for this type of splicing with VCAM-1 expressed by MDA-MB-435S cells by western blotting where a doublet was clearly observed (Figure 3.2). Furthermore, Abe et al. have demonstrated that endothelial cells treated with a combination of IL-4 and TNF–α express VCAM-1 based on western blotting and ELISAs, but this VCAM-1 does not recognize its ligand on circulating T-cells (Abe *et al.*, 1996). To further support this hypothesis, we found that only one of three commercially available mAbs to VCAM-1 recognized the molecule expressed on breast carcinoma cells, whereas, all three recognized VCAM-1 present on TNF–α stimulated endothelial cells (data not shown).

Breast carcinoma cells bind eosinophils through interactions with α_4-integrins. Adhesion to BT-20 cells was in part due to interactions between α_4-integrins and VCAM-1, whereas, α_4- and β_2-integrins were responsible for adhesion to MDA-MB-435S cells. The α_4-integrins also seemed to be involved in eosinophil adhesion on control MDA-MB-435S since adhesion was significantly reduced. Interestingly, we found that eosinophils could also bind to the exposed matrix of MDA-MB-435S cells. Adhesion on control BT-20 cells was minimal and no effect was seen with any of the antibodies used. Assuming that the α_4-integrins were binding to VCAM-1 on the surface of breast carcinoma cells, it is interesting to note that aVCAM-1 antibody had no effect on reducing eosinophil adhesion on MDA-MB-435S cells when an α_4 mAb did. There are a

number of possible explanations for these observations. One explanation could be that the α_4-integrins were binding to other ligands than VCAM-1, therefore blocking VCAM-1 wouldn't inhibit eosinophils adhering to another ligand. Eosinophils express both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ on their surface. Both can bind to VCAM-1 and MAdCAM-1, however $\alpha_4\beta_1$ is the primary receptor for VCAM-1 and $\alpha_4\beta_7$ primarily binds to MAdCAM-1. There are no reports in the literature stating that MAdCAM-1 is expressed on breast carcinoma cells, however it is possible that $\alpha_4\beta_7$ on eosinophils could be binding to MAdCAM-1. Fibronectin released by breast carcinoma cells could also potentially be another ligand for both $\alpha_4\beta_1$ and $\alpha_4\beta_7$. In addition, osteopontin, a secreted bone matrix protein, has been shown to be a ligand for $\alpha_4\beta_1$ (Bayless *et al.*, 1998). Osteopontin can be produced by mammary epithelial cells and the presence of this protein has been shown to increase invasiveness of mammary epithelial cell lines through Matrigel (Tuck *et al.*, 1999).

Another possible explanation for this observation is that the VCAM-1 antibody used was not blocking the binding site. The VCAM-1 antibody used in these assays was purchased from Serotec and the epitope in which the antibody binds to is unknown. However, according to the manufacturer and previous antibody blocking studies done in our laboratory, this antibody blocks cellular adhesion. As mentioned earlier, we have reason to believe that the VCAM-1 expressed by breast carcinoma cells is somehow modified from VCAM-1 expressed on HUVEC, therefore it is possible that either domain 1 and/or domain 4 is left exposed after antibody treatment. The other two antibodies against the α_4- and β_2-integrins are known to block adhesion to their respective ligands (Schiffer *et al.*, 1995; Nortamo *et al.*, 1988) and as predicted were functioning properly.

The expression of adhesion and activation molecules by breast carcinoma cells may provide a mechanism for localizing eosinophils to tumor sites. Once localized at these sites, the role for eosinophils is unclear. There are several studies that suggest that eosinophil tumor infiltration is associated with improved prognosis (Goldsmith *et al.*, 1992; Bethwaite *et al.*, 1993; Ownby *et al.*, 1983; Iwasaki *et al.*, 1986). There are an equal number of studies showing either no role for eosinophils or an association with a poor prognosis (Leighton *et al.*, 1996; Fisher *et al.*, 1989; van Driel *et al.*, 1996). Recently it has been suggested that eosinophils may not play a direct role in tumor cell clearance, but may instead participate in tissue remodeling (Samoszuk, 1997; Kadin *et al.*, 1993). Eosinophils can synthesize cytokines, growth factors and proteases that are associated with both tissue remodeling and angiogenesis. These include transforming growth factors-α and -β1 (Elovic *et al.*, 1998; Minshall *et al.*, 1997; Eisma *et al.*, 1997), vascular endothelial cell growth factor (Horiuchi and Weller, 1997), and matrix metalloprotease-9 (Okada *et al.*, 1997; Ohno *et al.*, 1997). Thus, the role of eosinophils in tumor biology may be directed less toward direct tumoricidal activity and more to modulation of the tumor microenvironment.

3.3 Future Directions

Investigating other adhesion molecules on the surface of breast carcinoma cells would be of interest in understanding how eosinophils can bind to these cells. MAdCAM-1, ICAM-2, -3, -4, -5 and osteopontin are all potential ligands for eosinophils which warrant investigation. It would also be interesting to see if there was a difference in adhesion molecule expression between tumorgenic (BT-20) and non-tumorgenic (MDA-MB-435S) cancer cells. Further antibody blocking studies using a combination of

domain 1 and domain 3 antibodies against ICAM-1 would be useful in determining the role of LFA-1 versus Mac-1 in eosinophil adhesion to breast carcinoma cells.

In this study we investigated the effects TNF-α had on breast carcinoma cells, however there are a number of other cytokines identified within the tumor microenvironment such as IL-2 and IFN-γ that would be interesting to investigate. The tumor environment is a very complex network of various cytokines and we have just scratched the surface of the effects one cytokine, TNF-α, has on breast carcinoma cells. In the future, it would be interesting to examine the possible effects that a combination of cytokines would have on breast carcinoma cells.

Chapter Four: Specific Aim 2

Hypothesis: Breast carcinoma cells stimulated with TNF-α will release eosinophil specific chemoattractants and activators.

Objectives:

1. Do TNF-α-stimulated breast carcinoma cells release eosinophil activators and/or chemoattractants?
2. Do breast carcinoma cells release mediator(s) that can stimulate an increase in eosinophil adhesion to these cells?
3. Can these mediator(s) cause a superoxide burst in eosinophils?
4. Characterize and identify the activators and/or chemoattractants released by TNF-α-stimulated breast carcinoma cells.
5. Can breast carcinoma supernatants induce the expression of chemokine receptors not normally found on eosinophils?

4.1 Results

Both MDA-MB-435S and BT-20 cells stimulated with TNF-α release eosinophil activator(s). Deposition of eosinophil granule proteins at tumor sites suggests that mechanisms exist not only for eosinophil adhesion, but also for their activation (Samoszuk *et al.*, 1996; Samoszuk *et al.*, 1986). Furthermore, the observation that eosinophils were adhering both to MDA-MB-435S cells as well as to the exposed matrix suggested a role for eosinophil activators. This issue was addressed by harvesting the supernatants from either MDA-MB-435S or BT-20 cells treated with buffer alone or treated with TNF-α, then determining if there was a mediator in these supernatants that

could increase eosinophil adhesion to either gelatin- or fibronectin-coated surfaces. These surfaces were used to assess increased adhesiveness of the β_2-integrins or the α_4-integrins, respectively. Freshly isolated eosinophils were added to plates coated with gelatin or fibronectin. Eosinophils were then stimulated with a 1:10 dilution of supernatants from control or TNF-α-treated breast carcinoma cell lines.

Supernatants from 24-hour TNF-α-treated, but not control-treated, MDA-MB-435S cells increased eosinophil adhesion to fibronectin with little increase in binding to gelatin (Figure 4.1A). This was not because of the presence of TNF-α alone as neither TNF-α alone (Figure 4.2) nor the 4-hour TNF-α supernatant increased eosinophil adhesion in this assay (Figure 4.1A). As expected, an anti-α_4-integrin mAb blocked eosinophil adhesion to fibronectin (Figure 4.3) and a β_2-integrin mAb had no effect on adhesion (data not shown). Thus, MDA-MB-435S cells constitutively express adhesion proteins on their surface and can bind eosinophils, but only after stimulation with TNF-α for 24 hours do these cells release mediator(s) that can activate eosinophils.

We extended these experiments to examine the supernatant from BT-20 cells both before and after TNF-α stimulation. Unlike the MDA-MB-435S cells, the supernatants from BT-20 cells treated with TNF-α increased adhesion to both gelatin and fibronectin (Figure 4.1B). Interestingly, supernatant from both the 4-hour and the 24-hour TNF-α supernatants were able to increase eosinophil adhesion on fibronectin; whereas only the 24-hour supernatant had pro-adhesive effects on gelatin (Figure 4.1B). As with the supernatants from MDA-MB-435S, an anti-α_4-integrin mAb blocked adhesion to fibronectin (Figure 4.3). In addition, an anti-β_2-integrin mAb blocked most adhesion to

gelatin (Figure 4.4). These data suggest that mediators released from BT-20 cells activate both the α_4- and β_2-integrins on eosinophils promoting adhesion to both gelatin and fibronectin, whereas mediators released from MDA-MB-435S cells only activate the α_4-integrins promoting adhesion only to fibronectin.

Breast carcinoma cells release eosinophil chemoattractant(s). Knowing that breast carcinoma cells can release mediator(s) that promote binding of eosinophils to matrix proteins we next wanted to determine if the mediator(s) present in the supernatants were also chemotactic for eosinophils. We used an eosinophil transmigration assay to address this issue. Eosinophils were placed in the upper chamber of a transwell plate that was precoated with fibronectin. Supernatant from control or TNF-α-treated cells was diluted 1:10 and placed in the lower chamber. Supernatants from buffer-treated cells had no effect on eosinophil transmigration (Figure 4.5). In contrast, supernatants from both MDA-MB-435S and BT-20 cells treated with TNF-α elicited eosinophil transmigration (Figure 4.5). TNF-α alone at the same concentration present in the diluted supernatant (2 ng/ml) induced only minimal transmigration (Figure 4.5). Thus supernatants from TNF-α-stimulated breast carcinoma cells contain eosinophil chemotactic factors.

BT-20 cells stimulated with TNF-α release activators that increase eosinophil adhesion to BT-20 cells. Previously, we performed static adhesion assays in the absence of breast carcinoma supernatant, which allowed us to determine if eosinophils were capable of binding to breast carcinoma cells. Since breast carcinoma cells can support eosinophil adhesion and knowing that there are eosinophil activator(s) present in breast carcinoma supernatants, we next wanted to determine if the presence of breast carcinoma supernatants in static adhesion assays would effect eosinophil adhesion. We performed a

time course for eosinophil adhesion on both control and TNF-α-stimulated breast carcinoma cells in the presence or absence of supernatant. In the absence of supernatant, a significant increase in eosinophil adhesion was seen on TNF-α-stimulated MDA-MB-435S cells at 5 and 15 minutes compared to control (Figure 4.6A). Static adhesion assays performed in the presence of supernatant did increase eosinophil adhesion at the 5 minute time point compared to the 5 minute time point without supernatant (Figure 4.6). However, there wasn't a significant increase in eosinophil adhesion between control and TNF-α-stimulated cells in the presence of supernatant at each time point (Figure 4.6B). With the BT-20 cells, there was low eosinophil adhesion in the absence of supernatant with a significant increase on TNF-α-stimulated cells at 15 minutes (Figure 4.7A). Interestingly, eosinophil adhesion on TNF-α-stimulated cells dramatically increased in the presence of supernatant (Figure 4.7B). Adhesion on control cells in the presence of supernatant was maintained at the same level throughout the time course (Figure 4.7B). These data suggests that BT-20 cells release eosinophil activator(s) upon TNF-α stimulation, significantly increasing eosinophil adhesion on these cells. However, there was little or no increase in eosinophil adhesion on MDA-MB-435S cells in the presence of supernatant.

Supernatant from TNF-α-stimulated breast carcinoma cells induces the generation of superoxide from eosinophils. The ability of breast carcinoma supernatants to induce a superoxide burst in eosinophils was measured by the ability of these activated eosinophils to reduce ferric (Fe^{+3}) cytochrome c to the ferrous form (Fe^{+2}). Supernatants from control and TNF-α-treated breast carcinoma supernatants were used to stimulated eosinophils for varying times. Supernatant from control BT-20 cells induced more

superoxide than supernatant from control MDA-MB-435S whose superoxide generation was comparable to the negative control, RPMI (Figure 4.8A and B). Both MDA-MB-435S and BT-20 supernatants from TNF-α-stimulated cells produced more superoxide than equivalent amounts of TNF-α (Figure 4.8B). BT-20 supernatant induced more superoxide production from eosinophils than the MDA-MB-435S supernatant. These data show that eosinophils stimulated with breast carcinoma supernatants are stimulated to produce superoxide.

Since TNF-α alone could elicit some superoxide we next wanted to determine if the superoxide generation was due to the TNF-α in the supernatant. We used a TNFR1 mAb to see if superoxide could be blocked by inhibiting signals through TNFR1. Antibody inhibition assays were performed using supernatants from both cells lines, however data from the MDA-MB-435S cell line was inconsistent and therefore not reported. Before the superoxide assay, eosinophils were pre-treated with 50 μg/ml IgG mAb or 50 μg/ml TNFR1 mAb The concentrations used for TNFR1 was based on manufacturer's recommendation. The non-immune IgG mAb had no effect on superoxide production from eosinophils (Figure 4.9). Superoxide production was attenuated when eosinophils were pre-treated with a TNFR1 mAb (Figure 4.10). The TNFR1 antibody could not lower superoxide production to control levels, suggesting that there are other mediators in the supernatant activating the eosinophils.

Breast carcinoma cells express mRNA for both CC and CXC chemokines. RT-PCR was used to begin the search to identify the eosinophil activators released by breast carcinoma cells. Chemokines are small molecular weight chemical messengers that can activate eosinophils and cause a rise in intracellular calcium due to receptor activation.

Chemokines are also known to create chemotactic gradients that can localize eosinophils to specific sites. Expression of both CC and CXC chemokines were under investigation. Confluent breast carcinoma cells were stimulated with buffer alone or TNF-α for 24 hours and RT-PCR was performed with the appropriate primers. Both MDA-MB-435S and BT-20 cells constitutively express mRNA for MCP-1 and MIP-1α with some induction after stimulation with TNF-α (Figure 4.11A). There was no message for MCP-3 in MDA-MB-435S cells, however there was message present in TNF-α-stimulated BT-20 cells. MCP-4 mRNA was increased after TNF-α stimulation in both cell lines. Surprisingly, there was little to no expression of eotaxin, eotaxin-2 or eotaxin-3. Even though faint, there was a small induction of RANTES upon TNF-α stimulation.

Interestingly, abundant IL-8 message was present in both cell lines (Figure 4.11B). Gro-α, PF-4 and MIG mRNA levels were present at constant levels under control and TNF-α conditions. A strong IP-10 message was induced under TNF-α stimulation, which was apparent in both cell lines. Message for either SDF-1 or ENA-78 was absent.

In summary, for the CC chemokines MCP-1, MCP-3, MCP-4 and MIP-1α mRNA was present in both cell lines and IL-8, Gro-α, PF-4, MIG and IP-10 message was present for the CXC chemokines. Similar chemokine profiles were found between MDA-MB-435S and BT-20 cells. The only difference that could be made was the increased message for MCP-3 and MCP-4 in BT-20 cells upon TNF-α stimulation (Figure 4.11A).

RANTES, MCP-1 and IL-8 are released by TNF-α stimulated breast carcinoma cells. The presence of message does not always correlate with protein expression, so we used ELISA's to determine the presence and quantity of various chemokines in the

supernatant of control and TNF-α stimulated breast carcinoma cells. We found that supernatants from TNF-α-stimulated MDA-MB-435S and BT-20 cells contained RANTES, MCP-1 and IL-8 (Figure 4.12). There was approximately the same amount of RANTES in TNF-α supernatants from both cell lines (Figure 4.12A). Under the influence of TNF-α, MDA-MB-435S cells secrete approximately 95 ng/ml MCP-1, whereas BT-20 cells only secrete about 10 ng/ml (Figure 4.12B). Of the chemokines for which we performed ELISA's, IL-8 was released in the most abundant amount. MDA-MB-435S cells stimulated with TNF-α released about 260 ng/ml of IL-8 and BT-20 cells released 155 ng/ml (Figure 4.12C). Even control BT-20 cells released 15 ng/ml IL-8. We also assayed for other chemokines and neither eotaxin, MCP-3 or MIP-1α was released by these breast carcinoma cell lines (data not shown). Eosinophils also express receptors for lipid mediators such as leukotriene B_4 (LTB_4); there however was no LTB_4 present in the supernatants (data not shown).

The ability of breast carcinoma cells to release TNF-α was also under investigation. Breast carcinoma cells were stimulated with 20 ng/ml TNF-α for 24 hours, supernatants were collected and subsequently assayed for TNF-α by ELISA. Breast carcinoma cells stimulated with TNF-α did not release more TNF and in fact there was a reduction in the amount of TNF-α present in the supernatants (Figure 4.13). Control supernatants were also assayed and there was little TNF-α present (data not shown).

mAb's against RANTES and IL-8 attenuates eosinophil adhesion on fibronectin. To determine whether RANTES, MCP-1 or IL-8 present in the supernatant was participating in eosinophil activation, we examined the ability of mAb's against these chemokines to block eosinophil adhesion to fibronectin. Supernatants were pre-treated

with mAb's against RANTES, MCP-1 or IL-8 for 15 minutes. Eosinophil static adhesion on fibronectin was carried out as previously described. An anti-RANTES antibody was able to attenuate eosinophil adhesion in response to both MDA-MB-435S supernatants (23% inhibition) and to BT-20 supernatants (36% inhibition) (Figure 4.14A). An anti-MCP-1 antibody decreased adhesion with supernatants from TNF-α-stimulated MDA-MB-435S cells however the MCP-1 mAb had no effect on adhesion with BT-20 supernatant (Figure 4.14B). Interestingly, pre-treatment of TNF-α MDA-MB-435S supernatant with an IL-8 antibody reduced eosinophil adhesion on fibronectin by 63% (Figure 4.14C). However, eosinophil adhesion was only reduced by 24% with supernatant from TNF-α-stimulated BT-20 cells (Figure 4.14C). Pretreatment of eosinophils with an anti-CXCR3 mAb had no effect on adhesion on fibronectin (data not shown). fMLP was used as a positive control and any inhibition in eosinophil adhesion seen with fMLP was deemed non-specific.

Blocking CCR3 attenuates superoxide generation from eosinophils stimulated with supernatant from TNF-α stimulated BT-20 cells. With the identification of RANTES in the supernatant of TNF-α stimulated breast carcinoma cells we wanted to examine the effect a CCR3 mAb would have on superoxide production in eosinophils. RANTES can bind to four receptors, CCR1, CCR3, CCR-4 and CCR-5. Eosinophils express CCR1 and CCR3 on their surface and since there isn't a CCR1 mAb available, we only looked at the effects a CCR3 mAb would have on superoxide production. Before the superoxide assay, eosinophils were pre-treated with 5 μg/ml CCR3 mAb. Pretreatment with a CCR3 mAb attenuated superoxide burst from eosinophils stimulated with supernatant from TNF-α stimulated BT-20 cells (Figure 4.15).

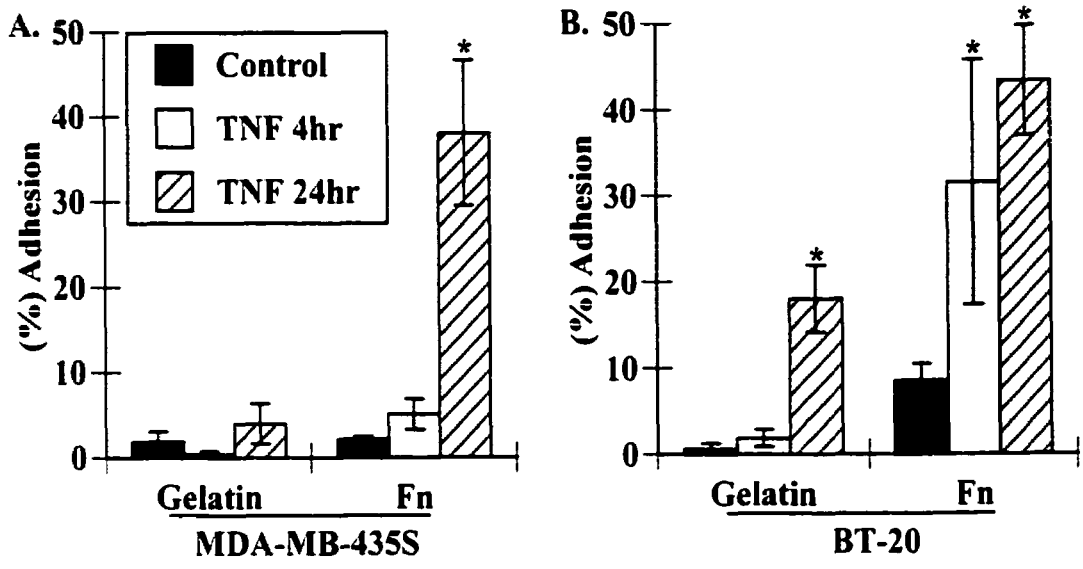


Figure 4.1. Both MDA-MB-435S and BT-20 cells release eosinophil activator(s). Supernatant were collected from either (A) MDA-MB-435S or (B) BT-20 cells treated with buffer alone or treated with TNF-α for 4 or 24 hours. 48-well plates were coated with either 0.2% gelatin or 25 μg/ml fibronectin (FN) for 2 hours at 37°C. Plates were washed once with HBSS and then freshly isolated eosinophils (1-2x10^6/ml) were added. Eosinophils were stimulated with a 1:10 dilution of the specified supernatants. After 10 minutes, the non-adherent and loosely adherent eosinophils were removed and adhesion was quantified using as eosinophil peroxidase activity assay as described in the methods. Data are mean ± SEM of at least three experiments. *$p<0.05$ as compared to control.

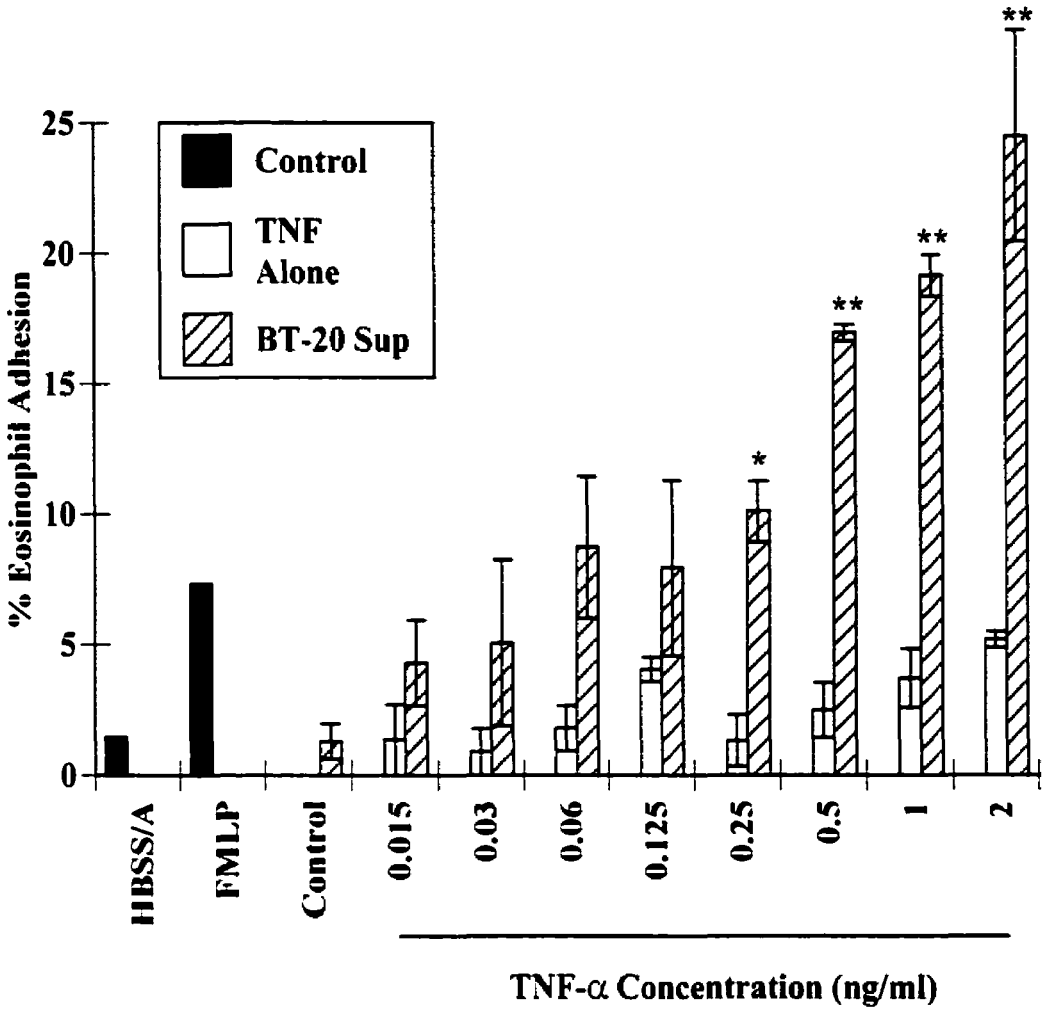


Figure 4.2. TNF-α alone does not increase eosinophil adhesion on fibronectin. BT-20 cells were treated with either buffer alone or with various concentrations of TNF-α and supernatants were collected after 24 hours. Plates were coated with 25 μg/ml fibronectin for 2 hours at 37°C. Plates were washed once with HBSS and then freshly isolated eosinophils ($2x10^6$/ml) were added. Eosinophils were stimulated with a 1:10 dilution of either TNF-α alone or supernatants collected from BT-20 cells. After 10 minutes, the nonadherent and loosely adherent eosinophils were removed and adhesion was quantified using eosinophil peroxidase activity as described in the methods. Data are mean ± SEM of three experiments. *$p<0.05$ and **$p<0.005$ as compared to control.

