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The Role of Soluble Fibrin in Lymphocyte and LAK Cell Adherence to and Migration across Vascular Endothelial Cells: Implications for Immunotherapy and Cancer

Brandy Lee Weidow
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To the Graduate Council:

I am submitting herewith a thesis written by Brandy Lee Weidow entitled "The Role of Soluble Fibrin in Lymphocyte and LAK Cell Adherence to and Migration across Vascular Endothelial Cells: Implications for Immunotherapy and Cancer." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Timothy Sparer, Major Professor

We have read this thesis and recommend its acceptance:

John Biggerstaff, Robert Mee

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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THE ROLE OF SOLUBLE FIBRIN IN LYMPHOCYTE AND LAK CELL ADHERENCE TO AND MIGRATION ACROSS VASCULAR ENDOTHELIAL CELLS: IMPLICATIONS FOR IMMUNOTHERAPY AND CANCER

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ABSTRACT

Although conventional therapies for metastatic cancers have made significant progress in recent years, they are relatively nonspecific and have many deleterious side-effects. Recently, novel therapies, including adoptive cellular immune therapies have had sporadic, but spectacular success in cancers such as malignant melanoma and renal cell carcinoma: tumors in which an immune response has been demonstrated. However, other physiological mechanisms, such as blood coagulation inhibit the immune response against cancers. Our previous work has shown that one of these coagulation proteins, soluble fibrin (sFn), inhibits unstimulated and activated lymphocyte adherence to tumor cells by blocking leukocyte integrin (CD11a/CD18) binding to tumor cell CD54, suggesting that sFn is an immunosuppressive agent in cancer. Since these receptors are also involved in lymphocyte/endothelial cell adherence and diapedesis (a necessary step in the immune response to cancer), it was hypothesized that sFn inhibits these functions, and that blockade of this inhibition using specific peptides would restore these immune responses. Fluorescently labeled lymphocytes and Interleukin-2 activated lymphocytes (LAK cells) were incubated with sFn (or its components; fibrinogen, Gly-Pro-Arg-Pro, or thrombin) in the presence or absence of specific blocking peptides. Lymphocyte and LAK cell adherence to endothelial cell monolayers was measured by perfusion at physiological shear rates in a microscope-mounted closed perfusion chamber, followed by image analysis using Image Pro Plus software. Diapedesis was measured by detection of fluorescence in 24-well microplates following immune cell incubation (18 h) with endothelial cell monolayers grown in transwells. SFn inhibited lymphocyte (54.1 + 11.3 %) and LAK cell (43.9 + 4.4 %) adherence to sFn pretreated endothelial cells, and intermediate values were obtained from sFn pre-treatment of only one cell type. Adherence was restored by peptide mediated blockade of sFn/CD54 binding, but not by CD11b blocking peptides. Diapedesis was also inhibited by sFn (lymphocyte 29.6 + 7.7 %; LAK 12.2 +

4.9 %) and restoration was observed using blocking peptides. These results confirm the stated hypotheses, and if physiologically relevant, suggest that sFn is an etiological agent in tumor growth and metastasis, and that blockade using fibrin specific peptides may enhance the effectiveness of adoptive immunotherapies.

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CHAPTER 1: LITERATURE REVIEW AND INTRODUCTION

1. 1 Introduction

Conventional treatments for cancer primarily include "broad spectrum" approaches such as chemotherapy, radiotherapy and invasive surgery. By contrast, the ability of the humoral, innate, and cellular immune responses to specifically target and treat cancer has attracted greater research and clinical interest in recent years. To this end, the field of immunotherapy in cancer has developed a number of therapeutic strategies in the treatment of cancer, including antibody and cytokine based treatments, combined chemotherapy and immunotherapy, and adoptive immunotherapy. Adoptive immunotherapy was pioneered by Stephen Rosenberg and others in the 1980's, and with various modifications and improvements, has resulted in a small but tantalizingly significant response in some melanoma and renal cell carcinoma patients, as evidenced by a recent Science paper (Morgan, 2006). However, the mechanisms involved in leukocyte and activated or modified leukocyte homing and specific tumoricidal responses to cancers is still not fully understood, in part due to the multi-disciplinary interactions which are highly probable in such therapies. These mechanisms necessarily involve leukocyte interactions with other molecular and cellular components of the blood, vascular endothelium, tissues and cancer cells. For example, our laboratory's previous work has clearly shown that one of the end products of blood coagulation, soluble fibrin (which is present in elevated levels in many cancer patients; Iverson, 1995), significantly inhibits leukocyte adherence and killing of cancer cells *in vitro*, and enhances experimental metastasis (Biggerstaff, 2006; 2007, 1999). Furthermore, the cell surface molecules which mediate this inhibition (leukocyte integrin CD11b/CD18 and its receptor CD54) are also highly important in leukocyte adherence and diapedesis across vascular endothelium. In bringing the subjects of immunotherapy and blood coagulation into cancer biology, we therefore hypothesized that soluble fibrin also inhibits unstimulated and activated

leukocyte adherence and diapedesis. It was further hypothesized that blockade of soluble fibrin binding to these receptors, using specific peptides (our lab has previously demonstrated peptide mediated restoration of leukocyte adherence to tumor cells; Biggerstaff, 2006), would restore the ability of leukocytes to adhere to and migrate across endothelial cell (EC) monolayers. Confirmation of this hypothesis would suggest that peptide therapies may be important as adjuvants to adoptive immunotherapies in the treatment of cancer.

The following sections describe work in the areas of cancer biology, cancer immunology, and fibrin biology which have led to the generation of the primary hypotheses for this project.

1. 2 Cancer

1. 2. 1 Epidemiology of Cancer

Cancer is the second leading cause of death in the United States, with more than one million Americans diagnosed with the disease each year, with this number likely to increase as the population increases. A recent report released by the nation's leading cancer organization finds that although Americans' risk of dying from cancer continues to drop, maintaining a trend that began in the early 1990s, the rate of new cancer development remains stable (Howe, 2003).

Localized cancer, or stage I of disease, is when tumor cells begin to exhibit invasion and disruption of local tissues to form a primary lesion. Tumor cells then invade the local lymphatic system and spread to the regional (stage II) or extended regional (stage III) draining lymph nodes as secondary tumors. Finally tumor cells invade into the blood stream where characteristic patterns of blood-borne metastasis indicate the onset of stage IV of disease. Particular tumors vary in the extent to which they follow these phases in sequence (Kroon, 2004).

1. 2. 2 Malignant Melanoma

Melanoma is the most serious form of skin cancer. According to the Cancer Research Institute, although melanoma accounts for only about 5% of all skin cancers, it is responsible for more than 75% of skin cancer-related deaths. Furthermore, melanoma cases in this country have more than doubled in the past 2 decades, with the rise expected to continue. The American Cancer Society projects that in 2007, the number of new cases of melanoma is estimated at 59,940, and the number of fatalities estimated at 8,110 (Jemal, 2007). Furthermore, melanoma is projected to affect 1 in 50 Americans by the year 2010 (Howe, 2003).

There are many risk factors that can lead to the onset of melanoma, including, but not limited to, exposure to chemicals or ultraviolet radiation (Austin, 1984; Holman, 1986), diet (Mackie, 1980), and genetic predisposition (Houlston, 1999). Exposure to sunlight appears to be the most significant factor involved in the observed increase though; many dermatologists believe there may be a link between childhood sunburns and melanoma later in life (Weinstock, 1989).

1. 2. 3 Clinical Presentation of Melanoma

Malignant melanoma is a neoplastic disease characterized by uncontrolled proliferation of melanocytes, or the pigment of the skin. There are various forms of cutaneous malignant melanoma, but the progression of disease in each form follows a similar pattern of growth and clinical deterioration (Roses, 1983). The most benign types of melanoma are very slow growing and, apart from a visible skin lesion, are generally symptomless. In many cases, it may take several years to become invasive. Generally, the first sign of invasion by melanoma is an increase in cell proliferation, or an increase of horizontal dimension across the surface of the skin, often displayed as an aberrant growth. Further progression occurs by cell proliferation in the

vertical plane of the skin, or subcutaneous growth. The final growth patterns observed in the progression of melanoma include the formation of local and distant metastases which often proceed rapidly following the vertical expansion of melanoma (Roses, 1983).

1. 2. 4 Metastasis of Melanoma

Melanoma, as all cancers, is characterized by abnormal division of cells, combined with malignant behavior of these cells. Metastasis is the unequivocal hallmark of the phenotypically malignant cell. Malignant melanoma cells tend to spread, either by direct growth into adjacent tissue through invasion, or by implantation into distant sites by metastasis, whereby the cancer cells break away from the primary tumor and move through the bloodstream or lymphatic system to other locations, and grow. Distant metastases can develop very rapidly, involving most internal organs. The progression of a tumor from benign and localized to invasive and metastatic growth is the major cause of poor clinical outcome in cancer patients (Hofman-Wellenhof, 1996).

1. 2. 5 Spontaneous Regression of Melanoma

Melanoma is an unusual cancer in that it exhibits a relatively high level of spontaneous regression of the primary growth (approximately 7-8%; Smith, 1965; McGovern, 1972). The only other malignant tumor that has demonstrated any degree of significant regression is renal cell carcinoma (de Riese, 1991). Unfortunately though, primary melanoma regression is not always considered a good prognostic sign, as metastases are often observed shortly thereafter (Kroon, 2004).

Histological observation of tissues present at the site of melanoma regression include a lymphocyte infiltration of the papillary dermis that can range from a sparse peri-vascular

population all the way to a thick band of lymphocytes on the surface of the dermis (McGovern, 1972). Observations indicate that these lymphocytes are capable of cytotoxicity to melanoma cells. The lymphocyte infiltrate also disappears following regression, implying that the response is specifically mounted against the melanoma cells (McGovern, 1972).

1. 2. 6 Conventional Treatment of Melanoma

If melanoma is recognized and treated early, it is much more manageable and results in better prognosis. If it remains undetected, it will spread or metastasize to other parts of the body. The prognosis and treatment options for the treatment of melanoma depend on the many factors including the stage of disease, the location and size of the tumor, the presence of bleeding or ulceration at the primary site, and the patient's general health. Conventional treatment of melanoma falls into three broad categories. The first method employed is surgery to remove the tumor, which is the primary treatment of all stages of melanoma and by far the most effective means of treatment provided it is detected early. In the presence of secondary deposits and/or uncontrollable primary growth that make surgery impractical, another option for treatment is chemotherapy. Chemotherapy is when drugs are used to stop the growth of cancer cells, either by killing the cells or by stopping them from dividing. The third treatment option for melanoma is radiation therapy, which uses high-energy x-rays or other types of radiation to kill cancer cells or prevent them from growing (Kroon, 2004).

These conventional treatments are often plagued by a number of side effects and problems. Most chemotherapy drugs are made to kill fast-growing cells, but because these drugs travel throughout the entire body, they can also affect normal, healthy cells. Although radiation is often localized, it can also damage normal cells and tissues. Damage to these healthy cells and

tissues is the cause of many side effects including, but not limited to, nausea and vomiting, fatigue, soreness, rash, mental depression and anxiety, and hair loss. Additionally, these treatments cause a decrease of a patient's immune system, which can often lead to secondary ailments. Similarly, surgery is often invasive and can lead to subsequent infection or other problems.

Emerging novel therapies, such as the adoptive transfer of activated or engineered lymphocytes for the treatment of certain cancers, are designed to overcome this vast array of side effects. These new treatments use the body's own immune cells to specifically target or "home" to the site of the tumor. Furthermore, these immune effector cells are removed from a patient prior to being manipulated and pretreated, followed by reintroduction back into the patient. This potentially eliminates or lessens the risk of side effects due to harsh treatments *in vivo.*

1. 3 Cellular Immunity and Cancer

1. 3. 1 Background

It has been over one hundred years since William Coley observed that tumor regression could be induced by stimulating the immune system with bacterial toxins (Coley, 1893). He reported that direct inoculation of bacteria into the site of a tumor, often led to high fever and other symptoms associated with an intense immune response, but ultimately led to complete regression in a fraction of patients with various types of advanced cancer. Coley's work was later reviewed by his daughter, where she detailed 30 complete remissions, which were representative of the 270 complete remissions out of 1200 patients treated with toxins by her father (Coley-Nauts, 1953). However, by the late 1930's, this form of treatment was largely

abandoned in favor of radiotherapy and chemotherapy, which together with surgery are still the major forms of cancer treatment today. In the mid 1970's, it was theorized that tumor necrosis factor and interferon(s) may have been the primary factors in the exhibited immune response, however Coley's results could not be reproduced to further study the exact mechanism.

In spite of the mixed acceptance of this work, the concept of a role for the immune response in the control and elimination of malignant cells survived, giving rise to a new theory. In 1959, it was proposed that a specific, adaptive immune response may evolve so as to protect the body from neoplasia. This theory was later termed the "immune surveillance" theory (Burnett, 1970), which stated that when aberrant cells with proliferative potential arise in the body they will carry new antigenic determinant on their cell surfaces, which will trigger an immune response to destroy the aberrant cells similarly to when an allograft (foreign tissue implant) is destroyed. According to this theory, cancer would only develop due to some failure of immune function, such as immunosuppression, or by adoption of immune evasion mechanisms by the tumor. The extent to which this actually occurs *in vivo* remains a topic for debate, but there is now considerable evidence to support this theory including spontaneous remission, tumor infiltrating lymphocytes, and increased disease in immunosuppressed individuals (Blattman, 2004). Therefore, many strategies for cancer treatment focus on augmenting the immune system so as to increase its surveillance for, and subsequent destruction of cancer cells.

More recently, some of the mechanisms that operate in the recognition and elimination of tumor cells has been worked out (reviewed in Greenberg, 1991), and have suggested a key role

for T lymphocytes in conferring the specificity of tumor rejections. There is also evidence for natural killer cells (NK), macrophages, and eosinophil involvement in immune mediated anti-tumor activity (Silverstein, 1999).

In humans, direct evidence for a role of the immune response in immunity to tumors was lacking for many years. The occurrence of spontaneous regressions of a number of tumors (especially malignant melanoma), the presence of mononuclear cellular infiltrates in many tumor types, and the response of certain tumors to immunotherapy all provided circumstantial evidence of the presence of an immune response to the tumor in a proportion of patients, and that immune intervention might provide a therapeutic strategy for human cancer.

1. 3. 2 Lymphocyte Recirculation and Infiltration

Lymphocytes are known to continuously circulate from blood to the lymphatic system via lymphoid and nonlymphoid tissues to fulfill the function of immune surveillance (Masuyama, 1992). In 1964, James Gowans demonstrated that lymphocytes spend between 2 and 12 hours in the blood before they appeared in the lymph or lymphoid organs (Gowans, 1964). However, in order for lymphocytes to recirculate through tissues, they must initially adhere to vascular endothelium and exit through the vessel walls in order to migrate through neighboring cells and extracellular matrix to a damaged tissue. The vascular endothelium is a single-cell layer that forms a continuous lining for the large container that holds circulating blood. The majority of this lining is found in the microcirculation of the capillary beds, where the surface area is very high, and the contact between the EC and blood components is maximized (Silverstein, 1999). Leukocyte emigration from the blood through the endothelial barrier during the inflammatory reaction is currently viewed as an adhesion cascade that

involves a coordinated function of a variety of adhesion receptors on the surface of leukocytes and EC (Carlos, 1994; Springer, 1994).

Acute inflammatory response mobilizes leukocytes to sites of inflammation, and results in increased adherence of lymphocytes and other inflammatory cells to endothelium (Silverstein, 1999). Thus, the migration of lymphocytes across the vascular endothelial cell wall is a prerequisite in the implementation of lymphocyte function (Oppenheimer-Marks, 1990). The majority of lymphocytes are capable of tissue selective trafficking (termed "homing"), recognizing organ-specific adhesion molecules on specialized EC (Butcher, 1980). A multi-step model for leukocyte homing has been proposed and confirmed by various *in vitro* and *in vivo* models. The first interactive step is the slow rolling of leukocytes along the endothelial lining, which subsequently may result in firm adhesion, diapedesis, and finally transmigration through endothelium into to surrounding tissue (Luscinska, 1996; Bevilacqua 1993; Springer, 1994; Butcher, 1991).

Clinical and pathologic evidence indicates that tumors can stimulate immune responses, such as the presence of these mononuclear cell infiltrates (Kroon, 2004). These immune cells respond to "danger" signals, which can be provided by growing tumors as a consequence of the genotoxic stress of cell transformation and disruption of the surrounding microenvironment. Under ideal conditions, these signals will induce inflammation, activate innate effector cells with anti-tumor activity, and stimulate professional antigen-presenting cells to subsequently trigger an adaptive response by T and B lymphocytes (Blattman, 2004). The most potent of these cells is considered to be the natural killer, or NK cell (Hanna, 1980), although other cells such as T-cells (Clark, 1988), monocytes (Tagliabue, 1979) and

polymorphonuclear neutrophils (Kindzelskii, 1999), show considerable anti-tumor cell activity after appropriate stimulation with cytokines or tumor cell products.

Further evidence for infiltration is provided by the observation of lymphocyte proliferation (hyperplasia) in lymph nodes draining sites of tumor growth (Vetto, 1997). In many tumors, there is evidence of cytokine effects, such as increased expression of class II major histocompatibility complex (MHC) molecules (Sikorska, 1999), and intercellular adhesion molecule-1 (ICAM-1; CD54) (Terol, 1998), suggesting an active immune response at the tumor site. The spontaneous regression of tumors such as melanoma (Halliday, 1995), and renal cell carcinoma (Jantzer, 1998), which are associated with dense peri- and intra-tumor lymphocytic infiltrates, is also suggestive of an immune mediated anti-tumor response.

1. 3. 3 Leukocyte and Endothelium Interactions

Inflammation is marked by the migration of leukocytes and plasma to a site. This is accomplished through an increased vascular permeability caused by retraction of EC and by enhanced migration of leukocytes across the local vascular endothelium and in the direction of the site of inflammation (Ugarova, 1998). Recirculating lymphocytes, monocytes, and granulocytes bear receptors that bind to cell-adhesion molecules on the vascular endothelium, enabling these cells to extravasate into the tissue (Shimizu, 1991; Diamond, 1990; Steeber, 1999).

Some of these adhesion molecules are expressed constitutively, while others are only expressed in response to localized concentrations of cytokines produced during inflammatory events. This process involves three main groups of adhesive mechanism; selectins (Abbassi,

1993; Barkalow, 2000; Carlos, 1994), integrins (Carlos, 1994; Adams, 1997; Beekhuizen, 1993), and immunoglobulins (Carreno, 1995). Selectins are thought to be involved in the initial rolling of leukocytes along the endothelium, after which integrin binding occurs, followed by diapedesis into the underlying tissue. The β1 and β2 integrins are also important mediators of leukocyte (and dendritic cell) adherence to CD54 (ICAM-1) and VCAM on EC (Steeber, 1999).

1. 3. 4 Leukocyte and Endothelial Cell Adhesion

The mechanisms by which vascular EC capture circulating leukocytes are now well characterized and many of the ligands that contribute to their adhesion and subsequent transvascular migration identified (Osborn, 1992; Springer, 1994). Lymphocyte transendothelial migration has been reported to be dependent on lymphocyte activation via signaling through the T cell and the interleukin-2 (IL-2) receptor (Pryce, 1997). Furthermore, to facilitate the retention and migration of leukocytes to an inflammatory site, both cell types (leukocytes and EC) express outer surface cellular adhesion molecules (CAM's). When these molecules are "activated", they undergo conformational changes increasing their affinity for binding to complementary ligands.

Previous studies have also shown that initial capture of immune cells from the circulation occurs via endothelial selectin molecules that result in weak attachment and, under conditions of flow, result in leukocyte rolling along the vessel wall (Springer, 1994). Leukocytes further bind to the adhesion receptors ICAM-1 and VCAM-1 on the endothelial surface before undergoing transendothelial migration (Luscinskas, 1995; Shimizu, 1991). ICAM-1 is also known to be involved in migration, independently of its role in adhesion, but the molecular

basis of this function is poorly understood (Millan, 2006).

To further facilitate the retention and migration of leukocytes to an inflammatory site, there are also a number of adhesion molecules present on the leukocytes that can mediate cell adhesion. Four members of the $ß_2$ subfamily of integrins have been identified: α_Mß2 (Mac-1, CD11b/CD18, and CR3), α_Lβ2 (leukocyte function–associated antigen and CD11a/ CD18), $\alpha_X ß_2$ (p150,95 and CD11c/CD18), and α_Dβ2 (CD11d/CD18) (Van der Vieren, 1995; Larson, 1990). Two of the leukocyte β2 integrins, CD11a and CD11b, have been reported to bind to ICAM-1 (and ICAM-2) on the tumor cell (Diamond, 1990, Male, 1994; Steeben, 1999). It has previously been shown that CD11a, a member of the integrin family, is present on lymphocytes and largely mediates adhesion between lymphocytes and EC. (Masuyama, 1992). Furthermore, a tight binding event and subsequent migration through the EC wall has further been shown to be mediated predominantly by the CD11a/ICAM-1 pairing (Oppenheimer, 1990; Oppenheimer, 1991; Kavanaugh, 1991; Yednock, 1992).

Many new strategies for the treatment of cancer focus on augmenting the immune system so as to increase its surveillance for, and subsequent destruction of cancer cells. Furthermore, the therapies are often based on augmentation or activation of such receptor/ligand pairs that are present in leukocyte infiltration and subsequent attack of tumor cells.

1. 3. 5 Immunotherapy of Cancer

Immunotherapy refers to any approach aimed at mobilizing or manipulating a patient's immune system to treat or cure disease (Steinman, 2004). It attempts to harness the power and specificity of the immune system for treatment of malignancy as previously described. Experiments in both mice and humans have established that the immune system can damage

and in some cases destroy even very large established tumors (Muranski, 2006).

The various forms of immunotherapy may be classified into two main groups: active and passive. Non-specific, active therapy involves activation of the immune system with agents such as PHA (adjuvant therapy). Specific, active therapy includes vaccinating cancer patients with tumor derived extracts. Unfortunately, clinical trials of tumor-specific active therapy (i.e. vaccination) have so far been disappointing, with objective response rates of 5% or less regardless of histology (Rosenberg, 2004).

Specific passive immunotherapy involves treatment with monoclonal antibodies directed against tumor antigens. Treatment with tumor antigens, or tumor infiltrating lymphocytes cultured with tumor antigens, are other forms of specific immunotherapy. Passive immunotherapy may be non-specific, specific, or a combination of both. Non-specific immunotherapy includes treatment with cytokines or LAK cells, which are discussed further below.

1. 3. 5. 1 Interleukin-2 Activation of Lymphocytes

A clear example of the effectiveness of immunotherapy to mediate cancer regression comes from studies of the administration of interleukin (IL)-2 to patients with metastatic melanoma and metastatic renal cancer (Rosenberg, 1998).

Human IL-2 is a 15-kDa glycosylated interleukin, or hormone, that is only produced by activated T-cells and functions primarily in the generation of cell proliferation and induction of cytotoxic activity in activated T-cells, macrophages, and NK cells that is instrumental in the body's natural immune response (Robb, 1981). IL-2 has no direct effect on cancer cells,

instead IL-2 mediates its effects by binding to IL-2 receptors, which are expressed by lymphocytes, the cells that are responsible for immunity (Smith, 1980).

There has been a total of 15% to 20% of patients with metastatic melanoma and metastatic kidney cancer experience objective regressions of metastatic disease, almost half of which are complete responses. With 20-year follow-up in some patients, it was shown that treatment with IL-2 probably "cures" patients who experience a complete response, because 80% of these patients have ongoing complete responses (Rosenberg, 2005). Further, only 6 of 33 patients with either metastatic melanoma or kidney cancer who had complete cancer regressions have ever had disease recurrence (Rosenberg, 1998). The treatment can be safely administered with a treatment-related mortality of <0.5% (Kammula, 1998). Thus, it seems that patients with metastatic melanoma and metastatic renal cancer join that very small group of patients with solid tumors that can be cured by systemic treatment. Similar results from many series led the Food and Drug Administration to approve the use of high-dose IL-2 for patients with metastatic kidney cancer in 1992 and for patients with metastatic melanoma in 1998. The durability of complete responses was a decisive factor in the approval of these treatment regimens, which should be offered to all eligible patients with these metastatic cancers (Rosenberg, 2005).

After the first descriptions of the effectiveness of IL-2 (Rosenberg, 1985; Lotze, 1986), immunotherapy has come of age, and substantial improvements based on increased scientific understanding have continued to develop.

1. 3. 5. 2 Adoptive Immunotherapy

In clinical trials associated with the administration of IL-2, one major side effect was toxicity using sufficient IL-2 concentrations to optimally activate the tumoricidal effect, presumably as a result of leukocyte activation. To overcome this effect, the peripheral blood lymphocyte population can be isolated from the patients using leukopheresis (or removal of leukocytes of blood followed by subsequent replacement of depleted plasma and red cells to donor). These cells are then activated *in vitro* with high doses of IL-2 to induce lymphokine activated killer cells (LAK), which exhibit non-antigen dependent, non-MHC restricted cytotoxicity. LAK cells were first described by Yron in 1980, and were originally thought to be a new class of lymphocyte derived cytotoxic cell (Grim, 1982; Guinan, 1989). Since LAK behavior can be derived from a variety of lymphoid cells, the term LAK is a description of a cellular activity, rather than a specific cell type (Maghazachi, 1988). Following cellular activation, the cells were washed and reintroduced into the patient's circulation, where they were expected to home to the tumors and kill them. This process is known as adoptive immunotherapy.
This adoptive transfer of autologous lymphocytes with antitumor activity, expanded *in vitro* and reinfused into patients, can mediate tumor regression (Rosenberg, 1988), and recent advances in this field have dramatically improved the effectiveness of this approach (Dudley, 2002, Dudley, 2003). In 2002, Rosenberg et al. reported some of their most promising results to date. The procedure involved removal of peripheral blood lymphocytes from melanoma patients, followed by reintroduction of LAK cells into the same patients. The results of their studies were that the infused cells usually took up longterm residence, and in 10 of the13 patients, their melanoma cells, including all metastases, regressed either partially or completely (Rosenberg, 2005). Lymphodepletion before the administration of autologous tumor reactive cells has led to objective responses in 18 (51%) of 35 patients with metastatic

melanoma, including those refractory to high-dose IL-2 and combination chemotherapy (Dudley, 2005). These studies demonstrate the effectiveness of immune T cells when administered to hosts actively depleted of T-regulatory cells, as well as cells that compete for homeostatic cytokines.

Unfortunately, multiple factors, including tumor immune evasion, homeostasis and induction of tolerance, as well as suboptimal quality of transferred T cells, have hampered the otherwise promising attempts at driving tumor rejection (Ross, 1993; Yee, 2000; Dudley, 2001).

To overcome some of the homing and other problems associated with LAK based immunotherapy, researchers have found immune cells deep inside some tumors, which have an enhanced "homing ability", and have named these cells tumor-infiltrating lymphocytes (TILs). Success with TILs in lab animals led researchers to devise a strategy to increase the anti-tumor activity of TILs. In the late 1980's, clinical trials were begun by Rosenberg and other groups (Rosenberg, 1985; Kawakami, 1988; Heo, 1987), in which tumor infiltrating lymphocytes (TIL) were removed from excised tumors, stimulated with IL-2, and reinjected into cancer patients to promote tumor regression.

Furthermore in a 2006 report, the Rosenberg group reported a 50% response rate according to RECIST criteria was reported in patients with metastatic melanoma treated with *in vitro* expanded TIL's and IL-2 following a lymphodepleting nonmyeloablative preparative regimen of cyclophosphamide and fludarabine (Gattinoni, 2006; Hughes, 2005; Morgan, 2006). Using this method, 2 out of 6 patients had a regression period of over 2 years following treatment (Gattinoni, 2006). This significant achievement was attributed to the key realization that the

host's immune system needs to be properly conditioned, in order to create an appropriate 'lymphoid space' that is devoid of regulatory mechanisms. However, immunodepletion and adoptive therapy have not yet been tested in randomized studies, but the results of recent clinical trials using these methods are notable for their unprecedented response rates and the fact that the patients studied had previously failed other modes of immunotherapy including high-dose IL-2 and ACT (Dudley, 2002; Morgan, 2006).

Although encouraging, the sporadic results obtained with adoptive immunotherapies may be explained if activated leukocytes interact in a more complex way with other molecules and cells in the circulation. These interactions have not been extensively studied, but there is mounting evidence that molecules of the blood coagulation system may play a major role in the modulation of the immune response. The following section brings together the interactions of the end products of the clotting system (fibrinogen and fibrin) with immune cells and EC with relevance to inflammation and cancer.

1. 4 The Role of Fibrin(ogen) in Immunity and Cancer

1. 4. 1 Background

A relationship between cancer and abnormalities of the coagulation system has been recognized for over 100 years (Trousseau, 1865). Thromboembolic disease (usually of unknown etiology), refractory to anticoagulant therapy, may be an early detectable sign of an underlying cancer, which could precede the onset of observable cancer by months or years. Although many cancer patients exhibit clinically significant hemostatic abnormalities, about 50% of all patients (> 90% with metastases) also have abnormal laboratory coagulation parameters (Trousseau, 1865). Such parameters include soluble fibrin (sFn) (Gouin-Thibault, 1999; Iversen, 1995; Nakagawa, 1994),

and its precursor fibrinogen (Fg), which may also be an early marker of undiagnosed malignancy (Andrassy, 1980).

A role for coagulation in tumor biology is further inferred by the anti-tumor effects of anticoagulant drugs, such as heparin or warfarin (Zacharski, 2005; Biggerstaff, 1997) and in a number of both experimental and spontaneous animal tumor models (Hilgard, 1976; McCulloch, 1987; Eichbaum, 1975; Biggerstaff, 1997). However, these therapies also increase the risk of bleeding due to inhibition of normal clotting.

1. 4. 2 Fibrin(ogen) and Soluble Fibrin in Disease

During the past two decades there has been an increasing appreciation of the multifaceted role fibrin(ogen) plays in the immune and inflammatory reactions. Although presumed for many years, many of the roles attributed to fibrin(ogen) have only recently been substantiated. In this regard, much of the available evidence indicates that fibrin(ogen) operates as an antagonist, or that the adhesive potential is realized in a host of diseases and contributes to the progression of those diseases. We, and others, have speculated that tumor growth and propagation by fibrin(ogen) may be an inadvertent consequence of the protein's role in inflammation. Furthermore, some researchers are willing to attribute some or most of the pathogenesis of cancer to adhesive inflammatory events involving fibrin(ogen) (Palumbo, 2002; Simpson-Haidaris, 2001; Retzinger, 1999). Presuming their ideas are correct, interfering with those events should mitigate disease.