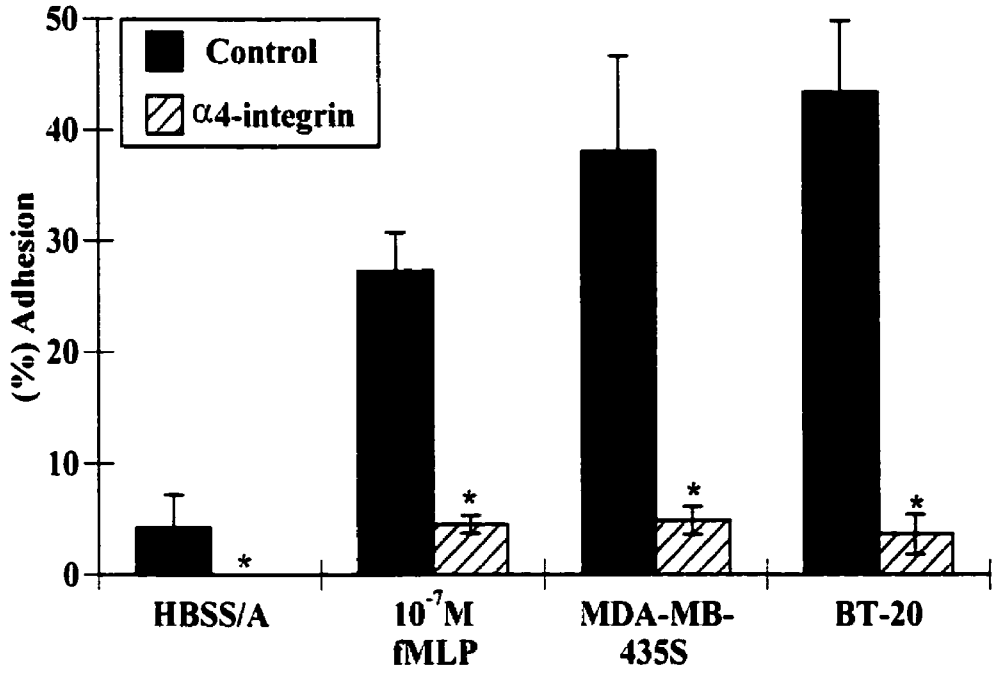


Figure 4.3. Breast carcinoma supernatants activate eosinophil α4-integrins. Eosinophils (1-2x10^6/ml) were added to 48- or 24-well plates coated with 25 μg/ml fibronectin. Supernatants from 24 hour TNF-α stimulated MDA-MB-435S or BT-20 cells were added in a 1:10 dilution. Eosinophils were pre-treated with 2 μg/ml of an anti-α4-integrin mAb for 10 minutes prior to stimulation. Adhesion was determined by eosinophil peroxidase assay as described in the methods. HBSS/A or 10^{-7}M fMLP were used as negative and positive controls for eosinophil adhesion. Data represent mean ± SD.

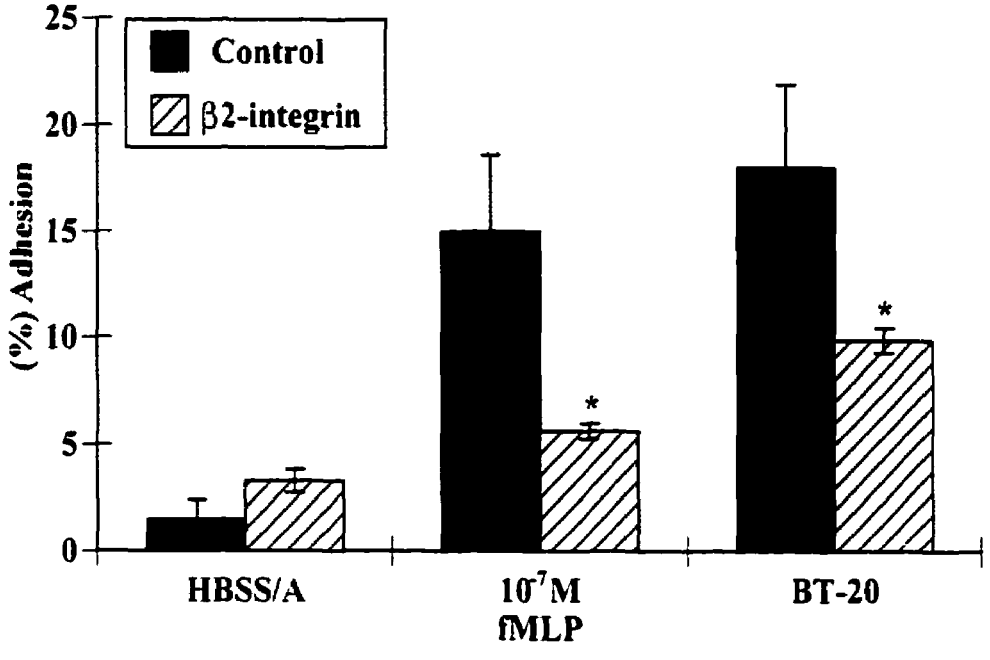


Figure 4.4. BT-20 supernatants activate eosinophil β2-integrins. Eosinophils (1-2x10^6/ml) were added to 48- or 24-well plates coated with 0.2% gelatin. Supernatants from 24 hour TNF-α stimulated BT-20 cells were added in a 1:10 dilution. Eosinophils were pre-treated with 5 μg/ml of an anti-β2-integrin mAb for 10 minutes prior to stimulation. Adhesion was determined by eosinophil peroxidase assay as described in the methods. HBSS/A or 10^{-7}M fMLP were used as negative and positive controls for eosinophil adhesion. Data represent mean ± SD.

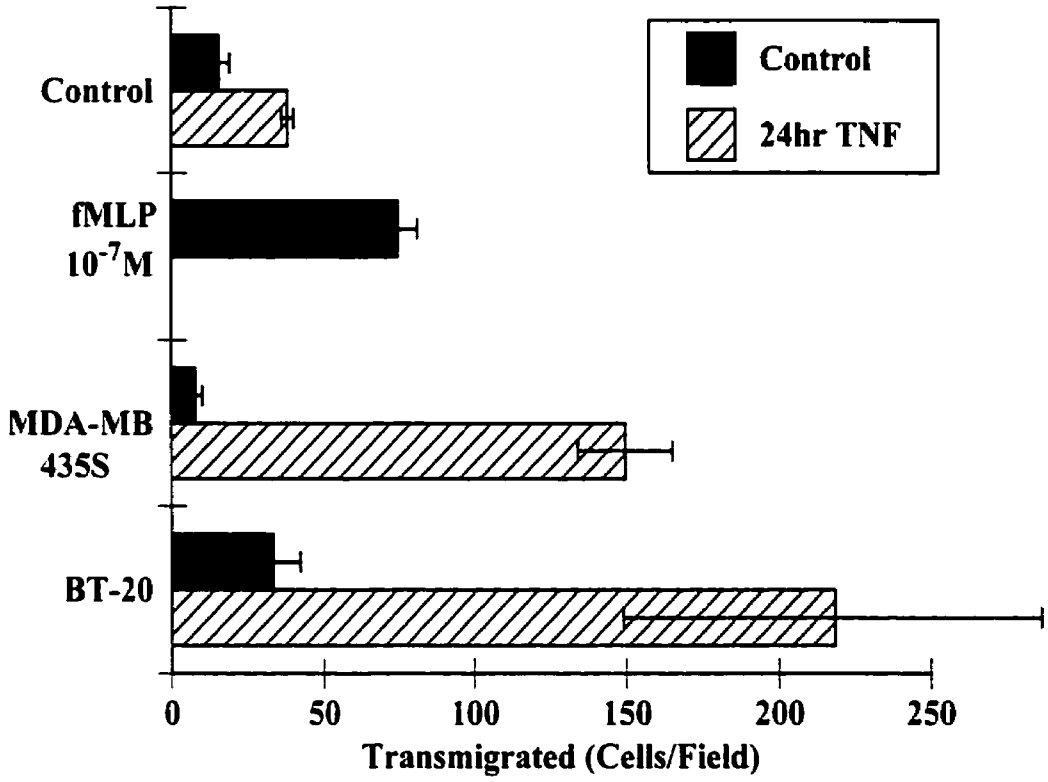


Figure 4.5. Breast carcinoma cells release eosinophil chemoattractant(s). Supernatants from either MDA-MB-435S or BT-20 cells treated with buffer alone or TNF-α for 4 or 24 hours were collected. Freshly isolated eosinophils ($4x10^6$/ml) were added to the upper chamber of a 3 μm-pore transwell dish coated with 25 μg/ml of fibronectin. Supernatants were placed in the lower chamber at a 1:10 dilution. M199/A or M199/A with an equivalent amount of TNF-α was used as controls (Control). Eosinophils that had migrated into the lower chamber after 90 minutes were counted as described in the methods. Experiments were performed twice with equivalent data. Data represent the mean ± range of a single experiment.

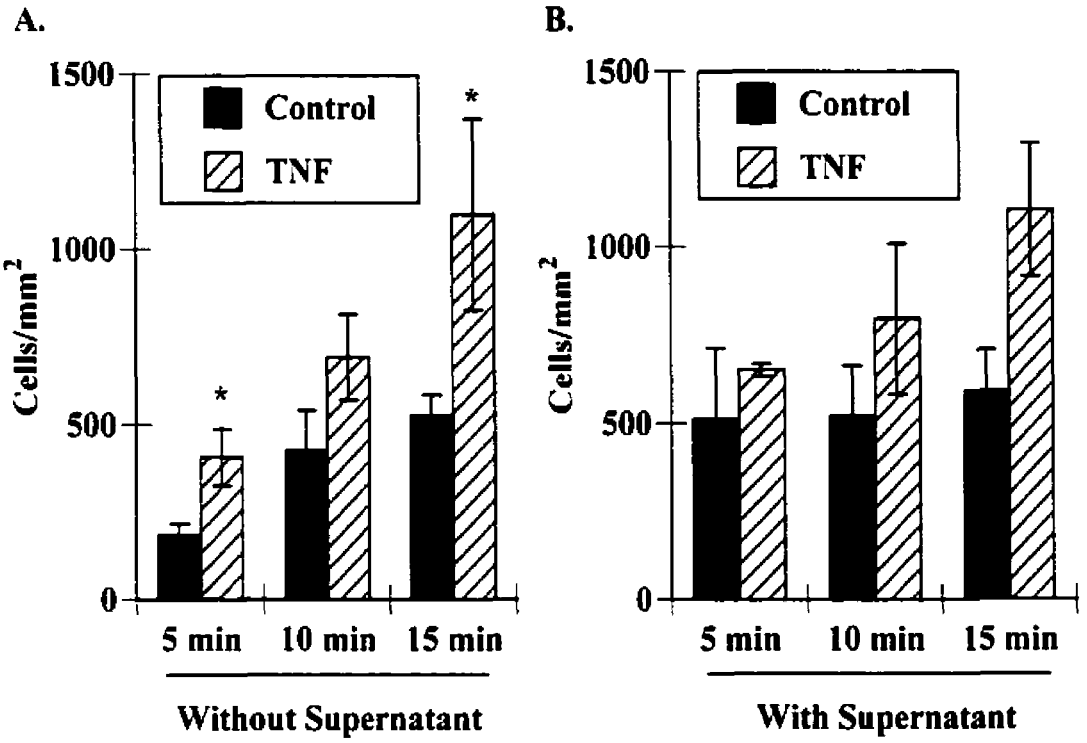


Figure 4.6. Conditioned supernatant has no significant effect on eosinophil adhesion to MDA-MB-435S cells at various time points. MDA-MB-435S cells were stimulated with either buffer alone or 20 ng/ml TNF-α for 24 hours. (A) Supernatants were either removed or (B) left in the well during the assay. Freshly isolated eosinophils ($2x10^6$/ml) were added to each well and incubated for either 5, 10 or 15 minutes at 37°C. Loosely and nonadherent cells were washed away and adherent eosinophils were visualized by microscope and counted by NIH Image. Data represent the mean ± SEM of at least three experiments. *$p<0.05$ as compared to control.

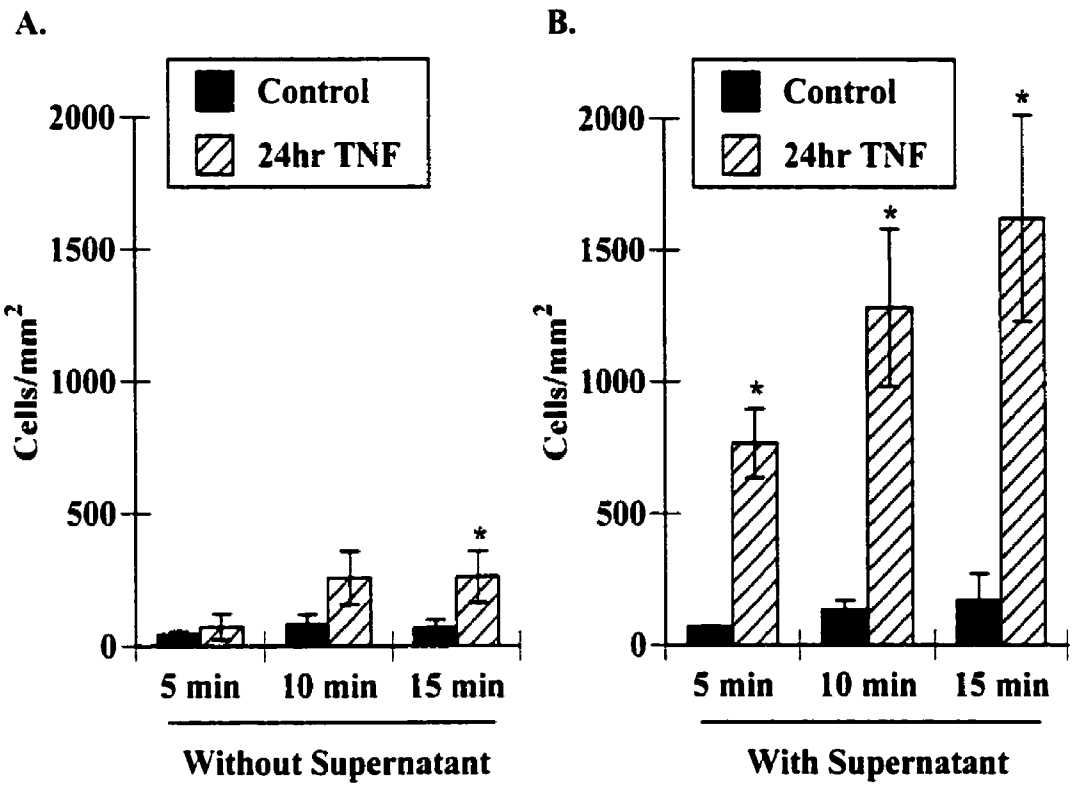


Figure 4.7. Conditioned supernatant increases eosinophil adhesion to BT-20 breast carcinoma cells at various time points. BT-20 cells were stimulated with either buffer alone or 20 ng/ml TNF-α for 24 hours. (A) Supernatants were either removed or (B) left in the well during the assay. Freshly isolated eosinophils ($2x10^6$/ml) were added to each well and incubated for either 5, 10 or 15 minutes at 37°C. Loosely and nonadherent cells were washed away and adherent eosinophils were visualized by microscope and counted by NIH Image. Data represent the mean ± SEM of at least three experiments. *$p<0.05$ as compared to control.

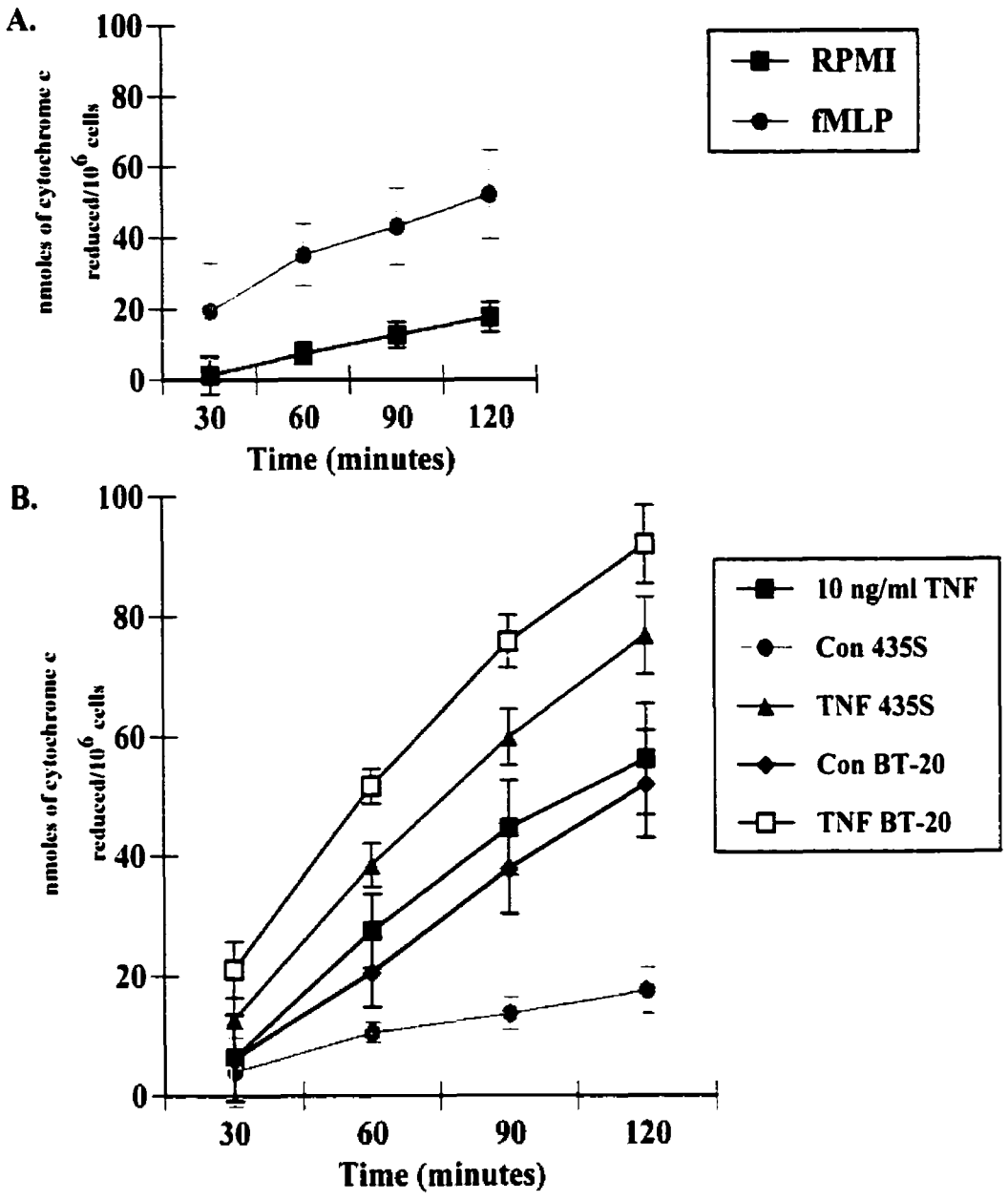


Figure 4.8. Breast carcinoma supernatant can stimulate superoxide burst in eosinophils. MDA-MB-435S and BT-20 breast carcinoma cells were stimulated with either RPMI/A or 20 ng/ml TNF-α in RPMI/A for 24 hours. 96-well plates were coated with 1% HBSS/A for 2 hours at 37°C. Plates were washed twice with HBSS and then freshly isolated eosinophils resuspended in 2.4 mg/ml cytochrome c ($0.5x10^6$/ml) were added. The appropriate stimulus was added in a 1:2 dilution and absorbance readings were taken at 30 minute intervals for 2 hours at 550 nm. (A) RPMI alone and 10^{-6}M fMLP served as the negative and positive controls, respectively. Data are mean ± SEM of at least three experiments.

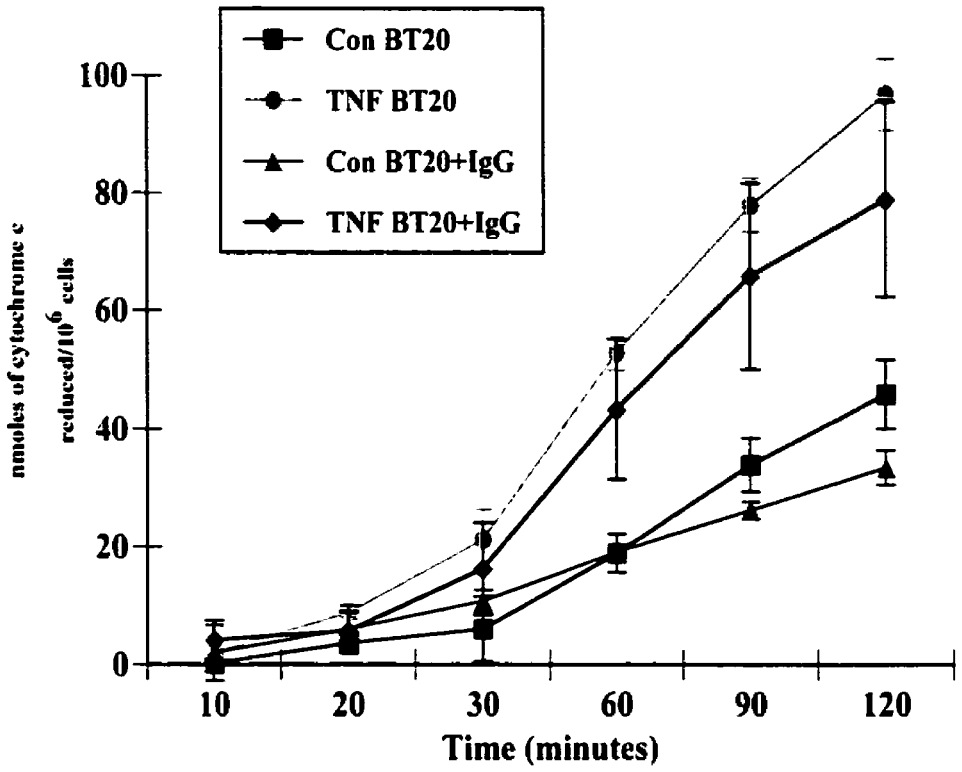


Figure 4.9. Adding a non-immune IgG mAb has no effect on superoxide production from eosinophils. BT-20 breast carcinoma cells were stimulated with either RPMI/A or 20 ng/ml TNF-α in RPMI/A for 24 hours. 96-well plates were coated with 1% HBSS/A for 2 hours at 37°C. Eosinophils were pre-treated with 50 μg/ml IgG mAb for 15 minutes. 96-well plates were washed twice with HBSS and pretreated eosinophils resuspended in 2.4 mg/ml cytochrome c ($0.5x10^6$/ml) were added. The appropriate stimulus was added in a 1:2 dilution and absorbance readings were taken at 10 minute intervals until 30 minutes passed and then readings were taken at 30 minute intervals for the remaining 2 hours at 550 nm. Data are mean ± SEM of at least three experiments.

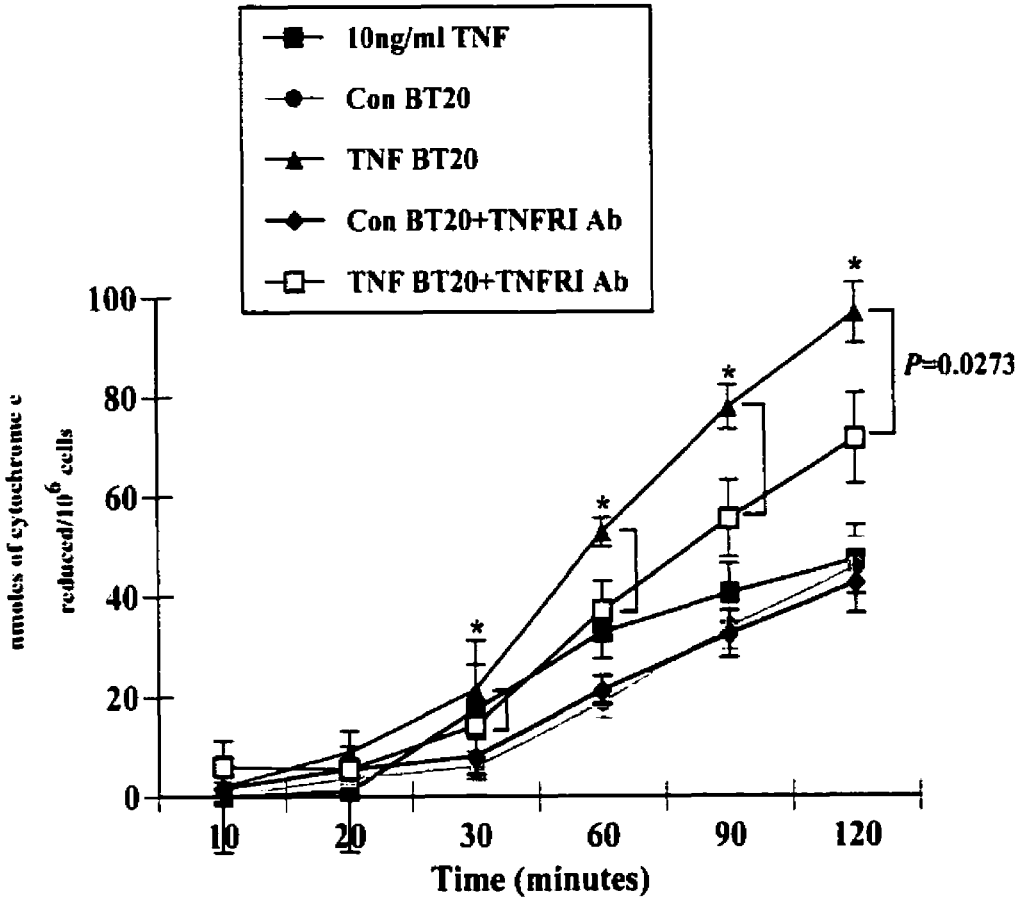


Figure 4.10. Blocking TNF receptor 1 significantly reduces superoxide burst in eosinophils stimulated with supernatant from TNF-α stimulated BT-20 cells. BT-20 breast carcinoma cells were stimulated with either RPMI/A or 20 ng/ml TNF-α in RPMI/A for 24 hours. 96-well plates were coated with 1% HBSS/A for 2 hours at 37°C. Eosinophils were pre-treated with 50 μg/ml TNFR1 mAb for 15 minutes. 96-well plates were washed twice with HBSS and pretreated eosinophils resuspended in 2.4 mg/ml cytochrome c ($0.5x10^6$/ml) were added. The appropriate stimulus was added in a 1:2 dilution and absorbance readings were taken at 10 minute intervals until 30 minutes passed and then readings were taken at 30 minute intervals for the remaining 2 hours at 550 nm. Data are mean± SEM of at least three experiments.

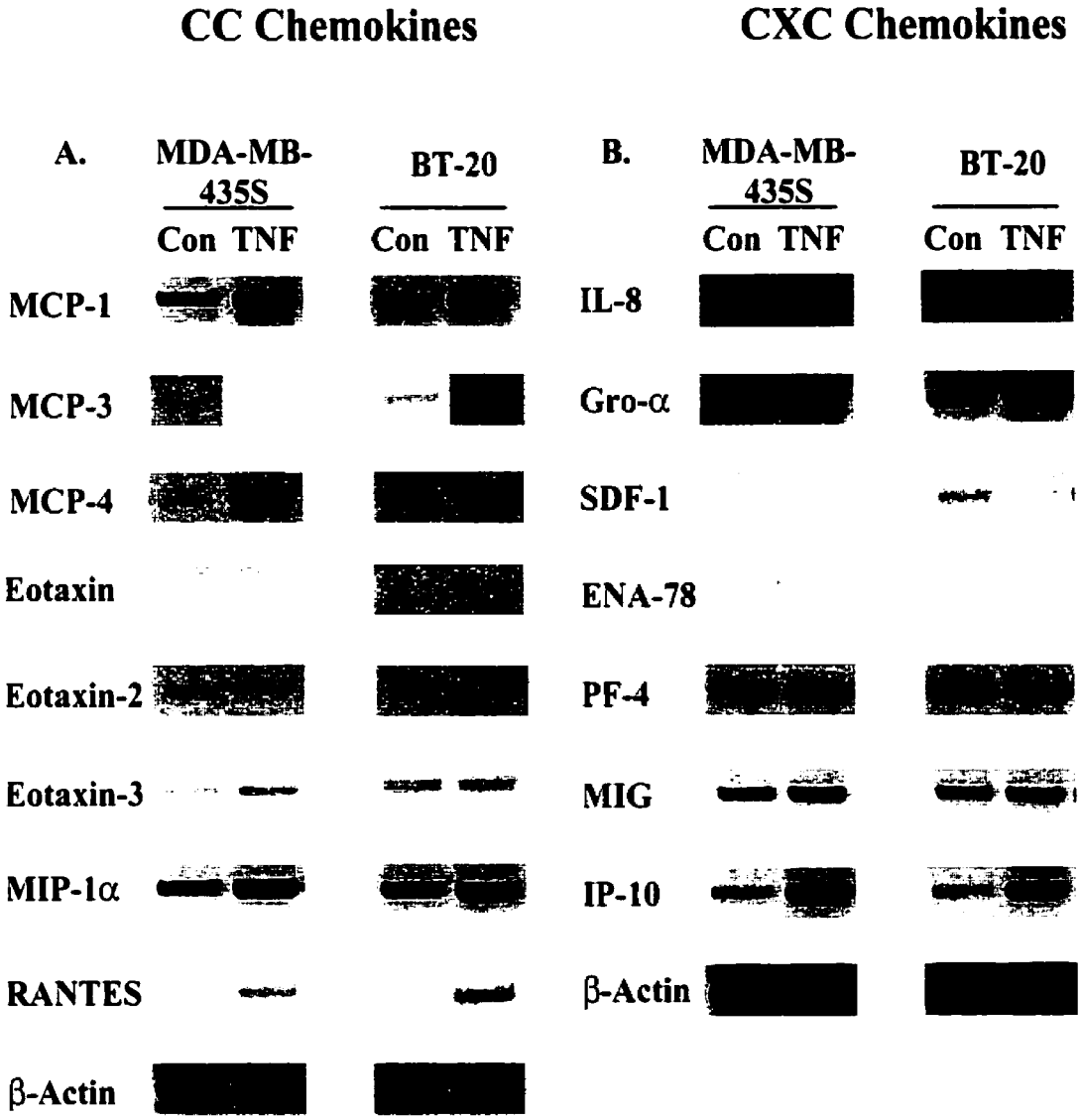


Figure 4.11. Breast carcinoma cells express mRNA for both CC and CXC chemokines. MDA-MB-435S and BT-20 breast carcinoma cells were either treated with M199/A alone (Con) or 20 ng/ml TNF-α (TNF) for 24 hours. Cells were lysed with TRIzol and RT-PCR was performed. Expression of (A) CC chemokines and (B) CXC chemokines were investigated. Each primer pair was used at least three times. Representative data is shown.

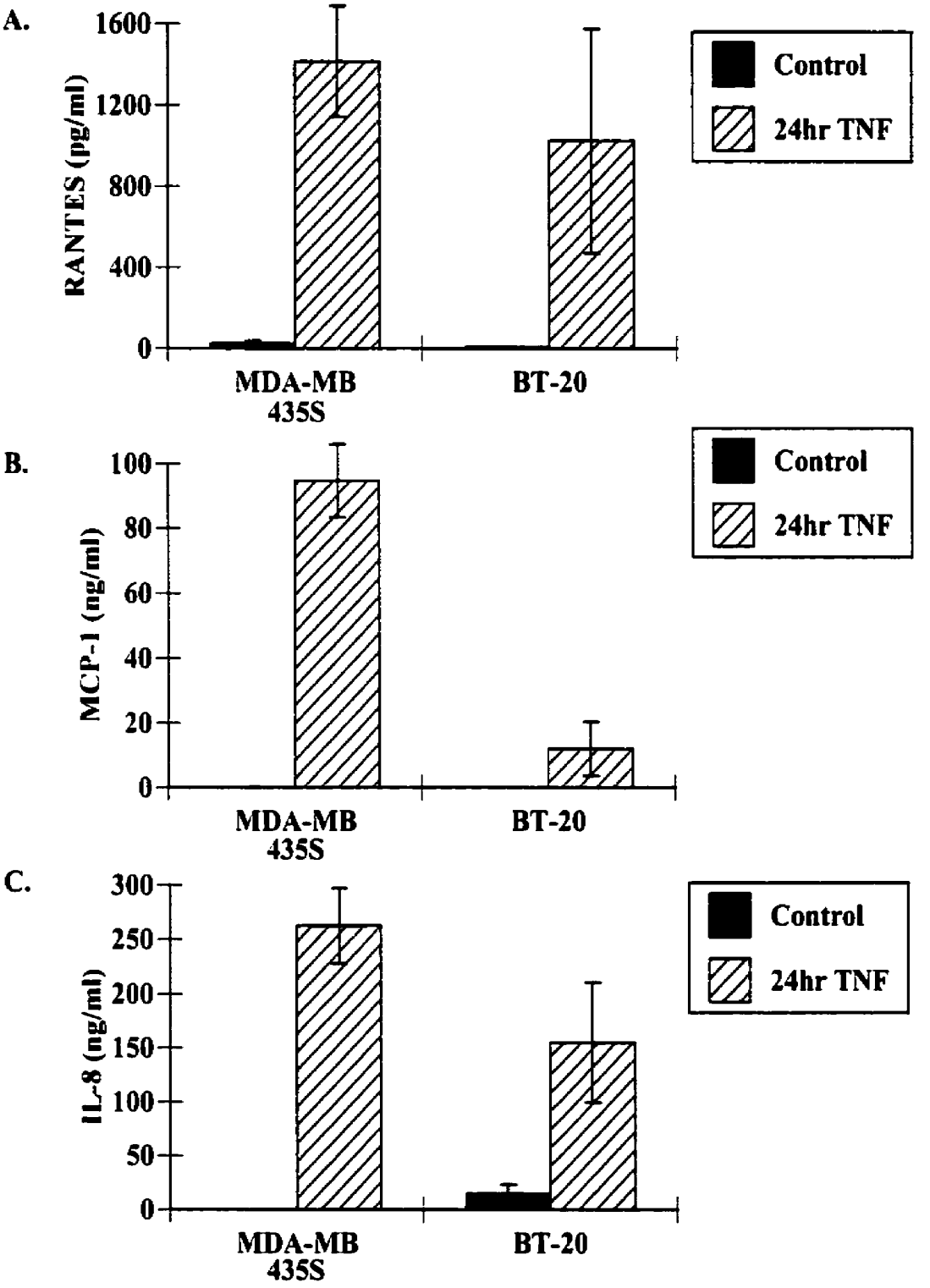


Figure 4.12. RANTES, MCP-1 and IL-8 are released by TNF-α stimulated breast carcinoma cells. Supernatants from MDA-MB-435S or BT-20 cells were collected as described in figure 10. (A) RANTES, (B) MCP-1 and (C) IL-8 were present in the supernatants of TNF-α stimulated breast carcinoma cells. Chemokines were assayed by ELISA according to the manufacturer's instructions. Data represent the mean ± SEM of at least three experiments.

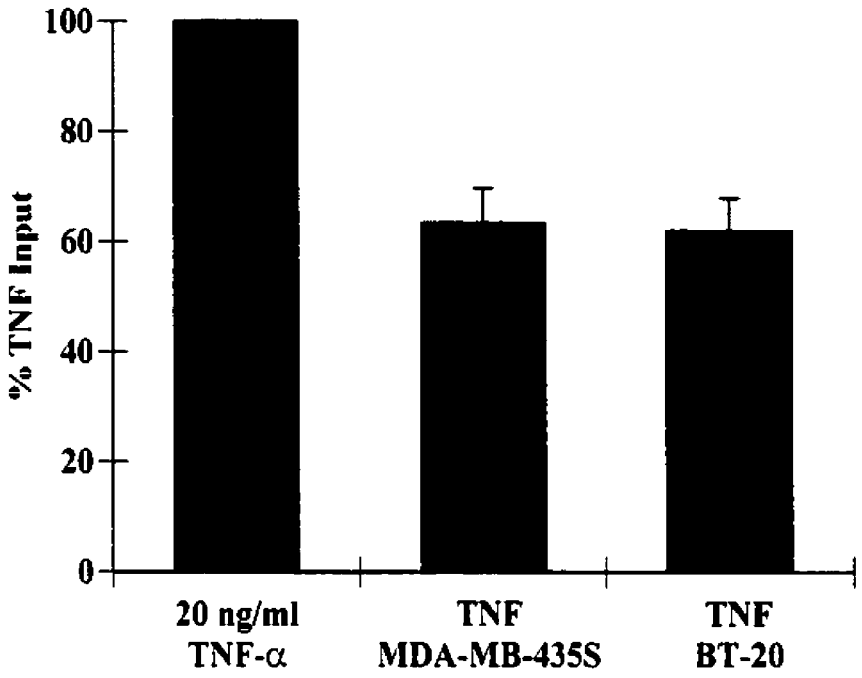


Figure 4.13. Breast carcinoma cells stimulated with TNF-α do not release more TNF. Breast carcinoma cells were stimulated with 20 ng/ml TNF-α for 24 hours. Stimulation buffer and supernatant from MDA-MB-435S and BT-20 were collected and assayed for TNF-α by ELISA. All manufacturer's instructions were followed. Data are presented as % Input which was calculated by dividing the amount of TNF present in the supernatant by the amount of TNF in the stimulation buffer. Data are representative of at least three experiments (mean±SEM).

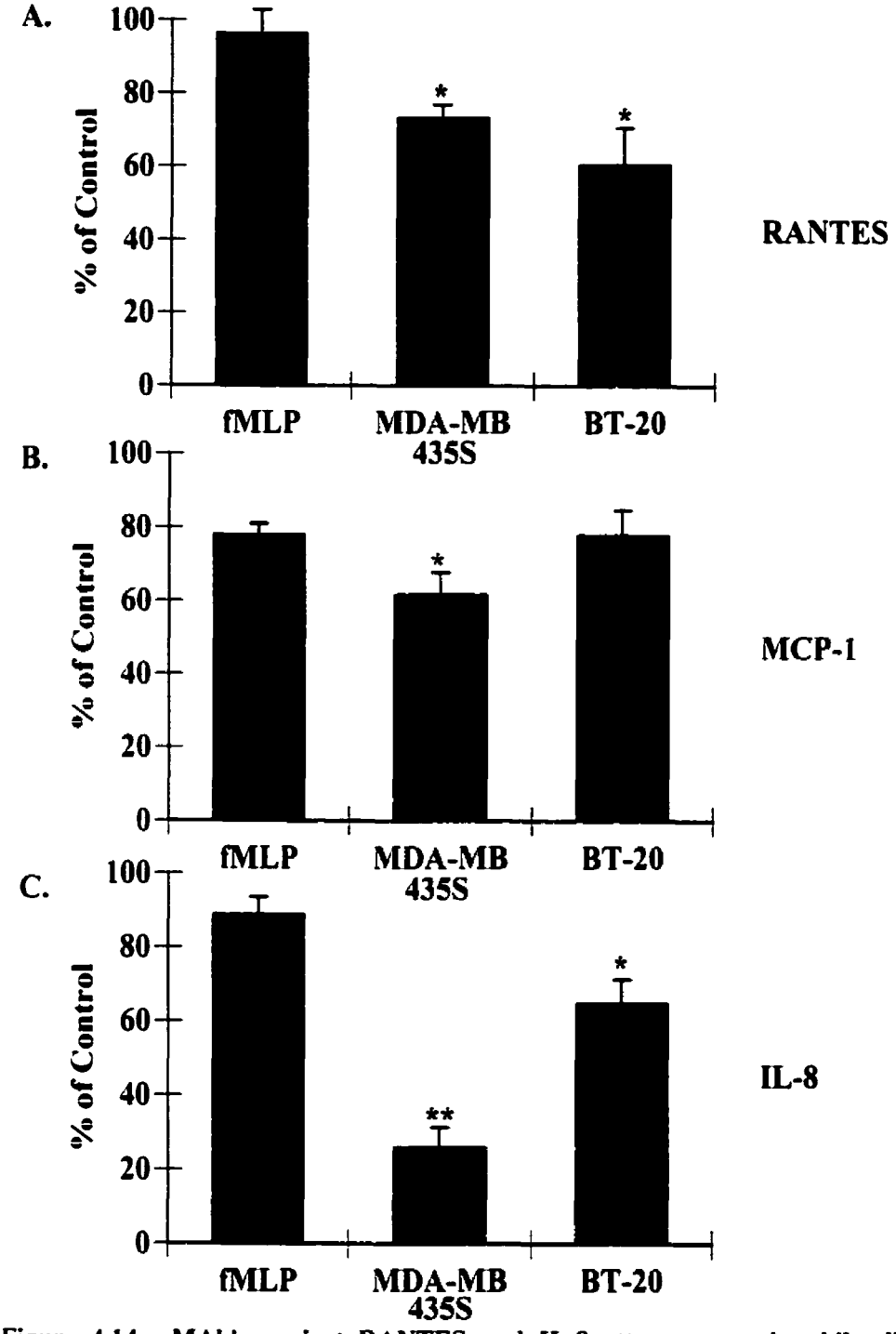


Figure 4.14. MAb's against RANTES and IL-8 attenuates eosinophil adhesion on fibronectin. Supernatants were colleced as previously described. Supernatants were pre-treated with 50 μg/ml of mAb's against (A) RANTES, (B) MCP-1 and (C) IL-8 for 15 minutes. Eosinophils ($1\text{-}2x10^6$/ml) were added to a 48-well coated with fibronectin and pre-treated supernatants were added in a 1:10 dilution. Plates were incubated for 10 minutes at 37°C. Non-adherent and loosely adherent eosinophils were removed and adhesion was quantified using an eosinophil peroxidase activity assay. Results from TNF-α stimulated supernatants are included in the figure. Percent control was calculated by dividing eosinophil adhesion with antibody by adhesion without antibody. Data are mean ± SEM of at least three experiments. *$p<0.05$ and **$p<0.005$ as compared to fMLP.

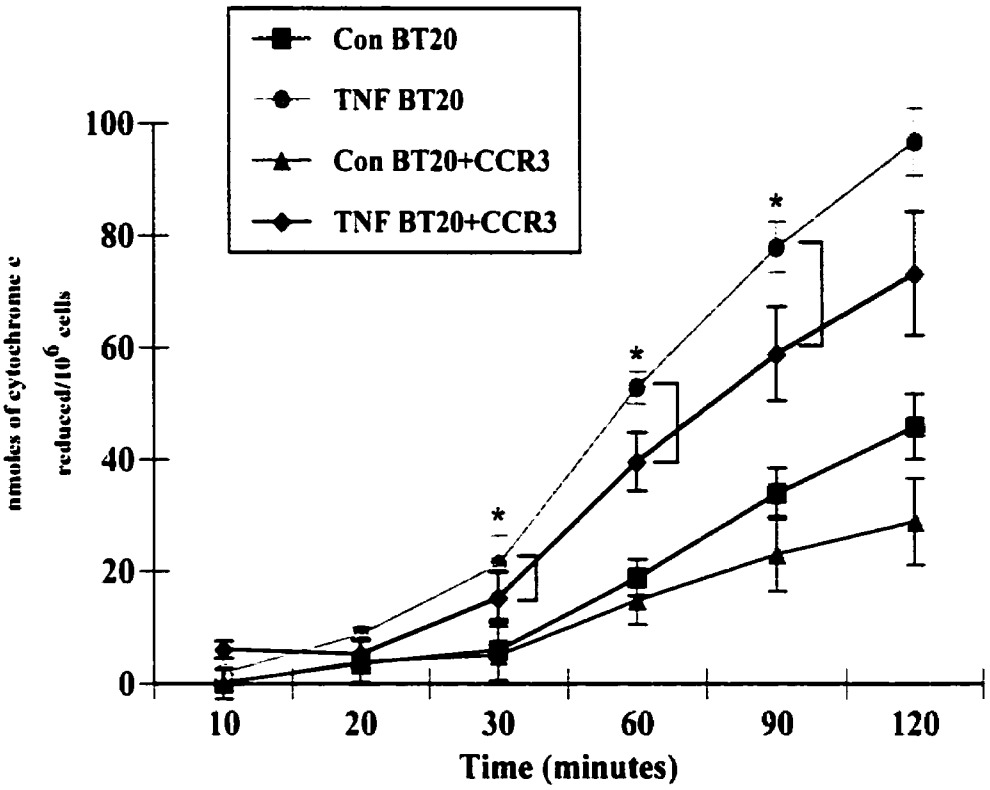


Figure 4.15. Blocking CCR3 attenuates superoxide generation from eosinophils stimulated with supernatant from TNF-α stimulated BT-20 cells. BT-20 breast carcinoma cells were stimulated with either RPMI/A or 20 ng/ml TNF-α in RPMI/A for 24 hours. 96-well plates were coated with 1% HBSS/A for 2 hours at 37°C. Eosinophils were pre-treated with 5 μg/ml CCR3 mAb for 15 minutes. 96-well plates were washed twice with HBSS and pretreated eosinophils resuspended in 2.4 mg/ml cytochrome c ($0.5x10^6$/ml) were added. The appropriate stimulus was added in a 1:2 dilution and absorbance readings were taken at 10 minute intervals until 30 minutes passed and then readings were taken at 30 minute intervals for the remaining 2 hours at 550 nm. Data are mean ± SEM of at least three experiments.

4.2 Discussion

The effects of TNF-α on breast carcinoma cells and their ability to activate eosinophils were under investigation. Our studies demonstrated that breast carcinoma cells release both eosinophil activators and chemoattractants. Also, the mediators present in the supernatant from TNF-α-stimulated breast carcinoma cells induced the generation of superoxide from eosinophils which was attenuated by either an antibody against TNFR1 or CCR3. We also showed that both MDA-MB-435S and BT-20 cells release RANTES, MCP-1 and IL-8 upon TNF-α stimulation and that antibodies against RANTES and IL-8 attenuated eosinophil adhesion on fibronectin.

Static adhesion assays on gelatin and fibronectin allowed us to determine that breast carcinoma cells release eosinophil activators. Supernatant from MDA-MB-435S cells stimulated with TNF-α for 24 hours increased eosinophil adhesion on fibronectin and not gelatin (Figure 4.1A). However, supernatant from BT-20 cells stimulated with TNF-α for 24 hours significantly increased eosinophil adhesion on gelatin and fibronectin. In addition, supernatant after 4 hours of stimulation increased eosinophil adhesion on fibronectin (Figure 4.1B). The inability of the 4 hour supernatant from MDA-MB-435S to induce eosinophil adhesion suggests that MDA-MB-435S cells do not store any preformed eosinophil activators in their granules. This also suggests that stimulation with TNF-α for 4 hours was inadequate to induce the new synthesis and release of eosinophil active proteins. Also, MDA-MB-435S cells do not release activators that modulate the affinity or avidity of the β_2-integrins on eosinophils since there was no increase in eosinophil adhesion on gelatin. However, after 24 hours of stimulation with TNF-α, both MDA-MB-435S and BT-20 cells released activators that

increased eosinophil adhesion to fibronectin suggesting that eosinophil active protein synthesis and release was occurring, which either increased the adhesiveness or up-regulated the expression of the α_4-integrins.

After determining that eosinophils can bind to breast carcinoma cells and that breast carcinoma cells release eosinophil activators, we determined if the presence of conditioned supernatant had an effect on eosinophil adhesion. Interestingly, the same amount of eosinophils bound to MDA-MB-435S cells after 15 minutes of adhesion whether the conditioned supernatant was present or absent (Figure 4.6). There was an increase in the number of eosinophils adherent on control MDA-MB-435S in the presence of supernatant at 5 minutes. These observations suggest that mediators released by MDA-MB-435S cells have little role in increasing eosinophil adhesion on these cells and that the observed adhesion is mainly due to the constitutive expression of VCAM-1 and ICAM-1 on the surface of MDA-MB-435S cells (Figure 3.3A). In the absence of conditioned supernatant, there was minimal eosinophil adhesion on BT-20 cells (Figure 4.7). However in the presence of conditioned supernatant, there was a dramatic increase in eosinophil adhesion on TNF-α stimulated BT-20 cells as compared to control. At the 10 and 15 minute time point, the amount of eosinophil adhesion to BT-20 cells surpassed the greatest amount of adhesion seen on MDA-MB-435S cells. This huge increase in eosinophil adhesion is probably due to eosinophil activators that BT-20 cells release after stimulation with TNF-α and to a lesser degree, the increased expression of VCAM-1 and ICAM-1 after stimulation with TNF-α since the same response was not seen in the absence of supernatant. These data show that the supernatant from TNF-α-stimulated BT-20 cells has a greater effect on activating eosinophils than supernatant from MDA-

MB-435S cells which agrees with the static adhesion assays on gelatin and fibronectin. From the identified chemokines in the supernatant, it is interesting to note that MBA-MB-435S have more RANTES, MCP-1 and IL-8 than BT-20 cells (Figure 4.12). We have yet to identify a mediator present in BT-20 cells that is not present in MDA-MB-435S cells.