Following its secretion from the liver, the protein exists in plasma, the lymph, and interstitial fluid (Chrobak, 1967; Le, 1998). Fibrinogen is a classic "acute-phase reactant," with serious

inflammatory challenges resulting in increased hepatic expression, the induction of extrahepatic expression, and increased circulating protein (Molmenti, 1993; Lee, 1996; Guadiz, 1997). In healthy individuals, the concentration of fibrinogen in plasma is reported to be between 4 and 10 μM (Retzinger, 1999). However, this concentration can increase by as much as 200 to 400% during times of physiological stress, such as disease (Shultz, 1990).

Fibrin(ogen) deposition is a universal feature within injured tissues and inflammatory foci. In the late 1950's, fibrin(ogen) was shown to be a component of tumor-induced inflammation, and more recently fibrin(ogen) (and derived materials like fibrin) have been shown to accumulate within tumor stroma and envelop tumor cells (Retzinger, 1999). Furthermore, i*n vitro* studies (Smiley, 2001; Sitrin, 1998; Rubel, 2002; Rubel, 2001; Shi, 2001) have shown that fibrin(ogen) can profoundly alter leukocyte function, leading to changes in cell migration, phagocytosis, NF-kappa B–mediated transcription, production of chemokines and cytokines, degranulation, and other processes. Furthermore, recent reports from *in vivo* studies suggest that pulmonary metastasis is reduced in fibrinogen deficient animals (Palumbo, 2002).

The presence of sFn in blood has, until recently, been considered a benign marker for the presence of an ongoing coagulopathy. However, others have reported a direct role for sFn in melanoma metastasis in an experimental animal model (Rickles, 1992; Biggerstaff, 1999). Several other studies suggest that sFn may be a prognostic marker in cancer (Beer, 2002; Spero, 1980), but no clinical studies have been performed to date to directly associate sFn with increased metastasis.

Furthermore, in solid tumors and in experimental systems, the presence of polymerized fibrin has

been reported to result in inhibition of the immune response to cancer. Gunji et al. showed that fibrin deposition on tumor cells during migration in the blood could protect them from elimination by NK cells or other cytotoxic cells (Gunji, 1988). Furthermore, this was also found particularly when using immunotherapies involving lymphokine (Interleukin-2) activated killer (LAK) cells (Gunji, 1999), mitogen (PHA) stimulated T cell blasts, or re-injected tumorinfiltrating leukocytes (TIL). Furthermore, immunosuppression induced by fibrin degradation products has also been reported (Girmann, 1976; Donnel 1989). Furthermore, our laboratory also recently demonstrated that sFn also inhibited lymphocyte and IL-2 activated LAK cell adherence and cytotoxicity against tumor cells (Figures A-1, A-2; Biggerstaff, 2007). All of these results collectively suggest that sFn may be an immunosuppressive agent in cancer, and that it is involved in the etiology of metastasis. Furthermore, successful transfer and treatment by these means are more commonly observed in those patients with less severe, early stage disease. This coincides with the findings that sFn is more elevated in those patients with severe prognosis. These pieces of information taken collectively, support the hypothesis that sFn is an immunosuppressive agent that inhibits lymphocyte recognition, binding, and diapedesis of endothelium, which allows for greater success of metastasis.

1. 4. 3 Fibrinogen Structure and Degradation

Fibrinogen is a dimeric protein, each half of which is composed of disulfide-bonded polypeptide chains designated Aα, Bβ, and γ. The designations "A" and "B" refer to fibrinopeptides A (FpA) and B (FpB) that constitute the amino terminal residues of the A and B chains (Retzinger, 1999).

The conversion of fibrinogen to fibrin manifests as a multi-step transformation of the original conformation into both water-soluble monomers and gel-like polymers. The initial steps of this

are catalyzed by thrombin, a trypsin-like serine protease, which hydrolyzes specific Arg-Gly bonds within the α and β chains of fibrinogen (Retzinger, 1999). Figure 1.1 is a diagram of fibrinogen structure and its sites of thrombin cleavage.

Fibrinogen is thought to be rather inert in circulation but it becomes reactive toward itself and a number of proteins and cell types upon conversion to fibrin (Medved, 2001). Conversion of fibrinogen to fibrin results in the exposure of multiple binding sites that provide its interaction with various other proteins and cells (Medved, 2001). Furthermore, fibrin(ogen) has been shown to suppress lymphocyte functions (Plow, 1986).

As earlier discussed, there are an abundance of cells present near those sites of inflammation, or cancer growth that have receptors for fibrin(ogen). Fibrin(ogen) binds to a wide range of cellular receptors, including two of the leukocyte β2 integrins, $\alpha_M\beta2$ (Mac-1) and $\alpha_X\beta2$ (p150,95) (Wright, 1998; Altieri, 1995; Altieri, 1988), and the β2 integrin receptor, CD54 (ICAM-1) (Languino, 1995; Gardiner, 1997), which are important mediators of leukocyte diapedesis. Many of the effects of fibrin(ogen) on leukocyte activity appear to be mediated by a specific receptor on leukocytes, the integrin receptor CD11b (α_Mß2) (Flick, 2004; Takami, 2002; Tang, 1993).

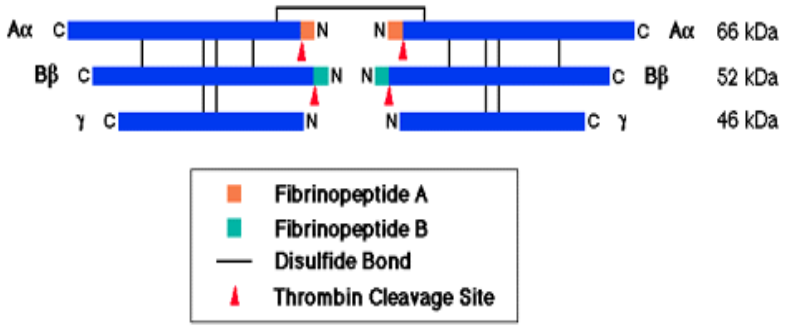


Figure 1-1. Fibrinogen structure (Tollefsen, online).

1. 4. 4 Anticoagulation Therapy of Cancer

Fibrin deposition around solid tumors may protect the host from tumor cell invasion by blocking their entrance into the circulation (Colucci, 1981). Furthermore, early studies using isotope labeled tumor cells showed that tumor cell deposition occurred regardless of whether animals were anticoagulated, but that subsequent metastatic growth was decreased by anticoagulant therapy, implying a role for fibrin in metastatic tumor growth (Fisher, 1967). Recently, Costantini and Zacharski reviewed the evidence addressing the significance of blood coagulation activation in fibrin deposition in tumor cells (Costantini, 1993). As for the involvement of fibrin(ogen) in malignancy, it has been proposed that the protein envelops tumor cells thereby protecting them from immune surveillance (Dvorak, 1981), and provides an intravascular matrix for trapping and anchoring malignant cells following their release from a parent tumor.(Markus, 1984). A role for coagulation in tumor biology is further inferred by the anti-tumor effects of anticoagulant drugs, such as warfarin and heparin (Zacharski, 2005), and other coumadin derivatives (Gasic, 1983), thrombin inhibitors (Hu, 2004; Asanuma, 2004), and in a number of both experimental and spontaneous animal tumor models (Hilgard, 1976; McCulloch, 1987; Eichbaum, 1975; Amirkhosravi, 1995).

Anticoagulant therapy, in combination with chemotherapy, has been applied with some success in the past. However, anticoagulation can be seen as a drawback in many patients, particularly those undergoing surgery, because excessive blood loss can result. Recently, alternative approaches have been applied in the treatment of cancer, that do not interfere with the natural coagulation patients.

1. 5 Peptide Therapy

1. 5. 1 Background

There are multiple examples in nature, where peptides are used as ligands to influence the function of specifically binding target proteins (Buerger, 2003). Furthermore, there is a wide spectrum of tools now used to interfere with the function of a given gene product, including selected inhibitory peptides (Borghouts, 2005). Most peptides being considered for therapeutic purposes at this time exert their action on the cell surface. Peptides bind to cell surface proteins and act by inducing or inhibiting one or more signal transduction pathways. Furthermore, small peptides are able to recognize specific protein domains and thus interfere with enzymatic functions or protein-protein interactions (Buerger, 2003). Proteins and peptides are experimentally selected for high-affinity cellular interactions with pre-determined target structures that are emerging as important molecules. These advances could serve to extend the conventional administration of drugs. In a few model systems, peptides have already been used to manipulate crucial regulatory networks in cancer cells (Buerger, 2003; Kardinal, 2001; Mai, 2001; Wang, 2000; Zhang, 2001). They can target specific intracellular proteins required by cancer cells for proliferation and invasion. It is conceivable that individual peptides can also be derived to inhibit function in a targeted fashion. These peptides can be used for mono therapy or in combination with conventional chemotherapeutic agents in order to better treat cancer (Borghouts, 2005).

1. 5. 2 Natural vs. Synthetic Peptides

Inhibitory peptides can either be designed from both naturally occurring binders or synthetically manufactured. Usually crystallographic data are required for the first approach, to study the interaction surface between the target protein and the binding partner (Borghouts, 2005). Several

findings (reviewed in Borghouts, 2005) indicate that peptides with high affinity already exist for various targets, which can be used for therapeutic approaches without screening large peptide libraries.

Furthermore, the use of small endogenous domains not only provides for high affinity, but also circumvents problems encountered with larger exogenous proteins, such as lower stability and higher immunogenicity if used *in vivo* (Borghouts, 2005). Unfortunately, the clinical fruits of many new therapeutic methods have not been abundant, in part because *in vitro* models used for screening often do not duplicate well *in vivo*. This too has been true of the past decade of peptide-drug design for the treatment of cancer. There are clear indications that smaller ligands are generally better for therapeutic use (Reilly, 1995). Compared with antibodies and their fragments, peptides are considerably smaller, and generally should not elicit an immune response upon repeated administration (Aina, 2002; Nilsson, 2000). Therefore, peptide ligands may overcome several limitations of antibody therapy and diagnosis (Mori, 2004).

Furthermore, what is needed is the therapeutic equivalent of what Muller et al. refer to as "smart bombs", or the ability to selectively inhibit inflammation where it is damaging without interfering with beneficial functions of the inflammatory response (Muller, 2002). Many current strategies lack this ability, and cause an added difficulty when dealing with any disease, particularly those associated with surgery.

1. 5. 3 Specific Peptide Therapeutics

As previously discussed, fibrin(ogen) is a ligand for many biological molecules. It has been reported to bind numerous integrins, including those present on many leukocytes (monocytes,

neutrophils, and lymphocytes), EC, and tumor cells. Many different studies have been performed to further identify the specific amino acid sequences on these molecules responsible for interactions. Furthermore, small peptides have been derived from fibrinogen, CD11b/CD18, and CD54, and tested for their ability to inhibit fibrinogen binding (Altieri, 1995; Ugarova, 1998; Yakubenko, 2001; D'Souza, 1996). Several other similarly derived peptides have been identified during the past several years, but these four specific sequences (and peptides) have been studied in detail by our lab (Biggerstaff, 2006). In recent studies, it was shown that the following four peptides sequences significantly restored monocyte adherence (and cytotoxicity) to tumor cells, following pretreatment with (and inhibition caused by) sFn (Biggerstaff, 2006). Furthermore, Table 1-1 details the sequences, molecules of origin, and ligands associated with each peptide.

1. 5. 3. 1 Peptide P1 Background

Peptide 1, (P1; ^{117}NQKIVNLKEKVAQLEA^{133}) present on the fibrinogen γ-chain, reportedly binds to the CD54 sequence present on the 1st immunoglobulin domain (Altieri, 1995). As previously discussed, ICAM-1 is expressed by many cell types including EC, leukocytes and many cancers. Furthermore, pretreatment of tumor cells with P1 was shown to block sFn from binding to tumor cells in our recent work (Figure A-3; Biggerstaff, 2006).

Table 1-1. Specific Peptide Sequences, Molecules of Origin, and Ligands (Biggerstaff, 2006).

Peptide #	Sequence	Molecule of Origin	Ligand
P1	NNQKIVNLKEKVAQLEA	sFn (g-chain)	CD54 (1st Ig)
P2	YKSMKKTTMKIIPFNRLTIF	sFn (g-chain)	$\alpha_M\beta2$ (αM I)
P3	KVILPRGGSVLVTC	CD54 (1stIg)	sFn (g-chain)
P4	KFGDPLGYEDVIPEADREG	$\alpha_M\beta2$ (αM I)	sFn (g-chain)

1. 5. 3. 2 Peptide P2 Background

Several peptides corresponding to potential fibrin(ogen)/CD11b binding domains have been identified over the past several years. Recently, Ugarova et al. revealed another possible binding site in the region of 377–395 of the fibrinogen chain that may be important (Ugarova, 1998). Furthermore, the P2 peptide (^{377}YKMKKTTMKIIPFNRLTIG^{395}), originally designated P2C, was shown to significantly inhibit CD11b-mediated cell adhesion to immobilized fibrinogen and directly supported saturable binding to the $_{M}$ I-domain (Ugarova, 1998). It is currently considered to be the major fibrin(ogen) γ-chain binding site for CD11b ($\alpha_M\beta2$) and CD11c ($\alpha_X\beta2$) (Ugarova, 1998). Furthermore, pretreatment of monocytes with P2 has recently been shown to block sFn from subsequently binding to cells, allowing restoration of binding to tumor cells (Figure A-3; Biggerstaff, 2006).

1. 5. 3. 3 Peptide P3 Background

The major fibrin(ogen) binding site on CD54 is in the 1st Immunoglobulin domain (^{8}KVILPRGGSVLVTC^{21}, designated P3 herein) (D'Souza, 1996), and binds to the fibrinogen γ-chain. Pretreatment of sFn with P3, prior to exposure to monocytes, has also been recently shown to restore monocyte binding to tumor cells (Figure A-4; Biggerstaff, 2006). Specifically, P3 binds the site taken to synthesize P1 herein (^{117}NQKIVNLKEKVAQLEA^{133}).

1. 5. 3. 4 Peptide P4 Background

The major fibrin(ogen) γ-chain binding site reported on CD11b ($\alpha_M\beta2$) is in the α_M I-domain (^{245}KFGDPLGYEDVIPEADR^{261}) (Yakubenko, 2001). Peptide 4 (P4) was created from this sequence, in order to block leukocyte binding to fibrin(ogen). Furthermore, pretreatment of sFn with P4 prior to exposure to monocytes, was recently shown to partially restore immune cell

binding to tumor cells (Figure A-4; Biggerstaff, 2006). Figure 1.2 is a schematic diagram showing the sequences and specificities of each of these peptides on the sFn γ-chain and on CD11b ($\alpha_M\beta 2$) and CD54.

As previously discussed, earlier studies (Biggerstaff, 2006), have shown that the addition of any/all of these peptides results in a significant restoration of adherence of sFn pretreated monocytes and tumor cells to one another (by a mechanism of CD11b and CD54; Figure A-5). However, since this project employs only lymphocytes and EC, the peptides designated P1 and P3 are being tested for their anticipated relevance to this model. Whereas, P2 and P4, which are specific to the monocyte receptor and complementary sequence on sFn, were not expected to exhibit the same therapeutic potential for these particular cell types (due to the different expression of receptors).

Additionally, previously performed experiments determined that the specific fibrin(ogen) adherence inhibitors did not affect fibrinogen clotting in the presence of thrombin *in vitro*. Furthermore, the peptides did not inhibit the normal coagulation cascade, as shown in re-calcified normal human plasma (Biggerstaff, 2006).

1. 6. Research Objectives and Hypotheses

There are seemingly many factors that are physiologically relevant to the epidemiology of disease and prognosis in melanoma patients. Furthermore, the relationship between host immune cells (leukocytes), the vascular system (endothelium), tumor cells, the cytokine network, and potential therapies is highly complex, as discussed. Therefore, the primary goal of this project was to further elucidate the details of these interactions. This will allow the identification of possible

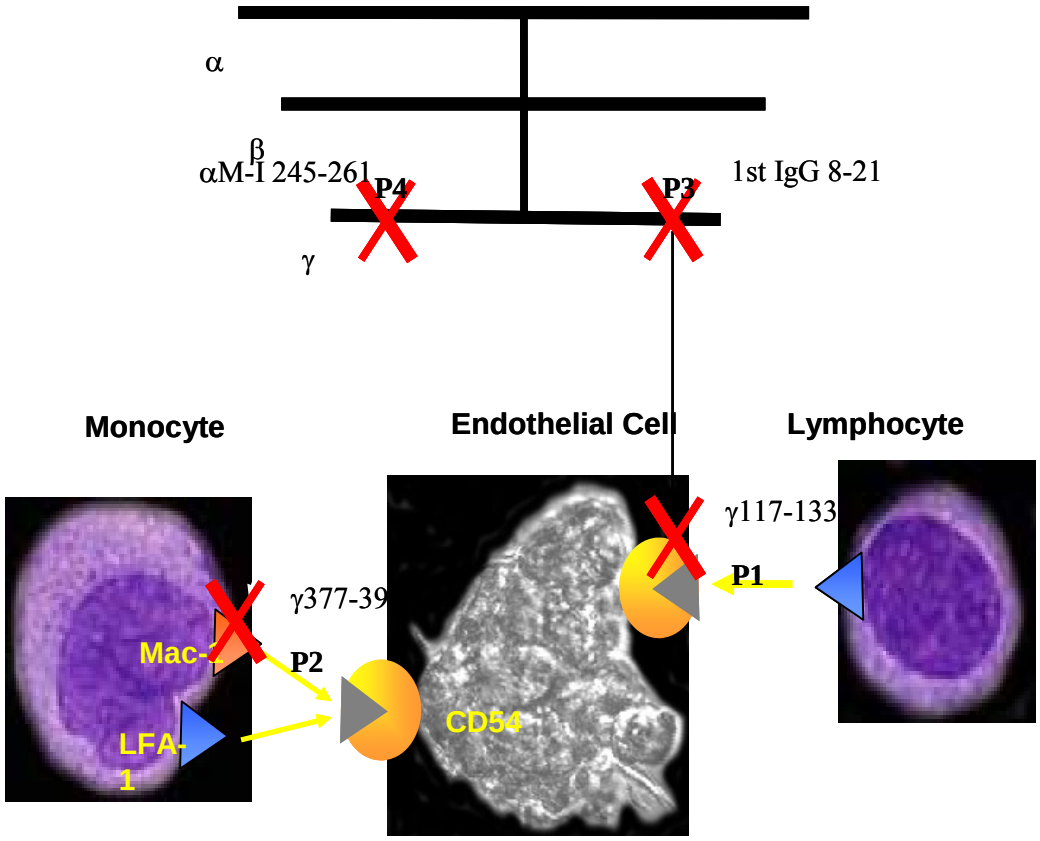


Figure 1-2. Model of Relevant Cellular and Fibrin(ogen) Receptor Sequences**.** Schematic diagram showing the amino acid sequences, sites of origin and effector molecules for four peptides (designated P1 – P4) reported to inhibit fibrin(ogen) binding to CD11b/CD18 or Mac-1 (orange) and CD54 (blue) (Biggerstaff, 2006 adaptation).

synergistic interactions and inhibitory factors involved in tumor cell progression and metastasis.

As presented above, fibrin(ogen) and sFn levels are often upregulated in cancer patients, and have been shown to act as immunosuppressors in many experimental animal models. Furthermore, our lab has recently shown that sFn inhibits monocyte adherence to and cytotoxicity of tumor cells. More recently, we have found that sFn also inhibits lymphocyte and LAK cell adherence to tumor cells.

There is also an abundance of evidence that these interactions are highly dependent on the CD11b/CD18, CD11b/CD18, and CD54 receptor interactions. More specifically, sequences within these receptors have been identified that actively mediate binding to fibrin(ogen). Furthermore, researchers have discovered complementary sequences that have been recently identified on the fibrin(ogen) molecule that bind specifically to these sequences. As a result, we have utilized these specific peptide sequences that have been shown to significantly restore levels of monocyte binding and cytotoxicity to tumor cells, even in the presence of sFn.

Based on these results, it was hypothesized that sFn may also inhibit lymphocyte and LAK cell adherence to and diapedesis through EC. We further hypothesized that a double blockade of sFn binding to CD54 on EC, using the above peptides (P1 and P3), would result in restoration of these effector functions without affecting normal clotting. Further pretreatment with P2 and P4, those peptides specific for monocyte interactions, are expected to minimally affect binding and migration, due to the incongruence of receptor and ligand in this model.

In this project, four specific aims have been identified that test these hypotheses including, 1) to

use immunochemistry and fluorescently labeled sFn to establish sFn binding to effector cells, 2) to determine the role of sFn in lymphocyte adherence to EC under flow conditions, 3) to determine the role of sFn in lymphocyte transmigration, or diapedesis through endothelium toward tumor cells in a standard microplate assay, and 4) to determine the effect of specific blocking peptides on sFn-induced inhibition of lymphocyte binding and diapedesis.

Inhibition of lymphocyte function by sFn would have important implications in adoptive immunotherapy of cancer in which activated, or modified lymphocytes are reintroduced into the circulation of cancer patients to kill metastatic cancer cells and to extravasate and home to solid tumors.

CHAPTER 2. EXPERIMENTAL DESIGN, MATERIALS, AND METHODS

2. 1 Experimental Design and Background

2. 1. 1 HMEC-1 Endothelial Cells

Human dermal microvascular endothelial cells (HMEC-1) were immortalized by transformation with SV40 virus (Ades, 1992). This particular cell line was chosen for its previously documented characteristic endothelial cell receptor biology, in order to further explore the complex relationship between lymphocytes, tumor cells, and endothelium.

HMEC-1 exhibits typical cobblestone morphology when grown in monolayer culture (Figure 2.1). It has previously been shown to bind to peripheral blood leukocytes and extra cellular matrix proteins via many specific cell adhesion molecules. HMEC-1 can either constitutively express or can be induced to express key integrins including alpha-1,-2,-3,-4,-5,-6, and –V, as well as beta-1,-3,-4 and -5. Furthermore, they have been shown to express immunoglobulin gene super family molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and a member of the selectin family, E-selectin (Xu, 1994).

2. 1. 2 A375 Melanoma Cells

The A375 human amelanotic malignant melanoma cell line was established from a solid tumor that was excised from a 54 year-old female with melanoma as described in Giard, et al. (Giard, 1973). This cell line was chosen due to its relevance to immunotherapy (melanoma) and due to its employment in our previous publications involving similar mechanisms. Furthermore, A375 cells express CD54, which has been reported on many cancerous and endothelial cell lines (Steeber, 1999).

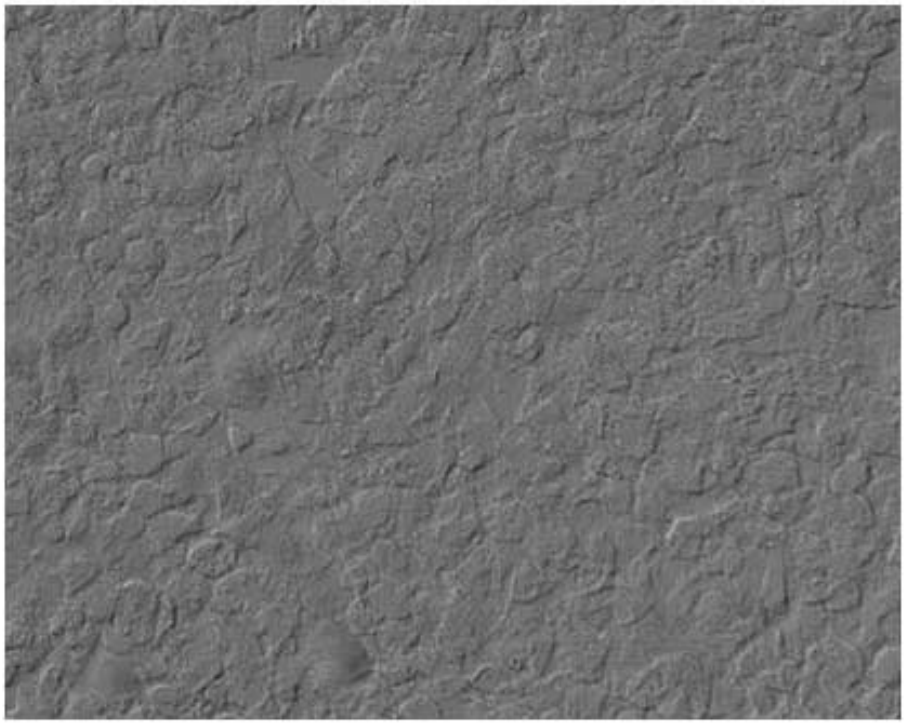


Figure 2-1. Confluent HMEC-1 endothelial cells (60x magnification).

2. 1. 3. Immunostaining and Fluorescently Labeled Soluble Fibrin Assays

2. 1. 3. 1 von Willebrand's Factor

Endothelial cell ultrastructure was initially described by Weibel et al. in the 1960's. The cells contain numerous unique, electron-dense, membrane-bound organelles, called Weibel-Palade bodies (Weibel, 1964). These particular structures contain a high concentration of von Willebrand factor (vWF), a large glycoprotein present in plasma. When EC are exposed to certain agonists, such as thrombin or histamine, these Weibel-Palade organelles "flow" to the surface of the cell and their contents are secreted into the blood, causing fusion between their membranes and the cellular surface. Therefore, HMEC-1 cells were stimulated with thrombin (which is necessary to convert Fg to sFn) and immunostained for vWF.

2. 1. 3. 2 CD54 (ICAM-1)

The CD54 antigen is a 90 kDa transmembrane glycoprotein, which is related to the Immunoglobulin family. As previously discussed, the CD54 antigen has been reported to mediate the adhesion of lymphocytes, monocytes, and neutrophils to both normal and activated endothelium. Therefore, HMEC-1 cells were stimulated with thrombin and stained for this antigen using indirect immunostaining to confirm presence of this receptor on this cell line.

2. 1. 3. 3 CD25

CD25, or Anti-IL-2R, recognizes an antigen that is the human low-affinity interleukin-2 receptor (IL-2R). Furthermore, CD25 expression is reported to be upregulated on activated lymphocytes, or LAK cells, compared to their lymphocyte precursors (Schlossman, 1995). Therefore, monoclonal antibodies directed against this receptor were applied to differentiate these different cell types after incubation for 72 hours.

2. 1. 3. 4 Oregon Green Labeled Soluble Fibrin on Effector Cells

In previous studies, we have shown that Oregon Green labeled soluble fibrin binds to monocytes, lymphocytes, and A375 tumor cells (Figures A-6, A-7; Biggerstaff 2006, 2007). In this project, we have employed the same method to show that sFn also binds to LAK and HMEC-1 cells.

2. 1. 4 Leukocyte Adherence to Endothelial Cells Under Flow Conditions

As earlier reported, postcapillary venules are major sites of leukocyte emigration in inflammation (Lawrence, 1990). Furthermore, physiological shear rates in these regions are generally reported to be between 35-560 s^{-1} (Lawrence, 1990; Heisig, 1968). In general, the movement of leukocytes to the vessell wall and subsequent efficiency of attachment both depend on the blood flow, therefore utilization of a perfusion model is more physiologically relevant than other static, cell binding methods that are often employed (Abbitt, 2003). Furthermore, our work previously included performing optimization assays for our particular flow model, in order to employ a shear rate that falls within this reported range while still observing a significant number of binding events (Figure A-8; Biggerstaff 2006).

To simulate the fluid shear stresses present *in vivo*, coverslips containing confluent endothelial cell monolayers were loaded into the FCS2 stage incubator (Bioptechs Inc., Butler, PA), on the stage of an inverted microscope to observe lymphocyte rolling and binding events. The FCS2 incubator is a closed, near-laminar flow, temperature-controlled perfusion chamber. Figure 2-2 shows the detailed setup of the apparatus.

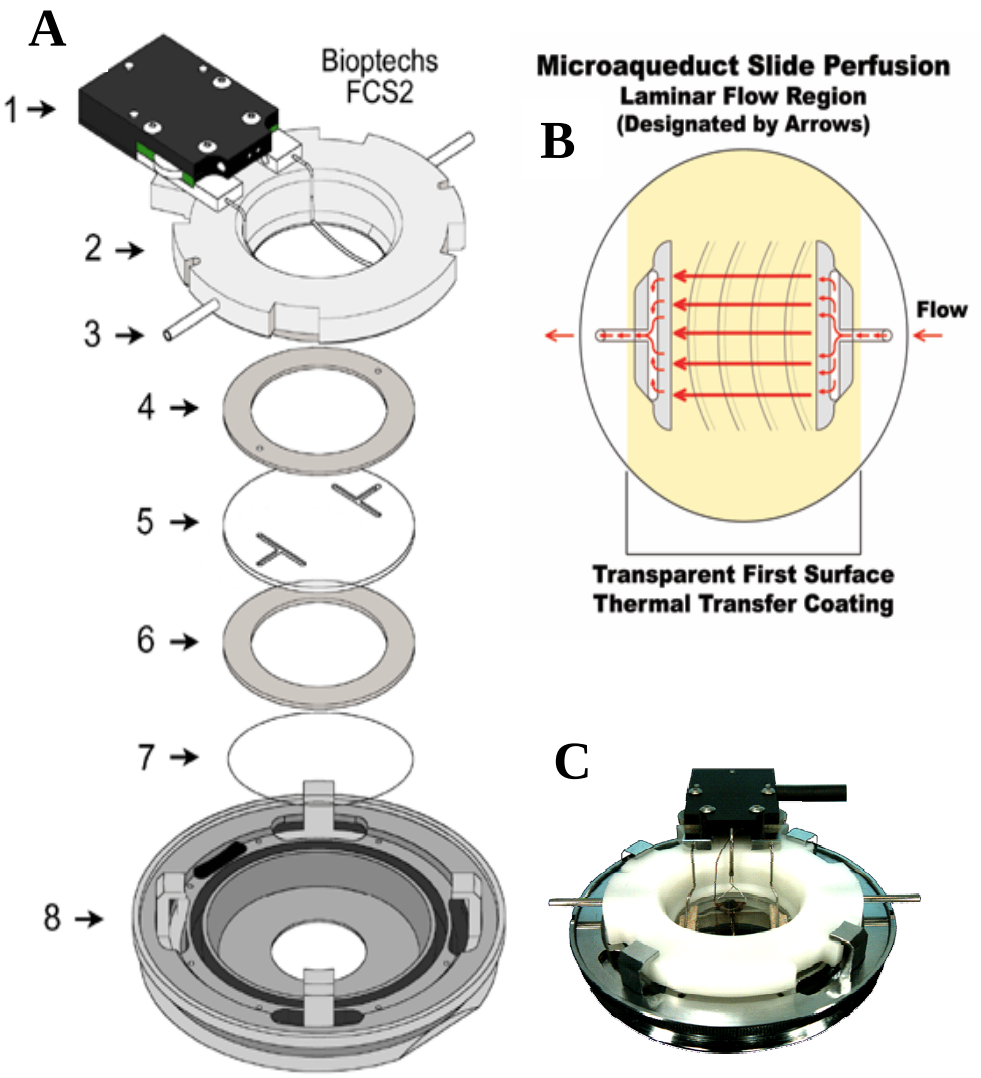


Figure 2-2. Bioptechs FCS2 System. A) Eight piece apparatus (1. Temperature sensors, 2. Heater contacts, 3. Perfusion connections, 4. Upper gasket, 5. microaqueduct slide (optical surface, perfusion guide and termperature control), 6. Lower gasket (defines flow geometry), 7. 40mm coverslip, 8. Self-locking base with internal temperature control), B) Microperfusion flow chamber, C) fully assembled apparatus.