Supernatant from both MDA-MB-435S and BT-20 cells stimulated with TNF-α are chemotactic for eosinophils (Figure 4.5). The identification of these chemoattractants began with mediators already identified for the specific recruitment of eosinophils into the tissue. Eotaxin, a member of the CC chemokine family, is a potent eosinophil-specific chemoattractant binding exclusively to CCR3 (Ponath *et al.*, 1996; Daugherty *et al.*, 1996). We assayed for all three members of the eotaxin family, eotaxin, eotaxin-2 and eotaxin-3, by RT-PCR and found little to no mRNA expression for these proteins (Figure 4.11). We next looked for the secreted protein and found no evidence of eotaxin in the supernatant of either control or TNF-α-stimulated breast carcinoma cells (data not shown). Since eotaxin is not the only eosinophil specific chemokine, we broadened our search to include chemokines known to be chemotactic for eosinophils such as RANTES (Alam *et al.*, 1993), MCP-3 (Dahinden *et al.*, 1994), MCP-4 (Uguccioni *et al.*, 1996; Stellato *et al.*, 1996) and MIP-1α (Rot *et al.*, 1992) and to chemokines that have no chemotactic response for unstimulated eosinophils such as members of the CXC chemokine family. Using commercially available ELISA kits we identified that both MDA-MB-435S and BT-20 cells under the influence of TNF-α release RANTES, MCP-1 and IL-8 (Figure 4.12). Blocking antibody studies showed that RANTES and IL-8 participate in activating eosinophils since adhesion on fibronectin was attenuated by these

antibodies (Figure 4.14). An antibody against MCP-1 had no effect on eosinophil adhesion when BT-20 supernatants were used, but it did have an effect when supernatants from MDA-MB-435S cells were used. The significance of RANTES in the supernatant is not surprising since it is well documented that RANTES can bind to both CCR1 and CCR3 (Neote *et al.*, 1993; Gao *et al.*, 1993; Ponath *et al.*, 1996) which are both found on eosinophils and cause chemotaxis (Alam *et al.*, 1993). We were surprised not only to find MCP-1 and IL-8 present in the supernatants from breast carcinoma cells, but to find that antibodies against these chemokines significantly reduced eosinophil adhesion on fibronectin when eosinophils are not thought to constitutively express the appropriate receptors for IL-8 or express any MCP-1 receptors.

The literature on eosinophils and responses towards IL-8 is contradictory. A number of studies have investigated the chemotactic behavior of eosinophils isolated from normal versus allergic donors towards IL-8. In general, normal eosinophils are not chemotactic towards IL-8, but eosinophils from allergic donors are (Sehmi *et al.*, 1993; Lampinen *et al.*, 1999; Schweizer *et al.*, 1994; Warringa *et al.*, 1991; Warringa *et al.*, 1992; Warringa *et al.*, 1993). The ability of IL-8 to induce migration of eosinophils from allergic donors suggests that these cells are in a primed state. To mimic *in vivo* priming, studies have shown that if eosinophils taken from a normal individual are incubated with either IL-5, IL-3 or GM-CSF they acquire the ability to migrate towards IL-8 (Sehmi *et al.*, 1993; Schweizer *et al.*, 1994, Warringa *et al.*, 1991; Warringa *et al.*, 1992). In contrast to these studies, Petering *et al.* showed that primed or unprimed eosinophils are not chemoattractant to IL-8 (Schroder *et al.*, 1987), they do not express the IL-8 receptors, CXCR1 or CXCR2, nor does IL-8 induce a rapid and transient release of

cytosolic free Ca^{2+} in eosinophils (Petering *et al.*, 1999). The donors used in our experiments were chosen from the general population and were not selected for particular characteristics such as allergy or asthma. This means that eosinophils were isolated from donors with no previous history of allergies or asthma and also from donors with allergies and/or asthma. Therefore it is possible that some of our experiments were performed with primed eosinophils that were capable of responding to IL-8 which could explain why we saw a reduction in adhesion on fibronectin with an IL-8 mAb.

Aside from the composition of our donor pool, the reduction in adhesion with an IL-8 mAb could possibly be justified if the IL-8 in our breast carcinoma cell supernatant was N-terminally truncated. IL-8 is generated from a 99 amino acid precursor and the most abundant forms of the mature protein consist of 77 and 72 amino acids (Baggiolini *et al.*, 1994). There are a number of naturally occurring aminoterminal variants of IL-8, including 1-77, 6-77, 7-77 (Van Damme *et al.*, 1989; Van den Steen *et al.*, 2000; Padrines *et al.*, 1994) and it has been shown that the truncated forms are more potent than full length IL-8 (Clark-Lewis *et al.*, 1991). It has been shown that aminoterminal modifications to chemokines can actually change the receptor it binds to and are therefore reactive to other cells types (Weber *et al.*, 1996). This has not been shown for IL-8, however it would be interesting to see if truncated forms of IL-8 are now active on eosinophils or if primed eosinophils have increased responsiveness to truncated forms of IL-8 as seen with neutrophils (Van den Steen *et al.*, 2000).

Eosinophils or even eosinophils pre-incubated with IL-5 have no chemotactic response towards MCP-1 (Schweizer *et al.*, 1994; Leonard and Yoshimura, 1990; Rot *et al.*, 1992; Dahinden *et al.*, 1994; Uguccioni *et al.*, 1996). Therefore the ability of an anti-

MCP-1 mAb to reduce eosinophil activation is interesting since eosinophils do not express CCR2 and are not responsive to MCP-1. There are two possible ways that MCP-1 can have an effect on eosinophils. First, MCP-1 can bind not only to CCR2 (Charo *et al.*, 1994; Myers *et al.*, 1995), CCR4 (Power *et al.*, 1995) and CCR10 (Bonini *et al.*, 1997), but also to CCR1 (Neote *et al.*, 1993) which is expressed by eosinophils. MCP-1 has also been shown to induce calcium mobilization through CCR1, specifically on eosinophils (Neote *et al.*, 1993; Fujisawa *et al.*, 2000). Therefore it could be possible that the small reduction seen with the anti-MCP-1 mAb with MDA-MB-435S supernatant could be due to MCP-1 binding to CCR1. The second mechanism that MCP-1 could be having an effect on eosinophils is based on the fact that aminoterminal truncation of MCP-1 has been shown to be a potent activator of eosinophils. MCP-1(2-76) has been shown to induce chemotaxis, actin polymerization, respiratory burst and calcium mobilization in eosinophils (Weber *et al.*, 1996). This truncated form, MCP-1(2-76), has now lost most of its potent effects on basophils but gained the ability to activate eosinophils. It is possible that MCP-1 found in breast carcinoma supernatant is of an aminoterminal variant that can now exert its activating abilities on eosinophils.

Eosinophils are known for the toxic proteins they release at inflammatory sites that cause tissue injury. As another measure of eosinophil activation, we investigated whether breast carcinoma cells could induce eosinophils to generate superoxide, a toxic oxygen species. Our studies showed that supernatant from breast carcinoma cells can induce superoxide production from eosinophils (Figure 4.8). This release was attenuated by blocking signals through TNFR1 or CCR3 (Figure 4.10 and 4.15). There are a number of known soluble activators that cause the release of reactive oxygen species

from eosinophils including, sIgA (Abu-Ghazaleh *et al.*, 1989), PAF (Kroegel *et al.*, 1989), GM-CSF (Horie *et al.*, 1996), IL-5 (Horie *et al.*, 1996) and PMA (Horie *et al.*, 1996). Consistent with published data, we found TNF-α alone can induce a moderate amount of superoxide from eosinophils (Horie *et al.*, 1996). The supernatants used to stimulate superoxide release from eosinophils contained TNF-α, however, the presence of TNF-α could not account for all of the superoxide released since a TNFR1 mAb only attenuated the response. To determine the role CCR3 had in inducing superoxide production we pre-incubated eosinophils with a CCR3 mAb. We found that CCR3 only attenuated release. It would be interesting to determine whether blocking both CCR3 and TNFR1 would abrogate the response.

The amount of superoxide generated from eosinophils stimulated with supernatant from breast carcinoma cells is noteworthy. The strongest known secretagogues for eosinophils are sIgA beads (Abu-Ghazaleh *et al.*, 1989) and PAF (Kroegel *et al.*, 1989). After 120 minutes of stimulation, eosinophils generated 58.6±7.4 superoxide in response to sIgA, 79.3±9.3 superoxide in response to PAF (Horie *et al.*, 1996) and stimulation with BT-20 TNF supernatant produced 92.0±6.55. Although side-by-side experiments need to be performed to make any comparisons, this data suggests that supernatant from TNF-α-stimulated BT-20 cells can induce eosinophils to generate large amounts of superoxide. This gives eosinophils a powerful tool in inducing tissue damage at breast carcinoma sites. Deducing the mediator(s) responsible for this robust release is difficult since TNF-α is a weak agonist for superoxide production and neither IL-8 or RANTES are capable of inducing superoxide generation on their own (Horie *et al.*, 1996). Perhaps a single mediator can not be held accountable for such a strong superoxide production from

eosinophils, rather a synergistic role between mediators may be the answer. TNF-α and IL-5 have been shown to have synergistic roles in inducing EDN release from eosinophils (Horie *et al.*, 1996). Further studies using different combinations of chemokine and cytokines may provide exciting insight into the mechanisms of eosinophil activation in the tissue.

In summary, our studies have shown that breast carcinoma cells activate eosinophils through a number of different mechanisms. In all of the activation assays performed, BT-20 supernatants activated eosinophils to a greater extent than MDA-MB-435S supernatant. The main difference between the two cell lines lies in their tumorgenicity; BT-20 cells are tumorgenic and MDA-MB-435S cells are non-tumorgenic. It is intriguing to speculate that there could be a possible link between tumorgenicity and to the extent of eosinophil activation by breast carcinoma cells and further studies in this area may shed some light on the extent of eosinophil activation at breast carcinoma sites.

4.3 Future Studies

In this study, we have just begun to understand the effector functions of eosinophils in response to mediators released by breast carcinoma cells *in vitro*. Lukacs *et al.* compared the chemokine receptor expression on peripheral versus elicited murine eosinophils and found peripheral eosinophils only expressed CCR1 and CCR3 whereas elicited eosinophils expressed CCR1 through 5 (Lukacs *et al.*, 1999). These eosinophils not only expressed receptors not normally found on eosinophils, but there was an enhanced expression of both CCR1 and CCR3 (Lukacs *et al.*, 1999). To understand the full potential of eosinophils in tumor sites, identifying chemokine receptor expression on

eosinophils that have already migrated into the tissue could prove to be a very interesting area of research.

Chapter Five: Specific Aim 3

Hypothesis: Eosinophils found in breast tumor sites are changing the tumor microenvironment through the release and/or activation of matrix metalloproteinases (MMPs).

Objectives:

1. Can eosinophils release matrix metalloproteinases?
2. Can breast carcinoma supernatants induce MMP-9 release from eosinophils?
3. Can TNF-α alone induce MMP-9 release from eosinophils?
4. Which TNF receptor is responsible for inducing MMP-9 release from eosinophils?
5. Can MMP-9 release from eosinophils stimulated with breast carcinoma cell supernatant be blocked with a TNF receptor antibody?
6. Can RANTES, MCP-1 or IL-8 induce MMP-9 release from eosinophils?
7. Can eosinophil adhesion on breast carcinoma cells stimulate MMP-9 release?

5.1 Results

Eosinophils stimulated with fMLP or TNF-α secrete MMP-9. Okada *et al.* (1997) have recently shown that eosinophils can release MMP-9 (92 kD type IV collagenase, gelatinase B) in response to cytokines and lipid mediators such as IL-5 and PAF. Based on our static adhesion data, we did not expect eosinophils to be responsive to TNF-α however, TNF-α alone did induce some superoxide generation from eosinophils. To determine if eosinophils could be stimulated to release MMP-9, we initially performed gelatin zymograms on cell-free supernatants from eosinophils

stimulated with buffer, fMLP or TNF-α. This method allowed us to determine the presence of gelatinase activity. Gelatin zymography showed a major gelatin degrading band at approximately 95 kD as well as two minor bands that migrated slower on the gel (Figure 5.1A). The major band co-migrated with an MMP-9 standard, but not with an MMP-2 standard (Figure 5.1A). The minor bands likely represent complexes with TIMP-1 and dimerized MMP-9. Consistent with other reports, we found no evidence of MMP-2 released from eosinophils (Figure 5.1A).

To further determine if eosinophils were actually releasing MMP-9 and not a nonspecific protease, we assayed for MMP-9 by ELISA. Eosinophils ($10x10^6$/ml) were stimulated with buffer or various concentrations of TNF-α for 30 minutes. Cells were spun and cell-free supernatants were used in a MMP-9 ELISA. Under TNF-α stimulation, we found that eosinophils could release anywhere from 5.6-62.8 ng/ml MMP-9 (data not shown). We found that MMP-9 release from eosinophils was sensitive to TNF-α stimulation since 0.1 ng/ml TNF-α was capable of releasing the same amounts of MMP-9 as eosinophils stimulated with 20-fold more TNF-α (2 ng/ml) (Figure 5.1B).

Supernatant from breast carcinoma cells stimulated for 24 hours with TNF-α induces MMP-9 release from eosinophils. We know that eosinophils are activated when exposed to supernatants from TNF-α-stimulated breast carcinoma cells as seen with an increase in adhesiveness on gelatin and/or fibronectin. We also wanted to determine if these mediators would be able to induce MMP-9 release from eosinophils. Since TNF-α alone could stimulate eosinophils to release MMP-9, we developed supernatants from TNF-α-stimulated breast carcinoma cells that were free of any exogenous added TNF-α. Breast carcinoma cells were stimulated for various times (i.e. 4, 8, 12, 16, 18 and 20

hours) with TNF-α, cells were washed four times with buffer and fresh buffer was added for the remaining 24 hour incubation period (i.e. after 4 hours with TNF-α, breast carcinoma cells were washed and incubated with fresh buffer for the remaining 20 hours: see methods). Supernatant harvested from breast carcinoma cells treated for 24 hours still contained the TNF-α used to stimulate these cells. Eosinophils were stimulated with supernatants from either control, 4, 8, 12 or 24 hour TNF-α-stimulated breast carcinoma cells for 30 minutes. Cells were spun and the supernatants were collected and run on a gelatin zymogram.

Supernatants from control MDA-MB-435S and BT-20 cells did not induce any MMP-9 degranulation and very faint bands were present after stimulation with the 4, 8 and 12 hour supernatant (Figure 5.2). Supernatant from 18 and 20 hours also produced little MMP-9 release (data not shown). There was however, abundant MMP-9 release after stimulating eosinophils with supernatant from breast carcinoma cells stimulated for 24 hours with TNF-α (Figure 5.2). Since the 24 hour supernatant was the only supernatant that contained TNF-α, two possible explanations could account for these observations. One possibility could be that mediators present in supernatants free from TNF-α do not induce MMP-9 release from eosinophils. However due to fact that both 24 hour supernatant and TNF-α control both contain 2 ng/ml TNF-α and that there was more MMP-9 released from eosinophils stimulated with 24 hour breast carcinoma supernatant, suggests either an additive or synergistic effect. Therefore, a second possible explanation could be that the mediators in the supernatant alone do not induce MMP-9 release but instead can synergize with TNF-α to maximize MMP-9 release. It should be noted however that some experiments showed equal MMP-9 release from

eosinophils stimulated with TNF-α alone and eosinophils stimulated with 24 hour supernatants.

MMP-9 release from TNF-α-stimulated eosinophils is time- and dose-dependent. After determining that TNF-α could stimulate eosinophils to release MMP-9, we next wanted to determine the time and dose dependence of this response. First eosinophils were stimulated with 2 ng/ml TNF-α for various times ranging from 5 minutes to 1 hour. TNF-α induced a time-dependent increase in MMP-9 release from eosinophils, with significant release observed as early as 25 minutes and with maximal release at 1 hour (Figure 5.3A). Figure 5.3B shows the dose-response curve at 30 minutes in response to TNF-α. There was no difference in the MMP-9 released at concentrations between 20 and 0.1 ng/ml of TNF-α (Figure 5.3B and data not shown) which agrees with the MMP-9 ELISA (Figure 5.1B). MMP-9 release diminished as concentrations dipped below 0.1 ng/ml. Notably, as little as 0.05 ng/ml of TNF-α was capable of eliciting MMP-9 release above background levels. Thus very low concentrations of TNF-α are sufficient to induce the release of MMP-9.

Eosinophils release pro-MMP-9 which can be activated by p-aminophenylmercuric acetate (APMA). To further characterize the MMP-9 released from eosinophils, we wanted to determine if the pro or activated form of the protease was being released. Eosinophils ($1x10^6$) were stimulated with buffer or 2 ng/ml TNF-α for 30 minutes. Cell-free supernatants were then assayed by a gelatinase activity assay. Any active gelatinases in the supernatant would cleave a biotinylated gelatinase substrate, reducing the amount of biotin on the plate. The remaining biotin molecules would be detected by the addition of a streptavidin-enzyme complex and enzyme substrate.

Therefore, small absorbance values reflects the presence of activated gelatinases in the sample. The results from the gelatinase activity assay showed that eosinophils release pro- or unactivated MMP-9 (Figure 5.4A).

Electrophoresis and renaturation can activate the gelatin degrading activity of pro-MMP-9, thus gelatin zymography detects both pro-MMP-9 (92 kD) and active MMP-9 (82 kD). Pro-MMP-9 from eosinophils has an apparent molecular weight of 95 kD (Okada *et al.*, 1997) and active MMP-9 is 84 kD. We next wanted to show that we could activate the pro-MMP-9 released from stimulated eosinophils. MMPs can be activated by a variety of proteases or by reaction with organic mercurials such as APMA. After eosinophils were stimulated with TNF-α, cell-free supernatants were treated with 0.5 mM APMA for 60 minutes and gelatin zymography was performed. APMA treatment of the pro-MMP-9 released by TNF-α stimulated eosinophils resulted in conversion to a 89 kD form (Figure 5.4B).

In summary, we found that eosinophils can be stimulated to release MMP-9 by TNF-α and depending on the donor, release could be as much as 63 ng/ml MMP-9. The MMP-9 released is in the pro-form and can be partially activated by an organic mercurial, APMA.

TNFR1 is required for MMP-9 release from TNF-α-stimulated eosinophils. TNF-α can bind and signal through two different receptors, TNFR1 (p55) and TNFR2 (p75). As previously shown, eosinophils express low levels of these receptors on their surface (Matsuyama *et al.*, 1998). After determining that TNF-α induced MMP-9 release from eosinophils, we wanted to determine if this was mediated through TNFR1, TNFR2 or both receptors. Eosinophils were incubated with neutralizing mAbs against TNFR1

and/or TNFR2 for 15 minutes prior to stimulation with 2 ng/ml TNF-α. After 30 minutes with TNF-α, the supernatant was collected and MMP-9 release was measured by gelatin zymography. An anti-TNFR1 mAb blocked virtually all MMP-9 release from eosinophils (Figure 5.5). In contrast, a TNFR2 mAb had little effect on MMP-9 release (Figure 5.5). In combination, the antibodies brought MMP-9 release to control levels. Non-immune IgG antibodies were used to eliminate the possibility of nonspecific antibody effects. There was no decrease in MMP-9 release with these antibodies (Figure 5.5). These data suggests that TNF-α acts primarily through TNFR1 to activate the release of MMP-9.

MMP-9 release from eosinophils stimulated with breast carcinoma supernatant can be attenuated by a TNFR1 antibody. Even though supernatant from breast carcinoma cells stimulated for 24 hours with TNF-α was capable of inducing MMP-9 degranulation, this however was the only supernatant that still contained the TNF-α used to stimulate the cells. To determine if the TNF-α present in the supernatant was responsible for the MMP-9 release we pre-treated eosinophils with a neutralizing antibody against TNFR1 and then stimulated with breast carcinoma supernatant. Supernatants from breast carcinoma cells treated with TNF-α were assayed for TNF-α by ELISA (Figure 4.13). TNF-α levels were actually lower in 24 hour supernatants from the stimulation buffer, thus the TNFR1 antibody should be sufficient to block all of the TNF-α in the supernatants. Pretreatment of eosinophils with TNFR1 mAb had no effect on MMP-9 release from eosinophils stimulated with control MDA-MB-435S and BT-20 supernatants indicating that background MMP-9 release was not signaling through TNFR1 (Figure 5.6). The antibody attenuated MMP-9 released from eosinophils

stimulated with supernatant from TNF-α treated breast carcinoma cells (Figure 5.6). The lack of complete inhibition suggests that there are other mediators present in breast carcinoma supernatant that could be inducing MMP-9 release from eosinophils.

Eosinophils stimulated with RANTES or IL-8 secrete pro-MMP-9. In chapter four we identified that breast carcinoma cells release RANTES, MCP-1 and IL-8 (Figure 4.12). Knowing that TNF-α alone is unable to induce all of the MMP-9 release from eosinophils stimulated with TNF supernatants, we wanted to determine if these chemokines alone could induce MMP-9 release. Chemokine concentrations used to stimulate eosinophils were based on quantities found in the breast carcinoma supernatant by ELISA (Figure 4.12). Eosinophils were stimulated with various concentrations of RANTES, MCP-1 and IL-8 and MMP-9 release was determined by gelatin zymography. Both RANTES and IL-8 induced MMP-9 release from eosinophils, however MCP-1 was unable to induce MMP-9 release above control levels (Figure 5.7). The inability of MCP-1 to induce MMP-9 degranulation was expected since control eosinophils do not express its receptor, CCR2.

Eosinophil adhesion on breast carcinoma cells does not induce MMP-9 release. Eosinophil adhesion to VCAM-1 has been shown to activate superoxide anion generation (Nagata *et al.*, 1995). We wanted to examine the possibility that eosinophil adhesion to breast carcinoma cells would induce MMP-9 release. Breast carcinoma cells were grown to confluence and stimulated with either buffer alone or TNF-α. Eosinophils ($1x10^6$) were added to either control or TNF-α-stimulated breast carcinoma cells and left to incubate for 2 hours at 37°C. MMP-9 release from breast carcinoma cells was assessed by washing the cells and incubating the carcinoma cells with buffer only for 2 hours. To

determine whether adhesion stimulated MMP-9 release, eosinophils were incubated with washed breast carcinoma cells. Adhesion on breast carcinoma cells was also performed in the presence of stimulation buffer, therefore mediators released by breast carcinoma cells could activate the eosinophils. We found that neither control or TNF-α treated MDA-MB-435S or BT-20 cells released MMP-9 on their own (Figure 5.8A and 5.9A). In addition, eosinophils adherent on washed breast carcinoma cells did not release any MMP-9 (Figure 5.8A and 5.9A). In the presence of supernatant from breast carcinoma cells however, eosinophils released MMP-9 in comparable amounts to the TNF control (Figure 5.8A and 5.9A).

The role of adhesion molecules in MMP-9 release was also examined. Eosinophils were pre-treated with antibodies against α_4, β_2 or a combination of the two. The eosinophils were added to breast carcinoma cells in the presence of supernatant for 2 hours. Blocking the α_4- and β_2-integrins had no effect on MMP-9 release from eosinophils adherent on breast carcinoma cells (Figure 5.8B,C and 5.9B,C). Thus, adhesion was not required for MMP-9 release in this system.