2. 1. 5 Lymphocyte Transmigration Through Endothelial Cells in Microplate Assays

A static, transwell migration system (adapted from Sancho, 1999) was used to perform experiments to determine the ability of lymphocyte and LAK cell preparations to cross endothelium in the presence or absence of an underlying tumor layer. Furthermore, the role of various levels of sFn in inhibiting lymphocyte/LAK cell diapedesis was also explored. Lastly, specific peptides were also added to the model, in addition to sFn, in order to test their abilities to restore the protein from inhibiting diapedesis.

2. 2 Sterile Cell Culture Methods

All cell culture was carried out under sterile conditions, and performed in a Class 2 biological safety cabinet following the University of Tennessee BSL-2 laboratory guidelines.

2. 2. 1 Endothelial Cell Culture

HMEC-1 (kindly provided by Dr. Tim Sparer) were cultured in high-glucose DMEM (Invitrogen, Carlsburg, CA) supplemented with 10% Fetal Bovine Serum (FBS; Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine (Hyclone, Logan, UT), and 100 µg/ml streptomycin/penicillin antibiotics (Hyclone). They were grown in both 25 cm^2 and 75 cm^2 sterile tissue culture flasks (Costar, Cambridge, MA) at 5% CO_2, at 37ºC in constant culture.

2. 2. 2 Tumor Cell Culture

A375 was purchased from ATCC (Rockville Pike, MD), and maintained in continuous cell culture in RPMI 1640 supplemented with Glutamax (Invitrogen) with 10% FBS, and 100 µg/ml streptomycin/penicillin antibiotics. They were grown in both 25cm^2 and 75cm^2 sterile tissue culture flasks at 37ºC at 5% CO_2.

2. 2. 3 Removal of Cells from Culture

Cells were detached from the plastic flasks using approximately 3-6 ml (depending on flask size) of trypsin/EDTA (0.25%; Hyclone). After trypsin was added, the flask was immediately placed into a 37ºC incubator, with 5% CO_2 for approximately 4-6 min, or until cells dislodged easily from the plastic. The cell suspension was transferred from the flask into a 15 ml centrifuge tube and topped off with RPMI 1640 with 10% FBS. Cells were then centrifuged at 200 x g for 10 min, to obtain cell pellets.

2. 3 Isolation of Peripheral Blood Lymphocytes

Venepuncture and disposal for this study was performed in accordance with the University of Tennessee Institutional Review Board approval (IRB# 6788B).

2. 3. 1 Venepuncture

Approximately 50 milliliters of peripheral blood were drawn from normal, healthy volunteers into 6 ml 3.2% lithium heparin vacutainers (Becton Dickinson VACUTAINER™ Systems, Rutherford, New Jersey).

2. 3. 2 Isolation of Lymphocytes

Whole blood was immediately transferred from 6 ml vacutainers into a 75cm^2 sterile tissue culture flask and diluted by approximately 50% with RPMI 1640. The cell suspension was separated using a density gradient centrifugation method created by Boyum in 1976, which isolates peripheral mononuclear cells from whole blood (Boyum, 1976). Approximately 8 ml of Lymphoprep (Axis-Shield, Oslo, Norway) was added to 8 separate universal containers (Dynalab, Rochester, NY). Subsequently, 13-15 ml of diluted blood was gently layered over media, using a

sterile pipette. All tubes were then centrifuged at 450 x g for 25 min. A distinct layer of mononuclear cells was extracted from each container, using a sterile, manual 2 ml pipet, and condensed into 4 fresh universal containers. The cell suspension was then topped off to fill the containers, and 3 additional centrifugation steps were performed to compound all cells present from the separation into one pellet. Isolated cells were transferred into another tissue culture flask containing 20 ml of RPMI 1640 with 10% FBS, and incubated at 37°C, in 5% CO_2, for 90 min in order to allow monocytes to adhere to the plastic, while the non-adherent cells (lymphocytes) remain in suspension. The lymphocyte suspension was decanted into a new universal container, topped off with RPMI 1640 and centrifuged at 200 x g for 10 min. A cell count was performed on the final cell suspension in an improved Neubauer counting chamber by diluting whole blood by adding 10 µl of blood into 90 µl of Trypan Blue stain (Sigma, St. Louis, MO). Cell purity was also determined by differential counting of lymphocytes and monocytes using May-Grunwald/Giemsa stain (Sigma, St. Louis, MO). In all experiments, the mean lymphocyte purity was determined to be 91 ± 5%.

2. 3. 3 Preparation of LAK Cells

Non-adherent lymphocytes derived from 50 ml of blood were further divided into two aliquots containing equal number of cells when experiments were planned to include LAK cells. The cells were centrifuged, the pellets resuspended, and counted to 1 x 10^6 cells/ml in either RPMI 1640 with 10% FBS (control cells) or RPMI 1640 with 10% FBS, supplemented with IL-2 (2.5 ng/ml final concentration; R&D Systems, Minneapolis, MN) to make LAK cells. Lymphocytes (both aliquots) were incubated at 37ºC upright in 25cm^2 tissue culture flask for approximately 72 hours. After this time, the cell suspensions were decanted into universal containers, washed by centrifugation at 200 x g two times in RPMI 1640 to remove any residual IL-2, and a cell count

and viability assessment performed prior to their inclusion in experiments.

2. 3 .4 Assessment of Cell Numbers and Viability

Leukocyte, tumor cell, and endothelial cell number and viability were determined by the addition of 10 µl of cell suspension to 90 µL of Trypan Blue (Sigma; St. Louis, MO). The cell suspensions were placed in an improved Neubauer hemocytometer chamber. The number of cells occupying the central 20 squares was counted and divided by 10, so as to obtain the concentration of cells required (in µl) to obtain a 10^6 cells/ml final concentration in one milliliter of media.

2. 4 Immunochemistry

2. 4. 1 Immunostain: von Willebrand's Factor on HMEC-1

HMEC-1 cells were adjusted to 5 x 10^6 cells/ml in one milliliter of RPMI 1640, in sterile 5 ml falcon tubes. The specific monoclonal antibody against von Willebrand's Factor (Clone 4F9; Beckman Coulter, Miami, FL), or isotypic control (IgG1 (mouse); Clone 679.1Mc7; Beckman Coulter) was added to the cell suspension and incubated at room temperature for 30 min. Equivalent concentrations/volumes of primary antibody and isotypic control were used (5 µg/µl). Following initial incubation, tubes were centrifuged at 200 x g for 10 min to wash cells. The supernatant was discarded and subsequently 10 µl (5 µg) of secondary antibody (Oregon Green labeled, mouse-anti-goat; Beckman Coulter) was added to 1 milliliter of RPMI 1640, which was added to the HMEC-1 pellet and incubated at room temperature for 30 min. The cells were again washed via centrifugation at 200 x g for 10 min. The supernatant was discarded and the cells were resuspended with 1 milliliter of 1% PFA in falcon tubes for 30 min, followed by an additional centrifugation step. The cell pellet was resuspended in 500 µl of RPMI 1640 and 10 µl of cell solution was transferred to standard microscope slides, mounted with Prolong Antifade

Reagent (Invitrogen, Carlsbad, CA), and sealed with 13mm x13mm pieces of cover glass.

2. 4. 2 Immunostain: CD54 (ICAM-1) on HMEC-1

HMEC-1 cells were adjusted to 5 x 10^6 cells in one ml of RPMI 1640, in sterile 5 ml falcon tubes. Ten µl of human-anti-mouse CD54 monoclonal antibody labeled with FITC conjugate (Clone 84H10; Beckman Coulter), or isotypic control (IgG1; Clone 679.1Mc7; Beckman Coulter) were added, and incubated for 30 min at room temperature. Equivalent concentrations/volumes of primary antibody and isotypic control were used (5 µg/µl). Following incubation, tubes were centrifuged at 200 x g for 10 min. The supernatant was discarded and the cells resuspended and fixed with 1 milliliter of 1% PFA in falcon tubes for 30 min, followed immediately by an additional centrifugation step. Fixed cells were then resuspended in 500 µl of RPMI 1640. Ten µl of cell solution was transferred to standard microscope slides, mounted with Prolong Antifade Reagent, and sealed with 13 mm x13 mm pieces of cover glass.

2. 4. 3 Immunostain: CD25 on Lymphocytes and LAK Cells

Lymphocytes/LAK cells were adjusted to 5 x 10^6 cells/ml in RPMI 1640, in 5 ml falcon tubes. Ten µl of CD25 specific monoclonal antibody (Clone 2A3; BD Biosciences, San Jose, CA) or isotypic control (IgG1; Beckman Coulter) was added to the cell suspension, and allowed to incubate for 30 min at room temperature. Equivalent concentrations/volumes of primary antibody and control were used (5 µg/µl). Following incubation, tubes were centrifuged at 200 x g for 10 min. The supernatant was discarded and subsequently 0.5 µL of secondary antibody (Oregon Green labeled, mouse-anti-human; Beckman Coulter) was added to 1 milliliter of RPMI 1640, which was added to the pellet and incubated at room temperature for 30 min. The cells were again washed via centrifugation at 200 x g for 10 min. The supernatant was discarded and

the cells were resuspended in 500 µL of RPMI 1640. Cells were then fixed with 1 milliliter of 1% PFA in falcon tubes for 30 min, followed immediately by an additional centrifugation. The pellet was resuspended in RPMI 1640 and 10 µL of cell solution was then transferred to standard microscope slides, mounted with Prolong Antifade Reagent, and sealed with 13mm x13mm pieces of cover glass.

2. 5 Preparation of SFn and Its Components, Fluorescent Probes, and Peptides

2. 5. 1 Preparation of Fibrinogen

Human, plasma derived fibrinogen (plasminogen-, fibronectin-, and factor XIII free) was obtained from American Diagnostica Inc. (Greenwich, Connecticut). The lyophilized stock amount (5 mg) was reconstituted in 2 ml of sterile deionized H_2O, and allowed to sit at room temperature for approximately 10 min while gently mixing solution to fully dissolve the reagent. Aliquots (25, 50, and 100 µl) of stock fibrinogen were stored in cryo-safe tubes and kept at -80ºC until time of experiment.

2. 5. 2 Preparation of Gly-Pro-Arg-Pro

The fibrin polymerization inhibitor Gly-Pro-Arg-Pro-amide (24 mM; GPRP-NH2) was obtained from Sigma Chemical Company (St. Louis, MO). The lyophilized stock amount (5 mg) was reconstituted in 2 ml of sterile, deionized H_2O, and allowed to sit at room temperature for approximately 10 min while gently mixing solution to fully dissolve the reagent. Aliquots (42 and 84 µl) of stock GPRP were stored in cryosafe tubes and kept at -80ºC until time of experiment.

2. 5. 3 Preparation of Thrombin

Thrombin (100 U/ml) was obtained from Sigma Chemical Company. The lyophilized stock amount (5 mg) was reconstituted in 2 ml of sterile, deionized H_2O, allowed to sit at room temperature for approximately 10 min while gently mixing solution to fully dissolve the reagent. Aliquots (5 and 10 µl) of stock thrombin were stored in cryo-safe vials and kept at -80º until time of experiment.

2. 5. 4 Preparation of Soluble Fibrin

SFn is made in the required amount for each experiment. All component reagents were briefly thawed to room temperature. To make one milliliter of sFn solution, 50 µl fibrinogen (0.5 mg/ml), 84 µl (4 mM) of GPRP-NH2, followed by 1.25 µl of thrombin were added to 865 µl of RPMI 1640 in a sterile, 5 ml falcon tube. This solution was allowed to sit at room temperature for 30 min to form sFn.

2. 5. 5 Preparation of Fluorescent Probes

Prepackaged, lyophilized probes, 1,1'-dioctadecyl-3,3,3',3'-tetra-methylindocarbocyanine perchlorate, or DiI (fluoresces red) and Calcein AM (fluoresces green), were obtained from Invitrogen (Eugene, OR). Each probe was reconstituted in 50 µl of sterile DMSO (Sigma) according to manufacturer recommendations (Akeson, 1993; Gant, 1992).

2. 5. 6 Preparation of Stock Peptides

Lyophilized peptides (50% purity) were obtained from Sigma-Genoysys (The Woodlands, TX). Reconstitution was performed according to the manufacturer recommendations for each. Peptides 1, 2, and 4 (designated in previous sections) were each made up to a 100 µM stock

concentration in sterile PBS. Peptide 3 was first solubilized in a small amount of DMSO (on account of its hydrophobicity), followed by subsequent suspension in PBS, and was made up to a 100 μM stock concentration. All peptides were divided into 50 μl aliquots, and stored at -20°C.

2. 6 Oregon Green Labeling of Effector Cells

EC, lymphocytes, and LAK cells were labeled with fluorescently labeled sFn and viewed microscopically.

2. 6. 1 Preparation of Oregon Green Labeled SFn

Oregon Green labeled fibrinogen was obtained from Molecular Probes (Oregon Green 488 human fibrinogen conjugate). Lyophilized stock fibrinogen (5 mg) was made up in RPMI 1640 to a total volume of 5 ml, aliquotted into cryovials (25 μl each), and stored at -20°C. At time of experiment, fluorescent sFn monomer (one milliliter) was made by thawing and adding 50 μl of Oregon Green labeled fibrinogen, 84 μl of the fibrin polymerization inhibitor GPRP (24 mM), followed by 1.25 μl thrombin (100 U/ml) to 865 μl of RPMI 1640 in a 5 ml falcon tube. This solution was incubated at room temperature for 30 min prior to use in assay.

2. 6. 2 Preparation of Effector Cells

Lymphocytes, LAK cells, and EC were all cultured and isolated as discussed in previous sections, counted to 5.0 x 10^6 cells per ml, and centrifuged at 200 x g for 10 min to obtain pellets. One ml of prepared, fluorescently labeled sFn was added to each cell type, and allowed to incubate for 30 min in a 37ºC, 5% CO_2 incubator. Ten μL of each cell type was added to a microscope slide, covered with Prolong Antifade Reagent, and sealed with a 13 x 13 mm coverslip for microscopy. Remaining cell suspensions were again centrifuged at 200 x g for 10 min, supernatants were

discarded, and 500 µl of RPMI 1640 added to each. Slides were again prepared as previously described.

2. 6. 3 Fluorescence Microscopy

An Olympus BX61 was used (100× oil immersion objective) to observe samples and Applied Spectral Imaging Software (Vista, CA) was used to capture images. Using the same microscope settings to observe differences in fluorescence between samples, five representative fields were captured of each slide for each pretreatment. Images were processed using Image Pro Plus™, Version 5.1, (Media Cybernetics, Silver Spring, MD) and deconvolved using AutoDeblur™ deconvolution software (Media Cybernetics).

2. 7 Binding of Lymphocytes and LAK Cells to Endothelial Cells Under Flow Conditions

2. 7 1 Preparation of Endothelial Cells on 40 mm Coverslips

Round, 40 mm coverslips were purchased from Bioptechs Inc. (Butler, PA). Coverslips were sterilized using a pure grade ethanol wash. HMEC-1 cells were prepared in media as described previously and counted to 1 x 10^6 cell/ml in DMEM with 10% FBS. One ml of cells, plus 4 additional ml of DMEM with 10% FBS was added to each sterile coverslip, in small Petri dishes. Coverslips were incubated in a 37ºC, 5% CO_2 incubator, for 24-48 hours, or until confluent. Confluence was checked using an inverted, phase-contrast Zeiss microscope.

2. 7. 2 Preparation of Fluorescently Labeled Lymphocytes

Lymphocytes and LAK cells were isolated as described in previous sections. Lymphocytes were labeled with 5 µl of DiI for 20 min at room temperature, centrifuged at 200 x g for 10 min to remove excess probe, and the supernatant decanted from the labeled cells.

2. 7. 3 Pretreatment of Effector Cells

Fluorescently labeled lymphocytes were suspended in 5 ml falcon tubes, (1 x 10^6 cells/ml each in RPMI 1640 with 10% FBS), and confluent HMEC-1 cells grown on coverslips were removed from incubators for experimentation. Cells were pretreated with sFn, peptides, or both as described below.

2. 7. 3. 1 Pretreatment of Effector Cells with SFn (and Components)

RPMI 1640 with 10% FBS and DMEM with 10% FBS were removed from lymphocytes (by centrifugation) and EC (by manual pipet), respectively. One milliliter of sFn solution, Fg, GPRP, or Thr was added to lymphocytes in tubes, and/or to EC on coverslips. All cells were incubated at 37ºC, with 5% CO_2 for 30 min prior to inclusion in assays.

2. 7. 3. 2 Pretreatment of Effector Cells with Peptides Prior to SFn

RPMI 1640 with 10% FBS and DMEM with 10% FBS were removed from lymphocytes (by centrifugation) and EC (by manual pipet), respectively. Cells were reconstituted in 1 ml peptide solutions; EC with P1 and P3 (4 mM) and lymphocytes with P2 and P4 (4 mM) for 30 min, followed by treatment with sFn for another 30 min, and incubated at 37ºC, with 5% CO_2.

2. 7. 4 Assembly of FCS2 Closed Perfusion System

Figure 2-1 above shows a detailed setup of the FCS2 apparatus. Coverslips with a confluent HMEC-1 cells were loaded into the FCS2 stage incubator (Bioptechs Inc.) and mounted on the stage of a Leica DMIRB inverted fluorescence microscope equipped with a Hamamatsu Color Chilled 3CCD camera (C5810 model) attached to a computer for data acquisition. A fluid

pathway was formed (Dimensions 0.5 mm × 14 mm × 25 mm) by separating the microaqueduct slide from the coverslip containing cells with a single silicone gasket with a rectangular bore, generating near-laminar flow conditions during perfusion. The stage incubator was initially connected to a peristaltic pump on the afferent side using 0.062 inch bore S/P medical grade silicone tubing (Fisher Scientific, Suwanee, GA). The efferent side was connected to waste initially. After connection of the electronic temperature control, the coverslips were perfused with RPMI 1640 to briefly buffer the cells at 37°C for ten min. The flow rate was set to 0.5 ml/min, which corresponds to a shear rate of 132.9 s^{-1}, which falls between the reported range as earlier discussed. The inlet was then connected to the tube containing the appropriately treated lymphocyte suspension (3 ml total). The efferent tubing was also transferred back to the same tube, and Di-I labeled lymphocytes were recirculated across the EC for 1 h, after which, the tubing was set up to the initial configuration and the cells were again washed with RPMI for 10 more min to remove unbound lymphocytes.

2. 7. 5 Image Capture and Quantitative Analysis

Individual still images in five randomly chosen fields of view were captured at 10 x magnification and stored on the computer. Image Pro Plus 5.1 software was used to quantitate the number of lymphocytes (labeled red) that were bound in each of the captured images. Software automatically selected for all fluorescent pixels present in each image, which separated the fluorescent lymphocytes from the black background. The counts for each treatment were manually added to a running spreadsheet (Microsoft Excel) for all experiments.

2. 8 Endothelial Transmigration Assay

2. 8. 1 Preparation of Polycarbonate Inserts

Corning Polycarbonate Transwell Permeable Supports (8 μm pore size; 6.5 mm insert diameter; 0.33 cm^2 growth area; 10 μm membrane thickness) were prepared in sterile, flat-bottomed 24-well microtiter plates (Fisher Scientific). HMEC-1 cells were detached from tissue culture flasks as previously described and counted to 1 x 10^6 cells/ml. 200 μl of this cell suspension (containing 2 x10^5 cells) were added to the top well of the inserts and grown for approximately 48 hours at 37ºC with 5% CO_2 conditions.

After cells were grown to probable confluence (approximately 48 h), a single representative insert was labeled with 2 μl of stock fluorescent probe Calcein AM for 20 min at room temperature. After incubation, the insert was gently washed with a sterile pipette three times with fresh DMEM media to remove excess probe, and fluorescence microscopy was performed, using an inverted Leica DMIRBE, with a 10x objective, to determine whether culture had grown to approximately 90%+ confluence for continuance of assay (using 488nm excitation; 535nm emission). Figure 2-3 is a representative image of a fluorescently labeled insert.

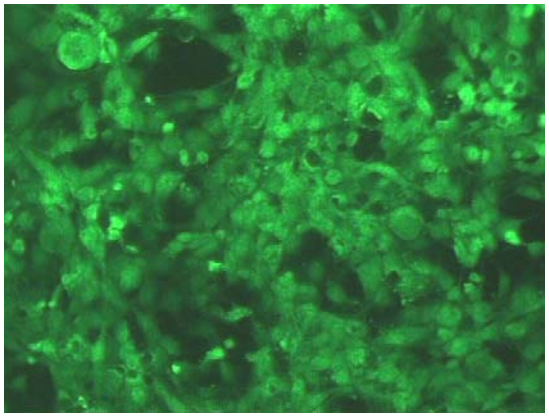


Figure 2-3 Calcein AM labeled HMEC-1 endothelial cells grown on polycarbonate inserts.

2. 8. 2 Preparation of Tumor Cells in 24-well Plates

The cell line A375 (1 x 10^5 cells/well) was grown on the bottom of 24-well tissue culture-treated microplates at 37ºC with 5% CO_2 conditions for approximately 24 hours, or until confluence.

2. 8. 3 Preparation of Tumor Cell Conditioned Media (TCCM)

A375 cell cultures were grown to approximately 75-80% confluence, media aspirated, cells rinsed with 5 ml of PBS, and incubated in 10 ml of fresh, serum free, RPMI 1640 for approximately 24 hours at 37°C/5% CO_2. After 24 hours, the media were collected and subjected to centrifugation (200 x g, 10 min) to remove debris, concentrated, and transferred to sterile, 15 ml conical tubes until time of experiment.

2. 8. 4 Preparation of eACA

6-Aminohexanoic acid (eACA) is a lysine analog that promotes rapid dissociation of plasmin from cells, which inhibits the activation of plasminogen and subsequent fibrinolysis. Furthermore, EACA has been used to inhibit fibrin degradation by cells. Therefore, this reagent was used during some experiments along with sFn in order to study its effect on diapedesis over an 18 hour incubation period.

Stock concentration of eACA (Sigma) was made up to 400 mM in RPMI 1640. 50 µL of stock solution was added to 1ml of sFn total solution, for a working concentration of 20 mM.

2. 8. 5 Preparation of Effector Cells for Transmigration Assays

Lymphocytes and LAK cells were isolated and labeled, and EC were isolated and seeded on inserts as previously described in previous sections. Cells were then pretreated with sFn (and components), peptides, or both as described below.

2. 8. 6 Pretreatment with Soluble Fibrin and Components

RPMI 1640 with 10% FBS and DMEM with 10% FBS were removed from lymphocytes and LAK cells (by centrifugation at 200 x g) and EC (by manual pipet), respectively. SFn was prepared as described earlier, and the 1 milliliter solutions of sFn, Fg, GPRP, or Thr was added to lymphocytes in 5 ml falcon tubes, and/or to EC on coverslips. All cells were incubated at 37ºC, with 5% CO_2 for 30 min prior to inclusion in assays.

2. 8. 7 Pretreatment with Peptides Prior to Soluble Fibrin

RPMI 1640 with 10% FBS and DMEM with 10% FBS were removed from lymphocytes and LAK cells (by centrifugation at 200 x g) and EC (by manual pipet), respectively. Cells were then reconstituted in one ml of peptide solution (40 µl of each peptide in pair with 920 µl RPMI;4 mM concentration each). EC were pretreated with P1 and P3 and lymphocytes with P2 and P4 for 30 min, followed by treatment with sFn (one milliliter total) for another 30 min, and incubated at 37ºC, with 5% CO_2.

2. 8. 8 Transmigration Assay Setup

Having completed all experimental preparation as described in the previous sections, the assay could be assembled as described below. The 24-well plate (containing the pretreated, confluent endothelial monolayers grown on inserts), was removed from incubation, and the excess DMEM present in the inserts was gently extracted using a 200 µl pipette and sterile tips. Inserts were gently washed with DMEM using the same method. Two hundred µl (containing 2.0 x 10^5) of lymphocytes or LAK cells (either untreated, or pretreated in sFn, Fg, GPRP, Thr, or peptides) were added to the top of the appropriate pretreated insert. Another 24-well plate was prepared to contain 600 µl of tumor cell conditioned media (TCCM) (or A375 cells in media) in the

appropriate wells (to match the number of inserts being tested). The inserts were then transferred from the original microplate to the new one with the media. All appropriate controls, including a total (maximal lymphocyte migration; 2 x 10^5 cells), TCCM, and RPMI 1640 media controls were also performed. Assays were incubated overnight at 37ºC, with 5% $C0_2$ for approximately 18 hours.

2. 8. 9 Transmigration Assay Quantitation

Following 18 hours of incubation, all inserts were gently removed (as to avoid any spilling from the insert into the well below) from the 24-well microplate. The remaining TCCM solution, containing the fluorescently labeled lymphocytes/LAK cells that have migrated through the endothelial cell monolayer/polycarbonate membrane was measured by a Perkin Elmer Victor 3 microplate reader equipped for fluorescence. The plate reader was set up to excite with a filter specific for excitation at 535 nm, and to emit with a 620 nm filter), to detect the DiI. The excitation lamp was set to 40000, the aperture to normal width, and the capture time lapse to 1 second.

2. 9 Statistical Analysis Methods

2. 9. 1 Statistical Analysis of Adherence Assays

On a given day, I evaluated between 2 and 5 treatment conditions. For each treatment condition performed, I obtained 5 fluorescent counts, taken from 5 randomly selected microscopy fields (using Image Pro Plus software to quantitate), and averaged these into a single fluorescence data point for that day. All data were stored on a running spreadsheet (in Microsoft Excel) until completion of all experiments.

Using JMP statistical software package (Cary, NC), paired sample t-tests (α=0.05) were performed on all relevant treatment combinations. This test looks at differences between two treatments each day the pair of treatments was performed (n = 3-15 days; depending on the pair). Two-sided student's t-tests were performed on all pairs because of uncertain expectations of results. Student's t-tests provide some useful information about these studies. However, their application is somewhat limited here, because of the low number of degrees of freedom (DF) for certain treatment pairs. In order to overcome this low DF, and provide additional analyses of each experimental setup, models were fit using JMP statistical software.

If we are willing to assume error variances are equal for the treatments, then a model can be fit to include additional data from other days of experimentation. Table 2.1 (A) is a representative data set that was modeled in JMP to calculate Least Square Means (LSM's), which estimates new averages (LSM's) assuming each treatment appeared on all dates. The LSM values for those treatments that have been performed all days (+/+ and -/-) will be the same as the standard means that were calculated for these same treatment (highlighted in blue; Table 2-1 A,B). However, the means of those treatments that were only performed on some days (+/- and -/+) were adjusted (in either an upward or downward direction) to project an LSM value for these treatments (highlighted in red; Table 2-1 (B)). The amount and direction in which these values are adjusted is dependent upon the original measurements obtained.

Also using JMP, Tukey's multiple comparisons tests were performed on all models (on LSM's) to control the overall risk for Type 1 errors, i.e. to control the risk of falsely declaring differences between treatments. This test also identifies significant differences between treatments, but does not always correspond with original t-tests results, due to the inclusion of different values.

Table 2-1 Computation of Least Square Means for models

A) Representative data set for fitting Least Square Means

	(-/-)	(+/+)	(-/+)	(+/-)
2/8/2007	3519	1131	.	.
2/15/2007	3453	1841	.	2382
2/18/2007	3497	1428	.	2508
2/22/2007	3811	2001	3050	.
2/24/2007	4658	1867	3038	.
2/27/2007	4094	2043	.	.
2/28/2007	4833	1564	.	.
3/1/2007	5171	1465	.	.
3/2/2007	3586	1433	.	.
3/6/2007	4359	2779	.	.
3/8/2007	3889	1470	.	.
3/10/2007	3002	1272	.	.
3/28/2007	3220	1733	.	.
3/30/2007	3678	2214	2374	2578
4/2/2007	3192	1938	.	.
Mean	3864.13	1745.27	2820.67	2489.33
St Dev	639.36	421.76	386.87	99.32

B) Representative Least Square Means table

Level	Least Sq Mean	Std Error	Mean
Mean (-/-)	3864.13	115.95	3864.13
Mean (-/+)	2597.91	302.47	2820.67
Mean (+/-)	2645.79	302.47	2489.33
Mean (+/+)	1745.27	115.95	1745.27

2. 9. 2 Statistical Analysis of Lymphocyte Transendothelial Migration Assays

On a given day, I evaluated between 4 and 8 different treatment conditions. I obtained one data point from each insert prepared, in arbitrary fluorescence units provided by plate reader software (depending on set parameters). When multiple inserts (2) of the same treatment were performed in a day (all experiments except optimization assays), individual data points of the same treatment were then combined (added) to obtain a mean and standard deviation of each treatment for that day. A total fluorescence measurement and a tissue culture conditioned media (TCCM) control were also performed each day. The mean of the TCCM measurement for each day was subtracted from all other treatments (to control out "background" from each measurement).