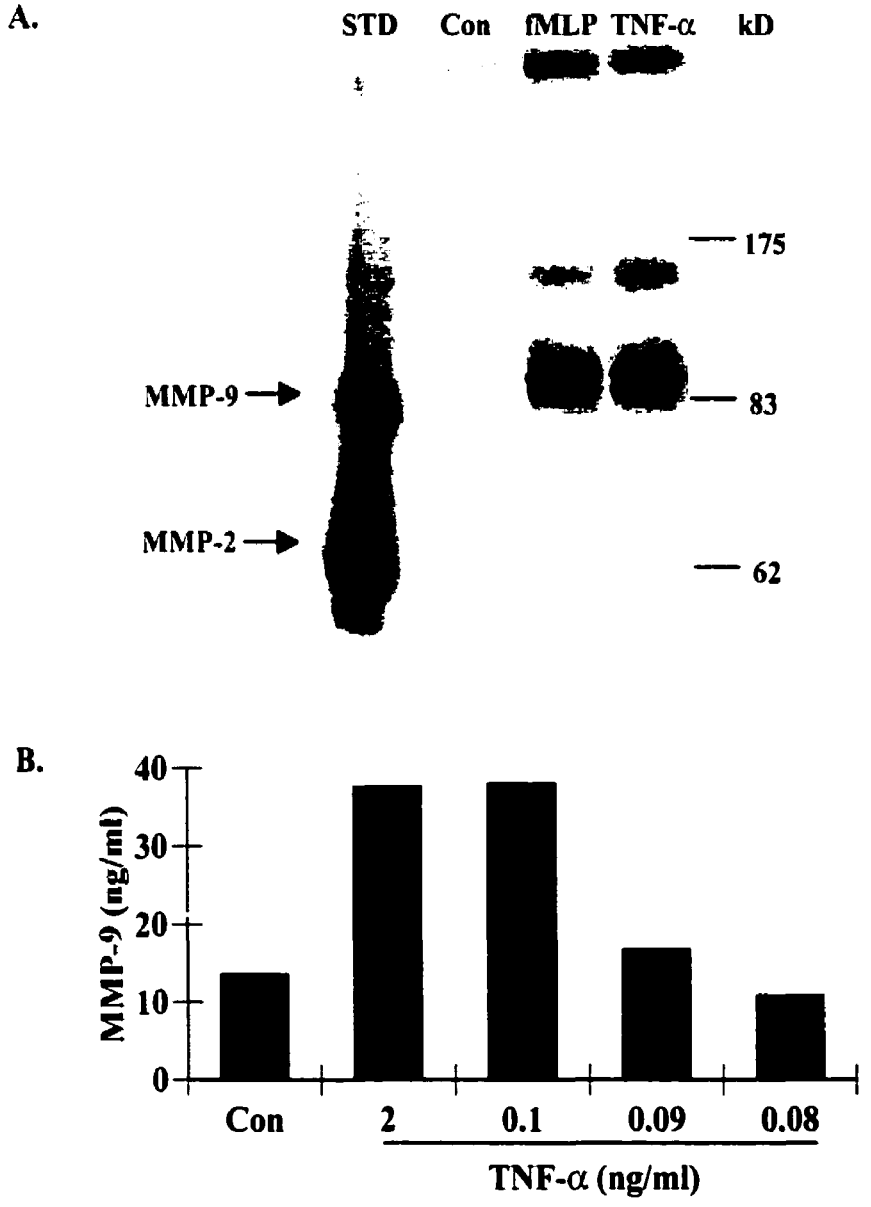


Figure 5.1. Eosinophils stimulated with fMLP or TNF-α secrete MMP-9. (A) Eosinophils ($10x10^6$/ml) were stimulated with buffer alone (Con) or buffer containing 2 ng/ml TNF-α (TNF-α) for 30 minutes. Cells were spun and supernatants were collected and gelatin zymography was performed as described in methods. Recombinant MMP-9 and MMP-2 were used as positive controls (STD). STD is from a different gel and is shown in the proper relative position. (B) Eosinophils were stimulated with buffer alone (Con), 2, 0.1, 0.09 or 0.08 ng/ml TNF-α. Cells were spun and the supernatants were collected. The supernatant was assayed for MMP-9 by ELISA according to manufacturer's instructions. Data in (A) are representative of at least ten experiments and data in (B) is from a representative experiment.

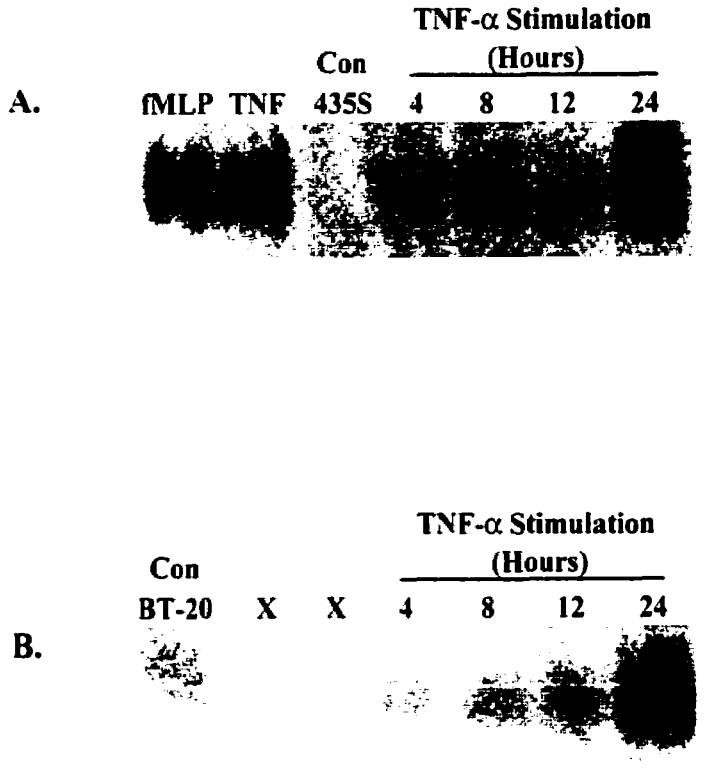


Figure 5.2. Supernatant from breast carcinoma cells stimulated for 24 hours with TNF-α induces maximal MMP-9 release from eosinophils. MDA-MB-435S and BT-20 breast carcinoma cells were stimulated with 20 ng/ml TNF-α for either 4, 8 or 12 hours and cells were washed and given new media. Alternatively, cells were stimulated for 24 hours with TNF-α. Supernatants were harvested after 24 hours of stimulation. Eosinophils ($10x10^6$/ml) were stimulated with 10^{-7}M fMLP, 2 ng/ml TNF-α, (A) MDA-MB-435S or (B) BT-20 supernatant for 30 minutes. Cells were spun and supernatants were collected. Gelatin zymography was performed as described in the methods. Data are representative of at least three experiments. "X" indicates an empty lane.

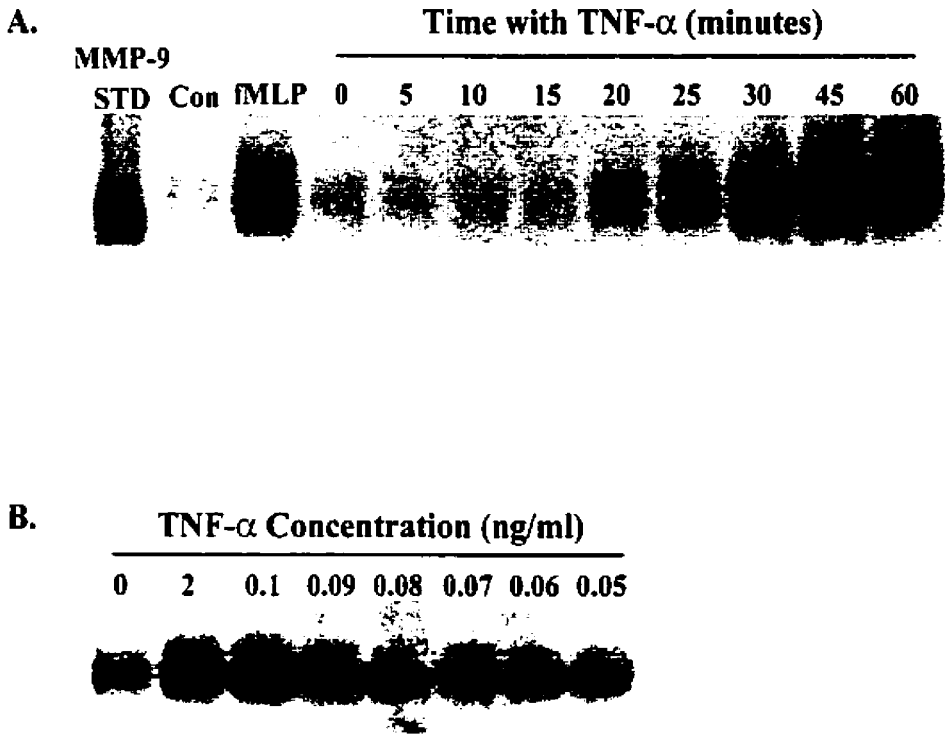


Figure 5.3. MMP-9 release from TNF-α-stimulated eosinophils is time- and dose-dependent. Eosinophils ($10x10^6$/ml) were treated with (A) 2 ng/ml TNF-α for the specified time or (B) the specified concentrations of TNF-α for 30 minutes. MMP-9 release was determined by gelatin zymography as described in the methods. A recombinant MMP-9 standard was used as a positive control (STD). 10^{-7}M fMLP was also used as a positive control. Data are representative of at least three experiments.

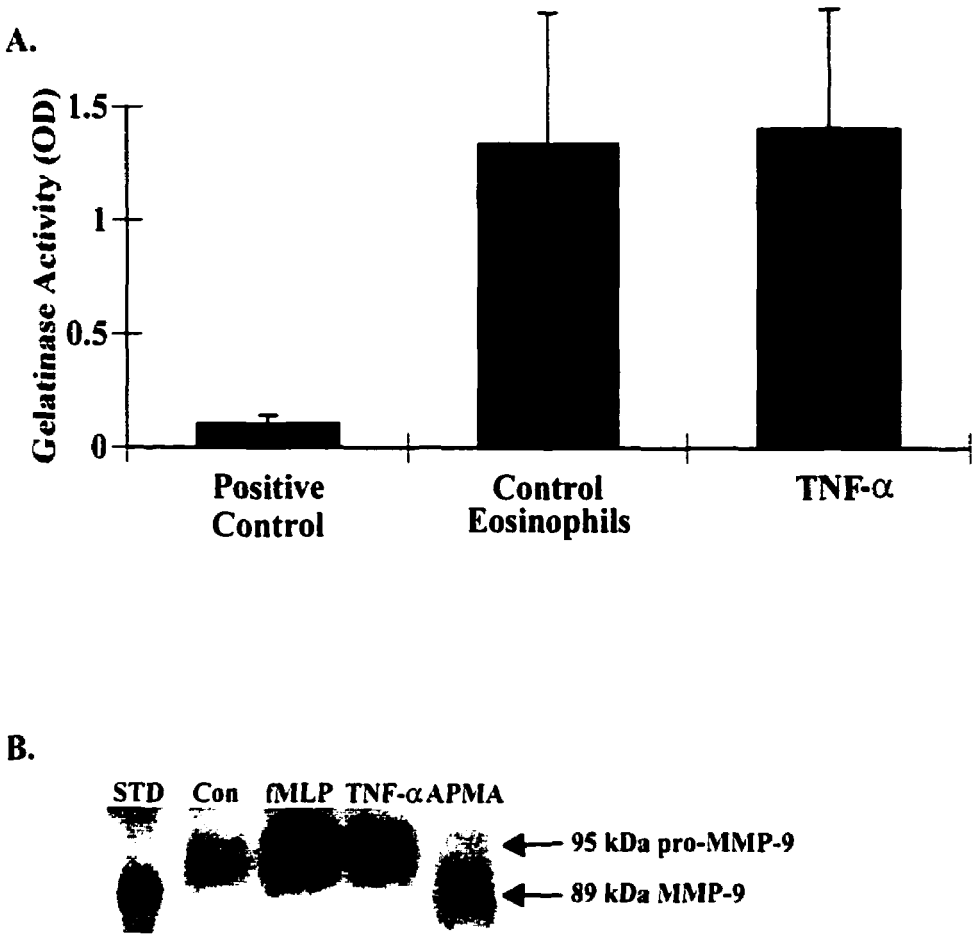


Figure 5.4. Eosinophils release pro-MMP-9 which can be cleaved by *p*-Aminophenylmercuric Acetate (APMA). (A) Eosinophils ($1x10^6$/ml) were either stimulated with buffer alone or TNF-α (2 ng/ml) for 30 minutes. Cells were spun and the supernatants were used in a gelatinase activity assay according to manufacture's instructions. Cleavage of a biotinylated substrate, as indicated by a reduction in absorbance (450nm), was used as a measurement of gelatinase activity. (B) Eosinophils were stimulated with buffer alone (Con), 10^{-7}M fMLP or 2 ng/ml TNF-α for 30 minutes. Supernatants from TNF-α-activated eosinophils were treated with 0.5mM APMA for 60 minutes at 37°C. Gelatin zymography was performed as outlined in the methods.

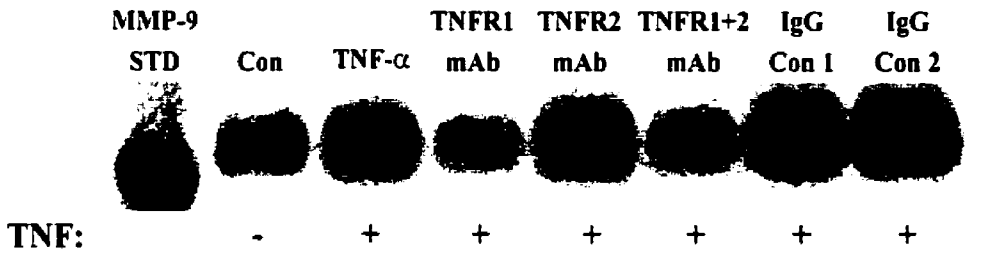


Figure 5.5. TNFR1 (p55), but not TNFR2 (p75), is required for TNF-α stimulated MMP-9 release from eosinophils. Eosinophils ($10x10^6$/ml) were either untreated or pretreated with neutralizing mAb to TNFRI (50 μg/ml), TNFRII (10 μg/ml) or both for 15 minutes. Eosinophils were also pretreated with a non-immune IgG_1 antibody (Con 1: 50 μg/ml; Con 2: 10 μg/ml) to eliminate the possibility of nonspecific antibody effects. Cells were then stimulated with buffer alone or 2 ng/ml TNF-α for 30 minutes. Cells were spun and the supernatants were collected. Gelatin zymography was performed as described in the methods. Data are representative of at least three independent experiments.

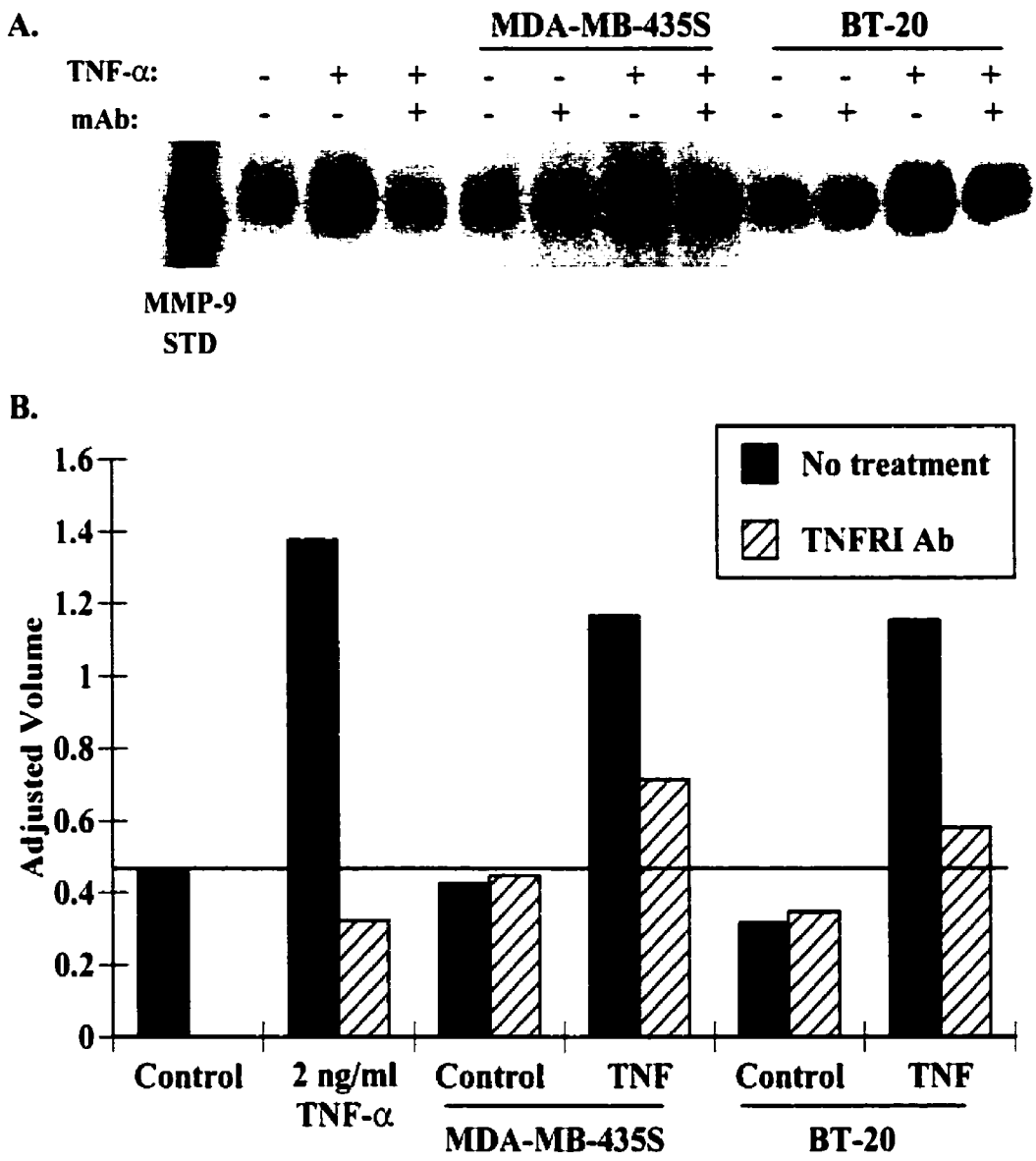


Figure 5.6. MMP-9 release from breast carcinoma supernatant-stimulated eosinophils can be attenuated by a TNFRI antibody. (A) Eosinophils ($10x10^6$/ml) were either untreated or pretreated with neutralizing mAb to TNFRI (50 μg/ml) for 15 minutes. Cells were then stimulated for 30 minutes with buffer alone, 2 ng/ml TNF-α, supernatant from control MDA-MB-435S or BT-20 cells, or supernatant from TNF-α stimulated MDA-MB-435S or BT-20 cells. Cells were spun and the supernatants were collected. Gelatin zymography was performed as described in the methods. Data are representative of at least three independent experiments. (B) The bands from the representative zymogram were analyzed by densitometry and are expressed as the adjusted volume from which background values have been subracted.

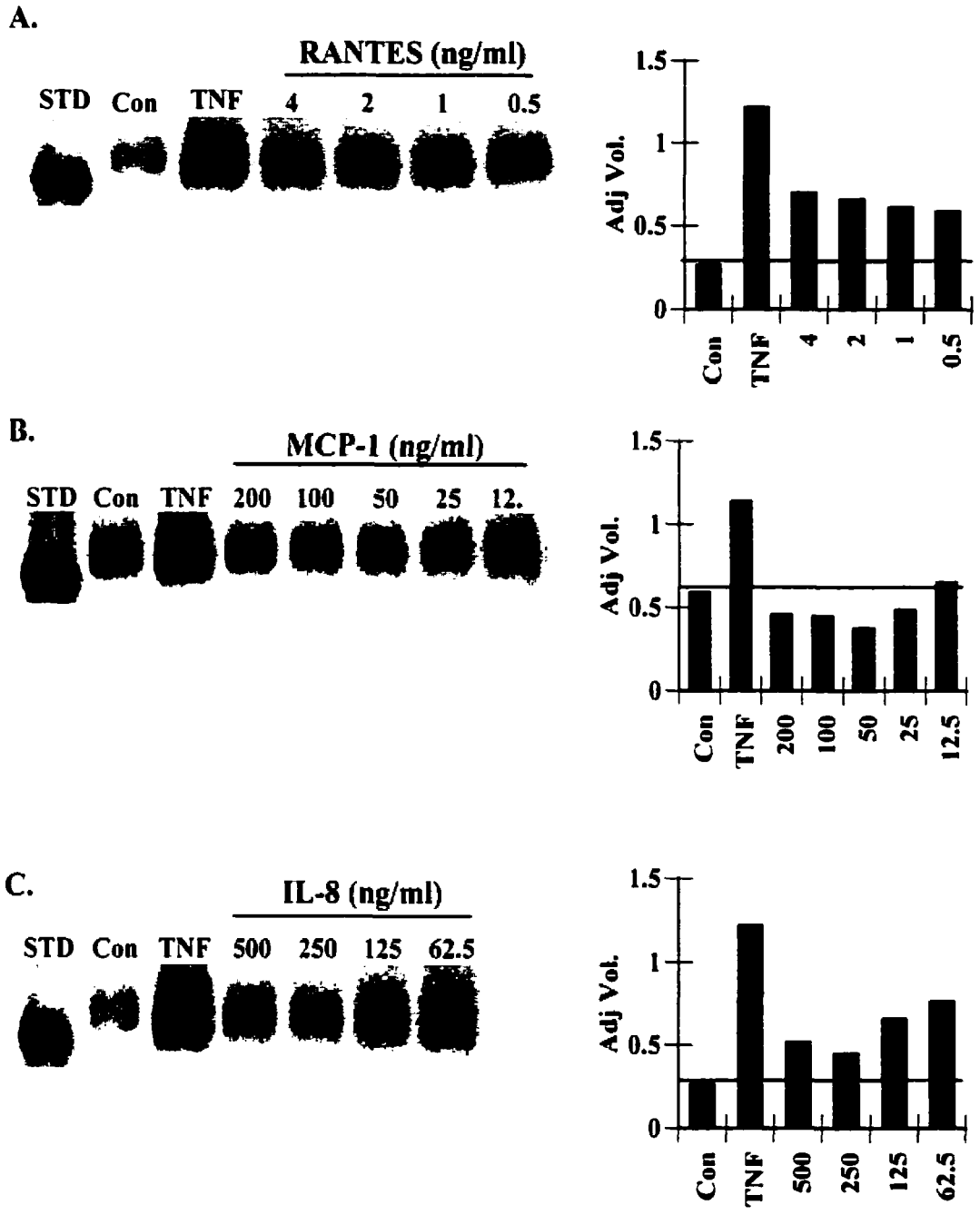


Figure 5.7. Eosinophils stimulated with RANTES or IL-8, but not MCP-1, secrete pro-MMP-9. Eosinophils ($10x10^6$/ml) were stimulated with various concentrations of (A) RANTES, (B) MCP-1 and (C) IL-8 for 30 minutes. Cells were spun and supernatants were collected. Gelatin zymography was performed as described in the methods. Densitometry was performed on the representative zymogram shown to the left. Background noise for each zymogram was subtracted from each band, therefore densitometry values are given in adjusted volume units. Data are representative of at least three experiments.

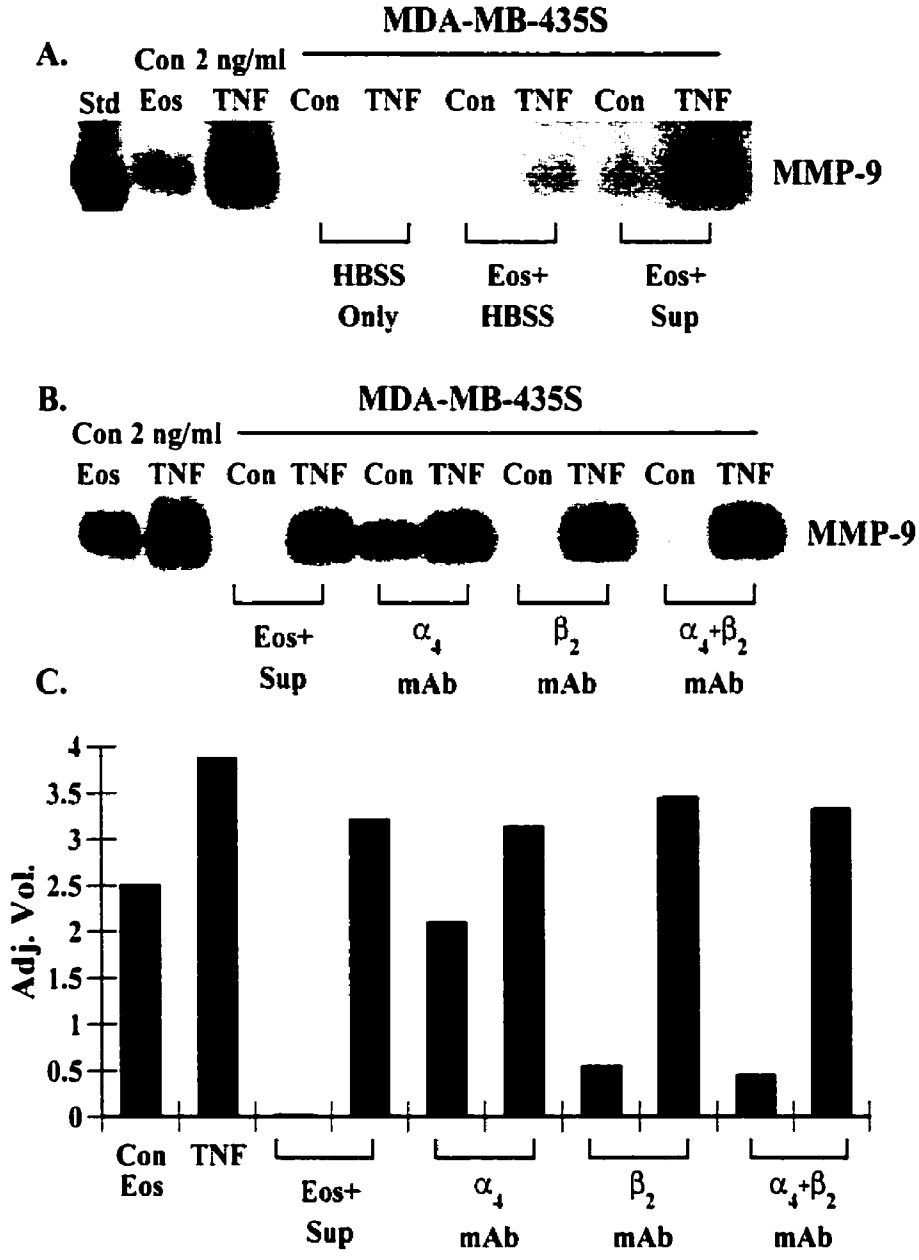


Figure 5.8. Pretreating eosinophils with antibodies against α_4- and β_2-integrins does not change the amount of MMP-9 released from eosinophils adherent to MDA-MB-435S cells. Confluent monolayers of MDA-MB-435S cells were stimulated with either M199/A alone (Con) or 20 ng/ml TNF-α (TNF) for 24 hours. (A) Breast carcinoma cells were either washed and HBSS was added (HBSS only) or they were washed and eosinophils ($1x10^6$) were added (Eos+HBSS). Eosinophils were also added to carcinoma cells with supernatant present (Eos+Sup). Eosinophils in suspension were either stimulated with buffer (Con eos) or 2 ng/ml TNF-α (2ng/ml TNF) and were performed side-by-side with the static adhesion assay. (B) Eosinophils were pretreated with anti-α_4mAb , anti-β_2 mAb or a combination of the two for 15 minutes. Eosinophils were then added to MDA-MB-435S cells and incubated for 2 hours at 37°C. (C) Densitometry was performed on the bands in (B). Data are representative of at least three experiments.

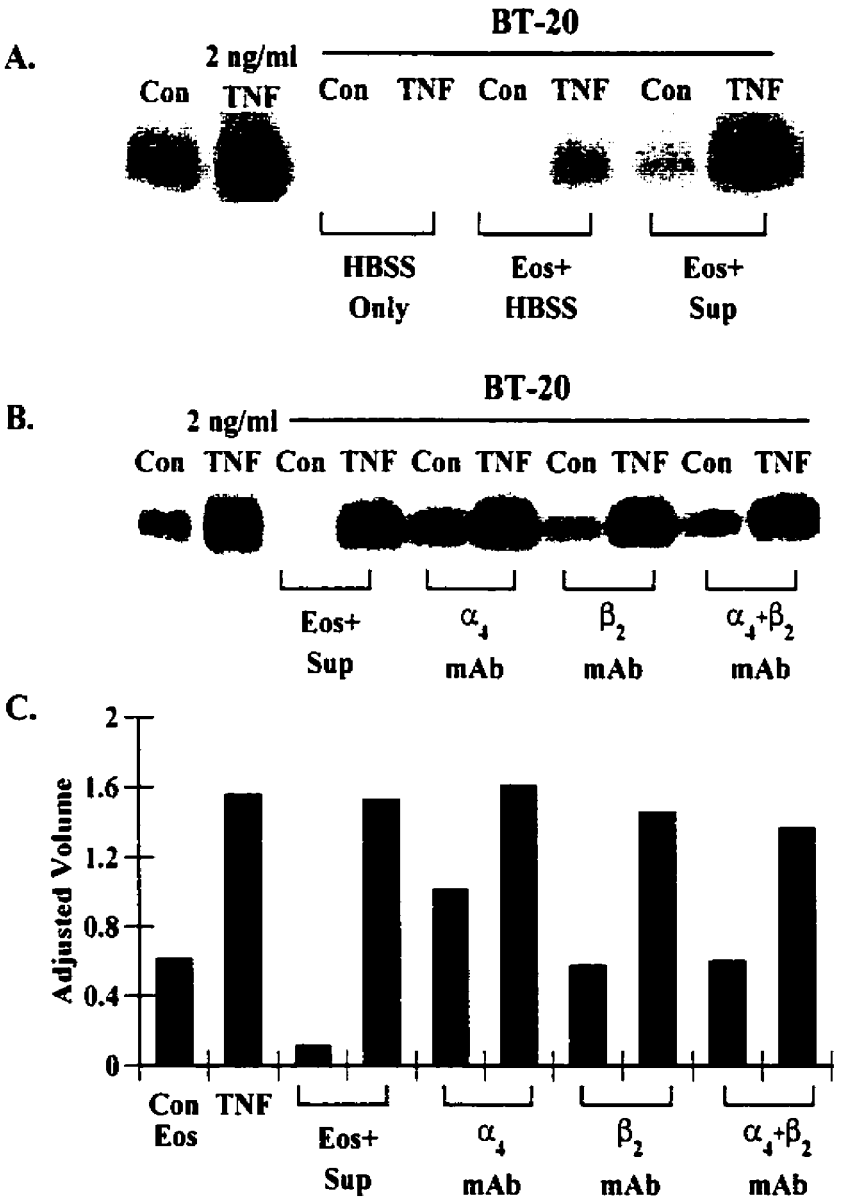


Figure 5.9. Pretreating eosinophils with antibodies against α_4- and β_2-integrins does not change the amount of MMP-9 released from eosinophils adherent to BT-20 cells. Confluent monolayers of BT-20 cells were stimulated with either M199/A alone (Con) or 20 ng/ml TNF-α (TNF) for 24 hours. (A) Breast carcinoma cells were either washed and HBSS was added (HBSS only) or they were washed and eosinophils ($1x10^6$) were added (Eos+HBSS). Eosinophils were also added to carcinoma cells with supernatant present (Eos+Sup). Eosinophils in suspension were either stimulated with buffer (Con eos) or 2 ng/mL TNF-α (TNF) and were performed side-by-side with the static adhesion assay. (B) Eosinophils were pretreated with anti-α_4mAb . anti-β_2 mAb or a combination of the two for 15 minutes. Eosinophils were then added to BT-20 cells and incubated for 2 hours at 37°C. (C) Densitometry was performed on the bands in (B). Data are representative of at least three experiments.

5.2 Discussion

The extracellular matrix (ECM) is composed of glycoproteins, glycosaminoglycans, collagens and proteoglycans and provides not only structural support for tissue and organs, but also modulates development, proliferation and migration. One family of proteases, called the matrix metalloproteases (MMPs), has the capability of degrading all components of the ECM. MMPs are a family of 17 structurally and functionally related zinc endopeptidases that are further categorized based on substrate specificity and structure (reviewed in Westermarck and Kahari, 1999). There are five subgroups consisting of: collagenases, gelatinases, stromelysins, membrane-type-like and other MMPs. MMPs have been shown to be important proteinases that are involved in many normal physiological processes such as uterine implantation, menstruation, embryonic growth, tissue morphogenesis and tissue repair (Shapiro, 1998). However, inappropriate or overexpression of certain MMPs has contributed to the disease progression of rhematoid arthritis, osteoarthritis, multiple sclerosis and cardiovascular disease (Shapiro, 1998). There is also a link between the overexpression of MMPs and an increase tumor progression (Liotta *et al.*, 1980; Westermarck and Kahari, 1999; Davies *et al.*, 1993; Stetler-Stevenson *et al.*, 1993; MacDougall and Matrisian, 1995; Mignatti *et al.*, 1993). In this study, we investigated the ability of eosinophils to release one class of MMPs, the gelatinases, in response to mediators released from breast carcinoma cells.

As with all of our other experiments, we first used TNF-α alone to determine if the cytokine has any effects on MMP-9 release. To our surprise, TNF-α induced significant release of MMP-9 from eosinophils. Despite low expression of TNF

receptors, eosinophils were very sensitive to TNF-α stimulation. We investigated the ability of eosinophils to release MMP-2 or MMP-9 in response to TNF-α and found that eosinophils released MMP-9 in both a time- and dose-dependent fashion (Figure 5.3). We found no evidence of MMP-2 released from eosinophils stimulated with TNF-α. MMP-9 release was seen with as low as 0.09 ng/ml TNF-α and as rapidly as 20 minutes after stimulation. Signals through TNFR1 were responsible for the majority of MMP-9 released from eosinophils due to complete inhibition seen with a TNFR1 mAb (Figure 5.5).

We next wanted to determine if supernatant from TNF-α-stimulated breast carcinoma cells could induce MMP-9 release from eosinophils. However, since TNF-α alone could stimulate eosinophils to release MMP-9, we had to develop supernatants from TNF-α-stimulated breast carcinoma cells that was free of any exogenous added TNF-α. To remedy this problem, we stimulated breast carcinoma cells for various times (i.e. 4, 8, 12, 16, 18 and 20 hours), washed the cells four times with buffer and added fresh buffer for the remaining 24 hour incubation period (i.e. after 4 hours with TNF-α, breast carcinoma cells were washed and incubated with fresh buffer for the remaining 20 hours). None of the TNF-free supernatants from the time course could induce MMP-9 release from eosinophils (Figure 5.2). In contrast, the 24 hour supernatants that contained TNF-α did stimulate MMP-9 release from eosinophils. In some experiments equivalent amounts of TNF-α stimulated less MMP-9 release suggesting that there were additional mediators present in the supernatant (Figure 5.2 versus 5.6).

To determine if there were other mediators capable of increasing MMP-9 release, we incubated eosinophils with a neutralizing TNFR1 mAb and then stimulated with

supernatant from breast carcinoma cells treated with TNF-α for 24 hours. Pretreatment with a TNFR1 mAb only attenuated MMP-9 release suggesting a role for additional mediators such as RANTES, IL-8 and MCP-1 which were previously identified in TNF supernatants. These results could also mean that there was incomplete blockade due to an increase in TNF-α production from either the breast carcinoma cells or from the eosinophils. This is unlikely since supernatants from breast carcinoma cells treated for 24 hours were assayed for TNF-α by ELISA (Figure 4.13) and our results showed that there was actually a decrease in TNF-α after stimulation suggesting that the TNF-α was being degraded. We also assayed supernatant from eosinophils adherent to breast carcinoma cells (in the presence of TNF supernatant) for 2 hours and TNF concentrations were the same as found in supernatant alone (data not shown). In addition, MMP-9 released from eosinophils stimulated with TNF-α alone, in equivalent amounts found in the supernatant, was completely inhibited after the eosinophils were pretreated with a TNFR1 mAb (Figure 5.6).

The ability of RANTES, MCP-1 and IL-8, which were identified in TNF-stimulated breast carcinoma supernatant, to stimulate MMP-9 release from eosinophils was examined. Our studies showed that the CC chemokine, RANTES and the CXC chemokine, IL-8 could stimulate MMP-9 release from eosinophils (Figure 5.8). It should be noted that two out of three experiments showed increased levels of MMP-9 released from control in response to IL-8. However, MMP-9 released varied between donors which was most likely due differential expression of CXCR1 and CXCR2 on eosinophils.

Taken together, breast carcinoma supernatants without TNF-α does not stimulate MMP-9 release, however mediators such as RANTES and IL-8 found in TNF-

supernatants can induce MMP-9 release. These results suggest that RANTES and/or IL-8 may act synergistically with TNF-α to induce MMP-9 release from eosinophils. One way to determine if synergy is actually occurring would be to add back TNF-α to time course supernatants and stimulate eosinophils with these supernatants. Suboptimal concentrations of IL-8 and TNF-α or RANTES and TNF-α could also be used to determine specific synergy roles of these chemokines with TNF-α. Future experiments incubating eosinophils with a TNFR1 mAb + CCR3 mAb could provide insight into the role RANTES is playing in stimulating MMP-9 release from eosinophils. Also, pretreating supernatants with an IL-8 or RANTES mAb and eosinophils with a TNFR1 mAb could identify the role these chemokines have with MMP-9 secretion from eosinophils.

Eosinophil adhesion has been shown to induce activation and alter functional responses in eosinophils (Nagata *et al.*, 1995). We wanted to determine if eosinophil adhesion to breast carcinoma cells would have any effect on the amount of MMP-9 released. Adhesion on washed breast carcinoma cells did not stimulate MMP-9 secretion from eosinophils (Figure 5.8A and 5.9A). MMP-9 was secreted when eosinophils were added to TNF-α-stimulated breast carcinoma cells in the presence of supernatant. Antibodies against α_4- and β_2-integrins had no effect on MMP-9 released from eosinophils (Figure 5.8B,C and 5.9B,C). These data suggest that adhesion alone has no effect on stimulating MMP-9 release from eosinophils and the MMP-9 release seen is mainly due to the presence of TNF-α and secondarily to other mediators in the supernatant.

Using zymograms, gelatinase activity assays and stimulation with APMA we determined that eosinophils released the proform of MMP-9 (Figure 5.1 and 5.4). This observation was expected since almost (Pei and Weiss, 1995) all MMPs are released in the latent form and require subsequent proteolytic cleavage of the prodomain for activation. It is interesting to note that after stimulation with APMA, there was a drop in molecular weight from 95 kDa to approximately 89 kDa. Although APMA is known to activate MMP-9 (Okada *et al.*, 1992; Shapiro *et al.*, 1995), APMA treated pro-MMP-9 from eosinophils only provided a truncated form with no gelatinase activity (data not shown). This suggests that APMA cleavage resulted in a shortened form of MMP-9 that still contained the prodomain cysteine interacting with the active zinc molecule in the catalytic domain.

Organomercurial agents such as APMA activate MMPs by chelating the cysteine residue in the prodomain leaving the zinc molecule in the catalytic domain accessible. Subsequent autolytic cleavage of the prodomain results in an intermediate protein of approximately 86 kDa and in the absence of TIMP-1, further autolytic cleavage occurs resulting in a 67 kDa product (Shapiro *et al.*, 1995; Okada *et al.*, 1992). However in the presence of TIMP-1, this intermediate protein of 86 kDa is the final product and lacks enzymatic activity due to TIMP-1 binding to the carboxy terminus of MMP-9 (Ogata *et al.*, 1995; Okada *et al.*, 1992; Shapiro *et al.*, 1995). Differences between the type of MMP-9 leukocytes secrete has already been noted in the literature. Neutrophils release MMP-9, TIMP free (Hibbs *et al.*, 1985), however macrophages secrete MMP-9 in complex with TIMP-1 (Mautino *et al.*, 1999). Therefore, one possible explanation for our results could be that eosinophils secrete MMP-9 as a proenzyme in association with

TIMP-1, therefore preventing full activation of pro-MMP-9 upon stimulation with APMA. This is consistent with work done in Moqbel's lab, where they showed that both TIMP and MMP-9 levels increased after stimulation with TNF-α (Schwingshackl *et al.*, 1999). Consequently, for eosinophil generated MMP-9 to have any effect in breast carcinoma sites, it would have to be activated in an environment where activated MMPs were in molar excess to TIMP-1. This balance of inhibitors and activated proteinases adds to the precisely controlled activities of MMPs. Specific stimulus must be present and in sufficient quantities for MMP release, activation of pro-MMPs by other proteinases, inhibitors bound to secreted MMPs and soluble inhibitors such as TIMPs and α_2-macroglobulins are all factors in regulating the activation and sequestering of MMPs.

The question of whether or not the environment surrounding breast carcinoma cells contains proteases that can activate eosinophil-derived MMP-9 is an important one. The expression of MMPs at breast carcinoma sites has been investigated and a number of MMPs have been identified that could potentially activate pro-MMP-9 released from eosinophils. A summary of the possible interactions between various MMPs at breast tumor sites is presented in Figure 5.10. MMP-3 (Heppner *et al.*, 1996), MMP-2 (Davies *et al.*, 1993; Dalberg *et al.*, 2000), MT1-MMP (Ueno *et al.*, 1997; Dalberg *et al.*, 2000) and MMP-13 (Freije *et al.*, 1994; Uria *et al.*, 1997) have all been identified at breast carcinoma sites and all of these MMPs are known to activate pro-MMP-9 *in vitro* into an enzymatic active form. Hahn-Dantona *et al.* proposed a multi-step mechanism of activating pro-MMP-9 in tumor-bearing tissue where plasminogen from the circulation would be activated by uPA released from the tumor. This would convert plasminogen into plasmin, a protease that can activate pro-MMP-3 released by stromal cells (Hahn-

Dantona *et al.*, 1999). Activated MMP-3 can now cleave the prodomain of pro-MMP-9, thereby activating it (Ogata *et al.*, 1992). The increase in MMP-9 activity in this system was shown to enhance the ability of a breast carcinoma cell line to migrate through Matrigel, a reconstituted basement membrane.

MMP-3 expression is however not always found at breast carcinoma sites and other mechanisms for MMP-9 activation do exist. MMP-13 has been shown to be expressed in breast carcinomas but not in normal tissue (Uria *et al.*, 1997; Freije *et al.*, 1994) and can activate MMP-9 *in vitro* (Knauper *et al.*, 1997). Pro-MMP-13 can be released by stromal cells, such as tumor-associated fibroblasts, and can be activated by a variety of different proteases, including MMP-2, MT1-MMP and plasmin (Knauper *et al.*, 1996). MT1-MMP, a membrane expressed protease, has been shown to be expressed by invasive breast carcinomas (Ueno *et al.*, 1997; Dalberg *et al.*, 2000) which could potentially activate pro-MMP-13 and pro-MMP-2 (Davies *et al.*, 1993) released at the tumor site. Activated MMP-2 can also activate pro-MMP-9 and has been identified to be expressed at breast carcinoma sites (Fridman *et al.*, 1995; Davies *et al.*, 1993). Stromelysin-3 (MMP-11) has been identified to be expressed exclusively by stromal cells in breast carcinomas (Basset *et al.*, 1990). This MMP is unique to other MMPs in that it can be activated intracellularly and can be released in an activated form (Pei and Weiss, 1995). Secreted activate stromelysin-3 could potentially initiate a cascade of MMP activation which ends in the activation of MMP-9, however the exact biological activity of active (45kDa) stromelysin-3 remains to be investigated further. Substrates for stromelysin-3 include, serine protease inhibitors α1-proteinase inhibitor (α1-PI) and α2-antiplasmin (Pei *et al.*, 1994). Degradation of α1-PI, an inhibitor of elastase, by

stromelysin-3 may contribute to elastase mediated tissue damage (Pei *et al.*, 1994). Stromelysin-3 may also contribute indirectly to the increase in plasmin levels due to activity against α2-antiplasmin (Pei *et al.*, 1994). This in turn can promote activation of resident pro-MMPs in the microenvironment.

Although MMP-3, MMP-13, MT1-MMP, plasmin and MMP-2 can all participate in activating pro-MMP-9 a couple of cautionary comments must be made. All of the activation assays were carried out *in vitro* and we can only speculate as to what happens in the tissue. Also, due the heterogeneous nature of cancer cells and the surrounding microenvironment we don't know if all or any of these MMPs are present in any given case. Given that these MMPs were present, attention must be given to the level of TIMPs in the tissue since activation of all MMPs can be prevented by these natural inhibitors.

With these comments in mind, increased expression of MMP-9 has been shown to correlate with advanced stages of disease (Davies *et al.*, 1993). We have shown that eosinophils can release pro-MMP-9 in response to TNF-α, RANTES and IL-8, all mediators identified at breast carcinoma sites. There is also evidence that pro-MMP-9 can be activated at tumor sites. Therefore, it is possible that extensive eosinophil recruitment to breast tumor sites, as seen by Samoszuk *et al.* (1996), could be contributing to the remodeling of the tumor microenvironment and perhaps increasing tumor progression.

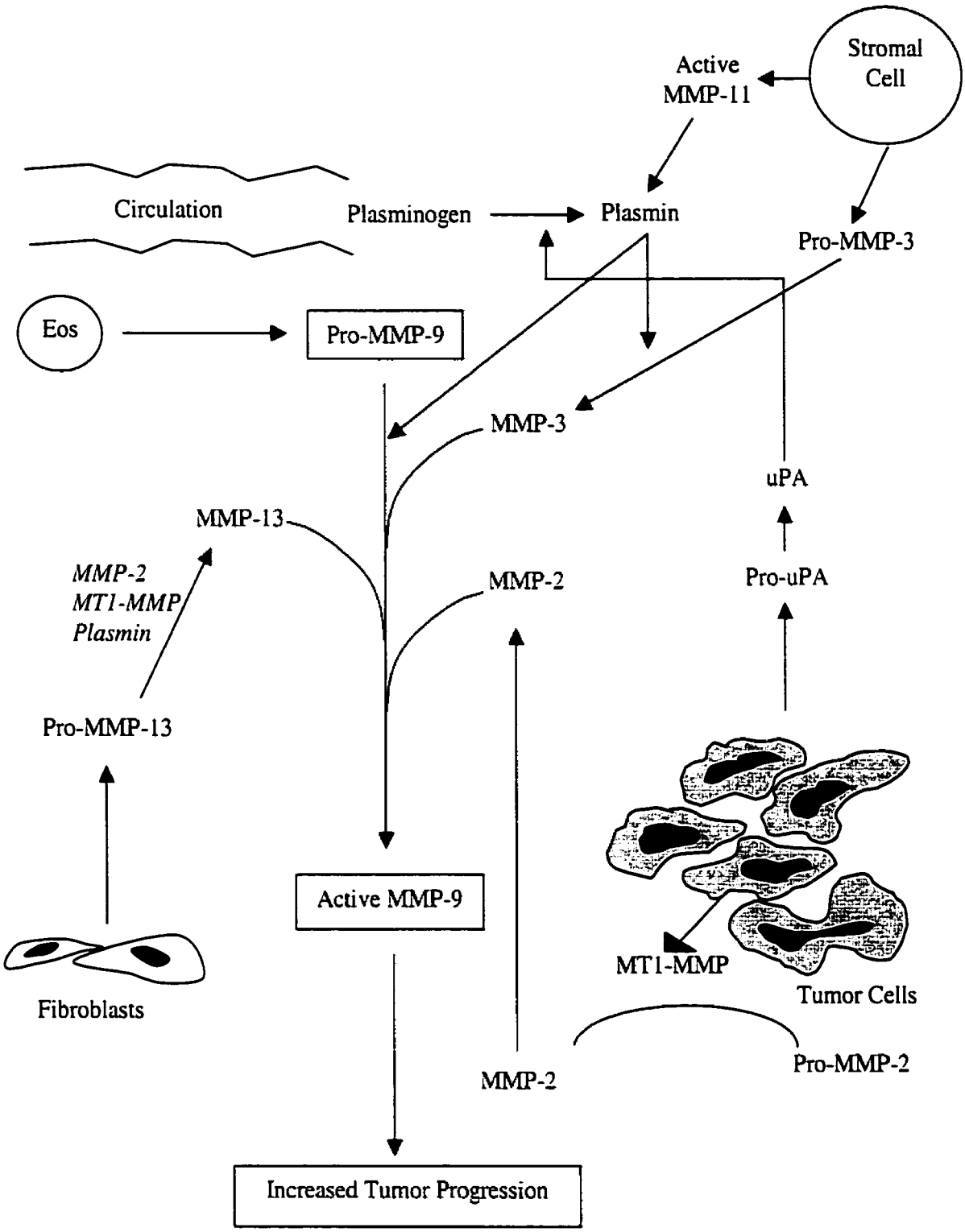


Figure 5.10. Possible mechanisms of MMP-9 activation at breast carcinoma sites.

5.3 Future Studies

Our studies exclusively used TNF-α to stimulate MMP-9 release from eosinophils, however there are other cytokines present at breast carcinoma sites. It would be interesting to investigate the response eosinophils have to other Th type 1 cytokines found at tumor sites. In addition, it would be interesting to see if there are any synergistic effects of TNF with either IL-8 or RANTES in MMP-9 production. Pinpointing the role RANTES and IL-8 play in inducing MMP-9 release from eosinophils stimulated with supernatant from TNF-α-treated breast carcinoma cells could shed light on the role these chemokines play in other systems.

Further investigation into the role eosinophils play in tumor progression would be very exciting. One experiment that could answer this question would be to incubate breast carcinoma cells with activated eosinophil-derived MMP-9 in a transwell coated with Matrigel and seeing if there is an increase migration of breast carcinoma cells. Also, taking the above experiment but adding eosinophils alone or eosinophils with macrophages and then looking at tumor migration may provide insight into cooperative behaviour between leukocytes at the tumor site.

Chapter Six: Summary

6.1 Summary

The aim of this thesis was to determine the molecular mechanisms involved in selective eosinophil recruitment at breast carcinoma sites and to reveal a functional role for eosinophils at tumor sites. Although we did not uncover eosinophil specific mechanisms of recruitment we took several steps forward in understanding the interactions between eosinophils and breast cancer cells. Breast tumor sites contain immunoreactive TNF-α. Using TNF-α we uncovered a number of novel findings in the course of these studies.

Under the influence of TNF-α, breast carcinoma cells can upregulate the surface expression of VCAM-1 and ICAM-1. Constitutive expression of VCAM-1 and ICAM-1 was found on MDA-MB-435S cells with a slight increase in expression upon TNF-α stimulation. However, under control conditions BT-20 cells had minimal VCAM-1 and ICAM-1 expression, with a significant increase in expression after 24 hours of stimulation with TNF-α. P- and E-selectin expression was also investigated, but we did not detect the presence of these adhesion molecules on breast carcinoma cells.

TNF-α-stimulated breast carcinoma cells were also shown to release mediators that could activate eosinophils and induce the release of superoxide. RANTES, MCP-1 and IL-8 released from TNF-α-stimulated breast carcinoma cells could potentially play a role in the recruitment of eosinophils, neutrophils and macrophages. Although we only showed that supernatant from TNF-α-stimulated breast carcinoma cells was chemotactic for eosinophils, it is possible that the abundant release of IL-8 could be attracting neutrophils and the presence of MCP-1 could be playing a role in attracting macrophages.