Using JMP software, matched pair student's t-tests (one or two-sided) were performed on appropriate pairs to determine any significant differences of fluorescence (migration) between treatments.

Similar statistical methods were employed as previously described for the binding section above. If we are willing to assume error variances are equal for the treatments, then a model can be fit to include additional data from other days of treatment. LSMs were computed assuming all treatments appeared on all dates. Also using JMP, Tukey's multiple comparisons tests were performed on all models to control the overall risk for Type 1 errors.

CHAPTER 3. RESULTS

3. 1 Cellular Immunostaining

3. 1. 1. von Willebrand's Factor on HMEC-1 Cells

HMEC-1 cells were immunostained for human factor VIII/V or von Willebrand's Factor (using specific monoclonal antibody directed against vWF, and a fluorescently labeled secondary antibody) showed significant expression of this cellular molecule (Figure 3-1) compared to the purified immunoglobulin matched isotypic control (IgG1). Furthermore, nonlabeled cells and a secondary antibody control were also performed (indicating no autofluorescence, and almost no nonspecific binding of secondary antibody, respectively). This signifies that HMEC-1 cells contain the storage organelles (Weibel-Palade Bodies), which contain key components of both inflammatory and haemostatic processes (vWF secretion).

3. 1. 2 CD54 on HMEC-1 Cells

HMEC-1 cells immunostained for CD54 showed significant expression of this cellular receptor (Figure 3-2) compared to the purified immunoglobulin matched isotypic control (IgG1) indicating almost no nonspecific labeling. This corresponds with reports that this particular cell line (which is representative of many vascular EC *in vivo*) that express this receptor.

3. 1. 3 CD25 on Lymphocytes and LAK Cells

Lymphocytes and LAK cells immunostained for CD25, both showed increased expression of this cellular molecule (Figure 3-4) compared to its purified immunoglobulin matched isotypic control (IgG1). However, as expected, LAK cell signal fluorescence (or fluorescence intensity) was higher than that of untreated lymphocytes.

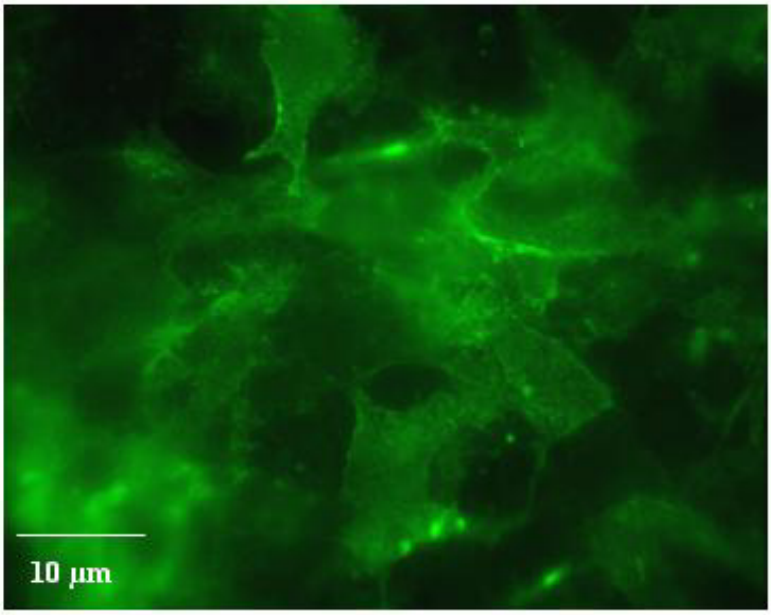


Figure 3-1. vWF expression on HMEC-1 endothelial cells.

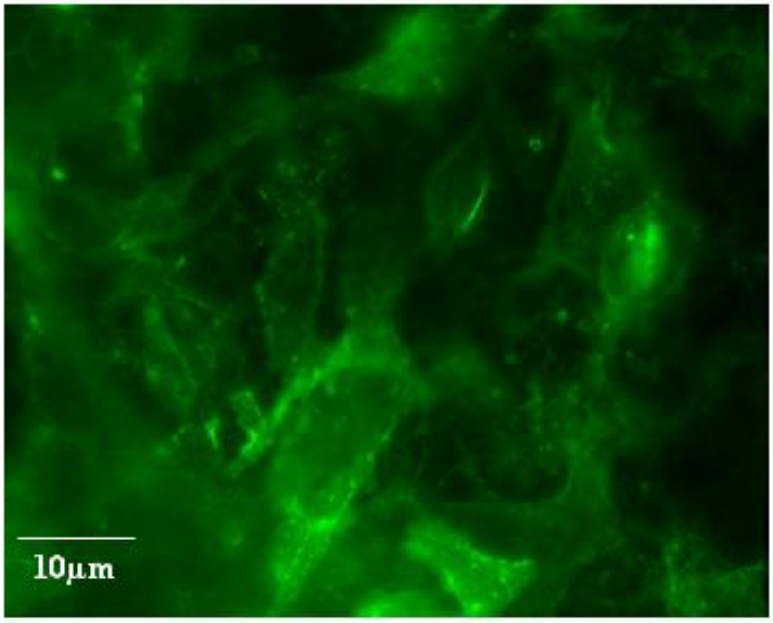


Figure 3-2. CD54 expression on HMEC-1 endothelial cells.

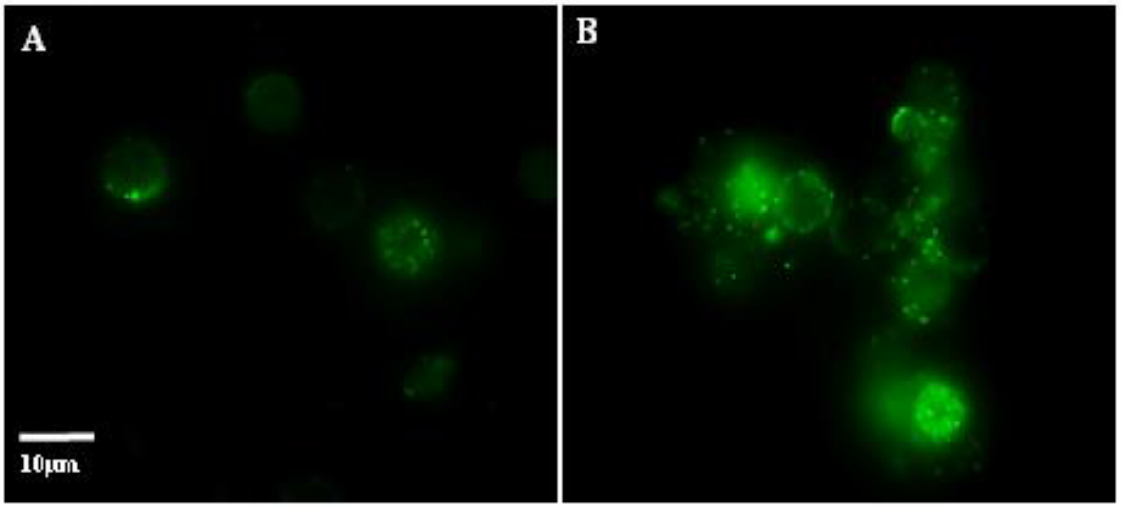


Figure 3-3 CD25 expression on lymphocytes (A) and LAK cells (B)

3. 2 Oregon Green Labeled SFn Pretreatment of LAK and Endothelial Cells

LAK cells and EC were incubated alone or in the presence of fluorescently labeled sFn. Figure 3-4 shows considerable binding of sFn to both LAK cells (A) and EC (B.), whereas no fluorescence was observed on cells incubated in the absence of sFn.

3. 3 Lymphocyte and LAK Cell Adherence to Endothelial Cells Under Flow Conditions

To simulate the fluid shear stresses present *in vivo*, lymphocytes or LAK cells were perfused across coverslips containing adherent HMEC-1 cells that were loaded into the FCS2 stage incubator, and mounted on the stage of an inverted fluorescence microscope equipped with a camera attached to a computer for data acquisition. Figure 3-5 is a representative image of fluorescently labeled lymphocytes that adhered to EC which was analyzed for quantitation.

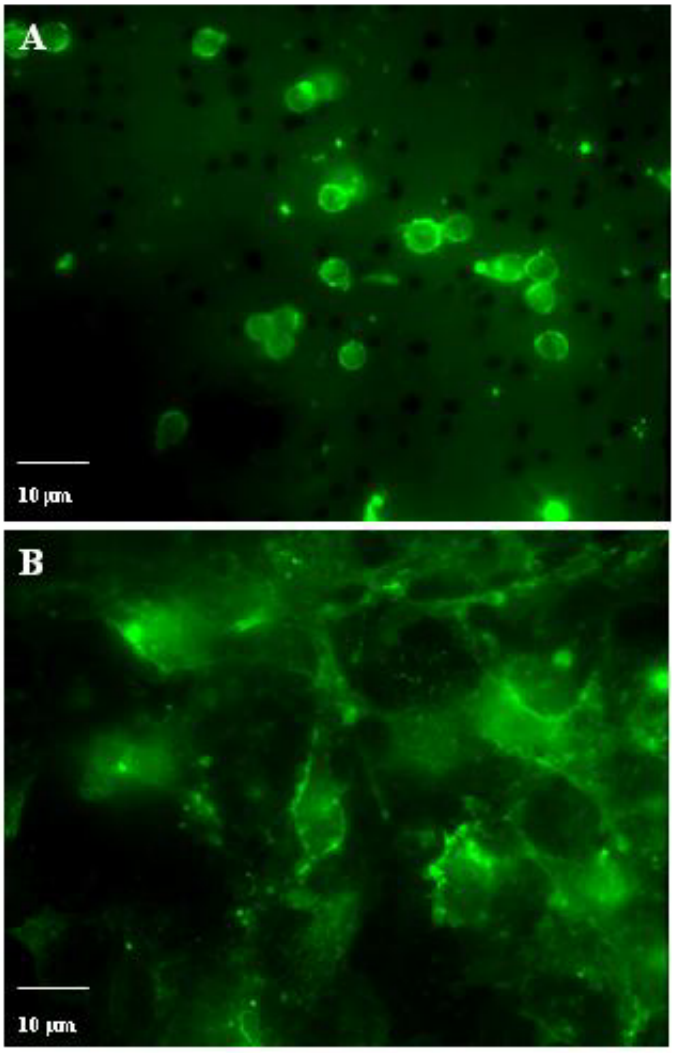


Figure 3.4 Oregon Green labeled sFn binding to LAK cells (A) and endothelial cells (B).

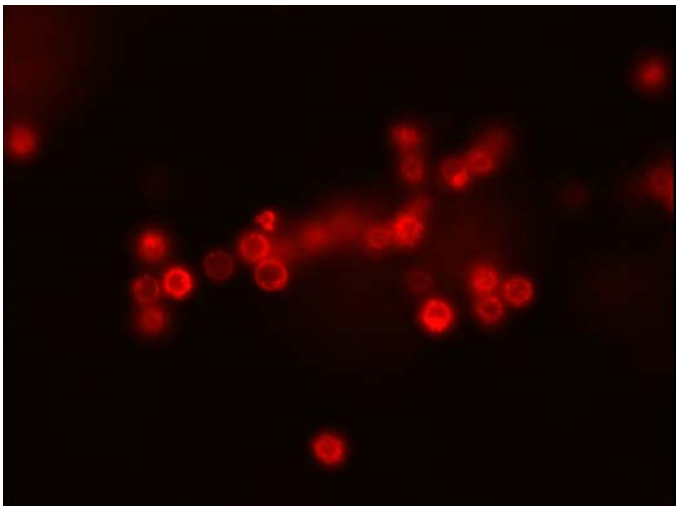


Figure 3-5 DiI labeled lymphocytes adherent to EC for fluorescence quantitation by Image Pro Plus software

3. 3. 1 Lymphocyte Adherence to Endothelial Cells in the Presence of Soluble Fibrin

SFn pretreatment of one or both cell types (EC/lymphocytes, respectively) significantly affected the binding events observed under flow conditions. Figure 3-6 shows the mean fluorescence units measured for each treatment (as determined by IPP software). Means for each day are listed in Table A-1 in the appendix. Untreated cells of each type (-/-) were used as a control value of the maximum number of fluorescence units measured for each experiment. Each other pretreatment measurement was then tested against the untreated control value in order to calculate percent inhibition of adherence. Furthermore, standard deviations for each pretreatment were further calculated including only those days that the treatment of interest was performed. Pretreatment of both cell types with sFn (+/+), resulted in the fewest number of bound lymphocytes to EC (or least fluorescence observed), resulting in a mean ± standard deviation of 54.1 ± 11.3% inhibition when compared to the control (n=15). Pretreatment of only EC (+/-) resulted in a 29.7 ± 1.4% decrease of binding events (n=3). Similarly, pretreatment of lymphocytes only (-/+) resulted in a 30.1 ± 8.8 % decrease of fluorescence (n=3).

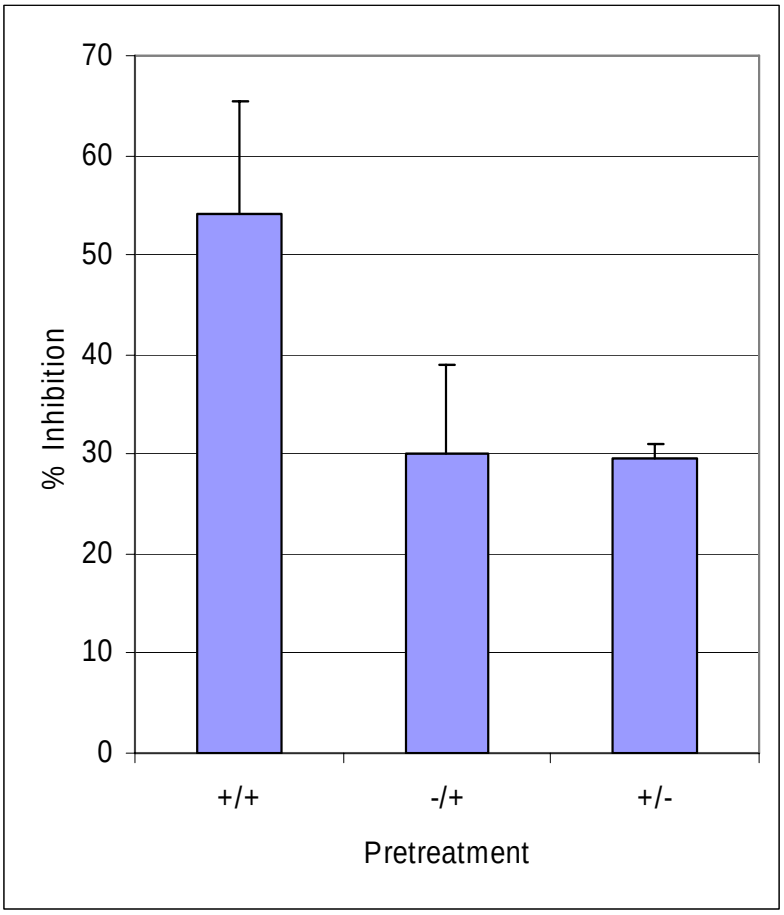


Figure 3-6. Effect of sFn pretreatment on lymphocyte adherence to endothelial cells (endothelial cells and lymphocytes, respectively) under flow conditions. All measurements are presented as percent inhibition compared to untreated cells. Values were produced by quantitation of adherent, fluorescently labeled lymphocytes to endothelial cells using Image Pro Plus software package.

3. 3. 2 Statistical Analysis: Lymphocyte Binding to Endothelial Cells in the Presence of Soluble Fibrin

Using JMP, matched pair student t-tests (α=0.05; Table A-2) were performed on the original data for the four possible pretreatment combinations of sFn (-/-, +/+, -/+, +/-). When neither cell type was pretreated with sFn (-/-), the mean fluorescence measured (binding events) was significantly larger than all other treatment means (+/+, +/-, and -/+). Furthermore, there were no additional significant differences found between any of the other treatment combinations.

Also using JMP, an additive model, including terms for date (15 days) and treatment (4 levels), was fit using Standard Least Squares. I obtained an R^2 value of 0.91 (Table 3-1; Part A), with treatment differences explaining the vast majority of the variation of fluorescence measurements. The Root Mean Square Error of 449.1 estimates the assumed standard deviation. The mean of response, and total observations are also listed.

Furthermore, the analysis of variance (ANOVA) (Table 3-1; Part B) provided an F ratio of 11.3324 ($P < .0001$) indicating a significant model. The model accounts for approximately 91% of variation (from R^2 value), whereas the unexplained variation accounts for the other 9%.

The residuals were also examined, and they were consistent with our assumption of a common variance. There are no "extreme outliers" that need additional attention for this model (Table 3-1; Part C).

Table 3-1. Statistical analysis: lymphocyte adherence to endothelial cells in the presence of soluble fibrin.

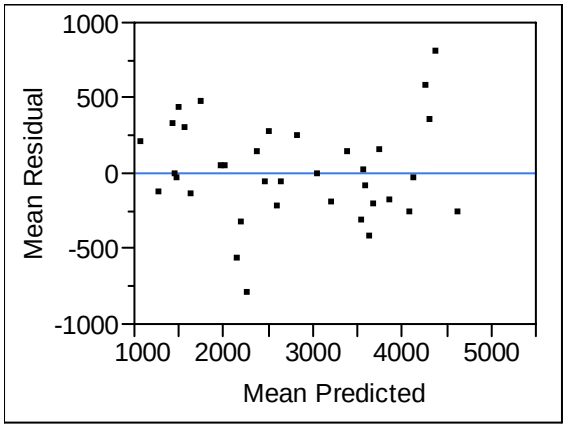
A) Summary of Fit

RSquare	0.914551
Root Mean Square Error	449.061
Mean of Response	2779.744
Observations (or Sum Wgts)	36

B) Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	17	38849167	2285245	11.3324
Error	18	3629805	201656	Prob > F
C. Total	35	42478971		<.0001

C) Residual by Predicted Plot



D) Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Date	14	14	4903330	1.7368	0.1345
Treatment	3	3	33794276	55.8613	<.0001

E) Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
Mean (-/-)	3864.0933	115.94706	3864.09
Mean (-/+)	2597.9093	302.46841	2820.60
Mean (+/-)	2645.7950	302.46841	2489.27
Mean (+/+)	1745.3200	115.94706	1745.32

The Effect Tests Table (Table 3-1; Part D) indicates that the date (DF=14) of the experiment was found to be an insignificant factor (P=0.1345; F Ratio=1.7638). However, pretreatment (DF=3) was found to be a significant factor (P < .0001; F Ratio=55.86).

Table 3-1, Part E is the Least Square Means table that was generated using this model. Furthermore, the specific LSM value is included for each, followed by the computed standard error and the simple mean computation. Note that both the LSM and mean are the same for both treatments (-/- and +/+) that were performed all days. However, the LSM value for (-/+) has been adjusted in a downward (negative) direction, and the LSM for (+/-) has been adjusted in an upward (positive) direction. This is due to fact that those days that (-/+) and (+/-) were tested, the (-/-) and (+/+) pretreatment measurements tended to be higher and lower (respectively) than other days.

Using the computed LSM values, Tukey's multiple comparison method was performed. Table 3-2 includes the specific details for each sFn pretreatment, including the lower and upper bounds (confidence intervals) with 95% certainty (CL Dif). Those values that are in red correspond to significant differences in the model.

Using this method, it was again shown that when neither cell was pretreated (-/-), the average fluorescence measured was significantly different than all other pretreatments involving sFn. However, when both cell types were treated with sFn (+/+) was not significantly different than other intermediate treatments' fluorescence. Furthermore, the intermediate treatments' measurements were not significantly different than one another. Table 3-2 (B) is a summary of these results.

T**able 3-2.** LSM differences Tukey's multiple comparison method: Lymphocyte adherence to endothelial cells in the presence of soluble fibrin. Those columns in red are significant.

A) LSMeans differences Tukey's table. α=0.050; Q=2.82629.

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	**Mean (-/-)**	**Mean (-/+)**	**Mean (+/-)**	**Mean (+/+)**
Mean (-/-)	0 0 0 0	1266.18 323.93 350.663 2181.71	1218.3 323.93 302.777 2133.82	2118.77 163.974 1655.34 2582.21
Mean (-/+)	-1266.2 323.93 -2181.7 -350.66	0 0 0 0	-47.886 415.75 -1222.9 1127.14	852.589 323.93 -62.932 1768.11
Mean (+/-)	-1218.3 323.93 -2133.8 -302.78	47.8857 415.75 -1127.1 1222.92	0 0 0 0	900.475 323.93 -15.047 1816
Mean (+/+)	-2118.8 163.974 -2582.2 -1655.3	-852.59 323.93 -1768.1 62.9323	-900.47 323.93 -1816 15.0466	0 0 0 0

B) Tukey's summary**.** Levels not connected by same letter are significantly different.

Level			**Least Sq Mean**
Mean (-/-)	A		3864.0933
Mean (+/-)		B	2645.7950
Mean (-/+)		B	2597.9093
Mean (+/+)		B	1745.3200

3. 3. 3 Lymphocyte Adherence to Endothelial Cells in the Presence of Fibrinogen, GPRP, and Thrombin

Untreated cells of each type (-/-) were used as a control value to obtain the maximum fluorescence (binding) measured (n = 15). Figure 3-7 shows the mean fluorescence units measured for each treatment (as determined by IPP software). Pretreatment of both cell types with sFn (+/+) resulted in the fewest number of bound lymphocytes to EC, resulting in a 54.1 ± 11.3% inhibition of fluorescence when compared to the control. Cells pretreated with fibrinogen resulted in a 6.8 ± 10.30% inhibition of binding events (n = 3). Cells pretreated with GPRP resulted in a 5.1 ± 3.3% decrease of binding events (n = 3). And when both cells were pretreated with thrombin, fluorescence was only decreased by 4.0 ± 5.8% (n=3). Means for each treatment combination for each day of experimentation are presented in Table A-1 in the appendix.

3. 3. 4 Statistical Analysis: Lymphocyte Adherence to Endothelial Cells in the Presence of Fibrinogen, GPRP, and Thrombin

Using JMP, matched pair student t-tests ($\alpha = 0.05$) were performed on the original data for appropriate pairs of the five possible pretreatment combinations (-/-, +/+, Fg/Fg, GPRP/GPRP, Thr/Thr). Individual tests for all appropriate tests are listed in Table A- 2 in the appendix. When both cell types were pretreated with sFn (+/+), the mean fluorescence measured was significantly different than all other treatments performed (-/-, Fg,Fg, Thr/Thr, GPRP/GPRP). Furthermore, there were no additional significant differences found between any of the other treatment combinations (Table A-2).

Also using JMP, an additive model, including terms for date (15 days) and treatment (5 levels), was fit using Standard Least Squares. I obtained an R^2 value of 0.92 (Table 3-3 (A)), with

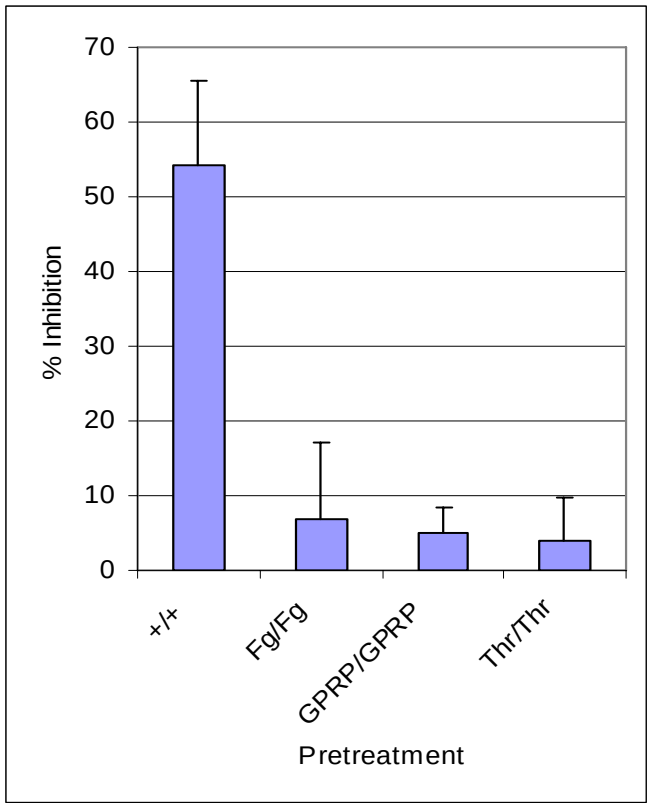


Figure 3-7. Effect of soluble fibrin components (fibrinogen, GPRP, and thrombin individually) on lymphocyte adherence to endothelial cells under flow conditions. All measurements are presented as percent inhibition compared to untreated cells. Values were produced by quantitation of adherent, fluorescently labeled lymphocytes to endothelial cells using Image Pro Plus software package.

Table 3-3. Statistical analysis: lymphocyte adherence to endothelial cells in the presence of fibrinogen, GPRP, and thrombin.

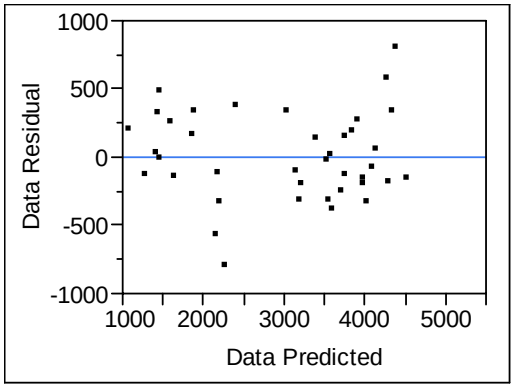
A) Summary of Fit

RSquare	0.920619
Root Mean Square Error	440.6393
Mean of Response	3003.533
Observations (or Sum Wgts)	39

B) Analysis of Variance

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	18	45035802	2501989	12.8860
Error	20	3883260	194163	**Prob > F**
C. Total	38	48919062		<.0001

C) Residual by Predicted Plot



D) Effect Tests

Source	**Nparm**	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Date	14	14	6184911	2.2753	0.0455
Label	4	4	35002211	45.0681	<.0001

E) Least Squares Means Table

Level	**Least Sq Mean**	**Std Error**	**Mean**
Mean(Fg/Fg)	3312.8067	301.01394	3575.67
Mean(GPRP/GPRP)	3417.7400	301.01394	3680.60
Mean(-/-)	3864.0933	113.77258	3864.09
Mean(+/+)	1745.3200	113.77258	1745.32
Mean(Thr/Thr)	3479.7400	301.01394	3742.60

treatment differences explaining the majority of the variation of fluorescence. The Root Mean Square Error of 440.6 estimates the assumed common standard deviation. The mean response and total observations (39) are also listed.

Furthermore, the ANOVA (Table 3-3 (B)) provided an F ratio of 12.89 ($P < .0001$), indicating a significant model. This model accounts for approximately 92% of variation (from R^2 value), whereas the unexplained variation accounts for the remaining 8%.

The residuals for the model were also examined, and they were consistent with the assumption of a common variance. There are no "extreme outliers" that need additional attention for this model (Table 3-3 (C)).

The Effect Tests Table (Table 3-3 (D)) indicates that the date (DF=14) of the experiment was found to be a significant factor (P=0.046; F Ratio=2.28). This could be due to the fact that different aliquots of reagents (blood, soluble fibrin, EC, probes, etc.) are used daily, which could influence final results. Pretreatment (DF=4) was also found to be a significant factor ($P < .0001$; F Ratio=45.07).

Table 3-3 (E) is the Least Square Means table that was generated using this model. Furthermore, the specific LSM value is included for each, followed by the computed standard error and the standard mean computation. Note that both the LSM and mean are again the same for both treatments (-/- and +/+) because they were performed all days. However, the LSM value for all other treatments (Fg/Fg, GPRP/GPRP, Thr/Thr) have all been adjusted in a downward (negative) direction. This is due to fact that those days that these treatments were tested, the fluorescence

measurements tended to be higher than for the other days.

Tukey's multiple comparison method ($\alpha = 0.05$) was then performed on the LSM values, for all pairs of the five possible pretreatment combinations (-/-, +/+, Fg/Fg, GPRP/GPRP, Thr/Thr). Table 3-4 (A) includes the specific details for each sFn component pretreatment, including the lower and upper bounds with 95% certainty (CL Dif). Those columns that are in red correspond to those values that are significant in the model. Furthermore, Table 3-4 (B) is a summary of these results. When pretreating both cell types with sFn (+/+), the average fluorescence measurement was significantly different than all other pretreatments. None of these controls' average fluorescence measurements were found to be significantly different than when neither cell type (-/-) was pretreated. Furthermore, there were no additional significant differences found between any of the other treatment combinations.

3. 3. 5 Lymphocyte Adherence to Endothelial Cells in the Presence Peptides and SFn

SFn pretreatment of one or both cell types significantly affected the binding events observed under flow conditions. Figure 3-8 represents the mean fluorescence measurements for each treatment performed. Untreated cells of each type (-/-) were used as a control value to determine maximum fluorescence measured (n=15). Pretreatment of both cell types with sFn (+/+) resulted in the fewest number of bound lymphocytes to EC (or least fluorescence observed), resulting in a 54.1 ± 11.3 % inhibition when compared to the control (n=3). EC and lymphocytes that were treated with both peptides 1 and 3 (P1/P3), prior to sFn exposure, resulted in only a 26.7 ± 4.1% inhibition of fluorescence, restoring some binding (n=3). However, EC and lymphocytes treated with peptides 2 and 4 (P2/P4), prior to sFn exposure, resulted in only a 46.1 ± 12.3% inhibition of fluorescence (n=3). Table A-1 in the appendix lists specific means for each day of experiments.

Table 3-4. LSMs differences Tukey's multiple comparison method (soluble fibrin components). Those columns in red are significant.

A) LSMeans differences Tukey's table. α=0.050; Q=2.99238.