A surprising finding was that TNF-α alone could directly stimulate eosinophils to release pro-MMP-9. Activation of eosinophil-derived pro-MMP-9 could potentially occur by a number of different mechanisms. Other MMPs such as MMP-2 and MMP-3 have been shown to activate pro-MMP-9 and could possibly activate pro-MMP-9 *in vivo*. Plasmin has also been shown to activate pro-MMP-9 directly as well as reactive free radicals. This is particularly intriguing as these supernatants also increase superoxide production by eosinophils. This could potentially serve as a means of pro-MMP-9 activation. Once recruited into the tissue and to the tumor site, eosinophils could also be involved in the destruction of tumor cells.

An aspect of tumor cell progression that we did not emphasize in the thesis is the process of angiogenesis. Most patients don't die from primary tumors, but from tumor metastasis. Angiogenesis or neovascularization is the formation of new blood vessels. The formation of these new blood vessels are required for primary tumors to grow beyond a certain size. They function to supply oxygen and nutrients needed for growth as well as a route for waste export. Certain chemokines, such as IL-8, have been linked with the formation of new blood vessels. An increase in MMP expression has also been linked to neovascularization and tumor progression. In this study we have shown that breast carcinoma cells release an abundant source of IL-8 and that eosinophils stimulated with TNF-α can release MMP-9 into the environment. Due to the scope of this project, we didn't investigate the possibility of eosinophils contributing to the formation of new blood vessels, however this would be a very interesting area to investigate due to the fact that mediators involved in tumor progression are present.

These data suggest that activated breast carcinoma cells could be releasing chemokines like IL-8 to stimulate self growth and attract new blood vessels. Leukocyte infiltration could represent either a direct attempt by the immune system to clear the tumor or it alternatively could be just a side effect from the released chemokines. Furthermore once in the tumor environment, activated granulocytes release superoxide. This superoxide is normally used to clear pathogens, but here superoxide could serve to increase tumor growth and activate MMPs.

6.2 Limitations

Although this study provided insight into the role of eosinophils at breast carcinoma sites there were some recognized limitations to our experiments. First, the issue regarding the use of cell lines versus primary cells needs to be addressed. We used breast carcinoma cells lines as a model for this study. There are advantages and disadvantages to both models. An apparent advantage to using primary cells is that samples can be obtained from a number of different patients at different stages of disease. This allows for useful comparisons between benign and metastatic tumors. However due to the difficulty in obtaining primary cells and the difficulty in propagating these cells in culture makes this a very difficult model to work with. Breast cancer cell lines are easily obtained and are easy to culture. However, late passage cells are routinely used and although different laboratories use the same cell line clonal variation of the tumor cells may provide contradicting results.

Another limitation involves the use of peripheral eosinophils versus elicited eosinophils in our studies. There are a number of studies that have shown that peripheral leukocytes are different from transmigrated leukocytes in the expression of adhesion

molecules and chemokine receptors on their surface (Poon *et al.*, 1999; Lukacs *et al.*, 1999). Since we were looking at the effects of eosinophils and breast carcinoma cells it would have been ideal to use transmigrated eosinophils in our experiments rather than peripheral cells. From a technical stand point, isolating enough peripheral eosinophils for a given experiment is a bit difficult due to low percentages circulating in the blood. In order to obtain transmigrated eosinophils we would have to allow eosinophils to transmigrate through a boyden chamber for 2 hours in which we would retrieve only about 40% of the input eosinophils. This yield would be insufficient to then use in an experiment.

Another limitation in this study was the fact that we primarily looked at recruitment mechanisms after the eosinophil had transmigrated through the endothelium. We didn't uncover any eosinophil specific chemokines that could be involved in selective recruitment however this does not necessarily mean that they don't exist. Selective recruitment may lie at the level of the endothelium and with specific adhesion molecules expressed. Mechanisms for selective recruitment of eosinophils has been shown on IL-4-stimulated endothelial cells (Patel, 1999). This interaction is mainly mediated by P-selectin and VCAM-1 expressed on the endothelium (Patel, 1999). Also, the possibility of other cell types in the tumor environment releasing eosinophil specific mediators can not be ruled out.

In summary, from the initial observation in 1996 by Samoszuk *et al.* (1996) we have uncovered possible mechanisms of eosinophil recruitment to tumor sites and possible roles that eosinophils may be playing in the tissue. Breast carcinoma cells can release eosinophil activators inducing chemotaxis, degranulation and the generation of

superoxide. Adhesion molecules expressed on the surface may provide a final stop signal for the release of EPO.

References

Abbas, A.K., Lichtman, A.H. and Pober, S. 1997. Cellular and Molecular Immunology, Third Edition. W.B. Saunders Company: Philadelphia.

Abe, Y., Ballantyne, C.M. and Smith, C.W. 1996. Functions of domain 1 and 4 of vascular cell adhesion molecule-1 in alpha4 integrin-dependent adhesion under static and flow conditions are differentially regulated. Journal of Immunology, 157: 5061-5069.

Abu-Ghazaleh, R.I., Fujisawa, T., Mestecky, J., Kyle, R.A. and Gleich, G.J. 1989. IgA-induced eosinophil degranulation. Journal of Immunology, 142(7): 2393-2400.

Alam, R., Stafford, S., Forsythe, P., Harrison, R., Faubion, D., Lett-Brown, M.A. and Grant, J.A. 1993. RANTES is a chemotactic and activating factor for human eosinophils. Journal of Immunology, 150: 3442-3447.

Auvinen, P., Lipponen, P., Johansson, R. and Syrjanen, K. 1995. Prognostic significance of TGF-beta 1 and TGF-beta 2 expressions in female breast cancer. Anticancer Research, 15(6B): 2627-2631.

Baggiolini, M. 1998. Chemokines and leukocyte traffic. Nature, 392(6676): 565-568.

Baggiolini, M., Dewald, B. and Moser, B. Inflammation: Basic Principles and Clinical Correlates (Third Edition). Edited by Gallin, J.I. and Snyderman, R. 1999. Lipponcott Williams & Wilkins, Philadelphia, pg 419-431.

Baggiolini, M., Dewalk, B. and Moser, B. 1994. Interleukin-8 and related chemotactic cytokines-CXC and CC chemokines. Advances in Immunology, 55: 97-179.

Bajaj P., Lawry J., Shenton G. and Rees R.C. 1993. Interleukin-6 and tumour necrosis factor alpha synergistically block S- phase cell cycle and upregulate intercellular adhesion molecule-1 expression on MCF7 breast carcinoma cells. Cancer Letters, 71:143-9.

Basset, P., Bellocq, J.P., Wolf, C., Stoll, I., Hutin, P., Limacher, J.M., Podhajcer, O.L., Chenard, M.P., Rio, M.C. and Chambon, P. 1990. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature, 348: 699-704.

Bayless, K.J., Meininger, J., Scholtz, M. and Davis, G.E. 1998. Osteopontin is a ligand from the $\alpha 4\beta 1$ integrin. Journal of Cell Science, 111: 1165-1174.

Bethwaite, P.B., Holloway, L.J., Yeong, M.L. and Thornton, A. 1993. Effect of tumour associated tissue eosinophilia on survival of women with stage IB carcinoma of the uterine cervix. Journal of Clinical Pathology, 46(11): 1016-1020.

Blumenthal, R.D., Samoszuk, M., Taylor, A.P., Brown, G., Alisauskas, R. and Goldenberg, D.M. 2000. Degranualting eosinophils in human endometriosis. American Journal of Pathology, 156(5): 1581-1588.

Bonecchi, R., Polentarutti, N., Luini, W., Borsatti, A., Bernasconi, S., Locati, M., Power, C., Proudfoot, A., Wells, T.N., Mackay, C., Mantovani, A. and Sozzani, S. 1999. Up-regulation of CCR1 and CCR3 and induction of chemotaxis to CC chemokines by IFN-gamma in human neutrophils. Journal of Immunology, 162(1): 474-479.

Bonini, J.A., Martin, S.K., Dralyuk, F., Roe, M.W., Philipson, L.H. and Steiner, D.F. 1997. Cloning, expression, and chromosomal mapping of a novel human CC-chemokine receptor (CCR10) that displays high-affinity binding for MCP-1 and MCP-3. DNA and Cell Biology, 16(10): 1249-1256.

Bousquet, J.B., Chanez, P., Lacoste, J.Y., Barneon, G., Ghavanian, N., Enander, I., Venge, P., Ahlstedt, S., Simony-Lafontaine, J., Godard, P. and Michel, F.B. 1990. Eosinophilic inflammation in asthma. New England Journal of Medicine, 323: 1033-1039.

Bradley, B.L., Azzawi, M., Jacobson, M, Assoufi, B., Collins, J.V., Irani, A.M.A., Schwartz, L.B., Durham, S.R., Jeffery, P.K. and Kay, A.B. 1991. Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. Journal of Allergy and Clinical Immunology, 88: 661-674.

Budinsky, A.C., Brodowicz, T., Wiltschke, C., Czerwenka, K., Michl, I., Krainer, M. and Zielinski, C.C. 1997. Decreased expression of ICAM-1 and its induction by tumor necrosis factor on breast-cancer cells in vitro. International Journal of Cancer, 71(6): 1086-1090.

Camp, B.J., Dyhrman, S.T., Memoli, V.A., Mott, L.A. and Barth, R.J. Jr. 1996. In situ cytokine production by breast cancer tumor-infiltrating lymphocytes. Annals of Surgical Oncology, 3(2): 176-184.

Carlos, T.M., Schwartz, B.R., Kovach, N.L., Yee, E., Rosa, M., Osborn, L., Chi-Rosso, G., Newman, B., Lobb, R. and Harlan, J.M. 1990. Vascular cell adhesion molecule-1 mediates lymphocyte adherence to cytokine-activated cultured human endothelial cells. Blood, 76(5): 965-970.

Carswell, E.A., Old, L.J., Kassel, R.L., Green, S., Fiore, N. and Williamson, B. 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proceedings of the National Academy of Sciences USA, 72(9): 3666-3670.

Charo, I.F., Myers, S.J., Herman, A., Franci, C., Connolly, A.J. and Coughlin, S.R. 1994. Molecular cloning and functional expression of two monocyte chemoattractant protein 1

receptors reveals alternative splicing of the carboxyl-terminal tails. Proceedings of the National Academy of Science U.S.A, 91(7): 2752-2756.

Charpin, C., Bergeret, D., Garcia, S., Andrac, L., Martini, F., Horschowski, N., Choux, R. and Lavaut, M.N. 1998. ELAM selectin expression in breast carcinomas detected by automated and quantitative immunohistochemical assays. International Journal of Oncology, 12:1041-1048.

Clark-Lewis, I., Schumacher, C., Baggiolini, M. and Moser, B. 1991. Structure-activity relationships of interleukin-8 determined using chemically synthesized analogs. Critical role of NH2-terminal residues and evidence for uncoupling of neutrophil chemotaxis, exocytosis, and receptor binding activities. Journal of Biological Chemistry, 266(34): 23128-23134.

Dahinden, C.A., Geiser, T., Brunner, T., von Tscharner, V., Caput, D., Ferrara, P., Minty, A. and Baggiolini, M. 1994. Monocyte chemotactic protein 3 is a most effective basophil- and eosinophil-activating chemokine. Journal of Experimental Medicine,179(2): 751-756.

Dalberg, K., Eriksson, E., Enberg, U., Kjellman, M. and Backdahl, M. 2000. Gelatinase A, membrane type 1 matrix metalloproteinase, and extracellular matrix metalloproteinase inducer mRNA expression: correlation with invasive growth of breast cancer. World Journal of Surgery, 24: 334-340.

Daugherty, B.L., Siciliano, S.J., DeMartino, J.A., Malkowitz, L., Sirotina, A. and Springer, M.S. 1996. Cloning, expression, and characterization of the human eosinophil eotaxin receptor. Journal of Experimental Medicine, 183(5): 2349-2354.

Davies, B., Miles, D.W., Happerfield, L.C., Naylor, M.S., Bobrow, l.G., Rubens, R.D. and Balkwill, F.R. 1993. Activity of type IV collagenases in benign and malignant breast disease. British Journal of Cancer, 67: 1126-1131.

DeChatelet, L.R., Shirley, P.S., McPhail, L.C., Huntley, C.C., Muss, H.B. and Bass, D.A. 1977. Oxidative metabolism of the human eosinophil. Blood, 50(3): 525-535.

de la Torre, M., Heldin, P. and Bergh, J. 1995. Expression of the CD44 glycoprotein (lymphocyte-homing receptor) in untreated human breast cancer and its relationship to prognostic markers. Anticancer Research, 15(6B):2791-2795.

Diamond, M.S., Staunton, D.E., Marlin, S.D. and Springer, T.A. 1991. Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. Cell, 65: 961-971.

Dippold, W., Wittig, B., Schwaeble, W., Mayet, W. and Meyer zum Buschenfelde, K.H. 1993. Expression of intercellular adhesion molecule 1 (ICAM-1, CD54) in colonic epithelial cells. Gut, 34: 1593-1597.

Donegan, W.L. and Spratt, J.S. 1995. Cancer of the Breast. Fourth Edition. W.B. Saunders Company: USA.

Eisma, R.J., Allen, J.S., Lafreniere, D., Leonard, G. and Kreutzer, D.L. 1997. Eosinophil expression of transforming growth factor-beta and its receptors in nasal polyposis: role of the cytokines in this disease process. American Journal of Otolaryngol, 18: 405-11.

Elovic, A.E., Ohyama, H., Sauty, A., McBride, J., Tsuji, T., Nagai, M., Weller, P.F. and Wong, D.T. 1998. IL-4-dependent regulation of TGF-alpha and TGF-beta1 expression in human eosinophils. Journal of Immunology, 160(12): 6121-6127.

Elsner, Hochstetterm, R., Kimmig, D. and Kapp, A. 1996. Human eotaxin represents a potent activator of the respiratory burst of human eosinophils. European Journal of Immunology, 26: 1919-1925.

Elsner, J., Petering, H., Kluthe, C., Kimmig, D., Smolarski, R., Ponath, P. and Kapp, A. 1998. Eotaxin-2 activates chemotaxis-related events and release of reactive oxygen species via pertussis toxin-sensitive G proteins in human eosinophils. European Journal of Immunology, 28(7): 2152-2158.

Ethier, S.P. 1996. Human breast cancer cell lines as models of growth regulation and disease progression. Journal of Mammary Gland Biology and Neoplasia, 1(1): 111-121.

Fisher, E.R., Paik, S.M., Rockette, H., Jones, J., Caplan, R. and Fisher, B. 1989. Prognostic significance of eosinophils and mast cells in rectal cancer: findings from the National Surgical Adjuvant Breast and Bowel Project (protocol R-01). Human Pathology, 20:159-63.

Forni, G., Giovarelli, M., Santoni, A., Modesti, Andrea and Forni, M. 1987. Interleukin 2 activated tumor inhibition in vivo depends on the systemic involvement of host immunoreactivity. The Journal of Immunology, 138(11): 4033-4041.

Forssmann, U., Uguccioni, M., Loetscher, P., Dahinden, C.A., Langen, H., Thelen, M. and Baggiolini, M. 1997. Eotaxin-2, a novel CC chemokine that is selective for the chemokine receptor CCR3, and acts like eotaxin on human eosinophil and basophil leukocytes. Journal of Experimental Medicine, 185(12): 2171-2176.

Fox, S.B., Turner, G.D.H., Gatter, K.C. and Harris, A.L. 1995. The increased expression of adhesion molecules ICAM-3, E- and P-Selectins on breast cancer endothelium. Journal of Pathology, 177: 369-376.

Freije, J.M.P., Diez-Itza, I., Balbin, M., Sanchez, L.M., Blasco, R., Tolivia, J. and Lopez-Otin, C. 1994. Molecular cloning and expression of collagenase-3, a novel human matrix metalloproteinase produced by breast carcinomas. The Journal of Biological Chemistry, 269(24): 16766-16773.

Fridman, R., Toth, M., Pena, D. and Mobashery, S. 1995. Activation of progelatinase B (MMP-9) by gelatinase A (MMP-2). Cancer Research, 55: 2548-2555.

Fujisawa, T., Kato, Y., Nagase, H., Atsuta, J., Terada, A., Iguchi, K., Kamiya, H., Morita, Y., Kitaura, M., Kawasaki, H., Yoshie, O. and Hirai, K. 2000. Chemokines induce eosinophil degranulation through CCR-3. Journal of Allergy and Clinical Immunology, 106(3): 507-513.

Fukushima, K., Hirota, M., Terasaki, P.I., Wakisaka, A., Togashi, H., Chia, D., Suyama, N., Fukushi, Y., Nudelman, E., Hakomori, S. 1984. Characterization of sialosylated Lewisx as a new tumor-associated antigen. Cancer Research, 44(11): 5279-5285.

Gao, J.L., Kuhns, D.B., Tiffany, H.L., McDermott, D., Li, X., Francke, U. and Murphy, P.M. 1993. Structure and functional expression of the human macrophage inflammatory protein 1 alpha/RANTES receptor. Journal of Experimental Medicine, 177(5): 1421-1427.

Geng, J.G., Bevilacqua, M.P., Moore, K.L., McIntyre, T.M., Prescott, S.M., Kim, J.M., Bliss, G.A., Zimmerman, G.A. and McEver, R.P. 1990. Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. Nature, 343(6260): 757-760.

Goldsmith, M.M., Belchis, D.A., Cresson, D.H., Merritt, W.D. and Askin, F.B. 1992. The importance of the eosinophil in head and neck cancer. Otolaryngol Head Neck Surgery, 106: 27-33.

Gonzalez, M.A., Pinder, S.E., Wencyk, P.M., Bell, J.A., Elston, C.W., Nicholson, R.I., Robertson, J.F., Blamey, R.W. and Ellis, I.O. 1999. An immunohistochemical examination of the expression of E-cadherin, alpha- and beta/gamma-catenins, and alpha2- and beta1-integrins in invasive breast cancer. Journal of Pathology, 187(5): 523-529.

Gonzalez-Amaro, R. and Sanchez-Madrid, F. 1999. Cell adhesion molecules: selectins and integrins. Critical Reviews in Immunology, 19: 389-429.

Gouon-Evans, V., Rothenberg, M.E. and Pollard, J.W. 2000. Postnatal mammary gland development requires macrophages and eosinophils. Development, 127: 2269-2282.

Gui, G.P., Wells, C.A., Browne, P.D., Yeomans, P., Jordan, S., Puddefoot, J.R., Vinson, G.P. and Carpenter, R. 1995. Integrin expression in primary breast cancer and its relation to axillary nodal status. Surgery, 117(1): 102-108.

Hahn-Dantona, E., Ramos-DeSimone, N., Sipley, J., Nagase, H., French, D.L. and Quigley, J.P. 1999. Activation of proMMP-9 by a plasmin/MMP-3 cascade in a tumor cell model. Annals of the New York Academy of Science, 878: 373-387.

Hakansson, L., Nielson, L.S. and Teder, P. 1994. Measurement of neutrophil and eosinophil adhesion to E-selectin, VCAM-1, and ICAM-1 by the use of transfected fibroblast cell lines. Journal of Immunological Methods, 176(1): 53-66.

Hanjer, J.S., Chandross, R.J., Solic, J.J., Weatherly, N.F., Laszlo, J., Moore, J.O. and Ottolenghi, A. 1981. Medusa cells: cytostructure and cytochemistry of amoeboid eosinophils with psuedopod-like processes. Histochemical Journal, 13: 905-919.

Hanker, J.S., Chandross, R.J., Weatherly, N.F., Laszlo, J., Moore, J.O., Buckley, R.H. and Ottolenghi, A. 1980. Medusa cells: the morphology and cytochemistry of common amoeboid variants of eosinophils. Histochemical Journal, 2: 701-706.

Harris. J.R., Morrow, M., Lippamn, M.E. and Hellman, S. 1996. Diseases of the Breast. Lippincott-Raven Publishers: Philadelphia.

Harrison. 1994. Principles of Medicine. Thirteenth Edition. The McGraw-Hill Companies, Inc: USA.

Harrison. 1998. Principles of Medicine. Thirteenth Edition. The McGraw-Hill Companies, Inc: USA.

Heppner, K.J., Matrisian, L.M., Jensen, R.A. and Rodgers, W.H. 1996. Expression of most matrix metalloproteinase family members in breast cancer represents a tumor-induced host response. American Journal of Pathology, 149(1): 273-282.

Hibbs, M.S., Hasty, K.A., Seyer, J.M., Kang, A.H. and Mainardi, C.L. 1985. Biochemical and immunological characterization of the secreted forms of human neutrophil gelatinase. The Journal of Biological Chemistry, 260(4): 2493-2500.

Horie, S., Gleich, G.J. and Kita, H. 1996. Cytokines directly induce degranulation and superoxide production from human eosinophils. Journal of Allergy and Clinical Immunology, 98: 371-381.

Horiuchi, T. and Weller, P.F. 1997. Expression of vascular endothelial growth factor by human eosinophils: upregulation by granulocyte macrophage colony-stimulating factor and interleukin-5. American Journal of Respiratory Cell Molecular Biology, 17: 70-77.

Hultborn, K.A., Larsen L.G. and Raghnult, I. 1955. The lymph draining from the breast to the axillary and parasternal lymph nodes: studied with the aid of colloidal AU198. Acta Radiology, 43: 53.

Hutchins, D. and Steel, C.M. 1994. Regulation of ICAM-1 (CD54) expression in human breast cancer cell lines by interleukin 6 and fibroblast-derived factors. International Journal of Cancer, 58: 80-84.

Idikio, H. 1998. Galectin-3 expression in human breast carcinoma: correlation with cancer histologic grade. International Journal of Oncology, 12(6): 1287-1290.

Itoh, T., Tanioka, M., Yoshida, H., Yoshioka, T, Nishimoto, H. and Itohara, S. 1998. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. Cancer Research, 58: 1048-1051.

Iwasaki K., Torisu M. and Fujimura T. 1986. Malignant tumor and eosinophils. I. Prognostic significance in gastric cancer. Cancer, 58: 1321-1327.

Jin, L., Yuan, R.Q., Fuchs, A., Yao, Y., Joseph, A., Schwall, R., Schnitt, S.J., Guida, A., Hastings, H.M., Andres, J., Turkel, G., Polverini, P.J., Goldberg, I.D. and Rosen, E.M. 1997. Expression of interleukin-1beta in human breast carcinoma. Cancer, 80(3): 421-434.

Jinquan, T., Jing, C., Jacobi, H.H., Reimert, C.M., Millner, A., Quan, S., Hansen, J.B., Dissing, S., Malling, H.J., Skov, P.S. and Poulsen, L.K. 2000. CXCR3 expression and activation of eosinophils: role of IFN-gamma-inducible protein-10 and monokine induced by IFN-gamma. Journal of Immunology, 165(3): 1548-1556.

Jong, E.C. and Klebanoff, S.J. 1980. Eosinophil-mediated mammalian tumor cell cytotoxicity: role of the peroxidase system. Journal of Immunology, 124(4): 1949-1953.

Kadin, M., Butmarc, J., Elovic, A. and Wong, D. 1993. Eosinophils are the major source of transforming growth factor-beta 1 in nodular sclerosing Hodgkin's disease. American Journal of Pathology, 142: 11-16.

Kameyoshi, Y., Dorschner, A., Mallet, A.I., Christophers, E. and Schroder, J.M. 1992. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. Journal of Experimental Medicine, 176(2): 587-592.

Kitaura, M., Suzuki, N., Imai, T., Takagi, S., Suzuki, R., Nakajima, T., Hirai, K., Nomiyama, H. and Yoshie, O. 1999. Molecular cloning of a novel human CC chemokine (Eotaxin-3) that is a functional ligand of CC chemokine receptor 3. The Journal of Biological Chemistry, 274(39): 27975-27980.

Kitayama, J., Fuhlbrigge, R.C., Puri, K.D. and Springer, T.A. 1997. P-selectin, L-selectin, and alpha 4 integrin have distinct roles in eosinophil tethering and arrest on vascular endothelial cells under physiological flow conditions. Journal of Immunology, 159: 3929-3939.

Knauper, V., Smith, B., Lopez-Otin, C. and Murphy, G. 1997. Activation of progelatinase B (proMMP-9) by active collagenase-2 (MMP-13). European Journal of Biochemistry, 248: 369-373.

Knauper, V., Will, H., Lopez-Otin, C., Smith, B., Atkinson, S.J., Stanton, H., Hembry, R.M. and Murphy, G. 1996. Cellular mechanisms for human procollagenase-3 (MMP-13) activation. The Journal of Biological Chemistry, 271(29): 17124-17131.

Kroegel, C., Yukawa, T., Dent, G., Venge, P., Chung, K.F. and Barnes, P.J. 1989. Stimulation of degranulation from human eosinophils by platelet-activating factor. Journal of Immunology, 142(10): 3518-3526.

Kugler, A. 1999. Matrix metalloproteinases and their inhibitors. Anticancer Research, 19: 1589-1592.

Kurizaki, T. Toi, M. and Tominaga, T. 1998. Relationship between matrix metalloproteinase expression and tumor angiogenesis in human breast carcinoma. Oncology Reports, 5: 673-677.

Kurtzman, S.H., Anderson, K.H., Wang, Y., Miller, L.J., Renna, M., Stankus, M., Lindquist, R.R., Barrows, G. and Kreutzer, D.L. 1999. Cytokines in human breast cancer: IL-1alpha and IL-1beta expression. Oncology Reports, 6(1): 65-70.

Lampinen, M., Rak, S. and Venge, P. 1999. The role of interleukin-5, interleukin-8 and RANTES in the chemotactic attraction of eosinophils to the allergic lung. Clinical and Experimental Allergy, 29(3): 314-322.

Leighton, S.E., Teo, J.G., Leung, S.F., Cheung, A.Y., Lee, J.C. and van Hasselt, C.A. 1996. Prevalence and prognostic significance of tumor-associated tissue eosinophilia in nasopharyngeal carcinoma. Cancer, 77: 436-440.

Leonard, E J. and Yoshimura, T. 1990. Human monocyte chemoattractant protein-1 (MCP-1). Immunology Today, 11(3): 97-101.

Lewis, C.E., Leek, R., Harris, A. and McGee, J.O. 1995. Cytokine regulation of angiogenesis in breast cancer: the role of tumor-associated macrophages. Journal of Leukocyte Biology, 57(5): 747-751.

Liapis, H., Flath, A. and Kitazawa, S. 1996. Integrin alpha V beta 3 expression by bone-residing breast cancer metastases. Diagnostic Molecular Pathology, 5(2): 127-135.

Liotta, L.A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C.M. and Shafie, S. 1980. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature, 284: 67-68.

Liu, C.M., Shun, C.T. and Cheng, Y.K. 1998. Soluble adhesion molecules and cytokines in perennial allergic rhinitis. Annals of Allergy and Asthma Immunology, 81(2): 176-180.

Lowe, D., Jorizzo, J. and Hutt, M.S. 1981. Tumour-associated eosinophilia: a review. Journal of Clinical Pathology, 34(12): 1343-1348.

Luboshits, G., Shina, S., Kaplan, O., Engelberg, S., Nass, D., Lifshitz-Mercer, B., Chaitchik, S., Keydar, I. and Ben-Baruch, A. 1999. Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. Cancer Research, 59(18): 4681-4687.

Lukacs, N.W., Oliveira, S.H. and Hogaboam, C.M. 1999. Chemokines and asthma: redundancy of function or a coordinated effort? Journal of Clinical Investigation, 104(8): 995-999.

Luster, A.D. 1998. Chemokines--chemotactic cytokines that mediate inflammation. New England Journal of Medicine, 338(7): 436-445.

MacDougall, J.R. and Matrisian, L.M. 1995. Contributions of tumor and stromal matrix metalloproteinases to tumor progression, invasion and metastasis. Cancer Metastasis Review, 14(4): 351-362.

Mantovani, A. 1999. The chemokine system: redundancy for robust outputs. Immunology Today, 20(6): 254-257.

Masson, R., Lefebvre, O., Noel, A., Fahime, M.E., Chenard, M.P., Wendling, C., Kebers, F., LeMeur, M., Dierich, A., Foidart, J.M., Basset, P. and Rio, M.C. 1998. In vivo evidence that the stromelysin-3 metalloproteinase contributes in a paracrine manner to epithelial cell malignancy. Journal of Cell Biology, 140(6): 1535-1541.

Matsuyama, G., Ochiai, K., Ishihara, C., Kagami, M., Tomioka, H. and Koya, N. 1998. Heterogeneous expression of tumor necrosis factor-alpha receptors I and II on human peripheral eosinophils. International Archives of Allergy and Immunology, 117(Suppl 1): 28-33.

Mautino, G., Henriquet, C., Gougat, C., Le Cam, A., Dayer J-M., Bousquet, J. and Capony, F. 1999. Increased expression of tissue inhibitor of matalloproteinase-1 and loss of correlation with matrix metalloproteinase-9 by macrophages in asthma. Laboratory Investigation, 79(1): 39-47.

Mayer, B., Lorenz, C., Babic, R., Jauch, K.W., Schildberg, F.W., Funke, I. and Johnson, J.P. 1995. Expression of leukocyte cell adhesion molecules on gastric carcinomas: possible involvement of LFA-3 expression in the development of distant metastases. International Journal of Cancer, 64: 415-23.

McBride, W.H., Thacker, J.D., Comora, S., Economou, J.S., Kelley, D., Hogge, D., Dubinett, S.M. and Dougherty, G.J. 1992. Genetic modification of a murine fibrosarcoma to produce interleukin 7 stimulates host cell infiltration and tumor immunity. Cancer Research, 52: 3931-3937.

Mignatti, P. and Rifkin, D.B. 1993. Biology and biochemistry of proteinases in tumor invasion. Physiology Review, 73(1): 161-195.

Minshall, E.M., Leung, D.Y., Martin, R.J., Song, Y.L., Cameron, L., Ernst, P. and Hamid, Q. 1997. Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. American Journal of Respiratory Cell and Molecular Biology, 17(3): 326-333.

Moqbel, R., Levi-Schaffer, F., and Kay, B. 1994. Cytokine generation by eosinophils. Journal of Allergy and Clinical Immunology, 94: 1183-1188.

Moy, J.N., Gleich, G.J. and Thomas, L.L. 1990. Non-cytotoxic activation of neutrophils by eosinophil granule major basic protein: effect on superoxide anion generation and lysosomal enzyme release. Journal of Immunology, 145: 2626-2632.

Myers, S.J., Wong, L.M. and Charo, I.F. 1995. Signal transduction and ligand specificity of the human monocyte chemoattractant protein-1 receptor in transfected embryonic kidney cells. The Journal of Biological Chemistry, 270(11): 5786-5792.

Nagase, H., Miyamasu, M., Yamaguchi, M., Fujisawa, T., Ohta, K., Yamamoto, K., Morita, Y. and Hirai, K. 2000. Expression of CXCR4 in eosinophils: functional analyses and cytokine-mediated regulation. Journal of Immunology, 164(11): 5935-5943.

Nagata, M., Sedgwick, J.B., Bates, M.E. Kita, H. and Busse, W.W. 1995. Eosinophil adhesion to Vascular Cell Adhesion Molecule-1 activates superoxide anion generation. Journal of Immunology, 155: 2194-2202.

Nagata, M., Sedgwick, J.B., Kita, H. and Busse, W.W. 1998. Granulocyte marcophage colony-stimulating factor augments ICAM-1 and VCAM-1 activation of eosinophil function. American Journal of Respiratory Cellular Molecular Biology, 19: 158-166.

National Cancer Institute of Canada, Canadian Cancer Statistics. 1998. Breast cancer facts. Http://www.cbcf.org/healfacts.htm.

Neote, K., DiGregorio, D., Mak, J.Y., Horuk, R. and Schall, T.J. 1993. Molecular cloning, functional expression, and signaling characteristics of a C-C chemokine receptor. Cell, 72(3): 415-425.

Nickel, R., Beck, L.A., Stellato, C. and Schleimer, R.P. 1999. Chemokines and allergic disease. Journal of Allergy Clinical Immunology, 104(4 Pt 1): 723-742.

Nortamo, P., Patarroyo, M., Kantor, C., Suopanki, J. and Gahmberg, C.G. 1988. Immunological mapping of the human leucocyte adhesion glycoprotein gp90 (CD18) by monoclonal anitbodies. Scandinavian Journal of Immunology, 28: 537-546.

Ogata, Y., Enghild, J.J. and Nagase, H. 1992. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. The Journal of Biological Chemistry, 267(6):3581-3584.

Ogata, Y., Itoh, Y. and Nagase, H. 1995. Steps involved in activation of the pro-matrix metalloproteinase 9 (progelatinase B)-tissue inhibitor of metalloproteinase-1 complex by 4-aminophenylmercuric acetate. The Journal of Biological Chemistry, 270(31): 18506-18511.

Ogawa, Y., Hirakawa, K., Nakata, B., Fujihara, T., Sawada, T., Kato, Y., Yoshikawa, K. and Sowa, M. 1998. Expression of intercellular adhesion molecule-1 in invasive breast cancer reflects low growth potential, negative lymph node involvement, and good prognosis. Clinical Cancer Research, 4: 31-36.

Ohno, I., Ohtani, H., Nitta, Y., Suzuki, J., Hoshi, H., Honma, M., Isoyama, S., Tanno, Y., Tamura, G., Yamauchi, K., Nagura, H. and Shirato, K. 1997. Eosinophils as a source of matrix metalloproteinase-9 in asthmatic airway inflammation. American Journal of Respiratory Cell and Molecular Biology, 16(3): 212-219.

Okada, S., Kita, H., George, T.J., Gleich, G.J. and Leiferman, K.M. 1997. Migration of eosinophils through basement membrane components in vitro: role of matrix metalloproteinase-9. American Journal of Respiratory Cell and Molecular Biology, 17(4): 519-528.

Okada, Y., Yukio, G., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K. and Hayakawa, T. 1992. Matrix metalloproteinase 9 (92-kDa gelatingase/type IV collagenase) from HT 1080 human fibrosarcoma cells. The Journal of Biological Chemistry, 267(30): 21712-21719.

Osborn, L., Vassallo, C. and Benjamin, C.D. 1992. Activated endothelium binds lymphocytes through a novel binding site in the alternately spliced domain of vascular cell adhesion molecule-1. Journal of Experimental Medicine, 176: 99-107.

O'Sullivan, C. and Lewis, C.E. 1994. Tumour-associated leucocytes: friends or foes in breast carcinoma. Journal of Pathology, 172(3): 229-235.

Ownby, H.E., Roi, L.D., Isenberg, R.R. and Brennan, M.J. 1983. Peripheral lymphocyte and eosinophil counts as indicators of prognosis in primary breast cancer. Cancer, 52(1): 126-130.

Padrines, M., Wolf, M., Walz, A. and Baggiolini, M. 1994. Interleukin-8 processing by neutrophil elastase, cathepsin G and proteinase-3. FEBS Letters, 352(2): 231-235.

Patel, K.D. 1999. Mechanisms of selective leukocyte recruitment from whole blood on cytokine-activated endothelial cells under flow conditions. Journal of Immunology, 162(10): 6209-6216.

Patel, K.D. 1998. Eosinophil tethering to interleukin-4-activated endothelial cells requires both P-selectin and vascular cell adhesion molecule-1. Blood, 92: 3904-3911.

Patel, K.D., Moore, K.L., Nollert, M.U. and McEver, R.P. 1995a. Neutrophils use both shared and distinct mechanisms to adhere to selectins under static and flow conditions. Journal of Clinical Investigation, 96(4): 1887-1896.

Patel, K.D., Nollert, M.U. and McEver, R.P. 1995b. P-Selectin must extend a sufficient length from the plasma membrane to mediate rolling of neutrophils. Journal of Cell Biology, 131(6 Pt 2): 1893-1902.

Paul, E.W.(Ed.). 1999. Fundamental Immunology, Fourth Edition. Lippincott-Raven: Philadelphia.

Pei, D. and Weiss, S.J. 1995. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. Nature, 375: 244-247.

Pei, D., Majmudar, G. and Weiss, S.J. 1994. Hydrolytic inactivation of a breast carcinoma cell-derived serpin by human stromelysin-3. The Journal of Biological Chemistry, 269(41): 25849-25855.

Petering, H., Gotze, O., Kimmig, D., Smolarski, R., Kapp, A. and Elsner, J. 1999. The biologic role of interleukin-8: functional analysis and expression of CXCR1 and CXCR2 on human eosinophils. Blood, 93(2): 694-702.

Ponath, P.D., Qin, S., Post, T.W., Wang, J., Wu. L., Gerard, N.P., Newman, W., Gerard. C. and Mackay, C.R. 1996. Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. Journal of Experimental Medicine, 183(6): 2437-2448.

Poston, R.N., Chanez, P., Lacoste, J.Y., Litchfield, T., Lee, T.H. and Bousquet, J. 1992. Immunohistochemical characterization of the cellular infiltration in asthmatic bronchi. American Review of Respiratory Disease, 145: 918-921.

Power, C.A., Meyer, A., Nemeth, K., Bacon, K.B., Hoogewerf, A.J., Proudfoot, A.E. and Wells, T.N. 1995. Molecular cloning and functional expression of a novel CC chemokine receptor cDNA from a human basophilic cell line. The Journal of Biological Chemistry, 270(33): 19495-19500.

Premack, B.A. and Schall, T.J. 1996. Chemokine receptors: gateways to inflammation and infection. Nature and Medicine, 2(11): 1174-1178.

Poon, B.Y., Ward, C.A., Giles, W.R. and Kubes, P. 1999. Emigrated neutrophils regulate ventricular contractility via alpha4 integrin. Circulation Research, 84(11): 1245-1251.

Proost, P., Wuyts, A. and van Damme, J. 1996. The role of chemokines in inflammation. International Journal of Clinical Laboratory Research, 26(4): 211-223.

Regidor, P.A., Callies, R., Regidor, M. and Schindler, A.E. 1998. Expression of the cell adhesion molecules ICAM-1 and VCAM-1 in the cytosol of breast cancer tissue, benign breast tissue and corresponding sera [In Process Citation]. European Journal of Gynaecology Oncology, 19: 377-383.

Roberts, R.L., Ank, B.J. and Stiehm, E.R. 1991. Human eosinophils are more toxic than neutrophils in antibody-independent killing. Journal of Allergy and Clinical Immunology, 87: 1105-1115.

Rot, A., Krieger, M., Brunner, T., Bischoff, S.C., Schall, T.J. and Dahinden, C.A. 1992. RANTES and macrophage inflammatory protein 1α induce the migration and activation of normal human eosinophil granulocytes. Journal of Experimental Medicine, 176: 1489-1495.

Samoszuk, M. 1997. Eosinophils and human cancer. Histology and Histopathology, 12: 807-812.

Samoszuk, M., Sholly, S. and Epstein, A.L. 1987. Eosinophil peroxidase is detectable with a monoclonal antibody in collagen bands of nodular sclerosis Hodgkin's disease. Laboratory Investigations, 56(4): 394-400.

Samoszuk, M.K., Nathwani, B.N. and Lukes, R.J. 1986. Extensive deposition of eosinophil peroxidase in Hodgkin's and non-Hodgkin's lymphomas. American Journal of Pathology, 125: 426-429.

Samoszuk, M.K., Nguyen, V., Gluzman, I. and Pham, J.H. 1996. Occult deposition of eosinophil peroxidase in a subset of human breast carcinomas. American Journal of Pathology, 148(3):701-706.

Schiffer, S.G., Hemler, M.E., Lobb, R.R., Tizard, R. and Osborn, L. 1995. Molecular mapping of functional antibody binding sites of α4 integrin. The Journal of Biological Chemistry, 270(24): 14270-14273.

Schroder, J.M., Mrowietz, U., Morita, E. and Christophers, E. 1987. Purification and partial biochemical characterization of a human monocyte-derived, neutrophil-activating peptide that lacks interleukin 1 activity. Journal of Immunology, 139(10): 3474-3483.

Schweizer, R.C., Welmers, B.A., Raaijmakers, J.A., Zanen, P., Lammers, J.W. and Koenderman, L. 1994. RANTES- and interleukin-8-induced responses in normal human eosinophils: effects of priming with interleukin-5. Blood, 83(12): 3697-3704.

Schwingshackl, A., Duszyk, M., Brown, N. and Moqbel, R. 1999. Human eosinophils release matrix metalloproteinase-9 on stimulation with TNF-α. Journal of Allergy and Clinical Immunology, 104: 983-990.

Sedgwick, J.B., Calhoun, W.J., Gleich, G.J., Kita, H., Abrams, J.S., Schwartz, L.B., Volovitz, B., Ben-Yaakov, M. and Busse, W.W. 1991. Immediate and late allergic airway response to segmental antigen challenge. Characterization of eosinophil and mast cell mediators. American Review of Respiratory Disease, 144: 1274-1281.

Segal, A.W., Garcia, R., Goldstone, H., Cross, A.R. and Jones, O.T. 1981. Cytochrome b-245 of neutrophils is also present in human monocytes, macrophages and eosinophils. Biochemistry Journal, 196(1): 363-367.

Sehmi, R., Cromwell, O., Wardlaw,A.J., Moqbel, R. and Kay, A.B. 1993. Interleukin-8 is a chemo-attractant for eosinophils purified from subjects with a blood eosinophilia but not from normal healthy subjects. Clinical and Experimental Allergy, 23(12): 1027-1036.

Shapiro, S.D. 1998. Matrix metalloproteinase degradation of extracellular matrix: biological consequences. Current Opinion in Cell Biology, 10: 602-608.

Shapiro, S.D., Fliszar, C.J., Broekelmann, T.J., Mecham, R.P., Senior, R.M. and Welgus, H.G. 1995. Activation of the 92-kDa gelatinase by stromelysin and 4-aminophenylmercuric acetate. The Journal of Biological Chemistry, 270(11): 6351-6356.

Shaw, J.O., Pinckard, R.N., Ferrigni, K.S., McManus, L.M. and Hanahan, D.J. 1981. Activation of human neutrophils with 1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (platelet activation factor). Journal of Immunology, 127: 1250-1255.

Spessotto, P., Dri, P., Bulla, R., Zabucchi, G. and Patriarca, P. 1995. Human eosinophil peroxidase enhances tumor necrosis factor and hydrogen peroxide release by human monocyte-derived macrophages. European Journal of Immunology, 25: 1366-1373.

Statistics Canada. 1998. Lifetime probability of developing and dying from cancer. Source: National Cancer Institute of Canada, *Canadian Cancer Statistics 1998, Toronto, 1998*. Http://www.statcan.ca/english/Pgdb/People/Health/health25a.htm.

Stellato, C., Beck, L., Schweibert, L., Li, H., White, J. and Schleimer, R.P. 1996. A novel C-C chemokine, CKβ10, is expressed in airway epithelial cells and is a potent eosinophil chemoattractant. Journal of Allergy and Clinical Immunology, 97: 304.

Sternlicht,M.D., Lochter, A., Sympson, C.J., Huey, B., Rougier, J-P, Gray, J.W., Pinkel, D., Bissell, M.J. and Werb, Z. 1999. The stromal proteinase MMP3/Stromelysin-1 promotes mammary carcinogenesis. Cell, 98: 137-146.

Stetler-Stevenson, W.G., Aznavoorian, S. and Liotta, L.A. 1993. Tumor cell interactions with the extracellular matrix during invasion and metastasis. Annual Review of Cell Biology, 9: 541-573.

Taub, D.D. 1996. Chemokine-leukocyte interactions. The voodoo that they do so well. Cytokine Growth Factor Review, 7(4): 355-376.

Tepper, R.I., Coffman, R.L. and Leder, P. 1992. An eosinophil-dependent mechanism for the antitumor effect of interleukin-4. Science, 257: 548-551.

Tepper, R.I., Pattengale, P.K. and Leder, P. 1989. Murine interleukin-4 displays potent anti-tumor activity in vivo. Cell, 57: 503-512.

Tuck, A.B., Aresenault, D.M., O'Malley, F.P., Hota, C., Ling, M.C., Wilson, S.M. and Chambers, A.F. 1999. Osteopontin induces increased invasiveness and plasminogen activator expression of human mammary epithelial cells. Oncogene, 18(29): 4237-4246.

Ueno, H., Nakamura, H., Inoue, M., Imai, K., Noguchi, M., Sato, H., Seiki, M. and Okada, Y. 1997. Expression and tissue localization of membrane-types 1, 2, and 3 matrix metalloproteinases in human invasive breast carcinomas. Cancer Research, 57: 2055-2060.

Ueno, T., Toi, M., Saji, H., Muta, M., Bando, H., Kuroi, K., Koike, M., Inadera, H. and Matsushima, K. 2000. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. Clinical Cancer Research, 6(8): 3282-3289.

Uguccioni, M., Loetscher, P., Forssmann, U., Dewald, B., Li, H., Lima, S.H., Li, Y., Kreider, B., Garotta, G., Thelen, M. and Baggiolini, M. 1996. Monocyte chemotactic protein 4 (MCP-4), a novel structural and functional analogue of MCP-3 and eotaxin. Journal of Experimental Medicine, 183(5): 2379-2384.

Uria, J.A., Stahle-Backdahl, M., Seiki, M., Fueyo, A. and Lopez-Otin, C. 1997. Regulation of collagenase-3 expression in human breast carcinomas is mediated by stromal-epithelial interactions. Cancer Research, 57: 4882-4888.

Valkovic, T., Lucin, K., Krstulja, M., Dobi-Babic, R. and Jonjic, N. 1998. Expression of monocyte chemotactic protein-1 in human invasive ductal breast cancer. Pathology Research and Practice, 194(5): 335-340.

Van Damme, J., Decock, B., Conings, R., Lenaerts, J.P., Opdenakker, G. and Billiau, A. 1989. The chemotactic activity for granulocytes produced by virally infected fibroblasts is identical to monocyte-derived interleukin 8. European Journal of Immunology, 19(7): 1189-1194.

Van Den Steen, P.E. , Proost, P., Wuyts, A., Van Damme, J. and Opdenakker, G. 2000. Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. Blood, 96(8): 2673-2681.

van der Pluijm, Vloedgraven, H., Papapoulos, S., Lowick, C., Grzesik, W., Kerr, J. and Robey, P.G. 1997. Attachment characteristics and involvement of integrins in adhesion of breast cancer cell lines to extracellular bone matrix components. Laboratory Investigations, 77(6): 665-675.

van Driel, W.J., Hogendoorn, P.C., Jansen, F.W., Zwinderman, A.H., Trimbos, J.B. and Fleuren, G.J. 1996. Tumor-associated eosinophilic infiltrate of cervical cancer is indicative for a less effective immune response. Human Pathology, 27: 904-911.

Velikova, G., Banks, R.E., Gearing, A., Hemingway, I., Forbes, M.A., Preston, S.R., Jones, M., Wyatt, J., Miller, K., Ward, U., Al-Maskatti, J., Singh, S.M., Ambrose, N.S., Primrose, J.N. and Selby, P.J. 1997. Circulating soluble adhesion molecules E-cadherin, E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in patients with gastric cancer. The British Journal of Cancer, 76(11): 1398-1404.

von Wasielewski, R., Seth, S., Franklin, R., Fischer, R., Hubner, K., Hansmann, M.L., Diehl,V. and Georgii, A. 2000. Tissue eosinophilia correlates strongly with poor prognosis in nodular sclerosing Hodgkin's disease, allowing for known prognostic factors. Blood, 95: 1207-1213.

Wardlaw, A.J., Moqbel, R. and Kay, A.B. 1995. Eosinophils: biology and role in disease. Advances in Immunology, 60: 151-266.

Wardlaw, A.J., Moqbel, R. and Kay, A.B. 1986. Platelet-activating factor. Journal of Clinical Investigations, 78: 1701-1706.

Warringa, R.A., Koenderman, L., Kok, P.T., Kreukniet, J. and Bruijnzeel, P.L. 1991. Modulation and induction of eosinophil chemotaxis by granulocyte-macrophage colony-stimulating factor and interleukin-3. Blood, 77(12): 2694-2700.

Warringa, R.A., Mengelers, H.J., Raaijmakers, J.A., Bruijnzeel, P.L. and Koenderman, L. 1993. Upregulation of formyl-peptide and interleukin-8-induced eosinophil chemotaxis in patients with allergic asthma. Journal of Allergy and Clinical Immunology, 91(6): 1198-1205.

Warringa, R.A., Schweizer, R.C., Maikoe, T., Kuijper, P.H., Bruijnzeel, P.L. and Koendermann, L. 1992. Modulation of eosinophil chemotaxis by interleukin-5. American Journal of Respiratory Cell and Molecular Biology, 7(6): 631-636.

Weber, C., Alon, R., Moser, B. and Springer, T.A. 1996a. Sequential regulation of alpha 4 beta 1 and alpha 5 beta 1 integrin avidity by CC chemokines in monocytes: implications for transendothelial chemotaxis. Journal of Cell Biology, 134(4): 1063-1073.

Weber, C., Kitayama, J. and Springer, T.A. 1996b. Differential regulation of b1 and b2 integrin avidity by chemoattractants in eosinophils. Proceedings of the National Academy of Sciences USA, 93: 10939-10944.

Weber, M., Uguccioni, M., Baggiolini, M., Clark-Lewis, I. and Dahinden, C.A. 1996. Deletion of the NH2-terminal residue converts monocyte chemotactic protein 1 from an activator of basophil mediator release to an eosinophil chemoattractant. Journal of Experimental Medicine, 183(2): 681-685.

Weber, M., Uguccioni, M., Ochensberger, B., Baggiolini, M., Clark-Lewis, I. and Dahinden, C.A. 1995. Monocyte chemotactic protein MCP-2 activates human basophil and eosinophil leukocytes similar to MCP-3. Journal of Immunology, 154(8): 4166-4172.

Westermarck, J. and Kahari, V. 1999. Regulation of matrix metalloproteinase expression in tumor invasion. FASEB Journal, 13: 781-792.

Wong, P.Y., Staren, E.D., Tereshkova, N. and Braun, D.P. 1998. Functional analysis of tumor-infiltrating leukocytes in breast cancer patients. Journal of Surgical Research, 76(1): 95-103.

Yamaguchi, Y., Hayashi, Y., Sugama, Y., Miura, Y., Kasahara, T., Kitamura, S., Torisu, M., Mita, S., Tominaga, A., Takatsu, K. and Suda, T. 1988. Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. Journal of Experimental Medicine, 167: 1737-1742.