Mean[i]-Mean[j] **Std Err Dif** **Lower CL Dif** **Upper CL Dif**	**(+/+)**	**(-/-)**	**Fg/Fg**	**GPRP/GPRP**	**Thr/Thr**
(+/+)	0	-2118.9	-1567.3	-1672.3	-1734.6
	0	160.89	321.779	321.779	321.779
	0	-2600.3	-2530.2	-2635.2	-2697.5
	0	-1637.4	-604.38	-709.38	-771.72
(-/-)	2118.87	0	551.6	446.6	384.267
	160.89	0	321.779	321.779	321.779
	1637.42	0	-411.28	-516.28	-578.62
	2600.31	0	1514.48	1409.48	1347.15
Fg/Fg	1567.27	-551.6	0	-105	-167.33
	321.779	321.779	0	359.76	359.76
	604.382	-1514.5	0	-1181.5	-1243.9
	2530.15	411.284	0	971.538	909.204
GPRP/GPRP	1672.27	-446.6	105	0	-62.333
	321.779	321.779	359.76	0	359.76
	709.382	-1409.5	-971.54	0	-1138.9
	2635.15	516.284	1181.54	0	1014.2
Thr/Thr	1734.6	-384.27	167.333	62.3333	0
	321.779	321.779	359.76	359.76	0
	771.716	-1347.2	-909.2	-1014.2	0
	2697.48	578.618	1243.87	1138.87	0

B) Summary of Tukey's table. Levels not connected by same letter are significantly different.

Level			**Least Sq Mean**
(-/-)	A		3864.0933
Thr/Thr	A		3479.8667
GPRP/GPRP	A		3417.5333
Fg/Fg	A		3312.5333
(+/+)		B	1745.3200

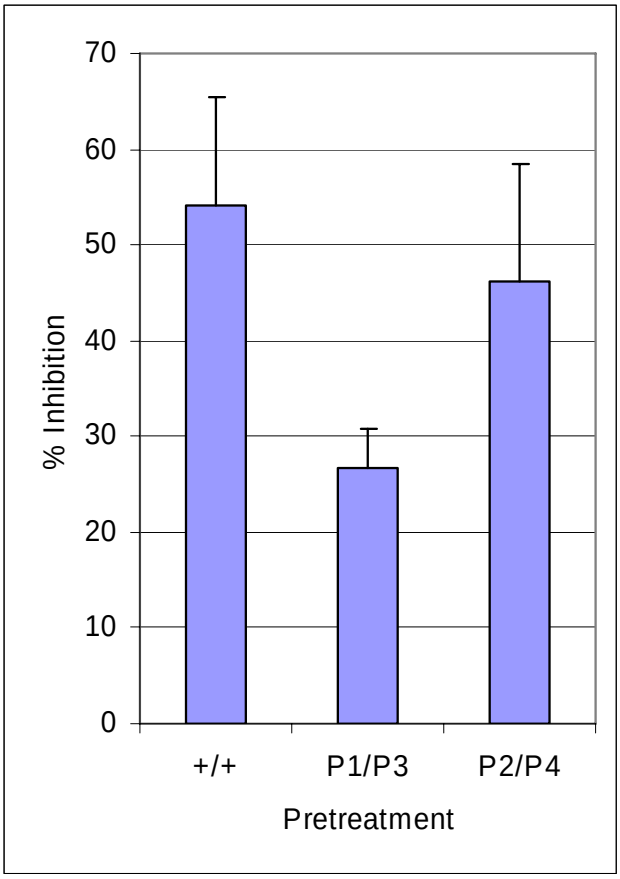


Figure 3-8. Effect of peptide and sFn pretreatment on lymphocyte adherence to endothelial cells under flow conditions. All measurements are presented as percent inhibition compared to untreated cells. Values were produced by quantitation of adherent, fluorescently labeled lymphocytes to endothelial cells using Image Pro Plus software package.

3. 3. 6 Statistical Analysis: Lymphocyte Adherence to Endothelial Cells in the Presence of Peptides and Soluble Fibrin

Using JMP, matched pair student t-tests ($\alpha = 0.05$) were performed on all possible pairs of the original data for the four pretreatment combinations (-/-, +/+, P1/P3, and P2/P4). Specific t-test values are listed in Table A- 2 in the appendix. When neither cell type was pretreated with sFn (-/-), the mean fluorescence was significantly larger than when both cells were pretreated (+/+), and larger than when cells were pretreated with peptides P2/P4 prior to sFn. However, when cells were pretreated with P1/P3, prior to sFn, the mean fluorescence was found to be significantly different than when neither cell was untreated (-/-). Pretreatment of both cells (+/+) was also significantly different than pretreatment with peptides P1/P3.

Also using JMP, an additive model, including terms for date (15 days) and treatment (4 levels), was fit using Standard Least Squares. An R^2 value of 0.92 was obtained (Table 3-5 (A)), with treatment differences explaining the majority of the variation within fluorescence. The Root Mean Square Error of 447.2 estimates the assumed common standard deviation. The mean response (fluorescence), and total observations (n=36) are also listed.

Furthermore, the ANOVA (Table 3-5; Part B) provided an F ratio of 12.60 ($P < .0001$), indicating a significant model. This model accounts for approximately 92% of variation (from R^2 value), whereas the unexplained variation accounts for the remaining 8%.

The residuals were also examined, and they were consistent with our assumption of a common variance. There are no "extreme outliers" that need additional attention for this model (Table 3-5 (C)).

Table 3-5. Statistical analysis: lymphocyte adherence to endothelial cells in the presence of peptides and soluble fibrin

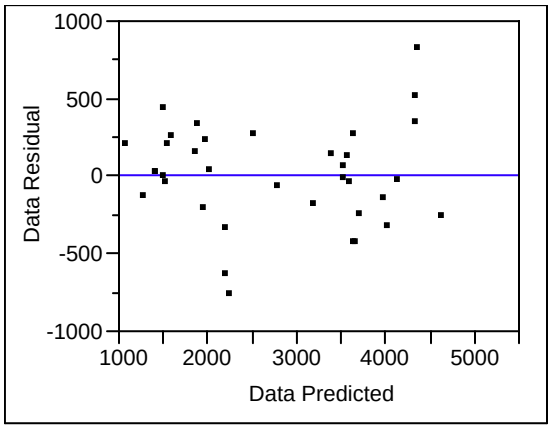
A) Summary of Fit

RSquare	0.922488
Root Mean Square Error	447.1762
Mean of Response	2762.667
Observations (or Sum Wgts)	36

B) Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	17	42837068	2519828	12.6012
Error	18	3599398	199967	Prob > F
C. Total	35	46436466		<.0001

C) Residual by Predicted Plot



D) Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Date	14	14	5434470	1.9412	0.0929
Label	3	3	34650112	57.7598	<.0001

E) Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
(+/+)	1745.2667	115.46040	1745.27
(-/-)	3864.1333	115.46040	3864.13
P1/P3	3102.7000	305.47951	3306.67
P2/P4	2172.0333	305.47951	1798.33

The Effect Tests Table (Table 3-5 (D)) indicates that the date (DF=14) of the experiment was not found to be a significant factor (P=0.093; F Ratio=1.94). Pretreatment (DF=3) was found to be a significant factor (P < .0001; F Ratio=57.76).

Table 3-5 (E) is the Least Square Means table that was generated using this model. The specific LSM value is included for each treatment, followed by the computed standard error and the standard mean computation. Note that both the LSM and mean are again the same for both treatments (-/- and +/+) because they were performed all days. However, the LSM values for the other treatments (P1/P3 and P2/P4)) have both been adjusted. P1/P3 has been adjusted in a downward (negative) direction, and P2/P4 in an upward (positive) direction. This is due to fact that those days that these treatments were tested, their fluorescence measurements tended to be higher and lower, respectively, than projected for the other days.

Tukey's multiple comparison method ($\alpha = 0.05$) was then performed on the LSM values, for all pairs of the four possible pretreatment combinations (-/-, +/+, P1/P3, P2/P4). Table 3-6 (A) includes the specific details for each pretreatment, including the lower and upper bounds with 95% certainty (CL Dif). Those columns that are in red correspond to those values that are found to significant in the model. When neither cell type was pretreated (-/-), the average fluorescence measurement was found to be significantly higher than when both cell types were pretreated with sFn (+/+), and when pretreated with P2/P4 prior to exposure to sFn. However, when cells were left untreated (-/-), the average fluorescence was not significantly different than pretreatment with P1/P3 peptide pair. In addition, pretreatment of both cells (+/+) was significantly different than treatment with P1/P3, however it was not significantly different than P2/P4 pretreatment measurements according to this model. Table 3-6 (B) is a summary of these results.

Table 3-6. LSM differences Tukey's multiple comparisons method. Those columns in red are significant.

A) LSMeans differences Tukey's HSD. α=0.050; Q=2.82629.

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	**(+/+)**	**(-/-)**	**P1/P3**	**P2/P4**
(+/+)	0 0 0 0	-2118.9 163.286 -2580.4 -1657.4	-1357.4 326.571 -2280.4 -434.45	-426.77 326.571 -1349.8 496.219
(-/-)	2118.87 163.286 1657.37 2580.36	0 0 0 0	761.433 326.571 -161.55 1684.42	1692.1 326.571 769.114 2615.09
P1/P3	1357.43 326.571 434.448 2280.42	-761.43 326.571 -1684.4 161.552	0 0 0 0	930.667 447.176 -333.18 2194.52
P2/P4	426.767 326.571 -496.22 1349.75	-1692.1 326.571 -2615.1 -769.11	-930.67 447.176 -2194.5 333.184	0 0 0 0

B) Summary of Tukey's results. Levels not connected by same letter are significantly different.

Level				**Least Sq Mean**
(-/-)	A			3864.1333
P1/P3	A	B		3102.7000
P2/P4		B	C	2172.0333
(+/+)			C	1745.2667

3. 3. 7 LAK Cell Adherence to Endothelial Cells in Presence of Soluble Fibrin and Peptides

SFn pretreatment of one or both cell types (endothelial/LAK cells, respectively) significantly affected LAK cell binding to EC observed under flow conditions. Figure 3-9 shows the mean fluorescence units measured for each treatment (-/-, +/+, P1/P3, P2/P4). Untreated cells of each type (-/-) were used as a control value of maximum number of fluorescence units measured for each experiment. Pretreatment of both cell types with sFn (+/+), resulted in the fewest number of bound LAK cells to EC (least fluorescence observed), resulting in a 43.9 ± 4.4% inhibition when compared to the control (n=2). Pretreatment of cells with peptides P1/P3 resulted in a 25.1 ± 6.2 % decrease of binding events (n=2), and pretreatment of cells with peptides P2/P4 resulted in a 37.05 ± 4.7% decrease of fluorescence (n=2). Specific measurements for each treatment are listed in Table A-3 in the appendix.

3. 3. 8 Statistical Analysis: LAK Cell Adherence to Endothelial Cells Under In the Presence of Soluble Fibrin and Peptides

Using JMP, matched pair student t-tests ($\alpha = 0.05$) were performed on all pairs for the four pretreatment combinations (-/-, +/+, P1/P3, and P2/P4). All specific t-test results are listed in Table A-4 in the appendix. When neither cell type was pretreated (-/-), the mean fluorescence was not significantly greater than when both cells were pretreated (+/+), or when cells were treated with P2/P4 or P1/P3 peptide pairs, prior to sFn. This result can be partially explained by the low DF employed for the t-test for this comparison. However, when both cell types were prestreated with sFn (+/+), the mean fluorescence was significantly different than pretreatment with P1/P3 and P2/P4 peptide pairs. Finally, when cells were pretreated with either peptide pair, the mean fluorescence was found to be significantly different than one another.

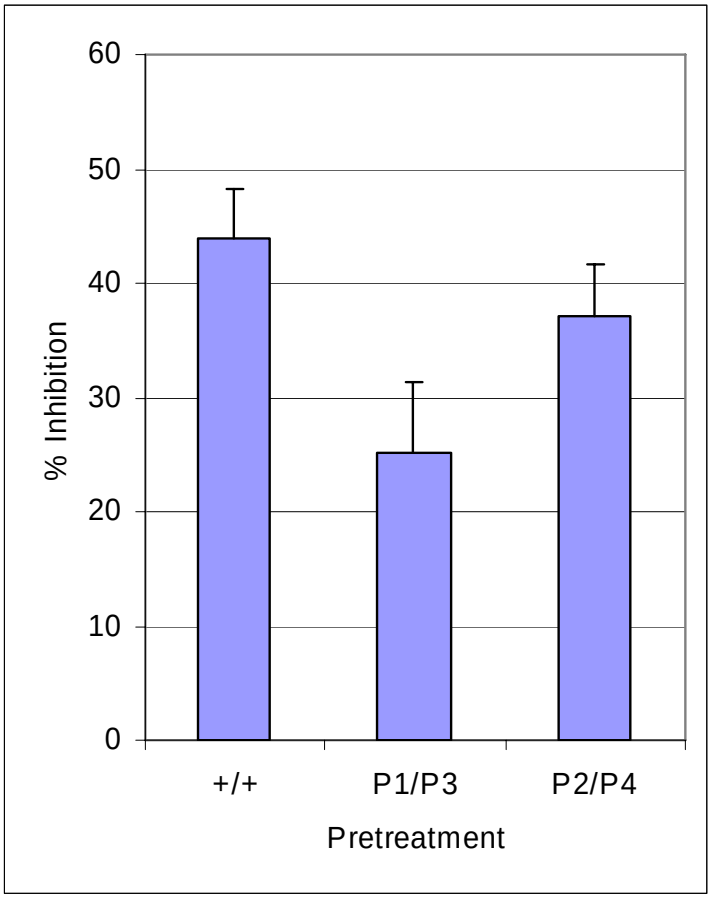


Figure 3-9. Effect of soluble fibrin and peptides on LAK cell adherence to endothelial cells under flow conditions. All measurements are presented as percent inhibition compared to untreated cells. Values were produced by quantitation of adherent, fluorescently labeled lymphocytes to endothelial cells using Image Pro Plus software package.

Also using JMP, an additive model, including terms for date (2 days) and treatment (4 levels), was fit using Standard Least Squares. I obtained an R^2 value of 0.99 (Table 3-7 (A)), with treatment differences explaining virtually all of the variation in fluorescence. The Root Mean Square Error of 179.7 estimates the assumed common standard deviation. The mean of response and total observations (8) are also listed.

Furthermore, the ANOVA (Table 3-7 (B)) provided an F ratio of 50.60 ($P < .0044$), indicating a significant model. The model accounts for approximately 99% of variation (from R^2 value), whereas, those unexplained variation accounts for the remaining 1%.

The residuals were also examined, which were consistent with our assumption of common variance. There are no "extreme outliers" that need additional attention for this model. (Table 3-7 (C)).

The Effect Tests Table (Table 3-7 (D) indicates that the date (DF=1) of the experiment was not found to be a significant factor (P=0.68; F Ratio=0.21). Pretreatment (DF=3) was found to be a significant factor ($P < .0003$; F Ratio=67.39).

Table 3-7 (E) is the Least Square Means table that was generated using this model. Furthermore, the specific LSM value is included for each, followed by the computed standard error and the standard mean computation. Note that both the LSM and mean are again the same for all treatments because all combinations were performed all days included in the model.

Table 3-7. Statistical analysis: LAK cell adherence to endothelial cells in the presence of soluble fibrin and blocking peptides.

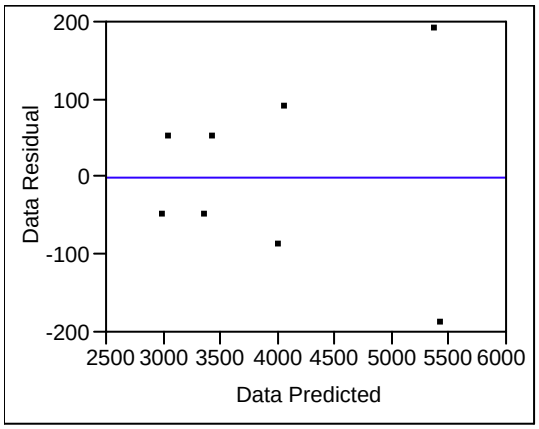
A) Summary of Fit

RSquare	0.985394
Root Mean Square Error	179.656
Mean of Response	3958.7
Observations (or Sum Wgts)	8

B) Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	4	6532434.2	1633109	50.5978
Error	3	96828.9	32276	Prob > F
C. Total	7	6629263.1		0.0044

C) Residual by Predicted Plot



D) Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Date	1	1	6867.9	0.2128	0.6760
Label	3	3	6525566.3	67.3928	0.0030

E) Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
(-/-)	5392.4000	127.03601	5392.40
(+/+)	3021.6000	127.03601	3021.60
P1/P3	4031.2000	127.03601	4031.20
P2/P4	3389.6000	127.03601	3389.60

Tukey's multiple comparison method ($\alpha = 0.05$) was then performed on the LSM values, for all pairs of the five possible pretreatment combinations (-/-, +/+, P1/P3, P2/P4). Table 3-8 (A) includes the specific details for each pretreatment, including the lower and upper bounds with 95% certainty (CL Dif). Those columns that are in red correspond to those values that are significant in the model. Table 3-8 (B) is a summary of these results. When neither cell type was pretreated (-/-) with sFn, the average fluorescence was significantly larger than all other treatments' means. Furthermore, when both cell types were pretreated with sFn (+/+), the average fluorescence was significantly different than when neither cell was treated with sFn (-/-), and when pretreated with P1/P3 prior to exposure to sFn. Furthermore, when cells were pretreated with the P1/P3 pair, the average fluorescence was not significantly different than P2/P4 pretreatment according to this model. Pretreatment with P2/P4 was significantly different than when neither cell was pretreated, but not different than when both cell types were pretreated with sFn.

3. 4 Lymphocyte Transendothelial Migration Optimization Assays

This section addresses the results of the transendothelial migration assays that were performed in order to investigate the ability of lymphocytes and activated LAK cells to permeate through confluent monolayers of EC grown on polycarbonate inserts under many different conditions. Each section below reports a separate experiment using different methodology.

3. 4. 1 Effect of Tumor Cells and TCCM on Migration

Lymphocyte migration through EC was measured in the presence and absence of either A375 tumor cells grown in 24-well microplates, or tissue culture conditioned media (TCCM) solution. A total count of fluorescence was also performed, including the maximum number of

Table 3-8. LSM differences Tukey's multiple comparisons method (peptides and soluble fibrin). Those columns in red are significant.

A) LSMean[i] By LSMean[j]. α=0.050; Q=4.82567.

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	(-/-)	(+/+)	P1/P3	P2/P4
(-/-)	0 0 0 0	2370.8 179.656 1503.84 3237.76	1361.2 179.656 494.238 2228.16	2002.8 179.656 1135.84 2869.76
(+/+)	-2370.8 179.656 -3237.8 -1503.8	0 0 0 0	-1009.6 179.656 -1876.6 -142.64	-368 179.656 -1235 498.962
P1/P3	-1361.2 179.656 -2228.2 -494.24	1009.6 179.656 142.638 1876.56	0 0 0 0	641.6 179.656 -225.36 1508.56
P2/P4	-2002.8 179.656 -2869.8 -1135.8	368 179.656 -498.96 1234.96	-641.6 179.656 -1508.6 225.362	0 0 0 0

B) Summary of Tukey's test. Levels not connected by same letter are significantly different.

Level				Least Sq Mean
(-/-)	A			5392.4000
P1/P3		B		4031.2000
P2/P4		B	C	3389.6000
(+/+)			C	3021.6000

lymphocytes (2 x 10^5 per well). Figure 3-10 shows all treatments that were performed as a percentage of the total fluorescence count measured (100%). When lymphocytes were added to those wells with A375 tumor cells grown in the plates, 95.3% fluorescence was observed after 18 hours of incubation with EC. When cells were incubated in the presence of one of two different batches of A375 conditioned TCCM (TCCM 1 and TCCM 2), 83.2% and 85.5% fluorescence was measured, respectively. In the absence of tumor cells or TCCM, cells incubated with RPMI media instead, resulted in only 17.4% total fluorescence. Controls including A375 cells grown in wells (39.9%), TCCM 1 (33.31%) and TCCM 2 (37.0%), and RPMI media alone (10.6%) were also performed.

Since this setup was not replicated, no statistical analysis was performed. However it is clear from Figure 3-10 that the presence of A375 tumor cells and TCCM derived from A375 tumor cells acts as a chemoattactant toward the lymphocytes present in inserts (lymph/A375, lymph/TCCM1, or lymph/TCCM2 versus the lymph/RPMI treatment). Additionally, the background readings (A375, TCCM1, and TCCM2) were found to be comparable to one another, but more than RPMI media alone.

Furthermore, an 18 hour assay was found to be appropriate, as the fluorescence observed from the lymphocytes incubated with A375 cells grown in the microplate was quite similar to the total (100%), which was one major goal of the exercise. Furthermore, both batches of lymphocyte/TCCM exhibited a similar measurement to one another, and a comparable value as compared to the total and the lymphocyte/A375 measurements. The other control measurements (TCCM1, TCCM2, RPMI, and A375) all produced measurements less than half of all other treatments.

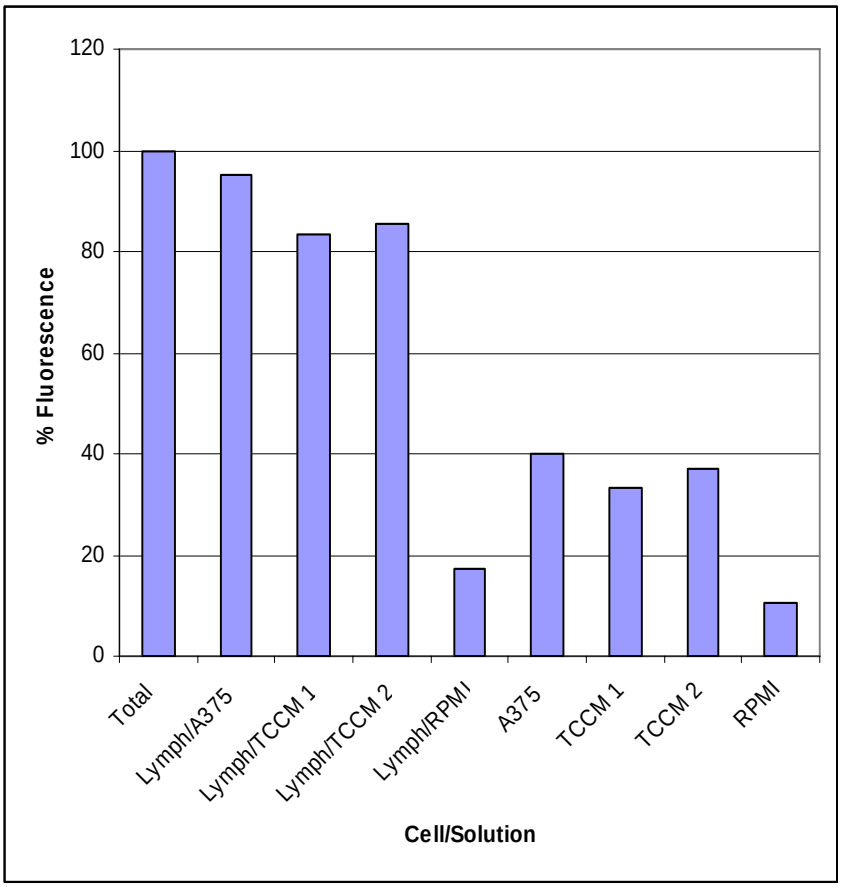


Figure 3-10. Effect of tumor cells and TCCM on total fluorescence observed.

3. 4. 2 Effect of Various Concentrations of Fibrin(ogen)/Soluble Fibrin on Lymphocyte Transendothelial Migration

Pretreatment with 4 different concentrations of sFn reagents (fibrinogen concentrations included 0.25, 0.50, 0.75, 1.0 mg/ml, with corresponding increases in GPRP and thrombin concentrations) were performed. Figure 3-11 shows the corresponding levels of inhibition caused by pretreatment with each. Pretreatment with 0.25 mg/ml of Fg (in sFn made with corresponding levels of GPRP and thrombin) resulted in a 14.10 ± 6.11% inhibition compared to migration observed of untreated cells. Pretreatment with 0.50 mg/ml (and corresponding levels of GPRP and thrombin) resulted in a 23.55 ±0.78% inhibition, 0.75 mg/ml resulted in a 23.26 ±2.09 inhibition, and 1.0 mg/ml (and corresponding levels of GPRP and thrombin) resulted in 20.73 ±5.73% inhibition. Table A- 9 in the appendix lists the specific means of each treatment.

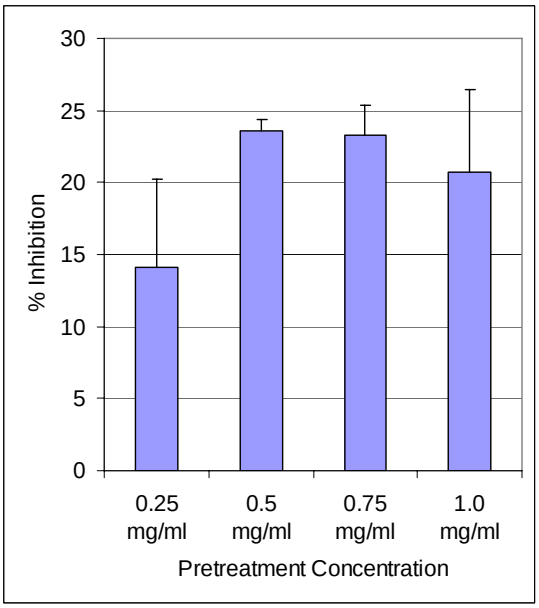


Figure 3-11. Effect of fibrin(ogen)/soluble fibrin (at different concentrations) on lymphocyte transendothelial migration(measurement represents % inhibition of pretreatment versus the negative sFn control (-/-) measurement (fluorescence).

3. 4. 3 Effect of eACA and SFn on Lymphocyte Transendothelial Migration

Figure 3-12 is a summary of results obtained from all treatments performed compared to when neither cell type was pretreated (-/-). When both cell types were pretreated with sFn prior to incubation (+/+), 18.0% inhibition of migration was observed. When cells were pretreated with sFn, and incubated with EACA (to slow fibrinolysis), 18.5% inhibition was observed. When EACA was incubated with the cells alone, only a 0.1% inhibition was observed (n=1). Clearly, pretreatments with sFn, in the presence or absence of EACA, were not significantly different than one another. However, both of these pretreatments were different than EACA alone. No additional statistical analysis was performed for this data. Specific data is listed in Table A-10 in the appendix.

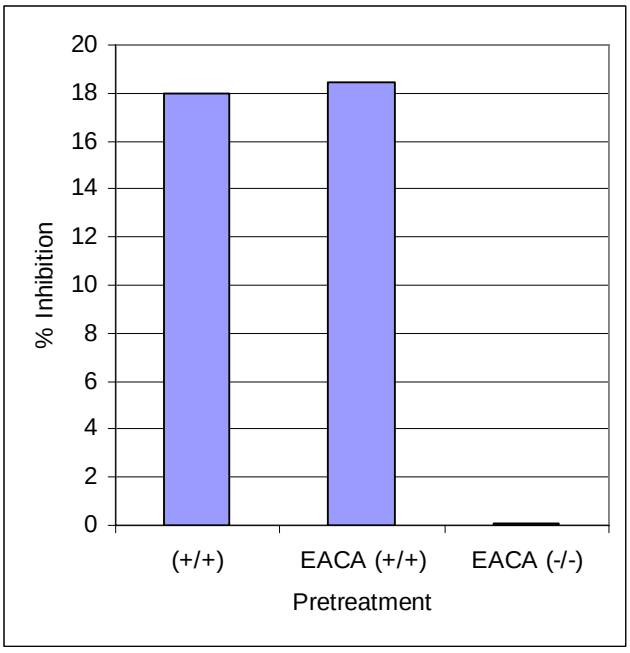


Figure 3-12. Effect of sFn pretreatment (in the presence of eACA) on lymphocyte transendothelial migration(measurement represents % inhibition of pretreatment versus the negative sFn control (-/-) measurement (fluorescence).

3. 4. 4 The Effect of Various Peptide Concentrations on Lymphocyte Transendothelial Migration

Here, cells were pretreated with each pair of peptides, at three different concentrations (2, 4, and 8 mM), prior to observation of migration. Figure 3-13 is a summary of these treatments. Pretreatment of both cells with sFn resulted in the maximum level of inhibition at 22.28% (n=1). Pretreatment with P1/P3 (at 2, 4, and 8 mM) prior to exposure to sFn, resulted in 9.88, 10.82, and 7.98%, respectively. Pretreatment with P2/P4 (at 2, 4, and 8 mM) prior to exposure to sFn, resulted in 17.5, 16.83, and 20.38%, respectively. Figure A-11 in the appendix lists all specific measurements for each treatment.

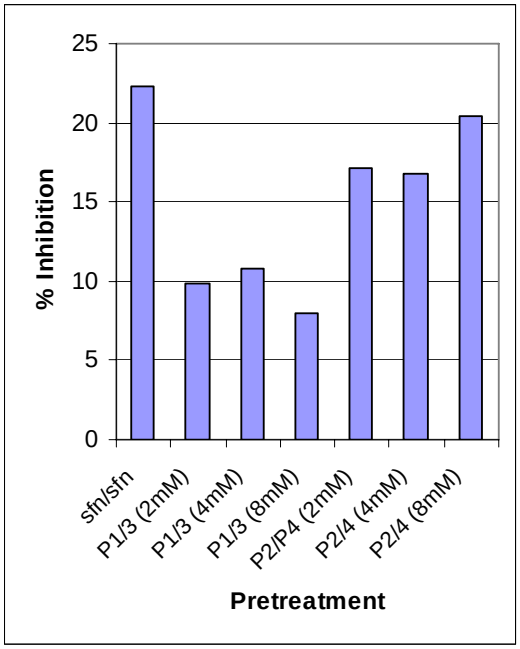


Figure 3-13. Effect of peptide concentration on lymphocyte transendothelial migration (measurement represents % inhibition of pretreatment versus the negative sFn control (-/-) measurement (fluorescence)).

3. 5 Lymphocyte and LAK Cell Transendothelial Migration in the Presence of Soluble Fibrin, Peptides, Fibrinogen, GPRP, and Thrombin

3. 5. 1 Lymphocyte Transendothelial Migration in the Presence of Soluble Fibrin

SFn pretreatment of one or both cell types significantly ($P < 0.05$) affected the number of cells that migrated through confluent monolayers of EC grown on 8 μm inserts incubated in 24-well plates. Figure 3-14 is a summary of the mean fluorescence units observed for each pretreatment. Untreated cells of each type (-/-) were used as a control value of fluorescence measured (n = 6). Furthermore, all other pretreatments were compared against this measurement in order to calculate a percent inhibition for each treatment. Pretreatment of both cell types with sFn (+/+, EC/lymphocytes respectively; n = 6) resulted in the fewest number of lymphocytes that migrated through the EC (or least fluorescence observed), resulting in 29.60 ± 7.67% inhibition when compared to the control (n = 6). Pretreatment of only EC (+/-) resulted in a 21.31 ± 7.83% decrease of migration (n = 2), and pretreatment of lymphocytes only (-/+) resulted in a 17.87 ± 2.30% decrease of migration (n = 2). Specific data are listed in Table A-5 in the appendix.

3. 5. 2 Statistical Analysis: Lymphocyte Transendothelial Migration in the Presence of Soluble Fibrin

Using JMP, matched pair student t-tests ($\alpha = 0.05$) were performed for all possible pairs of the four pretreatment combinations of sFn (-/-, +/+, -/+, +/-). When neither cell type was pretreated with sFn (-/-), the mean fluorescence (migration) was significantly greater than double pretreatment of cells with sFn (n=6). However, when only one cell type was pretreated with sFn (+/-) and (-/+), the mean fluorescence was not found to be significantly different than either double sFn (+/+) pretreatment or untreated cells (-/-). Details for the t-tests appear in Table A-6 in the appendix.

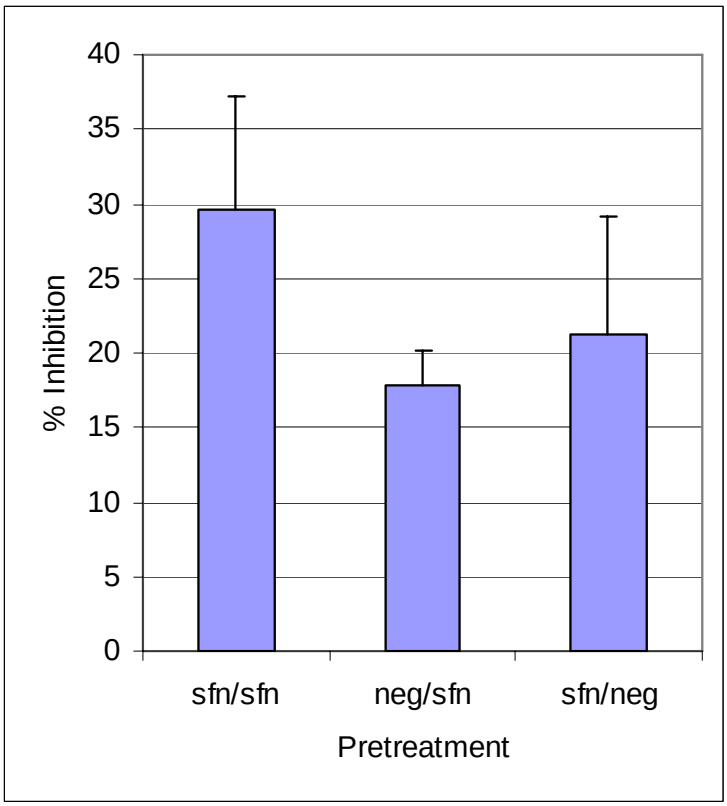


Figure 3-14. Effect of soluble fibrin on lymphocyte transendothelial migration (measurement represents % inhibition of pretreatment versus the negative sFn control (-/-) measurement (fluorescence).

Also using JMP, an additive model, including terms for date (6 days) and treatment (4 levels), was fit using Standard Least Squares (Table 3-9 (A-E)). I obtained an R^2 value of 0.98 Table 3-9 (A), with treatment differences explaining the vast majority of the variation within fluorescence. The Root Mean Square Error of 12624.0 estimates the assumed common standard deviation. The mean of response and total observations (16) are also listed.

Furthermore, the analysis of variance (ANOVA) (Table 3-9 (B)) provided an F ratio of 49.8 ($P < .0001$), indicating a significant model. The model accounts for approximately 98% of variation (from R^2 value), whereas the unexplained variation accounts for the other approximate 2% of variance.

The residuals were also examined, and were consistent with our assumption of a common variance. There are no "extreme outliers" that need additional attention for this model (Table 3-9 (C)).

The Effect Tests Table (Table 3-9(D)) indicates that both date (DF=5) of the experiment and pretreatment (DF=3) were found to be significant factors ($P<0.0001$; F Ratio=39.6 and $P < .0001$; F Ratio=50.4, respectively). Day could again be a significant factor due to the use of different reagents from day to day of experiments.

Table 3-9(E) is the Least Square Means table that was generated using this model. Furthermore, the specific LSM value is included for each, followed by the computed standard error and the standard mean computation. Note that the LSM and mean values are the same for both

Table 3- 9. Statistical analysis: Lymphocyte transendothelial migration in the presence of soluble fibrin.

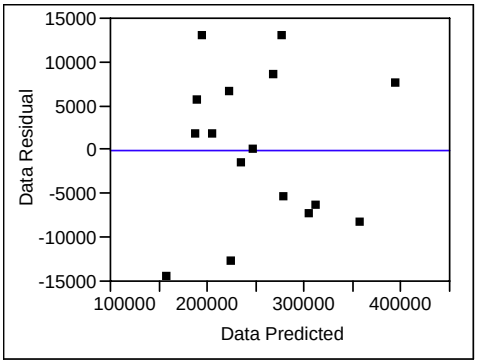
A) Summary of Fit

RSquare	0.982728
Root Mean Square Error	12624.02
Mean of Response	253010.5
Observations (or Sum Wgts)	16

B) Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	6.3472e+10	7.9339e+9	49.7845
Error	7	1115560289	159365756	Prob > F
C. Total	15	6.4587e+10		<.0001

C) Residual by Predicted Plot



D) Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Date	5	5	3.154e+10	39.5825	<.0001
Label	3	3	2.408e+10	50.3671	<.0001

E) Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
(-/-)	310501.00	5153.733	310501
(-/+)	268712.42	10307.465	219635
(+/-)	258686.92	10307.465	209609
(+/+)	221112.33	5153.733	221112

treatments (-/- and +/+) because they were performed all days. However, the LSM values for both intermediates (+/- and -/+) have been adjusted in an upward (positive) direction. This is due to fact that those days that (-/+) and (+/-) were tested, the mean fluorescence measurement tended to be lower (respectively) than other days included.

Using the computed LSM values, Tukey's multiple comparison method was performed. Table 3-10(A) includes the specific details for each sFn pretreatment, including the lower and upper bounds (or confidence intervals) with 95% certainty (CL Dif). Those values that are in red correspond to significant differences in the model. Using this method, it was shown that when neither cell was pretreated with sFn (-/-), the average fluorescence measurement was significantly different than all other pretreatments involving sFn. Furthermore, when both cell types were treated with sFn (+/+), the average fluorescence measurement was significantly different from the (-/+) pretreatment mean. The intermediate treatments' average measurements were not significantly different than one another. Table 3-10(B) is a summary of these results.

3. 5. 3 Lymphocyte Transendothelial Migration in the Presence of Fibrinogen, GPRP, and Thrombin

Figure 3-15 is a summary of inhibitions observed due to pretreatment of lymphocytes and EC with those components that are combined to create sFn *in vitro*: fibrinogen, GPRP, and thrombin. EC and lymphocytes that were pretreated with fibrinogen resulted in a 14.61 ± 3.79% decrease of migration (fluorescence) through endothelial monolayers (n=2). EC and lymphocytes treated with GPRP resulted in only a 3.99 ± 2.89% decrease of fluorescence (n=2). And when both cells were pretreated with thrombin, the average fluorescence measurement was decreased by 5.93 ± 6.86% (n=2). The original data for each date is listed in Table A-5 in the appendix.

Table 3-10. LSMeans Differences Tukey's HSD Multiple Comparison Method. Those columns in red are significant.

A) α=0.050 Q=3.31024 LSMean[i] By LSMean[j]

Mean[i]-Mean[j] **Std Err Dif** **Lower CL Dif** **Upper CL Dif**	**(-/-)**	**(-/+)**	**(+/-)**	**(+/+)**
(-/-)	0 0 0 0	41788.6 11524.1 3641.02 79936.1	51814.1 11524.1 13666.5 89961.6	89388.7 7288.48 65262 113515
(-/+)	-41789 11524.1 -79936 -3641	0 0 0 0	10025.5 12624 -31763 51814.1	47600.1 11524.1 9452.52 85747.6
(+/-)	-51814 11524.1 -89962 -13667	-10026 12624 -51814 31763.1	0 0 0 0	37574.6 11524.1 -572.98 75722.1
(+/+)	-89389 7288.48 -113515 -65262	-47600 11524.1 -85748 -9452.5	-37575 11524.1 -75722 572.98	0 0 0 0

B) Summary of Tukey's test.Levels not connected by same letter are significantly different.

Level				**Least Sq Mean**
(-/-)	A			310501.00
(-/+)		B		268712.42
(+/-)		B	C	258686.92
(+/+)			C	221112.33

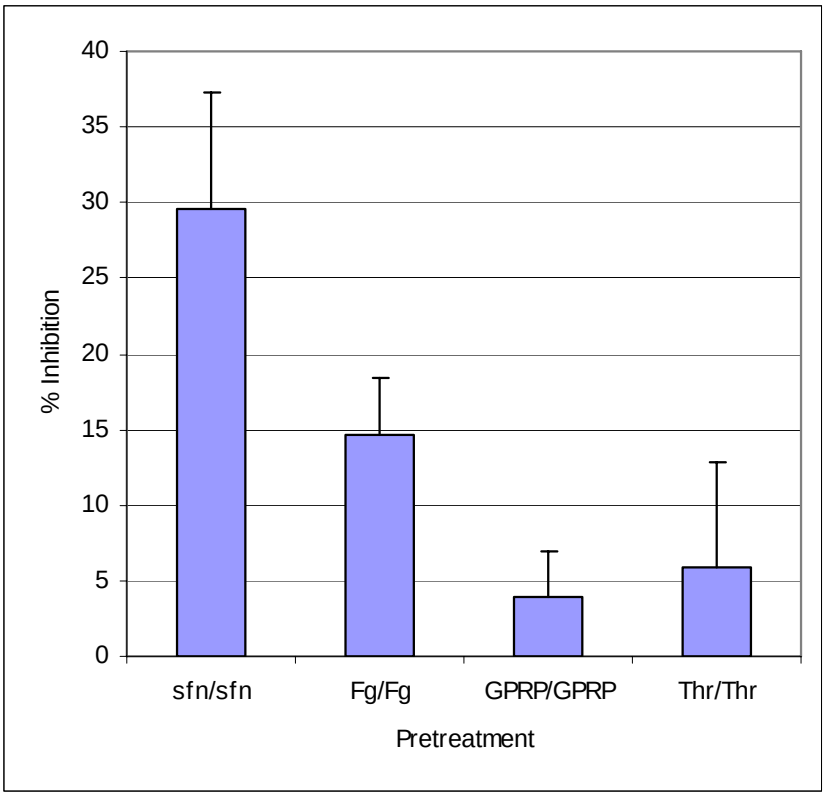


Figure 3-15. Effect of soluble fibrin, fibrinogen, GPRP, and thrombin pretreatment on lymphocyte transendothelial migration(measurement represents % inhibition of pretreatment versus the negative sFn control (-/-) measurement (fluorescence)).

3. 5. 4 Statistical Analysis: Lymphocyte Transendothelial Migration in the Presence of Fibrinogen, GPRP, and Thrombin

Using JMP, matched pair student t-tests ($\alpha = 0.05$) were performed on all pairs of interest for the five possible pretreatment combinations (-/-, +/+, Fg/Fg, GPRP/GPRP, Thr/Thr). Individual tests are listed in Table A- 6 in the appendix. When both cells were pretreated with sFn (+/+), the mean fluorescence was significantly different than when neither cell was pretreated (n=6). When neither cell type was treated with sFn (-/-), the average fluorescence was not significantly different than all other treatments. Similarly, there were no additional significant differences found between any of the other treatment combinations. These not-significant differences are not surprising, given that we only have 1 DF for error for each test.

Also using JMP, an additive model, including terms for date (6 days) and treatment (5 levels), was fit using Standard Least Squares. I obtained an R^2 value of 0.98 (Table 3-11(A)), with treatment differences explaining the vast majority of the variation within fluorescence. The Root Mean Square Error of 11,539 estimates the assumed common standard deviation. The mean of response and total observations (18) are also listed.

Furthermore, the ANOVA (Table 3-11(B)) provided an F ratio of 47.8 ($P < .0001$), indicating a significant model. This model accounts for approximately 98% of variation (from R^2 value), whereas the unexplained variation accounts for the remaining 2%.

The residuals were also examined, and they were consistent with our assumption of a common variance. There are no "extreme outliers" that need additional attention for this model. (Table 3-11(C)).

Table 3- 11. Statistical analysis: lymphocyte transendothelial migration in the presence of

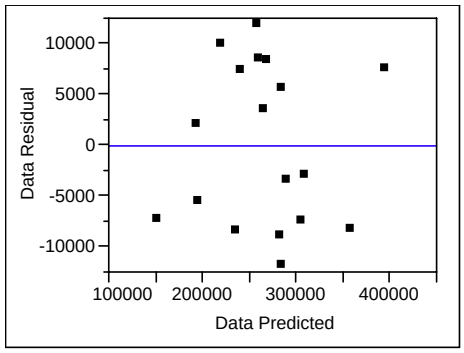
A) Summary of Fit

RSquare	0.981749
Root Mean Square Error	11538.61
Mean of Response	265537.4
Observations (or Sum Wgts)	18

B) Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	9	5.7295e+10	6.3661e+9	47.8150
Error	8	1065115386	133139423	Prob > F
C. Total	17	5.836e+10		<.0001

C) Residual by Predicted Plot



D) Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Date	5	5	3.2319e+10	48.5490	<.0001
Label	4	4	2.5503e+10	47.8877	<.0001

E) Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
(-/-)	310501.00	4710.6161	310501
(+/+)	221112.33	4710.6161	221112
Fg/Fg	262728.04	9421.2322	247060
GPRP/GRRP	292657.79	9421.2322	276989
Thr/Thr	286616.04	9421.2322	270948

The Effect Tests Table (Table 3-11(D)) indicates that the date (DF=5) of the experiment was found to be a significant factor (P<0.0001; F Ratio=48.5). Pretreatment (DF=4) was also found to be a significant factor (P < .0001; F Ratio=47.9). The use of different reagents could contribute to the fact that day was found to be significant as discussed earlier.

Table 3-11(E) is the Least Square Means table that was generated using this model. Furthermore, the specific LSM value is included for each, followed by the computed standard error and the standard mean computation. Note that both the LSM and mean are again the same for both treatments (-/- and +/+) because they were performed all days. However, the LSM value for all other treatments (Fg/Fg, GPRP/GPRP, Thr/Thr) have all been adjusted in a upward (positive) direction. This is due to fact that those days that these treatments were tested, the fluorescence measurements tended to be lower than for the other days.

Using the computed LSM values, Tukey's multiple comparison method was performed. Table 3-12(A) includes the specific details for each sFn pretreatment, including the lower and upper bounds (or confidence intervals) with 95% certainty (CL Dif). Those values that are in red correspond to significant differences in the model.

Table 3-12(B) includes a summary of these results. Using this method, when both cell types were pretreated with sFn (+/+), the average fluorescence was significantly different than all other treatments. Furthermore, when neither cell type was pretreated (-/-), the fluorescence was not significantly different than pretreatment with GPRP or thrombin. However, when neither cell was pretreated was significantly different than fibrinogen pretreatment.

Table 3-12. LSMeans Differences Tukey's HSD multiple comparisons method. Those columns in red are significant.

A) LSMean[i] By LSMean[j]; α=0.050; Q=3.45476;

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	**(-/-)**	**(+/+)**	**Fg/Fg**	**GPRP/GRRP**	**Thr/Thr**
(-/-)	0 0 0 0	89388.7 6661.82 66373.7 112404	47773 10533.3 11383.1 84162.8	17843.2 10533.3 -18547 54233.1	23885 10533.3 -12505 60274.8
(+/+)	-89389 6661.82 -112404 -66374	0 0 0 0	-41616 10533.3 -78006 -5225.8	-71545 10533.3 -107935 -35156	-65504 10533.3 -101894 -29114
Fg/Fg	-47773 10533.3 -84163 -11383	41615.7 10533.3 5225.83 78005.6	0 0 0 0	-29930 11538.6 -69793 9933.37	-23888 11538.6 -63751 15975.1
GPRP/GRRP	-17843 10533.3 -54233 18546.7	71545.5 10533.3 35155.6 107935	29929.8 11538.6 -9933.4 69792.9	0 0 0 0	6041.75 11538.6 -33821 45904.9
Thr/Thr	-23885 10533.3 -60275 12504.9	65503.7 10533.3 29113.8 101894	23888 11538.6 -15975 63751.1	-6041.7 11538.6 -45905 33821.4	0 0 0 0

B)

Level				**Least Sq Mean**
(-/-)	A			310501.00
GPRP/GRRP	A	B		292657.79
Thr/Thr	A	B		286616.04
Fg/Fg		B		262728.04
(+/+)			C	221112.33

3. 5. 5 Lymphocyte Transendothelial Migration in the Presence of Peptides and Soluble Fibrin

Figure 3-15 is a summary of effects on lymphocyte/EC migration (% inhibitions), caused by pretreatment of peptides, prior to incubation with sFn. When cells and sFn were pretreated with both peptides 1 and 3, respectively, resulted in only a 7.92 ± 2.2% inhibition of average fluorescence (migration). And when cells and fibrin were pretreated with peptides 2 and 4, respectively, resulted in a 9.03 ± 0.58% inhibition of fluorescence. Specific data are listed in Table A-5 in the appendix.

3. 5. 6 Statistical Analysis: Lymphocyte Transendothelial Migration in the Presence of Peptides and SFn

Using JMP, matched pair student t-tests ($\alpha = 0.05$) were performed on all pairs of the original data for the four possible pretreatment combinations (-/-, +/+, P1/P3, and P2/P4). When neither cell type was pretreated with sFn (-/-), the average fluorescence was not significantly different than the average fluorescence from when both cells were pretreated (+/+). Furthermore, when cells were pretreated with either peptide pair, P1/P3 or P2/P4 prior to sFn, the fluorescence measurement was not significantly different than (-/-) or (+/+) fluorescence measurements. These not-significant results are not surprising given the low number of DF (1) for this comparison. Details of specific t-test are listed in Figure A-6 in the appendix.

Also using JMP, an additive model, including terms for date (2 days) and treatment (4 levels), was fit using Standard Least Squares. I obtained an R^2 value of 0.99 (Table 3-13(A)), with treatment differences explaining the vast majority of the variation within fluorescence. The Root Mean Square Error is the assumed standard deviation.

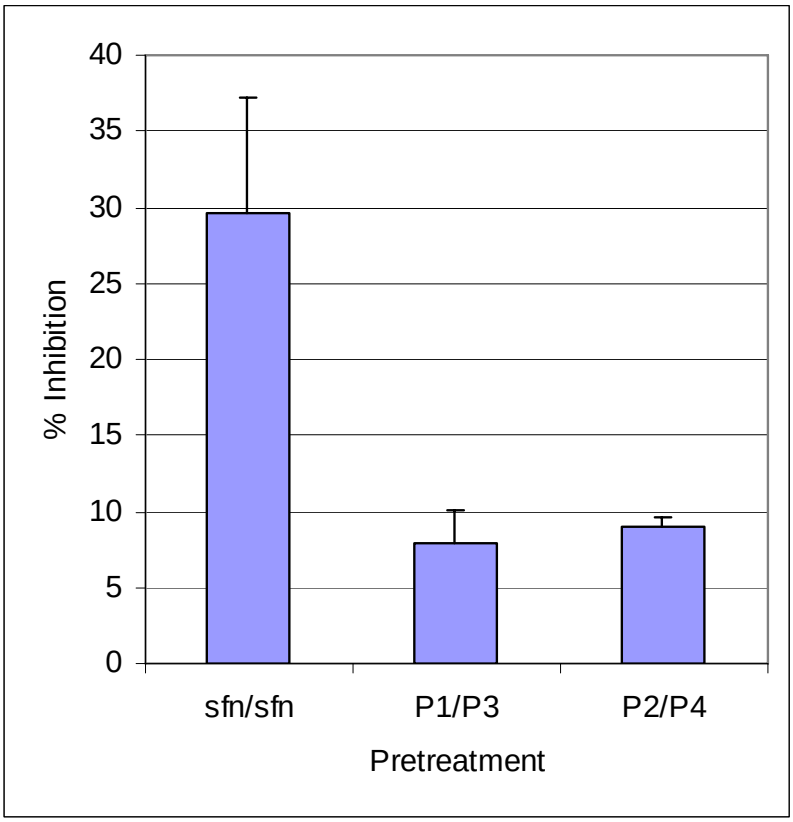


Figure 3-16. Effect of peptides and soluble fibrin on lymphocyte transendothelial migration (measurement represents % inhibition of pretreatment versus the negative sFn control (-/-) measurement (fluorescence).

Table 3-13. Statistical analysis: lymphocyte transendothelial migration in the presence of peptide and soluble fibrin.

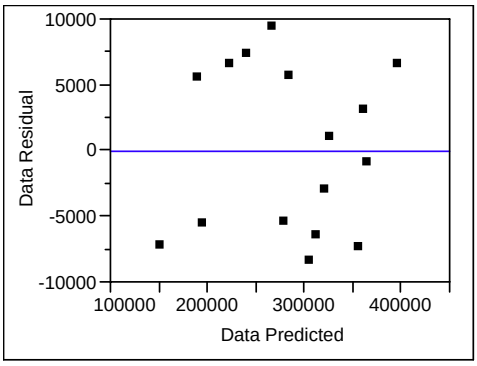
A) Summary of Fit

RSquare	0.992238
RSquare Adj	0.983368
Root Mean Square Error	9167.846
Mean of Response	285051.8
Observations (or Sum Wgts)	16

B) Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	8	7.5213e+10	9.4016e+9	111.8580
Error	7	588345795	84049399	Prob > F
C. Total	15	7.5801e+10		<.0001

C) Residual by Predicted Plot



D) Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Date	5	5	3.3449e+10	79.5938	<.0001
Label	3	3	2.4285e+10	96.3127	<.0001

E) Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
(-/-)	310501.00	3742.7574	310501
(+/+)	221112.33	3742.7574	221112
P1/P3	279958.29	7485.5149	344705
P2/P4	276123.79	7485.5149	340870

Furthermore, the ANOVA (Table 3-13(B)) provided an F ratio of 111.8 (P <.0001), indicating a significant model. This model accounts for approximately 99% of variation (from R^2 value), whereas, the unexplained variation accounts for the other 1% of variation.

The residuals were also examined, which were consistend with our assumption of a common variance. There are no "extreme outliers" that need additional attention for this model (Table 3-13(C)).

The Effect Tests Table (Table 3-13(D)) indicates that the date (DF=5) of the experiment was found to be a significant factor (P<0.0001; F Ratio=79.6). Pretreatment (DF=3) was also found to be a significant factor (P < .0001; F Ratio=96.3). The use of different reagents may have contributed to the fact that day was a significant factor.

Table 3-13(E) is the Least Square Means table that was generated using this model. Furthermore, the specific LSM value is included for each, followed by the computed standard error and the standard mean computation. Note that both the LSM and mean are again the same for both treatments (-/- and +/+) because they were performed all days. However, the LSM values for the other treatments (P1/P3 and P2/P4)) have both been adjusted: P1/P3 in a downward (negative) direction, and P2/P4 adjusted in an upward direction. This is due to fact that the days that these treatments were tested, their fluorescence measurement tended to be higher and lower, respectively, than the other days of the model.

Using the computed LSM values, Tukey's multiple comparison method was performed. Table 3-14 includes the specific details for each sFn pretreatment, including the lower and upper bounds

Table 3-14. LSM Differences Tukey's HSD multiple comparisons method (peptides and sFn)

A) α=0.05;Q=3.31024; LSMean[i] By LSMean[j]

Mean[i]-Mean[j] **Std Err Dif** **Lower CL Dif** **Upper CL Dif**	**(-/-)**	**(+/+)**	**P1/P3**	**P2/P4**
(-/-)	0 0 0 0	89388.7 5293.06 71867.4 106910	30542.7 8369.06 2839.08 58246.3	34377.2 8369.06 6673.58 62080.8
(+/+)	-89389 5293.06 -106910 -71867	0 0 0 0	-58846 8369.06 -86550 -31142	-55011 8369.06 -82715 -27308
P1/P3	-30543 8369.06 -58246 -2839.1	58846 8369.06 31142.3 86549.6	0 0 0 0	3834.5 9167.85 -26513 34182.3
P2/P4	-34377 8369.06 -62081 -6673.6	55011.5 8369.06 27307.8 82715.1	-3834.5 9167.85 -34182 26513.3	0 0 0 0

B) Summary of Tukey's test. Levels not connected by same letter are significantly different.

Level				**Least Sq Mean**
(-/-)	A			310501.00
P1/P3		B		279958.29
P2/P4		B		276123.79
(+/+)			C	221112.33

.

(confidence intervals) with 95% certainty (CL Dif). Those values that are in red correspond to significant differences in the model.

Using this method, it was again shown that when neither cell was pretreated with sFn (-/-), the average fluorescence was significantly different than the other pretreatment of double pretreatment with sFn (+/+). Furthermore, when cells were pretreated with either P1/P3 or P2/P4, the average fluorescence was significantly different than when both cell types were treated with sFn and than when neither was pretreated. Table 3-14(B) is a summary of these results.

3. 5. 7 IL-2 Activated LAK Cell/Lymphocyte Transendothelial Migration in the Presence of Soluble Fibrin and Peptides

Here, lymphocytes and LAK cells (activated with IL-2) were incubated for approximately 72 hours. SFn pretreatment of one or both cell types (EC and 3 day incubated lymphocytes) significantly affected the number of cells that migrated through confluent monolayers of EC on 8 μm inserts in 24-well plates. Untreated cells (-/-) were used as a control value of maximum number of events measured (n=3); a separate control was performed for untreated lymphocytes and untreated LAK cells. Figure 3-17 is a summary of means and standard deviations associated with each of these combinations. Pretreatment of lymphocytes and EC with sFn (+/+, EC/lymphocytes respectively; n=3) resulted in the least fluorescence observed, resulting in a 17.24 ± 0.99% inhibition when compared to its control. Pretreatment of both EC and IL-2 activated LAK cells with sFn **(**LAK **+/+)** resulted in an 12.24 ± 4.9% inhibition of fluorescence (migration), when compared to the control including LAK cells (n=3). EC and LAK cells that were pretreated with P1/P3 resulted in 1.85 ± 8.94% mean inhibition of migration (n=2). And cells treated with P2/P4 resulted in a 3.19 ± 3.51% decrease of migration (n=2). Specific means

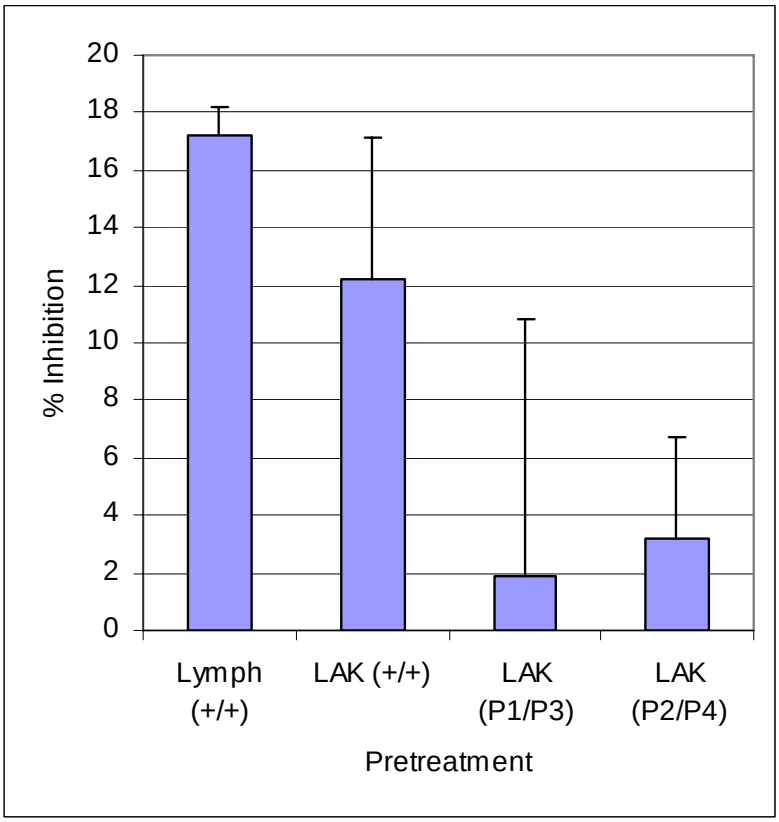


Figure 3-17. Effect of soluble fibrin and peptides on LAK cell transendothelial migration.

are listed in figure A-7 in the appendix.

3. 5. 8 Statistical Analysis: LAK Cell Transendothelial Migration in the Presence of Soluble Fibrin and Peptides

Using JMP, matched pair student t-tests ($\alpha = 0.05$) were performed on all pairs of the original data for the four possible pretreatment combinations (-/-, +/+, P1/P3, and P2/P4). When 3 day old lymphocytes and EC were pretreated with sFn (lymph +/+), the mean fluorescence was significantly different than when neither cell type was pretreated (lymph -/-). This combination was performed to test the level of migration achieved by these older lymphocytes (in the absence and presence of sFn). When both 3 day old, IL-2 incubated LAK cells and EC were pretreated with sFn (LAK +/+), the average fluorescence was not significantly different than when neither cell type was pretreated (LAK -/-). Furthermore, when LAK cells and EC were pretreated with either P1/P3 or P2/P4 prior to sFn, the mean fluorescence was not significantly different than either (LAK -/-) or (LAK +/+). These not-significant results are not surprising, given that we only have 1 DF for error for each test. Details of t-tests are listed in Table A-8 of the appendix.

Also using JMP, an additive model, including terms for date (2 days) and treatment (4 levels), was fit using Standard Least Squares (Figure 3-15). I obtained an R^2 value of 0.94 (Table 3-15(A)), with treatment differences explaining the vast majority of the variation within fluorescence. The Root Mean Square Error of 13678 estimates the assumed common standard deviation. The mean of response and total observations (16) are also listed.

Furthermore, the ANOVA (Table 3-15(B)) provided an F ratio of 20.36 ($P < .0002$), indicating a significant model. This model accounts for approximately 94% of variation, whereas unexplained

Table 3-15. Statistical analysis: LAK cell transendothelial migration in the presence of sFn and peptides.

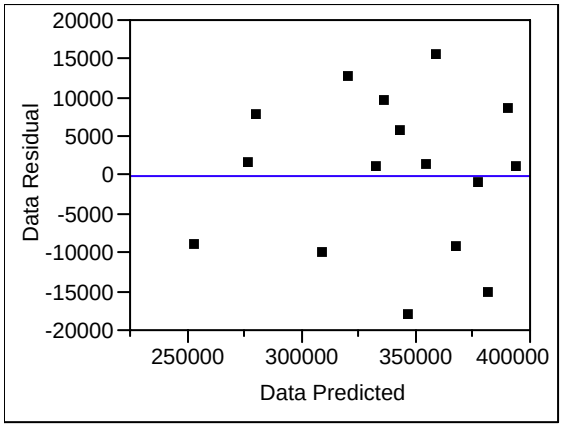
A) Summary of Fit

RSquare	0.946847
Root Mean Square Error	13677.96
Mean of Response	338778.5
Observations (or Sum Wgts)	16

B) Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	2.6661e+10	3.8087e+9	20.3582
Error	8	1496691733	187086467	Prob > F
C. Total	15	2.8158e+10		0.0002

C) Residual by Predicted Plot



D) Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Date	2	2	2187730589	5.8468	0.0272
Label	5	5	2.5074e+10	26.8044	<.0001

E) Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
LAK (-/-)	384103.50	7896.971	384104
LAK (+/+)	336453.83	7896.971	336454
LAK (P1/P3)	375289.00	10066.703	370287
LAK (P2/P4)	371002.75	10066.703	366001
Lymph (-/-)	325696.83	7896.971	325697
Lymph (+/+)	269705.83	7896.971	269706

variation accounts for the the remaining 6%.

The residuals were examined, which were consistent with our assumption of a common variance. There are no "extreme outliers" that need additional attention for this model (Table 3-15(C)). The Effect Tests Table (Table 3-15(D)) indicates that the date (DF=2) of the experiment was not found to be a significant factor (P<0.0272; F Ratio=5.85). Pretreatment (DF=3) was found to be a significant factor (P < .0001; F Ratio=26.8).

Table 3-15(E) is the Least Square Means table that was generated using this model. Furthermore, the specific LSM value is included for each, followed by the computed standard error and the standard mean computation. Note that both the LSM and mean are again the same for both lymphocyte and LAK cell sFn treatments (-/- and +/+) because they were performed all days. However, the LSM values for the other treatments (P1/P3 and P2/P4)) have both been adjusted: P1/P3 in an upward (positive) direction. This is due to fact that the days that these treatments were tested, fluorescence measurements tended to be lower than the other days of the model.

Using the computed LSM values, Tukey's multiple comparison method was performed. Table 3-16(A) includes the details for each sFn treatment, including the lower and upper bounds (confidence intervals) with 95% certainty (CL Dif). Those values in red correspond to significant differences in the model. Using this method, it was shown that when neither cell was pretreated with sFn (-/-), the average fluorescence was significantly different than all other pretreatments' average measurements. Furthermore, when cells were pretreated with either P1/P3 or P2/P4, the average fluorescence was significantly different than when both cell types were pretreated with sFn, and than when neither was pretreated. Table 3-16(B) is a summary of these results.

Table 3-16. LSM Differences Tukey's HSD multiple comparisons method (LAK cells).

A) LSMean[i] By LSMean[j]α=0.050 Q=3.65371

Mean[i]-Mean[j] Std Err Dif Lower CL Dif Upper CL Dif	**LAK (-/-)**	**LAK (+/+)**	**LAK (P1/P3)**	**LAK (P2/P4)**	**Lymph (-/-)**	**Lymph (+/+)**
LAK (-/-)	0 0 0 0	47649.7 11168 6845.06 88454.3	8814.5 12794.6 -37933 55562.1	13100.8 12794.6 -33647 59848.3	58406.7 11168 17602.1 99211.3	114398 11168 73593.1 155202
LAK (+/+)	-47650 11168 -88454 -6845.1	0 0 0 0	-38835 12794.6 -85583 7912.38	-34549 12794.6 -81296 12198.6	10757 11168 -30048 51561.6	66748 11168 25943.4 107553
LAK (P1/P3)	-8814.5 12794.6 -55562 37933.1	38835.2 12794.6 -7912.4 85582.7	0 0 0 0	4286.25 13678 -45689 54261.5	49592.2 12794.6 2844.62 96339.7	105583 12794.6 58835.6 152331
LAK (P2/P4)	-13101 12794.6 -59848 33646.8	34548.9 12794.6 -12199 81296.5	-4286.3 13678 -54261 45689	0 0 0 0	45305.9 12794.6 -1441.6 92053.5	101297 12794.6 54549.4 148044
Lymph (-/-)	-58407 11168 -99211 -17602	-10757 11168 -51562 30047.6	-49592 12794.6 -96340 -2844.6	-45306 12794.6 -92053 1441.63	0 0 0 0	55991 11168 15186.4 96795.6
Lymph (+/+)	-114398 11168 -155202 -73593	-66748 11168 -107553 -25943	-105583 12794.6 -152331 -58836	-101297 12794.6 -148044 -54549	-55991 11168 -96796 -15186	0 0 0 0

B) Summary of Tukey's test. Levels not connected by same letter are significantly different.

Level					**Least Sq Mean**
LAK (-/-)	A				384103.50
LAK (P1/P3)	A	B			375289.00
LAK (P2/P4)	A	B	C		371002.75
LAK (+/+)		B	C		336453.83
Lymph (-/-)			C		325696.83
Lymph (+/+)				D	269705.83

.

CHAPTER 4. SUMMARY OF RESULTS AND CONCLUSIONS

- OG labeled sFn (sFn) binds to IL-2 activated lymphocytes (LAK cells) and EC.
- SFn pretreatment inhibited lymphocyte (sign. Student's t-test and Tukey) and LAK cell (sign. Tukey) adherence to EC under flow conditions.
- SFn reagents (fibrinogen, GPRP, and thrombin individually) did not significantly affect lymphocyte/LAK cell adherence to EC under flow conditions.
- P1/P3 pretreatment, prior to sFn exposure, significantly (Student's t-test and Tukey) restored lymphocyte and LAK cell adherence to EC.
- P2/P4 pretreatment minimally restored lymphocyte and LAK cell adherence and was not a significant factor (Tukey).
- SFn pretreatment inhibited lymphocyte (sign. Student's t-test and Tukey) and LAK cell (sign. Tukey) transendothelial migration across EC (toward TCCM).
- SFn components (GPRP, and thrombin individually), did not significantly affect (Tukey's) lymphocyte migration. However, pretreatment with fibrinogen did significantly affect lymphocyte migration through EC.
- Pretreatment with either peptide pair (P1/P3 or P2/P4), prior to sFn exposure, significantly (Tukey) restored both lymphocyte and LAK cell diapedesis through EC.
- Fibrinolysis was determined to be minimal in the process of the transendothelial migration assay.
- Tested peptide concentrations (2, 4, 8 mM) resulted in comparable levels of inhibition (of diapedesis).
- Fibrinogen concentration (0.25, 0.50, 0.75, 1.0 mg/ml, and corresponding concentrations of other reagents to make sFn) was found to be insignificant factor above the concentration of 0.5 mg/ml.

CHAPTER 5. DISCUSSION

The results from these studies confirm the first of our hypotheses that sFn inhibits lymphocyte and LAK cell adherence to and diapedesis across endothelial monolayers.

The first aim of this study was to determine if sFn, which is elevated in the circulation of many cancer patients, affects the ability of peripheral blood lymphocytes to adhere to endothelium *in vitro*.

Initial experiments demonstrated that Oregon Green labeled sFn binds to both LAK cells and EC *in vitro*. These results correspond with our previously published data that sFn also binds to lymphocytes, monocytes, and tumor cells *in vitro* (Figures A-7 and A-8; Biggerstaff, 2007, Biggerstaff, 2006).

Subsequent experimentation, involving lymphocyte/EC binding assays under flow conditions, resulted in a significant inhibition of lymphocyte binding to EC. Maximal inhibition was observed when both cells were pretreated with sFn prior to inclusion in assays, however significant inhibition was also observed when only one cell type was pretreated. These results correspond well with our previous findings that sFn also inhibits lymphocyte (Figure A-1; Biggerstaff 2007) adherence (and cytotoxicity) to tumor cells by the same integrin mediated mechanism. This hypothesis is further supported due to the inability of individual sFn components (fibrinogen, GPRP, and thrombin) to significantly effected lymphocyte binding to EC, suggesting that the entire sFn molecule intact is required for inhibition to occur.

We have previously demonstrated that the predominant lymphocyte subset which interacts with

A375 melanoma cells is the CD8+ cytotoxic T cell (Biggerstaff, 2007). The predominant mechanism by which T lymphocytes adhere to EC is via CD11a/CD18 (which does not bind fibrin(ogen)) binding to EC CD54. Other leukocytes, such as monocytes, NK cells, and neutrophils also express an additional CD54 binding beta-2 integrin, CD11b/CD18, which also binds fibrin(ogen). Our observation that lymphocytes actually bound sFn, but do not express CD11b/CD18, would suggest that another receptor is responsible for fibrin binding to lymphocytes and LAK cells. Another leukocyte integrin expressed by lymphocytes, $\alpha_X\beta2$ does not bind to CD54, but does bind to fibrin(ogen) using the same binding motif as for CD11b. No experiments were performed in this study to investigate the role of $\alpha_X\beta2$ sFn/lymphocyte binding.

As discussed previously, Ugarova et al. have found particular peptide sequences corresponding to the gamma chain on fibrin(ogen) and complementary sequences on CD11b/Cd18 (P2/P4) and CD54 (P1/P3). In the present study, using lymphocytes as effector cells, only two of these peptides, P1 and P3, were anticipated to affect adherence significantly. However, peptides 2 and 4 were used in this study to control for other CD11b containing leukocyte subsets which may have remained after lymphocyte purification. As expected, lymphocyte adherence was significantly restored with the application of appropriate peptides (P1 and P3), but not by peptides P2 and P4. Thus, peptide blockade of either the CD54 binding site for fibrin, and the fibrin binding site for CD54, was confirmed to be an important mechanism in lymphocyte adherence to EC under these conditions.

Activated lymphocyte, or LAK cell, adherence to EC was similarly inhibited by sFn, confirming our previous observations (Biggerstaff, 2007) using LAK cell adherence to tumor cells (Figure A-1).

LAK cell adherence, in the presence of sFn, was restored with pretreatment of P1/P3, but not P2/P4.

Extrapolation of these results to the physiological setting would suggest that, in cancer patients with elevated levels of sFn, leukocytes (lymphocytes and monocytes), EC, and circulating tumor cells would bind sFn, resulting in the inability of lymphocytes and monocytes to adhere to, and subsequently kill, tumor cells, resulting in enhanced metastasis. The use of fibrin blocking peptides may represent a novel class of therapeutic agents to reduce sFn mediated immunosuppression, and decrease metastasis in many cancers, while avoiding the negative bleeding problems commonly associated with anticoagulant therapies, since they do not inhibit normal blood coagulation.

Since adherence is a necessary step leading up to diapedesis through endothelium, combined with the fact that sFn inhibits lymphocyte/LAK cell adherence, it was hypothesized that under comparable conditions, cellular diapedesis would similarly be inhibited. We have previously determined the optimal sFn and peptide conditions in previous studies, however since the diapedesis was novel, we optimized the conditions of this assay. The optimal concentration for sFn for inhibition was 0.5 mg/ml. The maximal restoration of diapedesis was seen using a 4 mM concentration of P1 and P3, and 8 mM concentration of peptides for P2/P4. However, all assays were subsequently performed at 4 mM to be comparable with previous studies and to accommodate the limited amount of peptide available to our laboratory.

Using an 18-hour static transendothelial migration assay, both lymphocyte and LAK cell diapedesis were inhibited by sFn in the same pattern observed for adherence. However, the

actual level of inhibition was lower than for adherence. There are two reasons for this: 1) Optimal cell number and experimental model parameters were different for binding and transendothelial migration assays. 2) Diapedesis was performed for 18 hours, compared with 1 hour for adherence. This may have allowed for these cells to overcome effects of fibrin by the employment of other molecular mechanisms such as fibrinolysis. To test this, we performed assays in the presence of the plasmin inhibitor, eACA. However, no effect was observed.

Although lymphocyte diapedesis was not affected by GPRP or thrombin, some inhibition was observed when cells were preincubated with fibrinogen, but to a significantly lower degree than sFn. This may have been because some "immobilization" of fibrinogen occurred over the 18 hour period in a similar to a fashion reported by Ugarova, et al, although this group demonstrated enhanced diapedesis when only EC were incubated with immobilized fibrinogen. Our previous results confirm this effect using sFn enhancement of monocyte adherence to tumor cells, but preincubation of both cell types with sFn resulted in pronounced inhibition. This would suggest that in the present study immobilization of fibrinogen on both cell types, would explain the observed inhibition. Since this study is concerned with sFn, further study of the mechanism(s) by which immobilized fibrinogen mediates leukocyte/EC interactions is outside the scope of this project.

SFn mediated inhibition of lymphocyte and LAK cell diapedesis was blocked by inclusion of peptides P1/P3, clearly implicating CD54 as an important mediator of diapedesis, which is consistent with previous literature.

In contrast to adherence assays, sFn mediated inhibition of lymphocyte and LAK cell diapedesis

was also blocked using peptides P2/P4 to a similar degree observed for P1/P3, implicating CD11b/CD18 in the mechanism of diapedesis. The increased incubation time, from 1 hour to 18 hours, may have allowed for upregulation of CD11b presence on lymphocytes and LAK cells. Alternatively, the αxβ2 integrin present on lymphocytes and LAK cells (to a lesser degree than CD11a/CD18) binds to sFn using the same sequence as CD11b. Although we did not study this effect further, our results could possibly suggest a role fort his integrin in diapedesis but not in initial binding. Furthermore, CD11b antigen is present on macrophages, granulocytes, natural killer cells, and monocytes. However, it has also been reported on activated lymphocytes (LAK cells)(Springer, 1979; Sanchez-Madrid, 1993). Therefore, this may somewhat explain how P2 and P4 inhibited LAK cells binding to sFn, compared to lymphocytes

These results demonstrated that sFn is immunosuppressive and may be directly involved in the etiology of metastasis. Clinically, elevated sFn levels in cancer patients may significantly reduce the effectiveness of currently employed immunotherapies targeted to kill cancer cells *in vivo*.

The fibrin(ogen)-CD11b/CD18 and -CD54 axes can now be viewed as a potentially useful target in the development of new therapeutic strategies for the treatment or prevention of inflammatory diseases. In fact, the potential usefulness of fibrinogen as a target in inflammatory disease has already been underscored in studies showing that the pharmacological or genetic depletion of fibrinogen in mice can diminish the progression of arthritis (Busso, 1998; and unpublished data).

An important implication of the findings in studies using fibrinogen $^{390–396A}$ (corresponding to the active peptide sequence within P4 which inhibits fibrin binding to CD11b) mice is that effective anti-inflammatory strategies focusing on fibrin(ogen)-leukocyte interactions could potentially be

devised that would not necessarily compromise hemostatic function. Therefore, in principle, inflammatory responses could be controlled at the level of hemostatic factors without increasing the risk of bleeding or thrombotic events (Busso, 1998).

Specifically, recent immunotherapy studies (Rosenberg et al.) have shown that many patients' tumors (generally stage 1) regress when activated LAK cells are introduced back into their bloodstreams. This correlates with the many findings suggesting that those patients in the later stages of disease, have elevated levels of sFn and its byproducts. We hypothesize that the reason for observing regression in patients in primarily stage 1 of disease is due, at least in part, to increased levels of sFn present in advanced stages of cancer.

This project addressed the role of sFn in specific lymphocyte adherence and diapedesis across endothelium to tumors. However, it would not necessarily be expected that tumor cell crossing of the endothelial barrier would be inhibited by sFn, since the mechanism of tumor cell extravasation is not β2 integrin mediated. However, other adhesive mechanisms involving integrins such as $\alpha_V\beta3$ (which also binds fibrinogen) may be affected by sFn, but most tumor/endothelial interactions involve preliminary lodgment in microcapillaries due to the large size of most tumor cells. Under such conditions, downstream hemostasis, as well as tumor cells and platelet release of cytokines such as VEGF, may cause endothelial death and partitioning, respectively. This would allow tumor cells to escape into secondary organs by a nonspecific extravasation through gaps in endothelium.

In summary, the results obtained in this project have demonstrated that sFn inhibits both lymphocyte and LAK cell adherence to and diapedesis across endothelial monolayers. In the

light of current immunotherapy protocols, elevated levels of sFn in the blood would be likely to interact with reintroduced, activated lymphocytes as well as precoating existing vascular endothelium. This would diminish the ability of therapeutic immune cells to adhere to blood vessel walls and home to tumors in order to kill them. Our data have shown that this inhibition can be blocked using specific peptides. The therapeutic use of such peptides may therefore enhance the ability of immunotherapeutic cells to reach their targets, resulting in decreased metastasis and possibly enhancing the natural immune response to tumors.

Together with our previous work, the data obtained in this project suggests novel mechanisms for the growth, progression, and treatment of certain cancers in the future. To this end, future work will concentrate on further defining the cellular mechanisms by which sFn, and its counterparts, influence cancer biology. These include experimental animal models to test the efficacy of potential peptide therapies, as well as studies investigating sFn mediated effects on cell signaling and receptor expression, directed towards a better understanding of the role of the immune response in cancer and other inflammatory diseases.

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APPENDIX

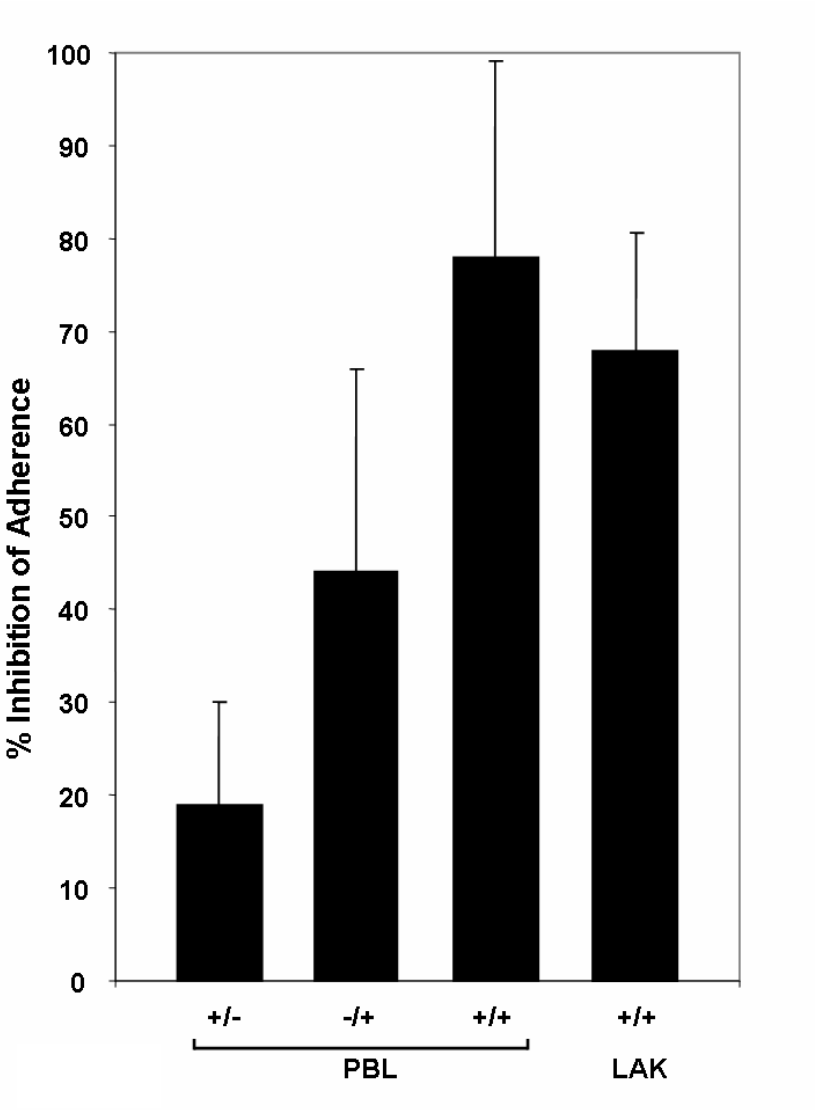

Figure A-1. Inhibition of lymphocyte adherence to tumor cells by sFn. Calcein AM labeled PBL were incubated with A375 cells after pre-treatment of A375 and/or PBL with RPMI or sFn prior to assay. sFn pre-treatment of cell type alone resulted in decreased adherence (+/-, -/+; $P<0.05$; n=3). Maximal inhibition occurred when lymphocytes or LAK cells and tumor cells (+/+) were treated with sFn ($P<0.05$ compared to single treatment; n=3)(Biggerstaff, 2007).

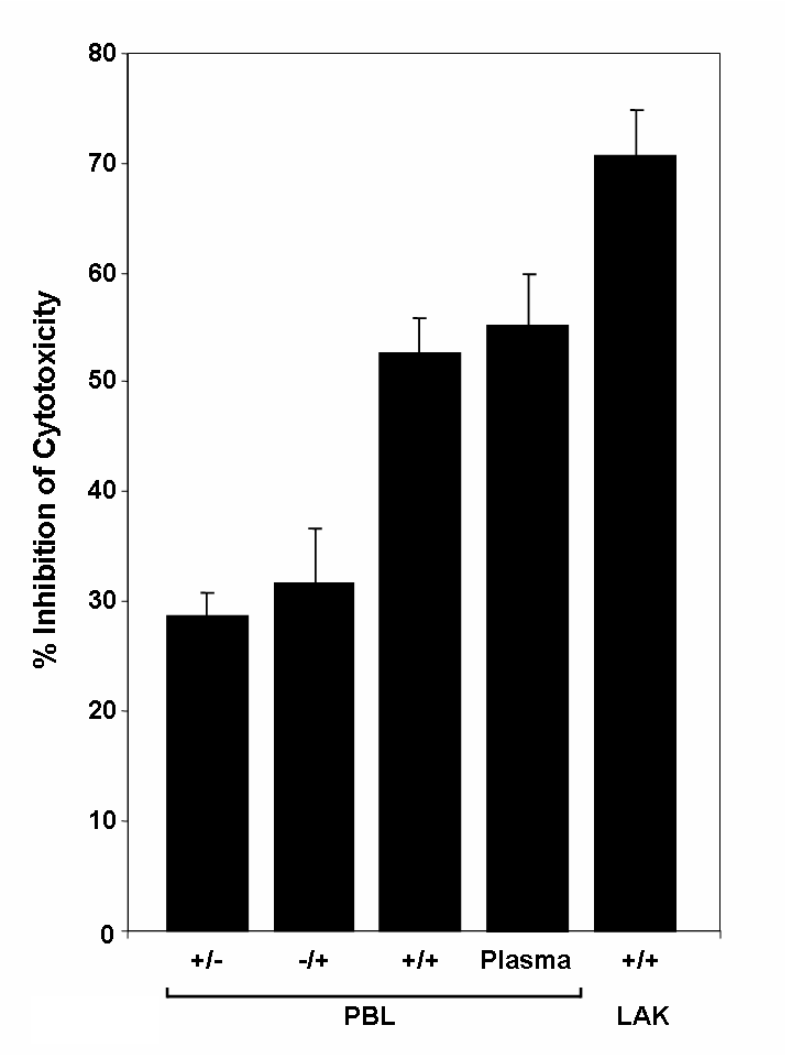

Figure A-2. Sfn inhibition of lymphocyte cytotoxicity against tumor cells**.** Calcein AM labeled PBL incubated with A375 cells after pre-treatment of A375 with or without sFn and pre-treatment of PBL with or without sFn prior to assay. sFn pre-treatment of either cell type alone resulted in decreased cytotoxicity (+/-, -/+; $P<0.05$; n=3). Maximal inhibition of PBL and LAK cell cytotoxic activity occurred when both cell types were treated with sFn (+/+; $P<0.05$ compared to single treatment; n=3). Pre-incubation of lymphocytes and tumor cells with recalcified plasma (+ GPRP-NH2) also significantly ($P < 0.05$ compared to untreated; n=3) inhibited lymphocyte cytotoxicity (Biggerstaff, 2006).

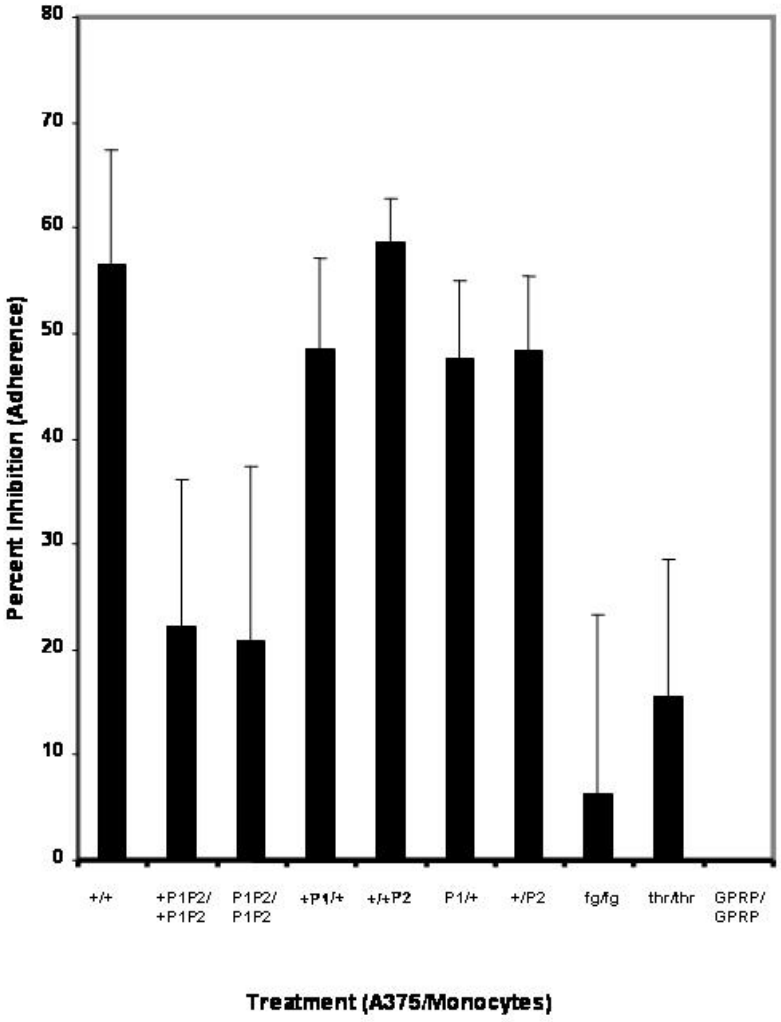

Figure A-3. Effect of specific blocking peptides designated P1 (binds to CD54) and P2 (binds to $\alpha_M\beta 2$) on sFn inhibition of monocyte/tumor cell adherence under flow conditions. (from left to right): sFn pre-treatment of monocytes and A375 cells (n=25) significantly ($P < 0.01$ compared to untreated control; n=30) inhibited monocyte adherence. Pretreatment of cells with P1 and P2 restored cell adherence to levels not significantly different to that of the untreated control ($P<0.05$ to fibrin (n=10); $P>0.05$ to control). Pre-treatment of tumor cells with P1 and monocytes with sFn or tumor cells with sFn and monocytes with P2 inhibited adherence to a similar level to that of sFn treatment of both cells ($P>0.05$ to fibrin; n=5 in each case). Pretreatment of effector and tumor cells with fibrinogen (Fg), thrombin or GPRP did not significantly inhibit adherence ($P > 0.05$ compared to untreated cells; n=5)(Biggerstaff, 2006).

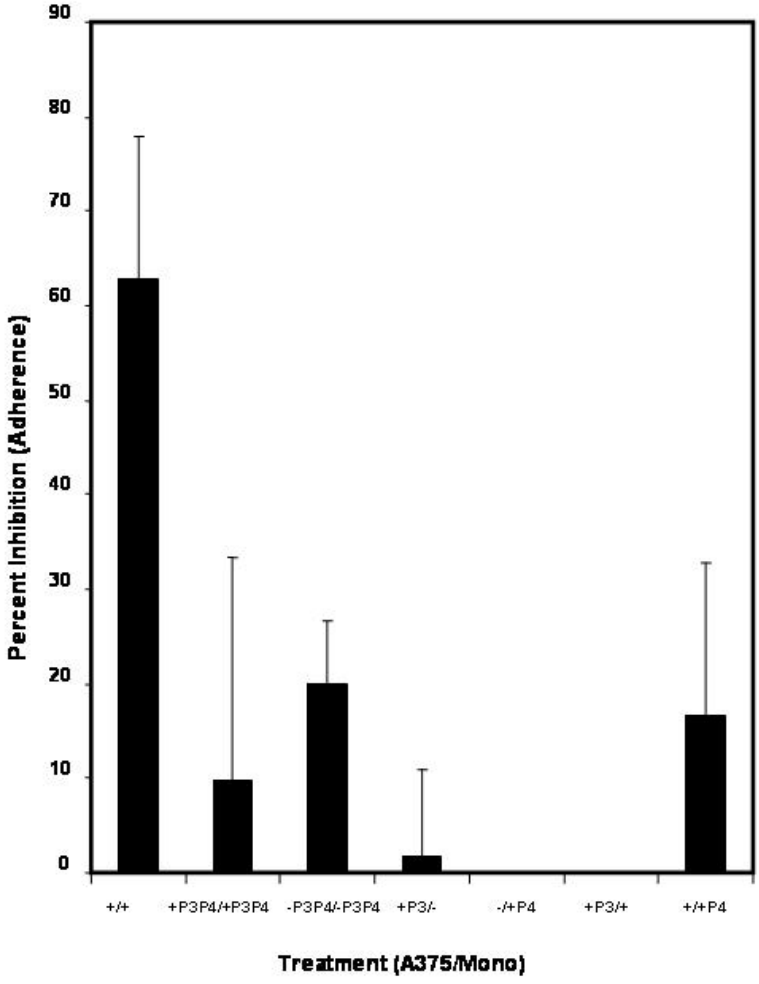

Figure A-4. Effect of specific blocking peptides designated P3 (binds CD54 binding site on sFn) and P4 (binds to $\alpha_M\beta 2$ binding site on sFn) on sFn inhibition of monocyte/tumor cell adherence under flow conditions. (from left to right): sFn treatment of monocytes and A375 cells (n=25) significantly ($P < 0.01$ compared to untreated control; n=10) inhibited cell adherence. Pretreatment of sFn with P3 and P4 restored cell adherence to levels not significantly different to the untreated control ($P<0.05$ to fibrin; n=5; $P>0.05$ to control). Pre-treatment of tumor cells and/or monocytes with either P3+P4, P3 alone or P4 alone were not inhibitory ($P > 0.05$; n=5 in each case, compared to untreated control)(Biggerstaff, 2006).

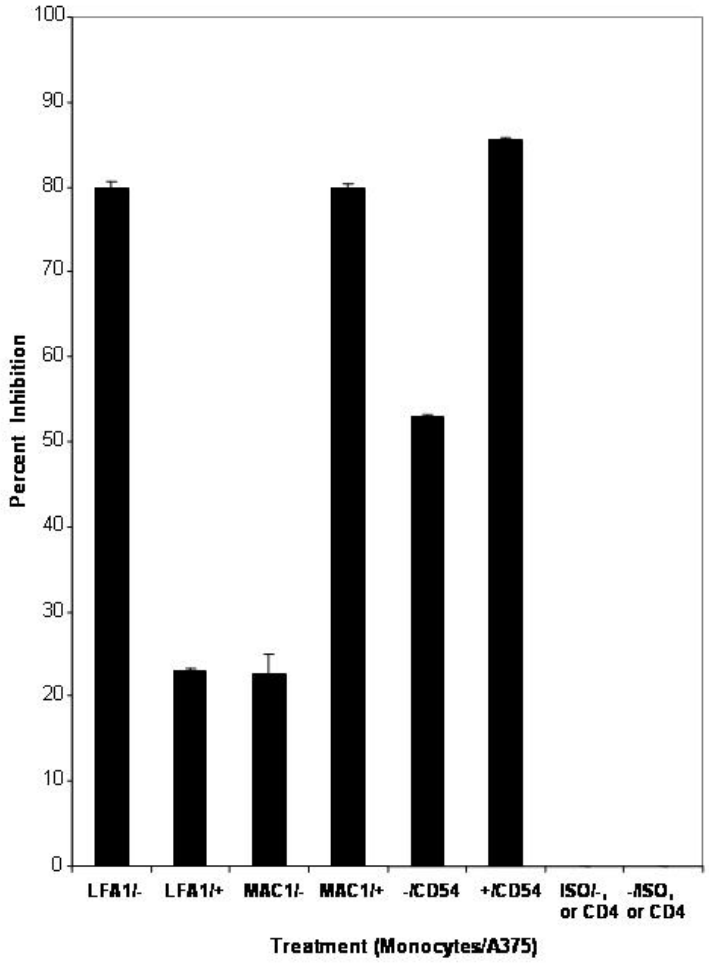

Figure A-5. Effect of monoclonal anti-$\alpha_L\beta2$, -$\alpha_M\beta2$ and CD54 on monocyte adherence to tumor cells under flow conditions in the presence or absence of sFn. Anti-$\alpha_L\beta2$ inhibited monocyte adherence to untreated cells ($P < 0.05$; n=3), but was significantly ($P<0.01$ compared to non-sFn treated cells; n=3) less effective in blocking monocyte binding to sFn pre-treated tumor cells. Conversely, anti-$\alpha_M\beta2$ inhibited monocyte adherence to sFn pre-treated tumor cells to a significantly ($P<0.01$) greater extent than to untreated tumor cells. Anti-CD54 inhibited monocyte adherence to untreated tumor cells by over 50%, and by over 80% when tumor cells were pre-incubated with sFn. Isotypic control IgGs or an irrelevant monoclonal antibody (CD4) did not affect monocyte adherence (Biggerstaff, 2006).

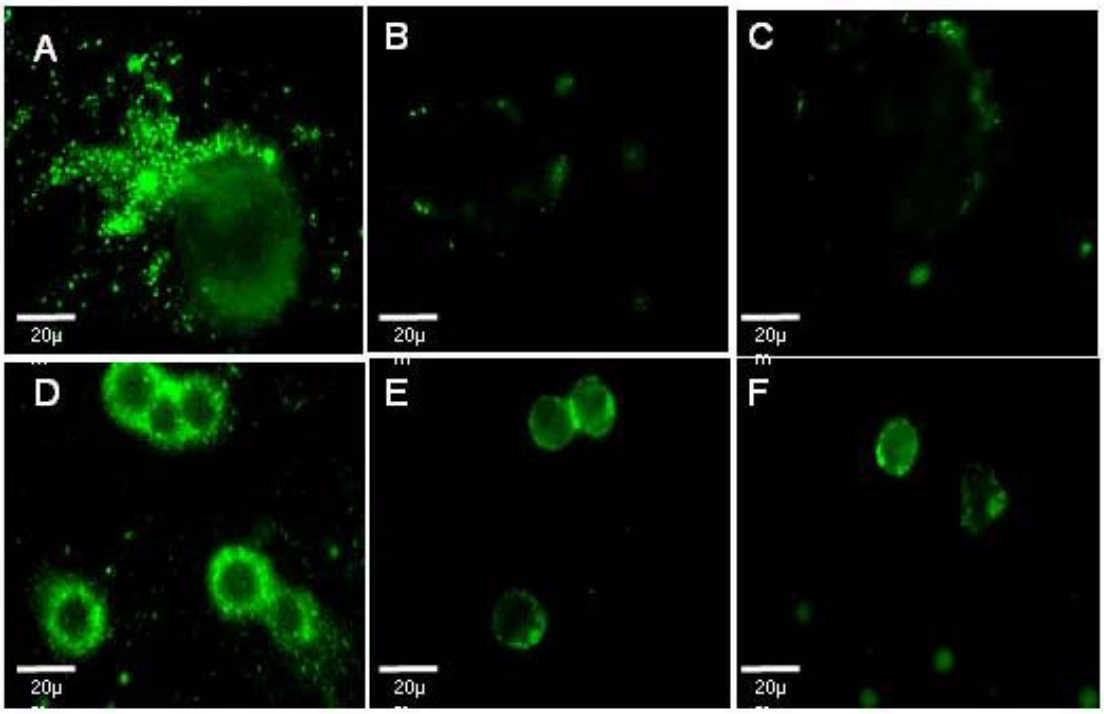

Figure A-6. Oregon Green Labeled Soluble Fibrin on A375 Melanoma Cells and Monocytes. Oregon Green labeled fibrinogen (0.5 mg/ml; Molecular Probes, Eugene, OR) was treated with thrombin (1.25 U) in the presence of 4 mM GPRP-NH2 to produce fluorescently labeled sFn. A375 cells were incubated with labeled sFn for 20 min in a Bioptechs FCS2 enclosed stage incubator. The residual sFn was washed away by perfusion and the cells were imaged on an Olympus BX61 fluorescence microscope equipped with a long pass 535 nm dichroic filter. Considerable binding of sFn was observed. A is a representative image showing tumor cell sFn binding. In contrast, little or no binding was observed when cells were pre-incubated with peptides P1 + P2 (B), or sFn with P3 + P4 (C). Similarly, sFn bound readily to monocytes (D), but was inhibited when cells were pre-incubated with P1 + P2 (E), or sFn was pre-treated with P3 + P4 (F) (Biggerstaff, 2006)

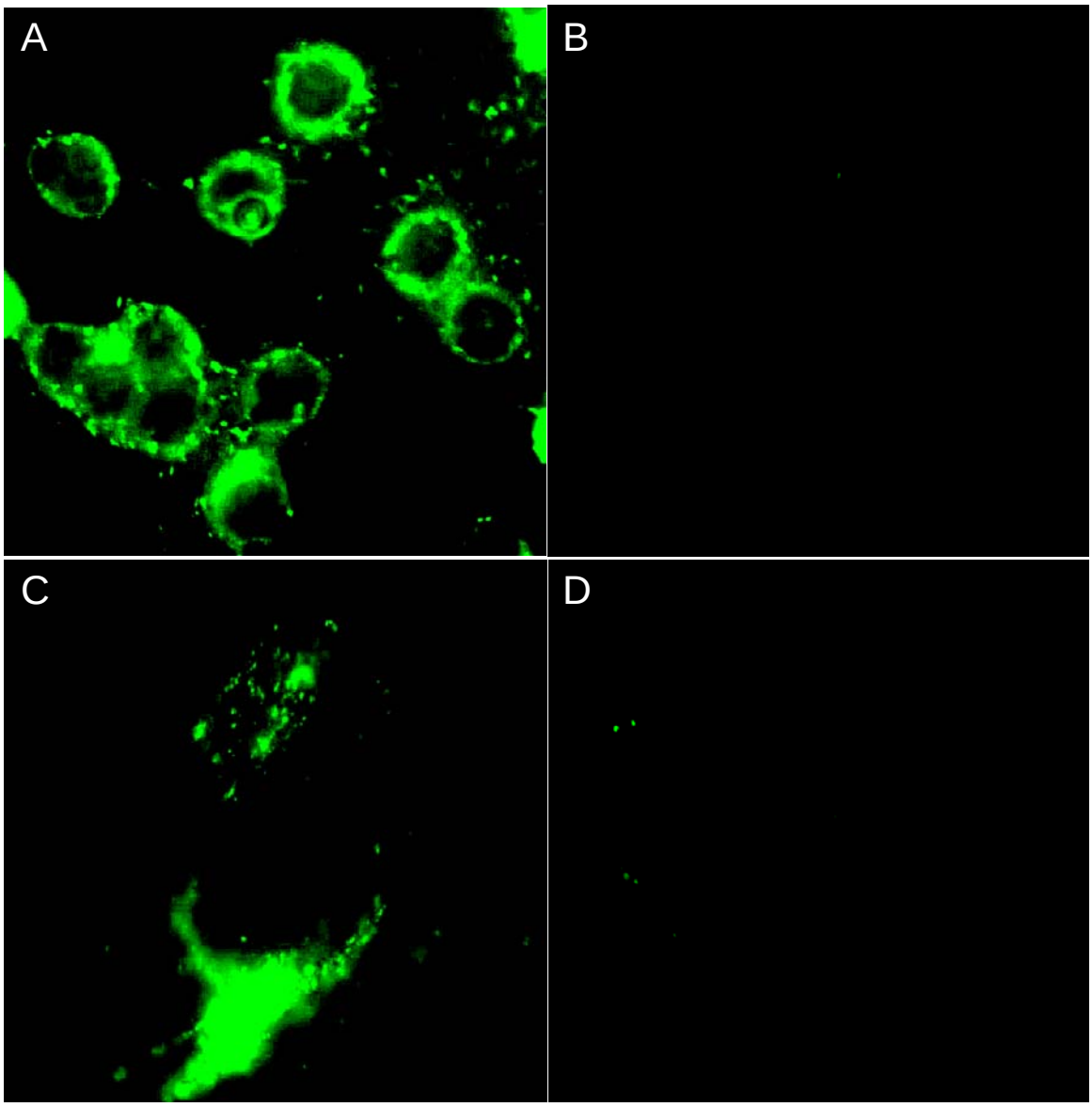

Figure A-7. Oregon Green labeled sFn binding to lymphocytes and tumor cells. Considerable binding of sFn was observed with both cell types. A) OG labeled sFn on lymphocytes, B) lymphocytes without OG sFn, C) A375 cells with OG sFn, D) A375 cell without OG sFn.(Biggerstaff, 2007).

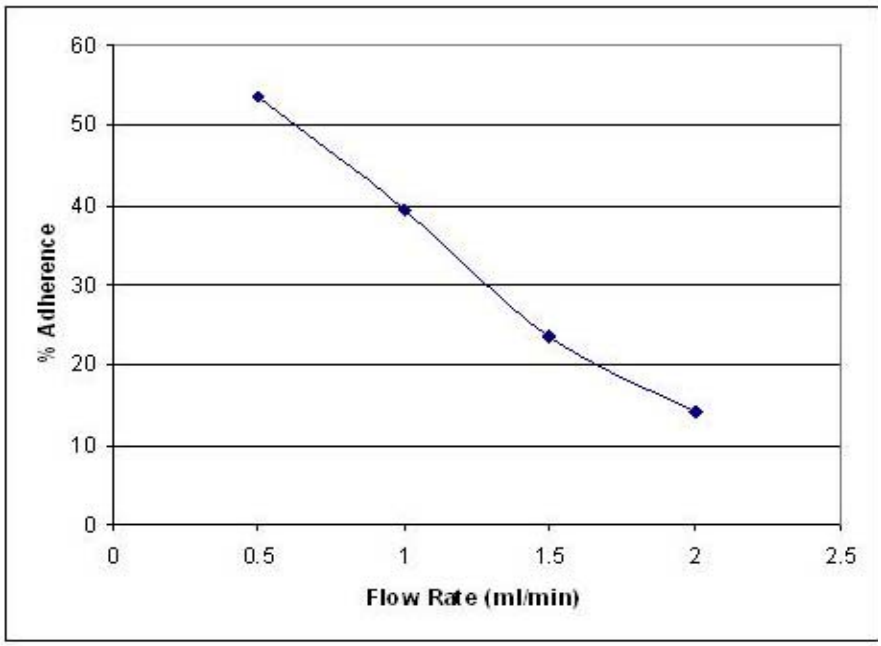

Figure A-8. Effect of perfusion flow rate on monocyte adherence to tumor cells**.** Monocytes (1 x10^6/ml) were perfused across a monolayer of A375 cells attached to a coverslip in a perfusion stage incubator for 1 h at 37^0C, and non-adherent cells were washed off by perfusion to waste for 10 min. Monocyte adherence was maximal at a flow rate of 0.5 ml/min, and linearly decreased as the flow rate was increased to 1, 1.5, and 2 ml/min. (Biggerstaff, 2007).

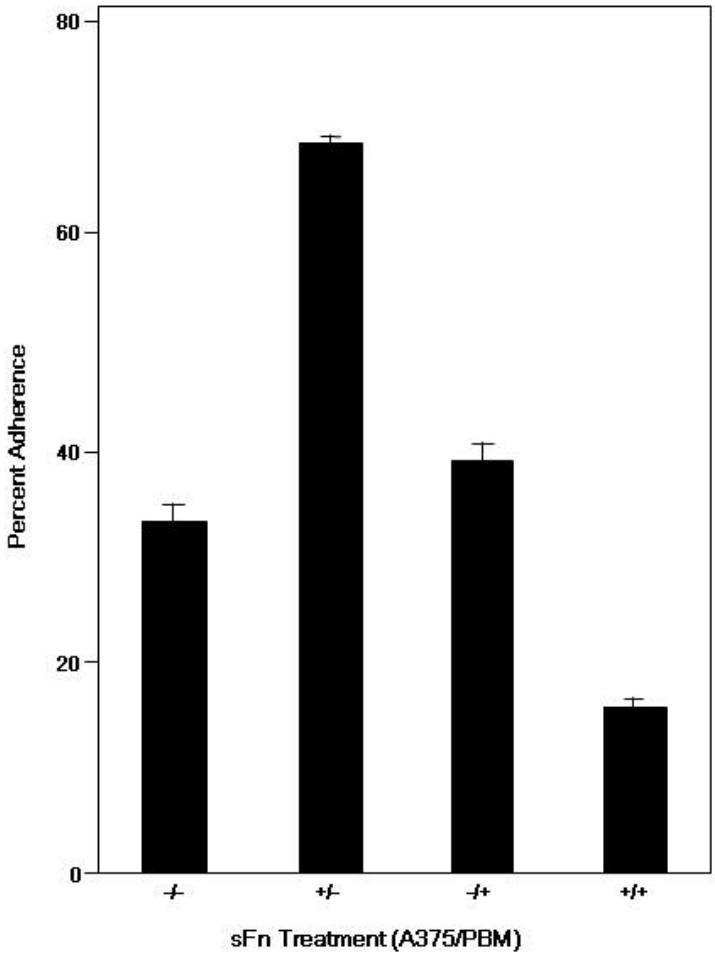

Figure A-9. Effect of sFn on monocyte adherence to tumor cells. Calcein AM labeled PBM incubated with A375 cells after pre-treatment of A375 and/or PBM with RPMI or sFn prior to assay. sFn pre-treatment of tumor cells significantly increased adherence to untreated A375 cells compared to the untreated control ($P < 0.01$: n=3). Preincubation of monocytes also marginally increased adherence to untreated A375 cells ($P < 0.05$: n=3) compared to the untreated control, but to a significantly lower degree than with sFn treated A375 cells ($P < 0.01$ compared to monocyte sFn). sFn pre-treatment of both effector and target cells resulted in a significant inhibition of adherence ($P<0.05$: n=3) compared to the untreated control.(Biggerstaff, 2006).

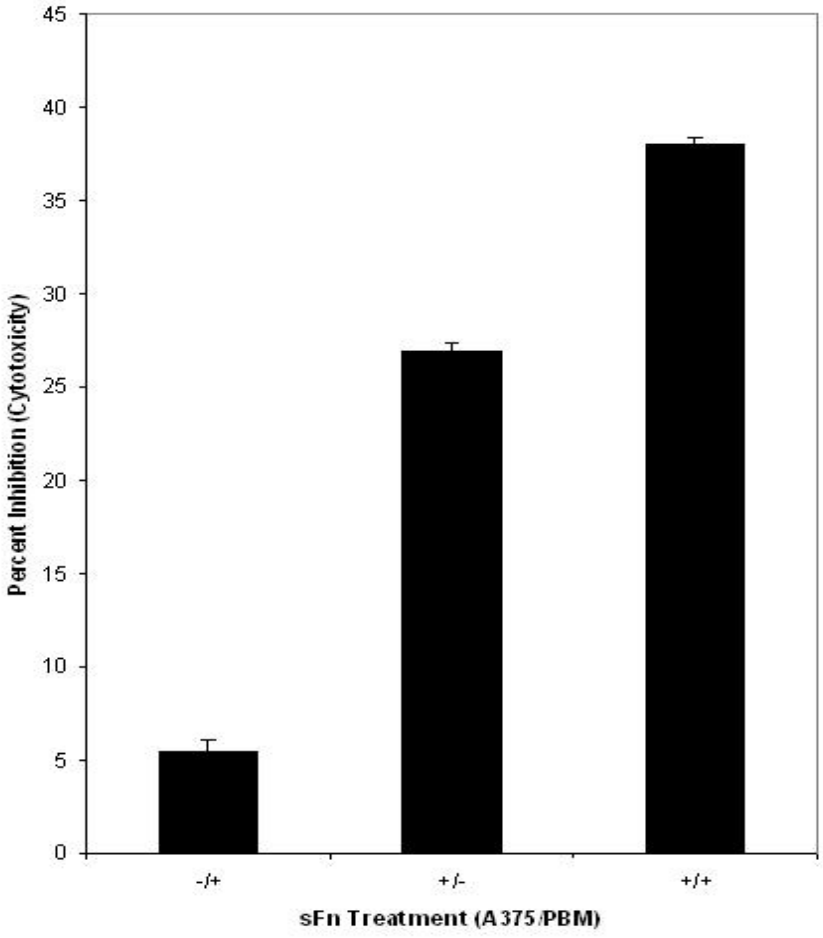

Figure A-10. Effect of sFn pre-treatment on monocyte cytotoxicity against tumor cells. Calcein AM labeled PBM incubated with A375 cells after pre-treatment of A375 with or without sFn and pre-treatment of PBM with or without sFn prior to assay. sFn pre-treatment of monocytes was slightly inhibitory ($P < 0.05$ compared to untreated control; n=3). Significantly greater inhibition was observed when A375 cells were sFn pre-treated ($P < 0.01$ compared to untreated and to monocyte treated cells; n =3). Maximal inhibition of PBM cytotoxic activity occurred when both cell types were treated with sFn ($P<0.01$ compared to untreated, monocyte treated or A375 treated cells: n=3).(Biggerstaff, 2006)

Table A- 1. Lymphocyte adherence to endothelial cells under flow conditions (measured in fluorescent pixels).

	(-/-)	(+/+)	(-/+)	(+/-)	P1/P3	P2/P4	Fg/Fg	GPRP/GPRP	Thr/Thr
2/8/2007	3519	1131	.	.	.	.	.	.	.
2/15/2007	3453	1841	.	2382	.	.	.	.	.
2/18/2007	3497	1428	.	2508	.	.	.	.	.
2/22/2007	3811	2001	3050	.	.	.	.	.	.
2/24/2007	4658	1867	3038	.	.	.	.	.	.
2/27/2007	4094	2043	.	.	.	.	3599	4021	4167
2/28/2007	4833	1564	.	.	3681	.	.	.	.
3/1/2007	5171	1465	.	.	3545	.	.	.	.
3/2/2007	3586	1433	.	.	2694	.	.	.	.
3/6/2007	4359	2779	.	.	.	.	3773	3992	4185
3/8/2007	3889	1470	.	.	.	1721	.	.	.
3/10/2007	3002	1272	.	.	.	1491	.	.	.
3/28/2007	3220	1733	.	.	.	2183	.	.	.
3/30/2007	3678	2214	2374	2578	.	.	.	.	.
4/2/2007	3192	1938	.	.	.	.	3354	3028	2876
Mean	3864.13	1745.27	2820.67	2489.33	3306.67	1798.33	3575.33	3680.33	3742.67
St Dev	639.36	421.76	386.87	99.32	534.92	352.42	210.50	565.12	750.61

Table A-2. Matched pair t-tests for lymphocyte binding to endothelial cells under flow conditions.

Comparison	n	Mean Diff	Std Error for Diff	t	two-sided p-value
Section 3.3.2					
(+/+) vs (-/-)	15	-2118.80	180.42	-11.74	< 0.0001
(-/+) vs (-/-)	3	-1228.40	250.82	-4.90	0.039
(+/-) vs (-/-)	3	-1053.30	33.13	-31.79	0.001
(-/+) vs (+/+)	3	-793.07	318.57	2.49	0.131
(+/-) vs (+/+)	3	-661.53	215.08	3.08	0.091
Section 3.3.4					
(Fg/Fg) vs (-/-)	3	-306.33	235.64	-1.30	0.323
(GPRP/GPRP) vs (-/-)	3	-201.33	86.70	-2.32	0.053
(Thr/Thr) vs (-/-)	3	-139.00	113.65	-1.22	0.346
(Fg/Fg) vs (+/+)	3	-1322.00	168.91	7.83	0.016
(GPRP/GPRP) vs (+/+)	3	-1427.00	277.78	5.14	0.036
(Thr/Thr) vs (+/+)	3	-1489.33	344.90	4.32	0.050
Section 3.3.6					
(-/-) vs (P1/P3)	3	-1223.80	214.89	5.69	0.029
(+/+) vs (P1/P3)	3	-1819.10	279.27	-6.51	0.023
(+/+) vs (P2/P4)	3	-306.33	72.23	-4.24	0.051
(P2/P4) vs (-/-)	3	-1571.90	327.93	-4.79	0.041

Table A-3.LAK cell adherence to endothelial cells under flow conditions (measured in fluorescent pixels.

	(-/-)	**(+/+)**	**P1/P3**	**P2/P4**
3/22/2007	5551.8	2941.8	3914	3310
6/20/2007	5233	3101.4	4148.4	3469.2
Mean	5392.4	3021.6	4031.2	3389.6
St Dev	225.43	112.85	165.75	112.57

Table A-4. Matched pair t-tests for LAK cell adherence to endothelial cells under flow conditions.

Comparison	n	Mean Diff	Std Error for Diff	t	two-sided p-value
Section 3.3.8					
(+/+) vs (-/-)	2	-2370.80	239.20	-9.91	0.064
(P1/P3) vs (-/-)	2	-1361.20	276.60	-4.92	0.128
(P1/P3) vs (+/+)	2	-1009.60	37.40	26.99	0.024
(P2/P4) vs (+/+)	2	-368.00	0.20	1840.00	<0.0001
(P2/P4) vs (-/-)	2	-2002.80	239.00	-8.38	0.076
(P1/P3) vs (P2/P4)	2	-641.60	37.60	-17.06	0.037

Table A-5. Lymphocyte transendothelial migration in the presence of all reagents (measured in fluorescence units).

DATE	neg/neg	sfn/sfn	neg/sfn	sfn/neg	TOTAL
4/4/07	452516.5	352012.5	396175	374956.5	537489.5
4/11/07	444845.50	340870.5	404759	405926.5	575453.5
4/14/07	416655.5	338311			513131.5
4/18/07	487573	411093			563522.5
4/20/07	514075.5	441411			585990.5
4/24/07	532001.5	427637.5			595144.5
Mean	474611.25	385222.5833	400467	390441.5	561788.6667
St Dev	44163.61	46682.24	6069.80	21899.10	31132.12

DATE	Fg/Fg	GPRP/GPRP	Thr/Thr
4/4/07			
4/11/07			
4/14/07	369434	411335	413702
4/18/07	451225	469183.5	454733
4/20/07			
4/24/07			
Mean	410329.5	440259.25	434217.5
St Dev	57834.97	40905.07	29013.30

DATE	P1/P3	P2/P4	TC/CM
4/4/07			163610
4/11/07			198054.5
4/14/07			143557.5
4/18/07			182982.5
4/20/07	491896	484068.5	165846
4/24/07	493970	494128.5	130611
Mean	492933	489098.5	164110.25
St Dev	1466.54	7113.49	24723.48

Table A-6. Matched pair t-tests for lymphocyte transendothelial migration results.

Comparison	n	Mean Difference	Std Error for Difference	t	two-sided p-value
Section 3.5.2					
(+/+) vs (-/-)	6	-89389	6134	-14.57	<0.0001
(-/+) vs (-/-)	2	-48214	8127.5	-5.93	0.106
(+/-) vs (-/-)	2	-58240	19320	-3.01	0.204
(-/+) vs (+/+)	2	-54025	9863	5.48	0.115
(+/-) vs (+/+)	2	-44000	21056	2.09	0.284
(+/-) vs (-/+)	2	-10026	11193	-0.9	0.535
Section 3.5.4					
(Fg/Fg) vs. (-/-)	2	-41785	5437	-7.69	0.082
(GPRP/GPRP) vs (-/-)	2	-11855	6535	-1.81	0.321
(Thr/Thr) vs (-/-)	2	-17897	14943	-1.2	0.443
(Fg/Fg) vs (+/+)	2	-35628	4505	7.91	0.08
(GPRP/GPRP) vs (+/+)	2	-65557	7467	8.78	0.072
(Thr/Thr) vs (+/+)	2	-59515	15875	3.75	0.166
Section 3.5.6					
(P1/P3) vs (-/-)	2	-30106	7926	-3.8	0.164
(P1/P3) vs (+/+)	2	-58408	7924	7.37	0.086
(P2/P4) vs (+/+)	2	-54574	11917	4.58	0.137
(P2/P4) vs (-/-)	2	-33940	3933	-8.63	0.073
(P2/P4) vs (P1/P3)	2	-3835	3993	-0.96	0.513

Table A-7. LAK cell transendothelial migration results (measured in fluorescence units).

DATE	Lymph (-/-)	LAK (-/-)	Lymph (+/+)	LAK (+/+)	LAK (P1/P3)	LAK (P2/P4)	TC/CM	TOTAL
5/17/07	502584.5	552568	444907	485738.5			157456	547261
5/26/07	444973.5	504168	390045.5	478595	520158.5	501627	146055.5	538398
5/30/07	479627	545669	424259.5	495122.5	513054.5	523013.5	146583	537056.5

Table A-8. Matched pair t-tests for 72 hour incubated LAK cell and lymphocyte transendothelial migration assays.

Comparison	n	Mean Difference	Std Error for Difference	t	two-sided p-value
Section 3.5.7					
Lymph (+/+) vs Lymph (-/-)	3	-55991	853	-65.64	0.0002
LAK (-/-) vs Lymph (-/-)	3	-58406	4652	12.56	0.0063
LAK (+/+) vs Lymph (+/+)	3	-66748	13927	4.79	0.0409
LAK (+/+) vs LAK (-/-)	3	-47650	11997.5	-3.97	0.0579
LAK (P1/P3) vs LAK (-/-)	2	-8312	24303	-0.34	0.7902
LAK (P1/P3) vs LAK (+/+)	2	-29748	11816	2.52	0.2407
LAK (P2/P4) vs LAK (-/-)	2	-12598	10057	-1.25	0.4289
LAK (P2/P4) vs LAK (+/+)	2	-25462	2430	10.48	0.0606
LAK (P2/P4) vs LAK (P1/P3)	2	-4286	14245	-0.30	0.8139

Table A-9. Lymphocyte transendothelial migration in the presence of various sFn concentrations (measured in fluorescence units).

DATE	(-/-)	0.25 mg/ml	0.5 mg/ml	0.75 mg/ml	1.0 mg/ml	TCCM	TOTAL
6/14/07	511695	450246	434956	439015	429014	179103	610593
6/22/07	514827	480156	429400	427139	455710	151050	587498
Mean	513261	465201	432178	433077	442362	165076.5	599045.5
St Dev	2214.66	21149.56	3928.69	8397.60	18876.92	19836.47	16330.63

Table A-10. Lymphocyte transendothelial migration in the presence of eACA (measured in fluorescence units).

DATE	(-/-)	(+/+)	EACA (-/-)	EACA (+/+)	TC/CM	RPMI	TOTAL
4/1/07	487958	429495	487637	427880	156708.5	69617	590753

Table A-11. Lymphocyte transendothelial migration in the presence of various peptide concentrations (measured in fluorescence units).

DATE	neg/neg	sfn/sfn	P1/P3 (2mM)	P1/3 (4mM)	P1/3 (8mM)	P2/P4 (2mM)	P2/4 (4mM)	P2/4 (8mM)	TCCM	TOTAL
5/7/07	590156	501333	550781	547011	558328	521786	523044	508926	191510.5	613746

VITA

Brandy L. Weidow was born September 29, 1979 in Binghamton, NY. She graduated from Beech Senior High School in Hendersonville, TN in 1998. She received her undergraduate degree in Microbiology, with a minor in English Literature, from the University of Tennessee in Knoxville, TN in 2003. She began working for the Center for Biomarker Analysis in 2004, under the appointment of Dr. John Biggerstaff. A desire to continue studies at the graduate level resulted in reenrollment at the University of Tennessee. She entered the Microbiology department and continued working in the laboratory of Dr. Biggerstaff. She completed her Master of Science degree in Microbiology, with a minor program in Statistics in August 2007.